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THE ATYPICAL RHO GTPASE RHOD AND ITS ROLE IN CELLULAR DYNAMICS

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The atypical Rho GTPase RhoD and its role in cellular dynamics
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Despite its discovery 20 years ago, the accumulated knowledge about the Rho member RhoD is scarce. Instead, the vast majority of studies concerning Rho GTPases has focused on the three classical members RhoA, Cdc42 and Rac1. In contrast, RhoD is considered an atypical Rho GTPase, with an aberrant GTP/GDP cycling and an unknown regulation.

Like most Rho GTPases, altered RhoD protein levels result in actin cytoskeleton reorganization. We found that increased RhoD protein levels lead to a less dynamic actin cytoskeleton, while RhoD silencing leads to more pronounced actin-containing structures, such as stress fibers, cortical actin and ruffles, depending on cell type. Actin-dependent processes, such as cell migration and cell proliferation, are significantly affected in absence of RhoD in fibroblasts.

Moreover, we have shown that endogenous RhoD, as well as its interaction partner WHAMM, localize to the Golgi apparatus. Silencing or overexpression of RhoD or WHAMM leads to a dispersion of the Golgi apparatus, suggesting a role of these proteins in Golgi homeostasis. In addition, protein transport from the ER to the plasma membrane is delayed both when overexpressing and silencing RhoD and WHAMM, as measured by the VSV-G protein transport assay.

One of the first described functions of RhoD was its regulatory role in endosome fusion and trafficking. We found that the localization to vesicles is independent of the nucleotide-bound status of RhoD. However, only the GTP-bound RhoD can localize to the plasma membrane. In contrast, RhoD must be inactivated for fusion of RhoD positive vesicles. Deleting the unique N-terminal of RhoD leads to an altered distribution and characteristics of RhoD positive vesicles.

Taken together, this thesis elucidates the role of RhoD in three different dynamic cellular processes; reorganization of the actin cytoskeleton, Golgi homeostasis and vesicle transport and fusion.

LIST OF SCIENTIFIC PAPERS

I. **M. Blom**, K. Reis, V. Nehru, H. Blom, A.K.B. Gad, P. Aspenstrom
RhoD is a Golgi component with a role in anterograde protein transport from the ER to the plasma membrane.
Exp Cell Res 2015 May 1;333(2):208-219

II. **M. Blom**, K. Reis, J. Heldin, J. Kreuger, P. Aspenstrom
The atypical Rho GTPase RhoD is a regulator of actin cytoskeleton dynamics and directed cell migration.
Exp Cell Res 2017 Mar 15;352(2):255-264

III. **M. Blom**, K. Reis, P. Aspenstrom
RhoD localization and function are dependent on the GDP/GTP-bound state and unique N-terminal motif.
Manuscript (2017)

CONTENTS

LIST OF ABBREVIATIONS

ADF	actin-depolymerization factor
ADP	adenosine diphosphate
Arp2/3 complex	actin related proteins 2/3 complex
ATP	adenosine triphosphate
Cdc42	cell division control protein 42 homolog
CP	capping protein
CRIB	cdc42/Rac-interactive binding motif
Dbl	diffuse B cell lymphoma
DH	Dbl-homology
DHR	Dock Homology Region
DRF	Diaphanous-related formin
ECM	extracellular matrix
EEA1	early endosome antigen 1
Ena/VASP	enabled/vasodilator-stimulated phosphoprotein
ER	endoplasmic reticulum
FA	focal adhesion
F-actin	filamentous actin
FH domain	formin homology
G2	gap phase 2
GA	Golgi apparatus
G-actin	globular actin
GalT	1,4-galactosyltransferease
GAP	GTPase activating protein
GDI	guanosine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GM130	Golgi matrix protein 130
GMF	glial maturation factor
GRASP65/55	Golgi reassembly-stacking protein of 65/55 kDa

GTP	guanosine triphosphate
IRSp53	insulin-receptor substrate p53
kDa	kilo Dalton
LIMK	Lin11, Isl-1, Mec-3 domain kinase
mDia	mammalian diaphanous protein
MLC	myosin light chain
MLCP	myosin light chain phosphatase
NPF	nucleation promoting factor
N-WASP	neuronal Wiskott-Aldrich syndrome protein
PDEδ	phosphodiesterase δ
PDGF	platelet derived growth factor
PH	pleckstrin homology
Rab	Ras-like proteins in brain
Rac	Ras-related C3 botulinium toxin substrate
Ras	Rat sarcoma
RBD	Rho binding domain
REM	Rho effector homology
Rho	Ras homolog
Rnd	Round
ROCK	Rho associated protein kinase
TC10	Teratocarcinoma 10
TGN46	trans-Golgi network 46
t-SNARE	target-soluble *N*-ethylmaleimide sensitive factor attachment protein receptor
VCA domain	verprolin, cofilin, acidic domain
VSV-G	vesicular stomatitis virus protein G
WAVE	WASP family-verprolin homologous protein
WHAMM	WASP homolog-associated protein with actin, membranes and microtubules
wt	wild-type

I. INTRODUCTION

This thesis aims to present data to elucidate the role of RhoD as a regulator of dynamic cellular processes. Being a member of the Rho GTPases, it is not surprising that RhoD exert a regulatory role on the actin cytoskeleton. However, this thesis also presents data to illuminate RhoD in its role as a regulator of vesicle dynamics and Golgi homeostasis.

1. Rho GTPases

1.1 The Rho GTPase family

Nature has developed a variety of ways to regulate the activity of cellular proteins. One common strategy is phosphorylation, often leading to activation of a protein. Other examples are ubiquitination, regulation by expression/degradation or confining the subcellular localization of a protein. The small GTPases, constituting around 150 protein members in mammals [1], are activated when binding a guanosine triphosphate (GTP). As the GTP is hydrolyzed to guanosine diphosphate (GDP), the protein becomes inactivated. This is the reason why small GTPases are considered "molecular switches". The binding and hydrolysis of the nucleotide is in turn tightly controlled, leading to an intricate regulatory system.[1]

Small GTPases can be further subdivided, depending on structural and functional similarities, into six subfamilies (Tab 1) [1].

Small GTPase subfamily	Nr of genes in human	Function
Rab	63	Vesicle transport
Ras	36	Cell proliferation, differentiation, survival
Arf	27	Vesicle transport
Rho	20	Actin cytoskeleton organization
Ran	1	Transport of RNA and proteins between nucleus and cytoplasm
Other, including Miro	9	Mitochondrial transport etc.

Table 1. The functions of the different small GTPase subfamilies.

The Rho family contains 20 members, known to regulate different aspects of actin cytoskeleton dynamics (Fig 1). The first Rho GTPases were found in 1985 as genes related to the already known Ras subfamily [2]. Out of the 20 Rho family members, only three of them have been studied in detail, RhoA, Cdc42 and Rac1. These three proteins have therefore been seen as the model for a bona fide Rho GTPase. Thus, Rho proteins possessing the characteristics of RhoA, Cdc42 and Rac1 and their isoforms are termed “classical Rho GTPases”, while the remaining have been referred to as “atypical Rho GTPases”. The dividing point between the two classes is the functionality of the GTP/GDP cycling [3]. To date, half of the Rho GTPases are considered atypical, which raises the question if they are really that atypical after all. The classical Rho GTPases have a slow intrinsic GTP hydrolysis and slow nucleotide exchange and requires additional regulatory proteins for the GTP/GDP cycling.

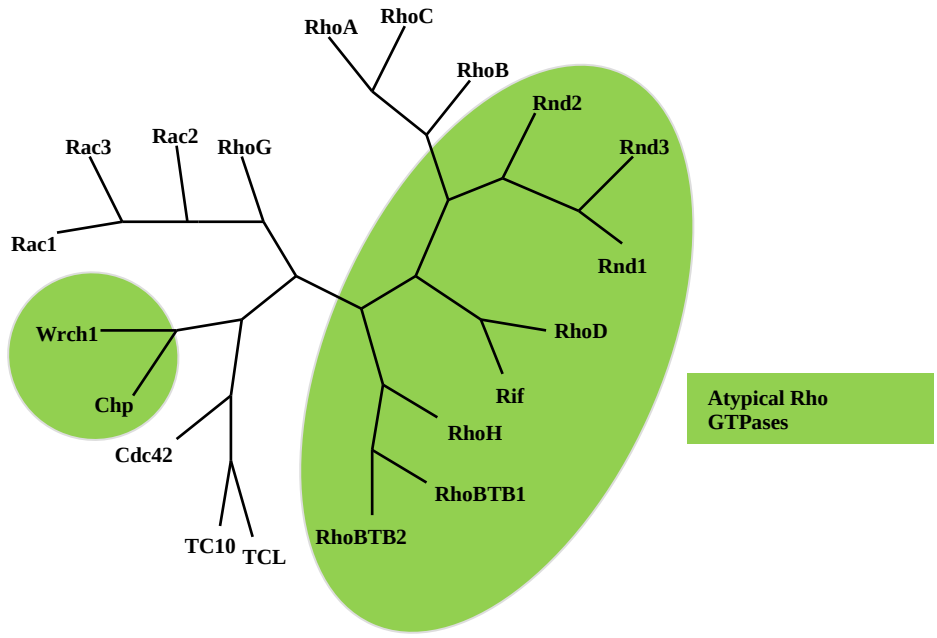

Figure 1. The Rho GTPase family tree based on similarity of the GTPase domain.

The atypical Rho GTPases have altered cycling properties, either aberrant ability to hydrolyze GTP or fast nucleotide exchange ability (tab 2). Rho BTB1 and 2 deviate even more among the Rho GTPases, as they have additional domains and therefore are more than three times the size of other Rho GTPases.

All Rho GTPases, except Rho BTB1 and 2, have a molecular weight of around 20 kilo Dalton (kDa) [4]. They contain a GTP binding domain that interacts with the guanosine nucleotide, containing the switch1 and 2 motifs (Fig 2) [5]. Switch 1 and 2 are regions that, when interacting with the γ-phosphate, alter their conformations. In the GTP-bound state, the Rho GTPase can interact with its effector proteins and induce downstream signaling. When GTP is hydrolyzed to GDP, the γ-phosphate is lost and switch 1 and 2 folds back to a relaxed conformation so that the interactions to the effector proteins are lost [6].

Atypical Rho GTPase	**Intrinsic GTP/GDP cycling**	**Consequence**
Chp	No data regarding intrinsic GTP/GDP cycling but structurally related to Wrch1 [3]	Possibly highly active
Wrch1	Fast nucleotide exchange [7, 8]	Highly active
RhoH	No GTP hydrolysis [9]	Constitutively active
RhoBTB1, RhoBTB2	Alternative amino acid in conserved region needed for GTP hydrolysis. RhoBTB2 may not bind nucleotide. [10, 11]	Not dependent upon activation by GTP binding?
Rnd1, 3	No GTPase hydrolysis, low GDP affinity [12, 13]	Constitutively active
Rnd2	Alternative amino acid in conserved region needed for GTP hydrolysis [12]	Theoretical constitutively active
RhoD, Rif	Fast nucleotide exchange (low GDP affinity) [14]	Highly active

Table 2. The atypical Rho GTPases.

Most Rho GTPases (as well as many other small GTPases) have a C-terminal CAAX-motif, where C stands for cysteine, A for aliphatic amino acids and X for variable amino acid. The motif is often post-translationally prenylated, in which a geranylgeranyl or a farnesyl lipid group is added to the protein. All Rho GTPases are localized to the plasma membrane and/or to an intracellular membrane [15], but they do not harbor any transmembrane domains. The modified CAAX-motif enables the protein to be inserted into a hydrophobic membrane and inhibition of this modification disturbs the localization and function of the protein [16]. The Rho GTPases are set apart from other small GTPases by the presence of the Rho insert region (Fig 2) [17].

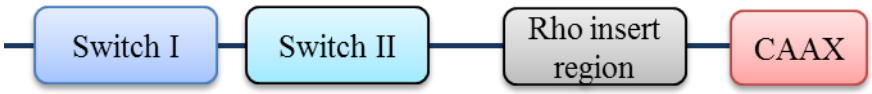

Figure 2. General structure of a Rho GTPase.

1.2 Regulation of the Rho GTPases

Similar to small GTPases in other Ras subfamilies, the intrinsic hydrolysis of GTP to GDP, and the dissociation of GDP for a GTP are very slow [18]. Another set of proteins therefore stimulate these processes, thereby acting as regulatory proteins. There are three main groups of regulatory proteins, GEFs, GAPs and GDIs (Fig 3).

Rho GTPase activator GEF (guanine nucleotide exchange factor)

GEFs act by facilitating dissociation of GDP so that a new GTP can bind, thereby functioning as an activator. There are two families of GEFs, one with a Dbl-homology domain (DH) and one with a Dock Homology Region (DHR) (Fig 4). The first such mammalian protein, discovered in 1985, was Dbl (diffuse B cell lymphoma) [19, 20]. Since then, around 70 members of this group have been found in humans [21]. A Rho GTPase is unstable when not binding a nucleotide. However, when interacting with a GEF, such as the DH domain in a Dbl protein, a stable Rho GTPase:GEF intermediate lacking nucleotide is possible. An excess of GTP over GDP in the cell will favor new binding of GTP to the GTPase [22]. Apart from a DH domain, almost all Dbl-family proteins contain pleckstrin homology (PH) domains. This domain interacts with phosphoinositides and localizes the proteins to the plasma membrane.

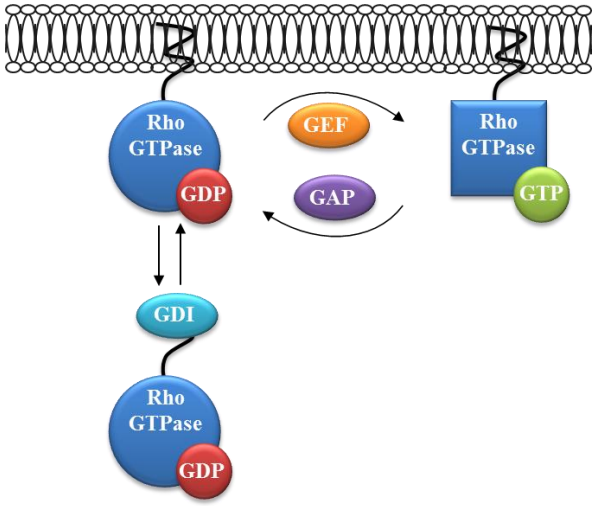

Figure 3. Regulation of the the classical Rho GTPases. Adapted from [23].

The Dbl proteins display a diverse range of additional domains, giving them ability to activate Rho GTPases in a localization-specific manner. Dbl proteins are themselves regulated e.g. by autoinhibition by its N-terminal. Cleavage of the N-terminal has been shown to result in constitutively active proteins of several DH domain-containing GEFs [24, 25].

A guanosine nucleotide binds to its GTPase with high affinity. It has been shown for Ras that the GEF Cdc25 acts by binding the GDP-bound small GTPase with low affinity, which leads to weakening of the GTPase-GDP interaction and strengthening of the GTPase:GEF interaction as the GDP dissociates. Binding of a new GTP will then displace the GEF from the GTPase, which becomes active [18, 26].

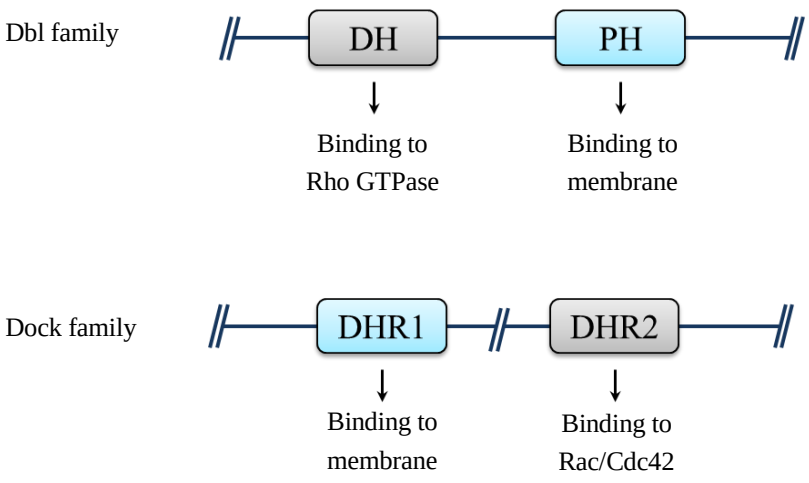

Figure 4. Protein structures of the Rho GEFs Dbl and Dock. Adapted from [27].

The second family of RhoD GEFs is the Dock family proteins, harboring a DHR2 domain which interacts with the GTPase. Although the mechanism to catalyze nucleotide exchange is similar between the DHR2 and DH domains, their amino acid sequences are unrelated. In addition, the Dock proteins have an upstream DHR1 domain, which, in analogy to PH domains, localizes the protein to the plasma membrane (Fig 4). Autoinhibition has been reported also for Dock GEFs [28, 29]. Whereas the Dbl-family GEFs activate many different Rho GTPases, the Dock GEFs are restricted to the Rac and Cdc42 families [27].

Rho GTPase repressor GAP (GTPase activating protein)

A GAP catalyzes the hydrolysis of GTP by stabilizing the GTPase in a conformation which helps a water molecule to hydrolyze the GTP to a GDP. The importance of regulation by GAPs *in vivo* is evident considering the abundance of mutations in the small GTPase Ras found in tumor samples. The mutations mainly lead to an altered amino acid at one of three positions in Ras; at position 12, 13 or 61. All three mutations result in a Ras protein in which the hydrolysis of the GTP by GAP is abolished, giving a constitutively active Ras-oncogene [30]. The regulations of the GAPs are not as clear as for the GEFs, but the RhoGAP β2-chimerin has been found to be autoinhibited [31].

Considering the fact that the activity of some GEFs and GAPs is not restricted only to one Rho GTPase, it is surprising that these regulatory proteins outnumber the Rho GTPases [18].

Rho GTPase inhibitor GDI (guanosine dissociation inhibitor)

The GDI adds another type of regulation; by binding and sequestering the Rho GTPase away from its site of action at the membrane (Fig 3). Compared to the GEFs and GAPs, the GDI protein family is very small and contains only three known members. Most Rho GTPases have C-termini that are prenylated after translation and a majority is geranylgeranylated [15]. The added lipid enables the Rho GTPase to be associated with membranes where it can be activated by GEFs and exert its action. GDIs bind to the geranylgeranyl lipid of the C-terminal Rho GTPase, and in so doing, extracting it from the membrane where the Rho GTPase was inserted. In this way, Rho GTPases are held as soluble inactive complexes in the cytosol. Some Rho GTPases are instead farnesylated, making them inert to GDIs [15]. Phosphorylation of GDI is known to decrease the interaction to the Rho GTPase, whereas phosphorylation of the Rho GTPase conversely leads to an increased interaction to the GDI [32].

PDEδ

The PDEδ protein binds preferably farnesylated proteins, but also geranylgeranylated, [33] and it has been shown to interact and extract farnesylated Rab13 and several Ras GTPases from membranes [34, 35]. PDEδ and GDIs work in a similar manner, by binding and capturing the prenylated group into a β-sandwich domain [36]. In contrast to GDIs, the PDEδ does not have a GTPase binding motif, explaining why it can interact with GTPase-unrelated proteins [33].

Additional regulation

In addition to the classical regulation by GEFs, GAPs and GDIs, Rho GTPases can be regulated by a number of post-translational modifications. The already mentioned **lipid modification** of the C-terminal regulates the subcellular localization [15]. The most common lipidation on Rho GTPases are geranylgeranylation (addition of a 20-carbon chain moiety) and farnesylation (addition of a 15-carbon chain moiety), both irreversible modifications. In addition, several Rho GTPases can undergo palmitoylation [37-39]. This process is reversible and, like prenylation, the palmitoylation enables the protein to be inserted into a membrane [40]. The prenylation and palmitoylation can occur on the same protein [39, 41].

Phosphorylation of Rho GTPases can cause a hampered intrinsic GTP/GDP cycling [42-44]. It can also interfere with the interactions to regulatory proteins or effector proteins. Addition of a ubiquitin moiety is a common way to mark a protein for degradation or targeting it to other subcellular compartments. One example is the **ubiquitination** of RhoA at the leading edge of a migrating cell, which inhibits the formation of stress fibers and enables the formation of lamellipodia and filopodia [45]. Rac1 can both undergo ubiquitination and sumoylation. **Sumoylation** of Rac1 leads to increased GTP-binding and has been shown to be important for optimal lamellipodia formation and cell migration after stimulation of hepatocyte growth factor [46]. Moreover, regulation of Rho GTPases occur at the level of **transcription** [47] and by the activity of **microRNAs** [48].

1.3 Rho effector proteins

As previously mentioned, Rho GTPases predominantly interact with their effector proteins when in a GTP-bound state. Mutations within the switch regions have revealed that different effector proteins bind to different amino acids within the switch regions. This means that a point mutation can abolish binding to one interaction partner, but leave the interaction to

another partner intact [49]. There are some domains that effector proteins which bind to Rho proteins share. One is the CRIB domain (Cdc42/Rac-interactive binding motif), which is present in many of the Cdc42 and Rac binding proteins. However, the CRIB domain itself is not sufficient to bind Cdc42 or Rac. Likewise, an REM, (Rho effector homology motif), has been found in several RhoA effectors [49]. One way Rho GTPases activate their effector proteins is by breaking an autoinhibitory intramolecular structure to expose functional domains [50, 51].

1.4 The Rho GTPase RhoD

One subgroup of the Rho GTPase family includes RhoD and Rif (Rho in filopodia) (Fig 1). Whereas Rif has a more tissue specific expression pattern, RhoD is expressed in most tissues, with high levels in kidney, liver, intestine and lung [52]. RhoD evolved late and is the only Rho GTPase expressed exclusively in mammals [53].

Since both RhoD and Rif have a functional GTP hydrolysis capacity, they were first considered to belong to the classical Rho GTPases. However, the finding that RhoD and Rif are capable to be activated without a GEF led to a reclassification to the atypical cohort. The binding of GDP to RhoD is more than 15 times weaker than to most Rho GTPases, suggesting that RhoD can be activated in absence of a GEF activator. The intrinsic nucleotide exchange activity of RhoD is increased in a way similar to that of Rac1b [14], a splice variant of Rac1 upregulated in breast and colorectal cancer [54, 55]. To date, RhoD has not been found upregulated or mutated in tumor samples.

Just as for most Rho GTPases, mutants disturbing the activity of RhoD have been used to examine the role of the protein. Mutation of the glycine at position 26 (corresponding to position 12 in Rac1) gives a hydrolysis-defective RhoD, which is constitutively active. Mutation of the threonine at position 31 (corresponding to position 17 in Rac) leads to a dominant negative mutant.

2. Actin

Together with the microtubule network and the intermediate filaments, the actin filaments give stability and dynamics to the cytoskeleton of the eukaryotic cell. Cells need to alter the morphology in order to adapt to the environment, to migrate and divide. This is possible due to a dynamic actin cytoskeleton, which can be rearranged into many supramolecular structures.

2.1 Polymerization and depolymerization

The basic building block of all actin-containing structures is the actin monomer. This polypeptide of 42 kDa is called G-actin for globular actin and can polymerize into filamentous actin, F-actin. The actin monomer binds an adenosine triphosphate (ATP) molecule in its nucleotide-binding cleft, which can be hydrolyzed to adenosine diphosphate (ADP). The binding of either nucleotide affects the conformation and thereby the properties of actin. The ATP-bound actin can more easily be incorporated into actin filaments, but is shortly after hydrolyzed to ADP. ADP-actin is prone to dissociate from the polymer, leading to depolymerization of the filament. ADP-bound G-actin can then exchange its ADP for ATP and be used again for insertion into F-actin (Fig 5).

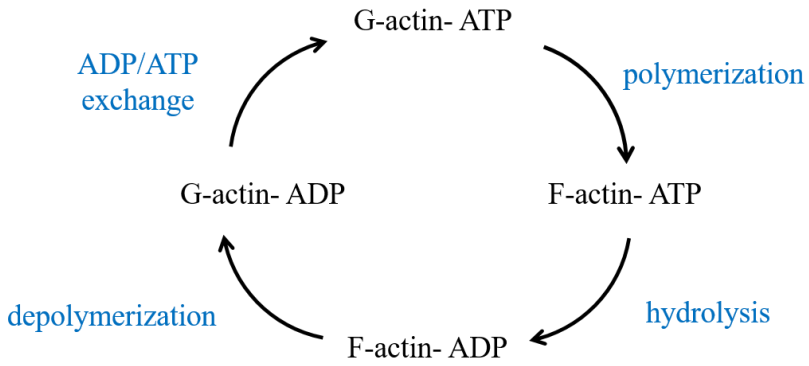

Figure 5. ATP-dependent actin polymerization.

Actin filaments have polarity, since the monomers orient the nucleotide-bounding cleft in the same direction. This direction is called minus or pointed end and contains GDP-bound actin. The polymerization takes place in the plus end, also called the barbed end, and contains mostly GTP-bound actin. Treadmilling describes the process in which an actin filament is polymerizing at the barbed end and, at the same time, depolymerizing at the pointed end. This

process is seen at the leading edge of a migrating cell, forcing the cell to move forward, (Fig 6) [56].

The concentration of actin monomers in a non-muscular cell is 8-250 μM [57]. This is much above the critical concentration *in vitro* for actin polymerization, which is 0.1 μM for ATP-bound actin and 5 μM for ADP-actin [58]. It has been shown that most G-actin in the *Xenopus* egg cell is ATP-bound, ready to be incorporated into F-actin [57]. The reason why a high concentration of ATP-bound G-actin is not instantly inserted into actin filaments is due to several regulatory mechanisms.

To avoid spontaneous actin polymerization, free actin monomers are sequestered by proteins such as **thymosin β4** [59]. This protein binds with high affinity to ATP-actin and thereby prevents polymerization of actin monomers [60]. The first actin sequestering protein identified was **profilin**, which hides the actin-actin binding site [61]. In fact, most cellular G-actin monomers are associated with profilin in *Acanthamoeba* [62]. This protein is, however, not only a negative regulator of actin polymerization, but contributes to a controlled polymerization mechanism. It facilitates ADP/ATP exchange of the G-actin monomer and binds to formins, thereby bringing polymerizable actin monomers to the growing end [61, 63].

Formation of actin polymers are limited by the nucleation, i.e. formation of actin trimers (the "nucleus"). This is a non-favorable process and is catalyzed by actin nucleation promoting factors (NPFs). Once a trimer is formed, the actin can elongate more rapidly [64]. Two important groups of actin NPFs are the WASP/WAVE proteins and the formins.

WASP/WAVE proteins contain a VCA domain which can bind and alter the conformation of the Arp2/3 complex [65]. The conformational change leads to the activation of this seven protein-complex, in which the proteins Arp2 and Arp3 are structurally related to actin (30-50 % sequence similarity) [66]. Binding to an actin filament, the Arp2/3 induces actin polymerization at an angle of 70 ° from the mother filament [67]. In this way, a branched actin network can be formed (Fig 6) [68].

Formins are multidomain NPFs that instead promote linear actin polymerization. Many formins have a C-terminal FH1 domain which interacts with profilin. In this way, profilin-bound actin monomers are recruited to the formin for polymerization. The FH2 domain shields the growing actin polymer end from capping proteins. The Diaphanous-related formins (DRFs) have a GTPase binding domain in the N-terminal, and interaction with Rho GTPases is one way in which the formins is activated [69, 70].

There are several proteins that control the degradation of the actin filaments. The actin-depolymerization factor family **ADF/cofilin** has three members in mammals; ADF, cofilin-1 and cofilin-2. These proteins sever the actin filaments leading to fragmentation or depolymerization to actin monomers. The binding of ADF/cofilin to ADP-actin is stronger than to ATP-actin, resulting in severing of "older" actin filaments. The actin monomers generated after depolymerization can be reused and incorporated in new actin filaments and longer fragments can function as nuclei to generate new actin polymerization sites [71].

Another negative regulating system is provided by **capping proteins** (CP), which bind to the actin barbed end with high affinity. This binding prevents further elongation as there is no longer access to the end monomer [72]. The formins and the actin elongation factors Ena/VASP in turn prevent binding of CP to the growing actin end, thus favoring polymerization [69, 73]

Gelsolin proteins bind to F-actin and kink the filament before breaking it in two. This protein is an efficient actin severing protein and remains attached to the filament after the breakage. In this way, it also acts as capping protein and prevents reannealing and polymerization from the new actin fragments [74].

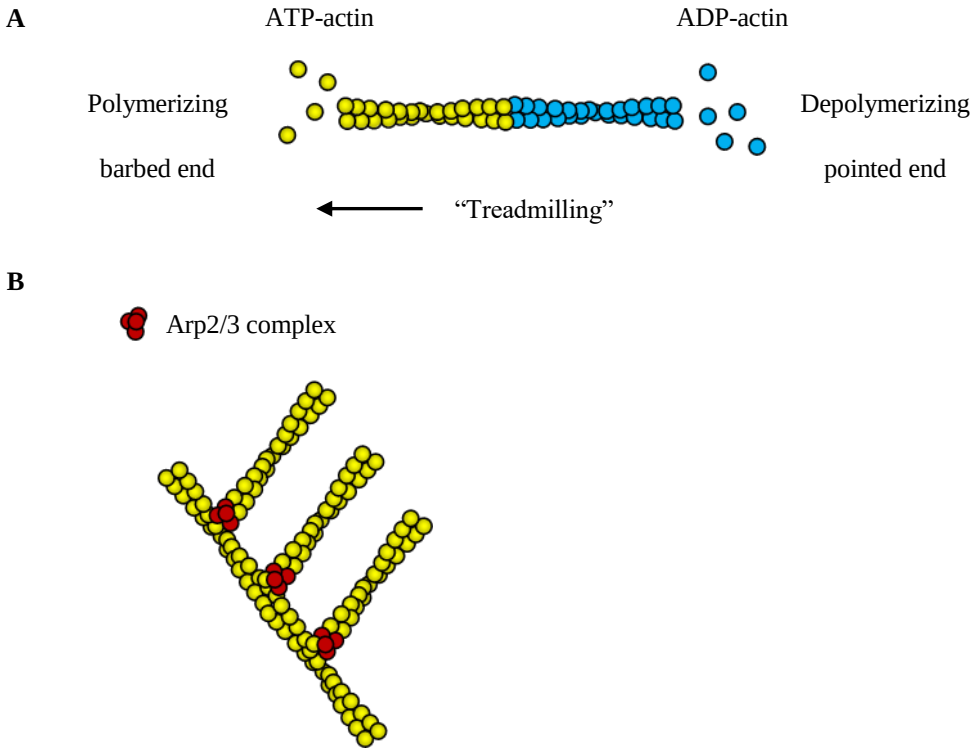

Figure 6. A) Linear actin polymerization and B) branched actin polymerization.

2.2 Actin structures

The actin polymers can be organized in a variety of ways. The simplest structure is formed when G-actin monomers bind to one another forming an actin **filament**, which has a right-handed helical structure (Fig 6A). When several filaments are packed together with the help of cross-linking proteins, they form an actin **bundle**. Since the filaments are polar, with one plus and one minus end, the bundles can either be parallel or anti-parallel (Fig 7). Parallel bundles containing around 15 filaments are found in filopodia [75]. **Filopodia** are dynamic fingerlike protrusions at the front of the cell (Fig 8). Its task is to sense the environment and to initiate contacts to the substratum or to the neighboring cells [76]. Filopodia are seen in a range of different cell types, but is an abundant structure in neuronal dendrites [77]. One possible mechanism by which filopodia could be formed is the association of a protein complex containing Ena/VASP with a set of actin filaments. Ena/VASP functions as actin elongation factors and so the filaments can grow and form a parallel actin bundle which is crosslinked by a crosslinking protein, often fascin [78]. There are several Rho GTPases shown to induce filopodia, such as Cdc42, TC10, Rnd3, Rif and RhoD [79-83] although not via the same pathway [84].

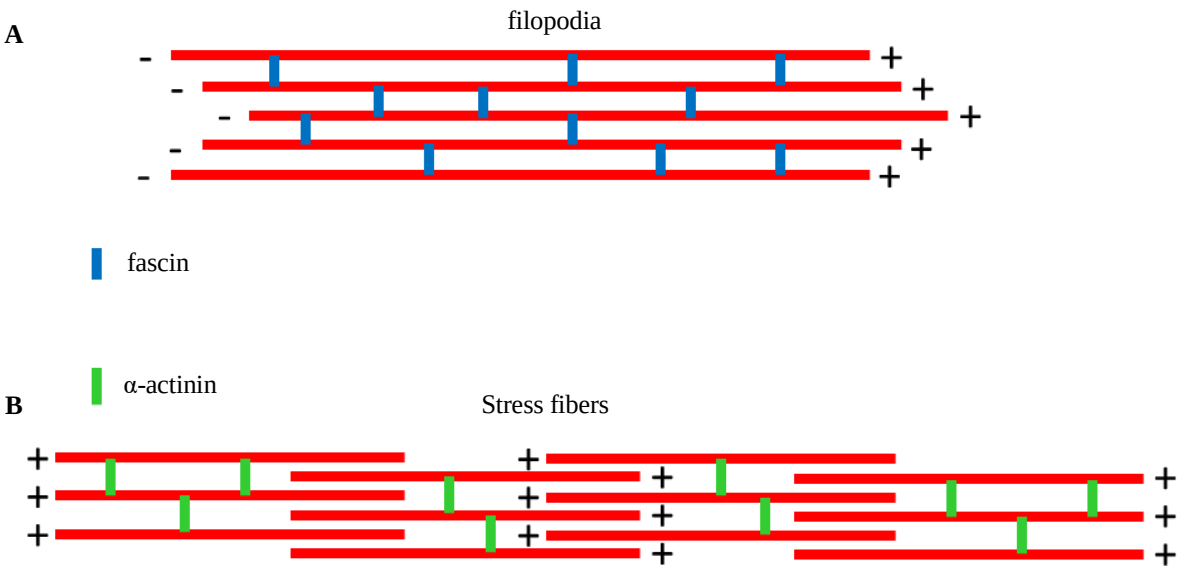

Figure 7. Crosslinked actin bundles in A) filopodia and B) stress fibers.

Bundles, with typically 10-30 anti-parallel actin filaments containing myosin II are called **contractile fibers** [85]. They are absent in filopodia and lamellipodia, but present in the rest of the cell. There are two types of contractile fibers; ventral stress fibers and transverse arcs. Ventral stress fibers run in the same direction as the cellular movement and are adhering to focal contacts. In contrast, transverse arcs run parallel to the leading edge, but are not anchored to focal contacts. There is also a third class of stress fibers called dorsal stress fibers, however they are not contractile (Fig 8) [86].

When actin filaments are elongated in a branched way, an actin **network** is formed. This actin structure is especially pronounced in the **lamellipodium**, the sheet-like membrane protrusion seen in the leading edge of a migrating cell (Fig 8) [87]. Rac1 activates the Arp2/3 complex at the plasma membrane to catalyze branched polymerization, which pushes the plasma membrane forward and creates cellular movement [88]. The lamellipodia is a highly dynamic structure that is constantly projecting and retracting. By means of integrins, the actin can be anchored to a substratum, which is required for a forward cellular migration and not just a retrograde flow of actin [89]. However, for efficient pushing against the membrane border, not only branched actin polymerization is required, but elongation of the branched filaments. This is facilitated by Ena/VASP elongation factors, which hinders capping proteins to bind, thus preventing stalled polymerization [90]. The balance between actin polymerization by Arp2/3 and formins determines formation of lamellipodia or filopodia. Silencing of CP has been shown to result in lamellipodia formation (although with less efficiency) rather than filopodia [91]. Ena/VASP proteins also recruit profilin-bound ATP-actin, which accelerates the filament elongation [92]. Examples of negative regulators that break down lamellipodia are GMFs (glial maturation factors), which sever branched actin filaments [93] and coronins, which inhibit the Arp2/3 activity [94]. Apart from in lamellipodia, branched actin polymerization is seen at the site of clathrin mediated endocytosis and mixed with linear actin filaments at the cellular cortex, called **cortical actin** [95, 96].

A **peripheral ruffle** has a structure similar to the lamellipodium; a sheet-like membrane protrusion, however, if the attachments at the leading edge are lost, the protrusions will fall back and form ruffles [78]. In fibroblasts, ruffles at the leading edge are, just as the lamellipodia, dependent upon Arp2/3 activation [97]. RhoA, Rac1 and Cdc42 signaling all contribute to the formation of ruffles at the leading edge [98].

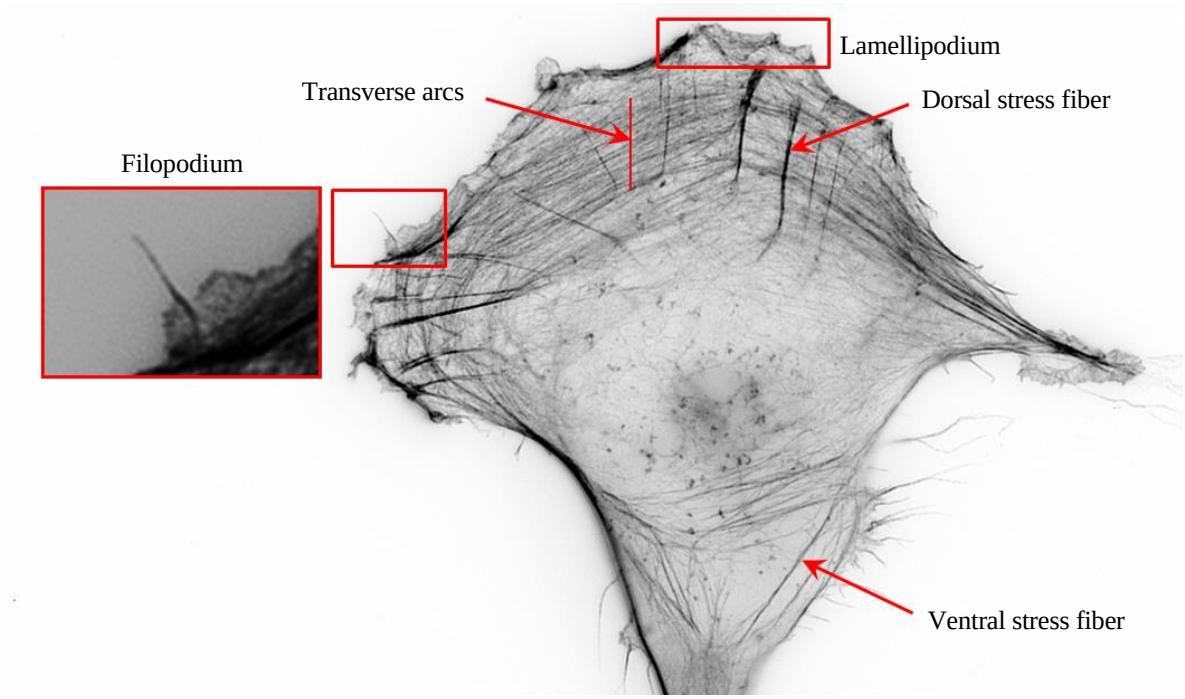

Figure 8. Cellular actin structures.

2.3 Actin myosin contraction

The contractile stress fibers in non-muscle cells are composed of anti-parallel actin filaments, myosin-II and cross-linking proteins (Fig 7B). Myosin-II has a globular domain ("head"), with interaction sites for actin and ATP in the N-terminal, while the C-terminal contains domains essential for homodimer formation [99]. When a myosin head binds ATP, it detaches from actin. Subsequent hydrolysis of ATP to ADP releases energy required for a conformational change of the myosin head. In this state, the myosin head can bind to actin, and once bound, it will fall back again, pulling the actin filament with it (called the "power stroke"). ADP is released and a new ATP is bound, causing the myosin head to detach from actin and a new cycle can start [100]. This movement of myosin heads will result in actin filaments sliding relatively to one another, thus shortening the fiber. Actin-myosin contraction in fibroblasts has been shown to shorten stress fibers with up to 25 % [101].

2.4 Focal adhesions

Focal adhesions (FA) are large protein complexes which mediate a link to the extracellular matrix (ECM). A cell must be able to adhere to a substratum in order to spread and to migrate. An important component in the FAs is the transmembrane protein integrin, which

interacts with the ECM in one end and an actin binding protein in the intracellular end (Fig 9A) [102]. In this way, actin-myosin contraction can exert traction force on the ECM [103].

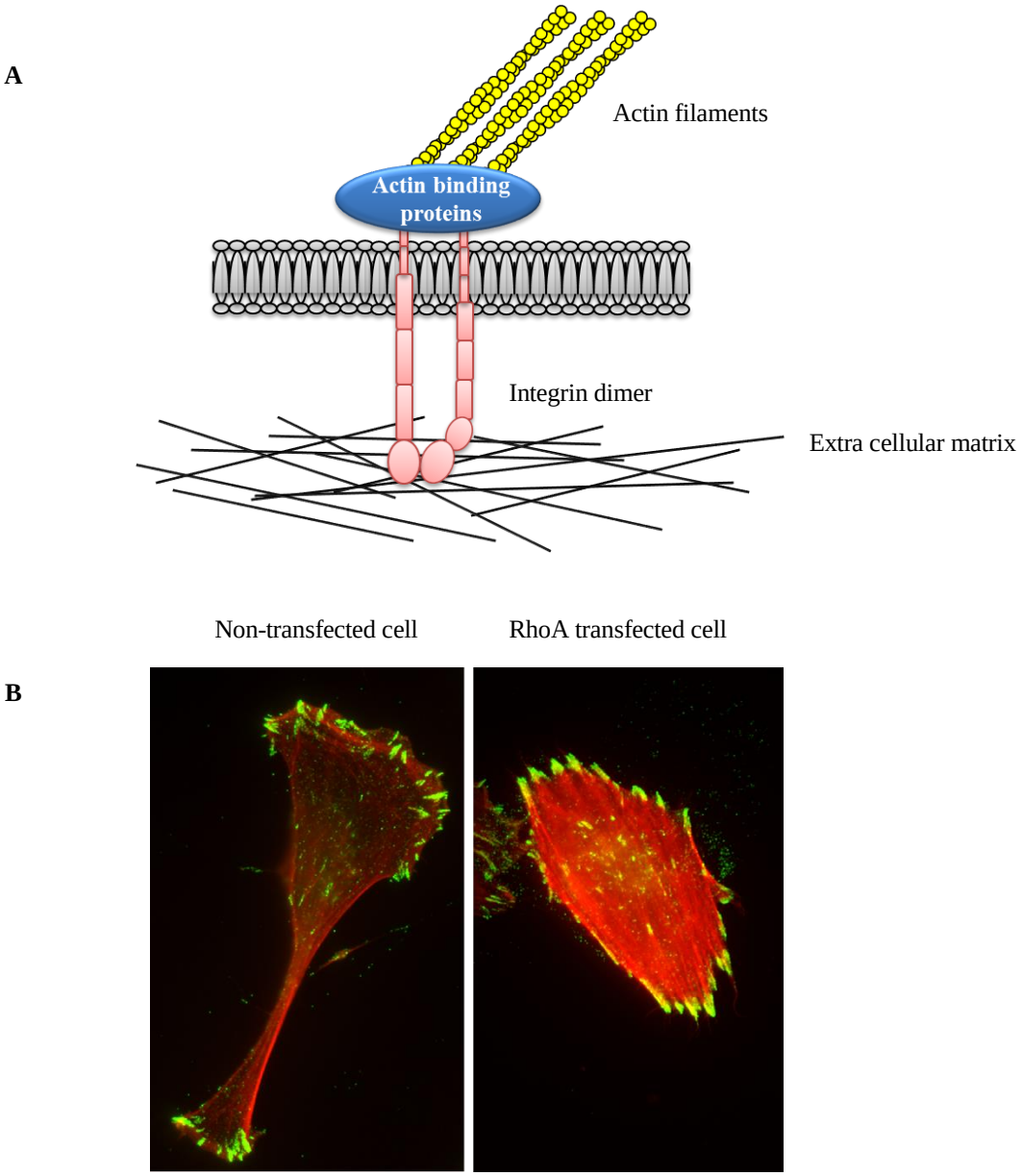

Figure 9. A) Schematic figure of a focal adhesion. Adapted from [104]. B) Focal adhesion (green) and F-actin (red) in non-transfected and RhoA transfected cell.

Integrins are heterodimers composed of one α- and one β-subunit, and due to isoforms of both subunits, 24 heterodimers can be found in humans. Different heterodimers display different binding preferences to ECM proteins, e.g. laminin and collagen [105]. Over 50 proteins have been reported to be part of FAs, leading to a complex structure which can be

regulated in a variety of ways [106]. FAs are highly dynamic structures, which is a prerequisite for a migrating cell where adhesions are constantly formed and dissolved [107]. The formation of FAs is stimulated by intracellular signals, such as activation of RhoA, leading to stress fiber formation and an increased size of FAs (Fig 9B). [108] However, external stimuli can lead to a similar response. FAs work as mechanosensors, and mechanical stress exerted on a cell leads to cell signaling transduction though the integrins, resulting in stress fiber formation and larger FAs [109].

2.5 RhoA, Cdc42 and Rac1 downstream pathways

RhoA, Cdc42 and Rac1 are the most studied Rho GTPases. Their effects were initially described in quiescent Swiss 3T3 fibroblasts as starvation of these cells leads to a very low background of organized F-actin fibers, making them optimal to use when studying actin dynamics. The original finding showed that RhoA is important for formation of stress fibers, Cdc42 for filopodia formation and Rac1 for lamellipodia formation. The downstream pathways of these three Rho GTPase archetypes have been studied thoroughly and are described in most simplified terms below.

RhoA

The ability of RhoA to regulate the actin cytoskeleton was found when RhoA G14V (constitutively active variant) was injected into Swiss fibroblasts, leading to stress fiber formation. In addition, stimulation with lysophosphatidic acid alone, or as a component in serum, induced similar stress fibers in starved cells. However, a simultaneous treatment with the RhoA inhibitor C3 transferase abolished stress fiber formation [110]. Binding of the active RhoA results in loss of the autoinhibited conformation of Rho associated protein kinase (ROCK). This leads to a phosphorylation of the myosin light chain phosphatase (MLCP) and inactivation of its phosphatase activity, subsequently resulting in a prolonged phosphorylation and activation of MLC. In addition, ROCK can phosphorylate and activate MLC directly [111]. MLC acts as a regulatory subunit of the myosin II motor protein and controls its interaction with actin. An activated myosin II can exert its function on actin filaments leading to cellular contraction [112]. Moreover, ROCK activates LIM kinase, which in turn phosphorylates and inhibits cofilin, thereby preventing actin depolymerization in favor of polymerization [113, 114]. RhoA interaction and activation of mDia1 stimulates formation of actin filaments, which are packed into stress fibers (Fig 10) [115].

Cdc42

Many Cdc42$^{-/-}$ cell types or cells expressing Cdc42 dominant negative show restrained or abolished filopodia formation. However, fibroblasts derived from Cdc42-null embryonic stem cells form normal filopodia, revealing that the importance of Cdc42 activity to form filopodia is cell type dependent [116]. Early work suggested that Cdc42 can activate both RhoA and Rac1. Therefore, in order to see the pure effect of injected active Cdc42 (Cdc42 G12V), the Swiss 3T3 cells were simultaneously injected with dominant negative Rac1 and treated with C3 transferase. This resulted in the formation of filopodia [117]. Several downstream targets have been shown to contribute to Cdc42-induced filopodia. The interaction and activation of the mouse Diaphanous protein 2 (mDia2) leads to stimulation of linear actin polymerization and has been shown to be required for Cdc42-induced filopodia formation [118]. The insulin-receptor substrate p53 (IRSp53) is another Cdc42 target protein contributing to filopodia formation in two ways; by assembling of actin filaments (via N-WASP and Ena/VASP) and by inducing membrane curvature (Fig 10) [119, 120]. Cdc42 also binds N-WASP directly, but this interaction is not required for filopodia formation [120, 121].

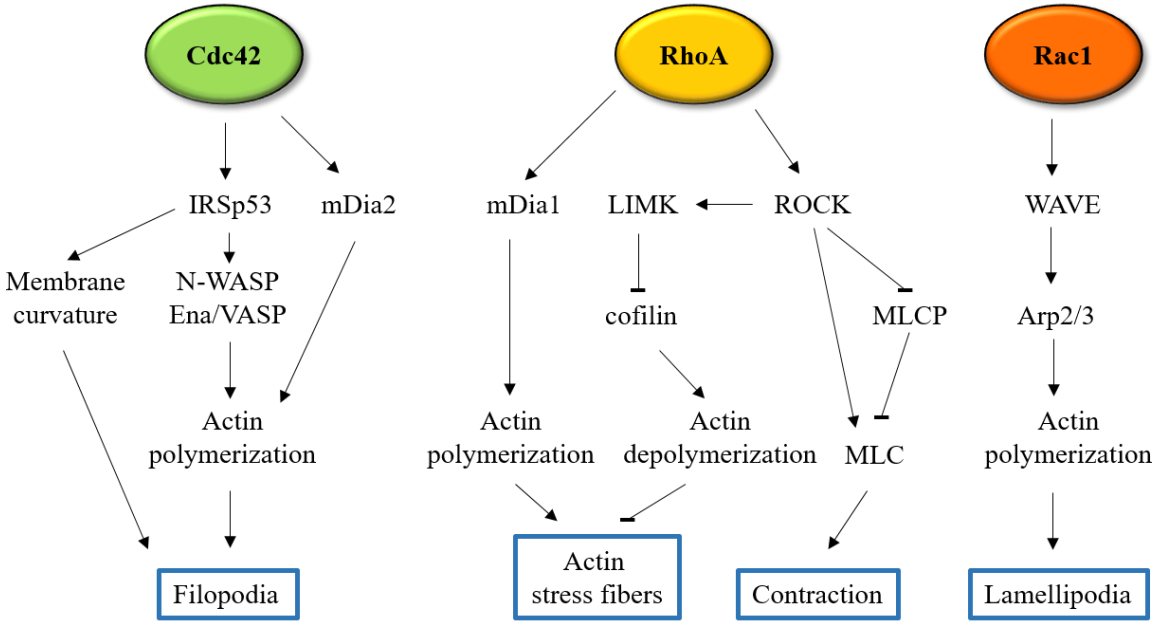

Figure 10. Signaling pathways of Cdc42, RhoA and Rac1 leading to filopodia formation, stress fibers and lamellipodia, respectively.

Rac1

The active Rac1 mutant G12V gave rise to membrane ruffling and lamellipodia formation when first injected into Swiss 3T3 cells. In addition, the dominant negative Rac1 inhibited

membrane ruffling stimulated by PDGF [122]. The main contribution of Rac1 signaling leading to formation of lamellipodia is the activation of the Arp 2/3 via the WAVE complex to induce a cross-linked actin network (Fig 10) [123].

2.6 Actin-driven cellular processes

There are innumerous processes in the cell, which are directly or indirectly dependent upon the actin cytoskeleton. Below are mentioned some major actin driven processes critical for proper cell function.

Cell migration

The procedure of cell migration can in many cell types be described as cyclic. (1) The cell must extend protrusions, filopodia and/or lamellipodia, in the direction of migration. (2) A protrusion that persists for a longer time can form mature FAs linking the protrusion to the substratum. (3) Contractility of actin-myosin filaments leads to a forward motion as (4) the FAs in the rear end dissolve and the newly formed in the front act as clutches. The cycle can then start over with new protrusions being formed [107].

All four steps in this cycle involve actin. As described above, the protrusions are dependent upon actin polymerization, contraction is possible due to myosin-dependent sliding of actin filaments, and the FAs are tightly coupled to the actin filaments. It is therefore fully understandable that an effect on the actin cytoskeleton will result in an altered cell migration, such as increased invasiveness, inability to move in a directed way and increased or decreased migratory speed [124-126].

Cell polarity

Cell polarity is found in most differentiated cells, meaning an unequal distribution of organelles, proteins or molecules (often regulatory ones). The establishment and maintenance of cell polarity is to a high degree dependent upon the inherited polarity of the actin cytoskeleton and the microtubule network [127]. The myosin motor proteins move on actin filaments mainly towards the barbed (growing) end. This contributes to cell polarity, as these motor proteins (especially myosin-V) can transport cargos over a long cellular distance. Furthermore, cell polarity is dependent upon the diverse actin structures in the cell. An external signal, which activates Rho GTPases locally in the membrane, will lead to a site-specific actin polymerization. This is an important concept in cell migration, where the

leading edge forms lamellipodia induced by Rac1, but the rear end forms stress fibers induced by RhoA [107].

Cytokinesis

The cytokinesis at the end of mitosis is enabled by the contractile ring made up of actin and myosin II filaments. The anaphase spindle recruits a RhoGEF to the so called equatorial zone. This leads to activation of RhoA at this site and a subsequent assembly of the contractile ring in the cell cortex. As the myosin containing fibers contract, the dividing cell is constricted and can eventually be pinched off to form two daughter cells [128]. The constriction is not only regulated by contraction, but a simultaneous cofilin-dependent actin depolymerization of the ring [129].

2.7 RhoD and actin

Most Rho GTPases exert effects on the actin cytoskeleton dynamics when exogenously expressed, though in a variety of ways [130, 131]. Overexpression of RhoD leads to stress fiber dissolution and filopodia formation [80]. A handful of RhoD binding partners have been described [132], but the mechanisms leading to the two actin phenotypes are still not known. No published data supports RhoD to act via the same signaling pathway as Cdc42 to induce filopodia. However, RhoD interaction with mDia3C has been proposed to be responsible for protrusions formed after fibroblast growth factor stimulation in mouse mesenchymal cells [133].

Exogenous expression of RhoD leads to a similar phenotype in many cell types (stress fiber dissolution and filopodia formation), while the actin phenotype resulting from RhoD silencing can vary depending on cell type. However, they all have in common more pronounced actin-containing structures, seen as increased amount of stress fibers, increased cortical actin and/or membrane ruffles. For example, silencing RhoD in HeLa cells leads to increased number of stress fibers and cortical actin (after 48 hrs), and later on ruffles (after 72 hrs) (Fig 11). The RhoD-SAAX mutant, which cannot be post-translationally prenylated has an altered localization and strongly induces actin stress fibers [134]. RhoD silencing leads to an increased size and reorganization of FAs [80, 134] whereas RhoD G26V expressing cells show decreased FA size [135].

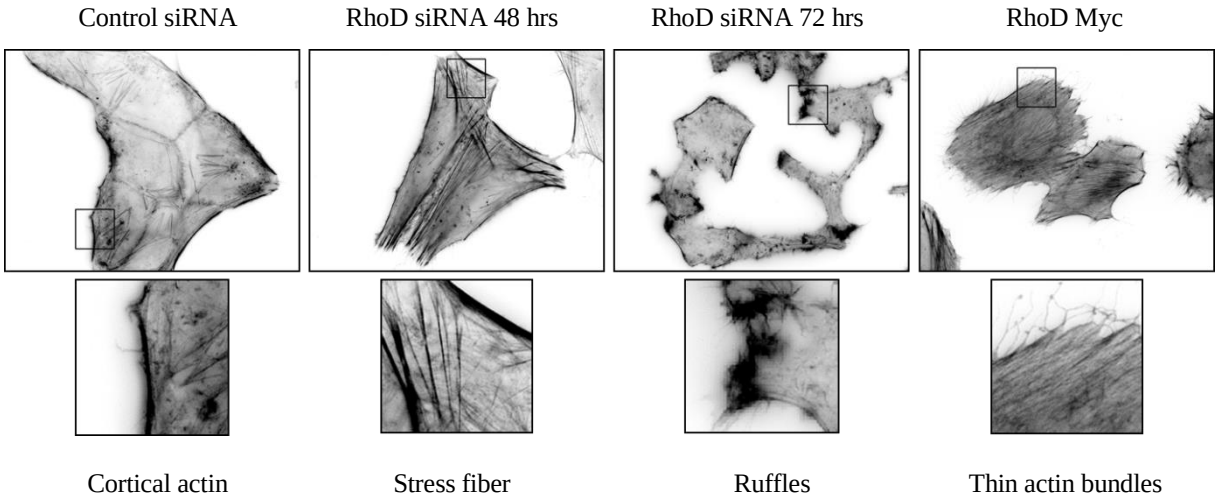

Figure 11. Dominant actin phenotype when RhoD is silenced or exogenously expressed.

3 The Golgi apparatus

3.1 Structure of the Golgi apparatus

The smallest functional unit of the Golgi apparatus (GA) is the cisterna, a flattened membrane disc. Cisternae are often found in layers, forming a Golgi stack, which usually contains three to five cisternae in the mammalian cell [136]. The cisternae can be functionally divided into four different classes, cis (closest to ER), medial, and trans cisternae and the trans-Golgi-network (from where the proteins exit Golgi). GRASP65 and 55 act as Golgi tethering factors holding the cisternae together in a stack, and co-silencing leads to disassembly of the whole stack [137]. GRASP65 and 55 form homo-oligomers that crosslink Golgi membranes from two different cisternae. In vertebrates, the stacks can be further organized into one Golgi ribbon localized at the centrosome. When forming the ribbon, GRASP65 and 55 together with golgins (a second class of Golgi tethering factors) facilitate a lateral membrane fusion to connect the stacks into one continuous ribbon (Fig 12) [138].

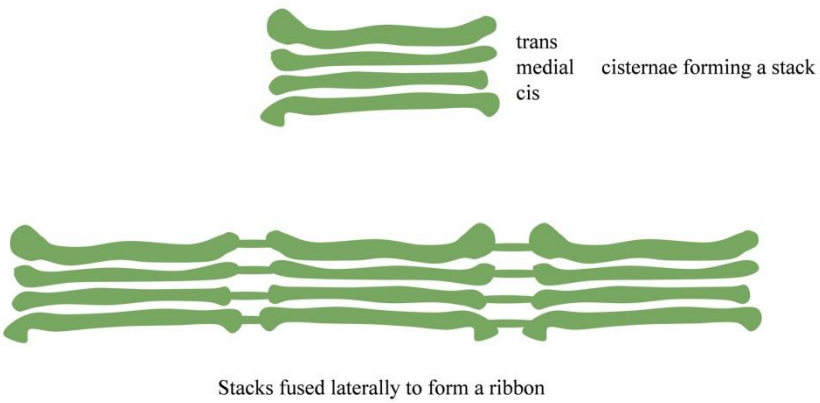

Figure 12. Stacked Golgi cisternae are fused to form a continuous Golgi ribbon.

3.2 Functions of the Golgi apparatus

The Golgi apparatus is a cellular organelle, with the main task to sort and post-translationally modify proteins and lipids in the secretory pathway. However, the GA has additional functions in controlling mitotic entry, microtubule organization and in calcium homeostasis [139-142].

The GA acts as a sorting station for transmembrane and soluble proteins. After synthesis at the endoplasmic reticulum (ER), proteins and lipids are transported to the GA. Many proteins and lipids are modified by a covalent addition of sugar molecules, a process which starts in the ER and is completed in the GA. Around 250 different glycosyltransferases are found in the mammalian GA, which add one sugar molecule to another [143]. As the protein or lipid travels through the Golgi, from the cis-, through the medial- and finally to the trans-Golgi, it is processed in a particular order, as the glycosyltransferases reside in specific compartments within the Golgi [143]. How the proteins and lipids are transported across the Golgi stack is still debated. Two models are proposed. (1) Cisternal maturation; where the cargo is maintained within the cisterna, which matures as it travels across the stack. The Golgi enzymes are recycled by retrograde transportation of vesicles from “later” to “earlier” cisternae [144]. (2) The cargo is transported forward in the Golgi stack by anterograde trafficking [145]. Finally, the proteins and lipids bud from the Golgi and are transported to their final subcellular destination or are secreted. The purpose of glycosylation is numerous. It can contribute to a stable protein conformation [146], regulate protein activity [147] or act as a transport sorting signal [148].

3.3 Golgi homeostasis

The Golgi apparatus is an organelle, which undergoes highly morphological changes during the cell cycle. The mammalian interphase GA is organized in a Golgi ribbon (Fig 12). It is believed that organization into stacks and ribbon render the function of the GA more efficient [149]. At cell division, the GA needs to be fragmented into tubular- and vesicular clusters, in order to form two new GA, one in each daughter cell [150].

In the G2 stage of the cell cycle, the Golgi ribbon is degraded into separate stacks. During prophase/prometaphase, the stacks undergo unstacking and vesiculation, leading to small vesicles and tubules, named Golgi “haze”. At telophase, the Golgi vesicles reassemble in each daughter cell, forming new stacks and finally a ribbon [151]. Inhibition of the Golgi

ribbon fragmentation will result in a G2 block, which is different from the block caused by DNA damage [140].

3.4 Actin at the Golgi complex

It is well known that the microtubule network plays a central role in Golgi morphology and function, a role that has been thoroughly studied [152]. To what extent the actin cytoskeleton influences the Golgi function is less known. When actin polymers are either stabilized, or destabilized by treatment of toxins, the Golgi apparatus becomes more compact. However, electron microscopy reveals important differences. When actin polymers are stabilized (after Jasplakinolide treatment), the Golgi stacks are fragmented, while the cisternae remain flat. When actin polymers instead are depolymerized (after Cytochalasin or Latrunculin treatment), large swelling of the cisternae is seen [153].

Several proteins associated with actin regulation and function are described to localize to and act at the Golgi, e.g. Rho GTPases (Cdc42, RhoA and TC10 [154-156]), actin motor proteins (myosin II, myosin VI [157, 158]) and the actin polymerization regulator Arp 2/3 [159]. Apart from stabilizing the structure of the Golgi complex, actin is involved in vesicle budding from the trans-Golgi-network and several actin motor proteins are known to mediate transport of Golgi-derived vesicles [158, 160].

3.5 RhoD and the Golgi apparatus

There is to date only one published study on the function of RhoD at the Golgi apparatus, which is included in this thesis (Paper I). Briefly, endogenous RhoD was found to co-localize with Golgi markers. Both overexpression and silencing of RhoD resulted in an altered Golgi morphology with a loss of a compact Golgi ribbon structure. RhoD was found to play a role in protein transport from ER to the plasma membrane via the GA. One interaction partner of RhoD, WHAMM, has previously been found to reside in Golgi membranes and similar to RhoD, WHAMM has been found to affect the Golgi morphology and ER to plasma membrane protein transport. It is, however, not established whether RhoD and WHAMM act via the same signaling pathway.

4 Vesicle dynamics

The different cellular vesicles enable protein trafficking between organelles and allow exchange of membrane components. Depending on the cellular route, the vesicles have different compositions. One class of vesicles is called endosomes, and is responsible for transport from the plasma membrane into the cell.

4.1 Early, late and recycling endosomes

When a soluble protein or a membrane-bound receptor at the plasma membrane is internalized, the plasma membrane buds inwards and eventually fuses with an early endosome, the first vesicle compartment in the endocytic pathway. At the early endosome, the cargo is sorted to one out of three routes; (1) recycled back to the plasma membrane via recycling endosomes, (2) sorted to late endosomes to be degraded in the lysosome or (3) delivered to the trans-Golgi network (Fig 13) [161].

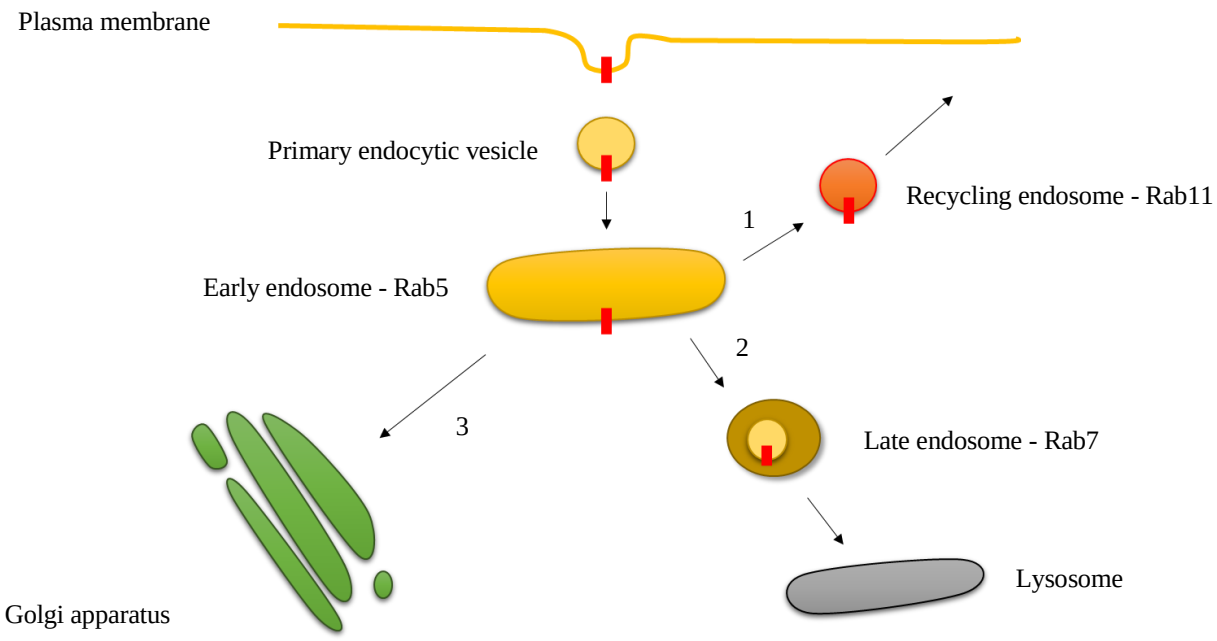

Figure 13. The endocytic pathway.

The small GTPases of the Rab family constitute an important group of endocytic regulators. Their localization to endocytic compartments directs vesicle trafficking in a specific way. When in active GTP-bound conformation, they facilitate vesicle budding, motility and tethering/fusion by recruiting effector proteins. Rab5 localizes to early endosomes and is commonly used as a marker for this compartment. In the same way, Rab11 is found predominantly in recycling endosome membranes and Rab7 in late endosomes [162] (Fig 13).

4.2 Vesicle trafficking

The transport of a vesicle can be divided into four steps; (1) formation of a vesicle from the donor membrane, (2) movement to the target compartment, (3) docking to the acceptor membrane and finally (4) fusion.

Rab proteins are involved in each of the four steps. (1) Rab9 can recruit a sorting adaptor to the membrane of a forming vesicle and increase the affinity between the sorting adaptor and its cargo [163]. (2) Vesicles are transported by motor proteins moving on actin filaments (for short-range transport) or microtubules (for long-range transport). A Rab11 effector protein can function as a myosin adaptor, linking the Rab-bound vesicle to the motor protein. Rab8a can interact directly with myosin proteins, whereas Rab6 can bind directly to a kinesin motor protein [164-166]. (3) In order for vesicles to fuse with the acceptor membrane, they need to be brought into close proximity. The Rab5 effector EEA1 acts as a tethering factor and can draw the two endosomes close together [167]. (4) In addition, EEA1 interacts with t-SNARE proteins which are required for the fusion of the vesicle with the acceptor membrane [168, 169].

4.3 RhoD and vesicle dynamics

One of the first described functions for RhoD was a role in endosome fusion and trafficking. Endosome fusion stimulated by the constitutively active mutant Rab5 Q79L was inhibited by the constitutively active mutant RhoD G26V but not by RhoD wt [52]. In up-following studies, RhoD G26V was shown to reduce both short and long-range endosome motility [170] in a Src-dependent way [171]. More specifically, RhoD has been described to co-localize mostly with Rab5 and Rab11 positive vesicle and less with Rab7 positive vesicles. [172]. An interaction between RhoD and the Rab5 effector Rabankyrin-5 was found in a

yeast two-hybrid screen. Confirming the interaction by co-immunoprecipitation, revealed that Rabankyrin-5 interacts both with RhoD-GDP and RhoD-GTP. When co-expressing RhoD G26V, Rab5 Q79L and Rabankyrin-5, they all co-localized at vesicles. Silencing of Rabankyrin-5 altered the localization of RhoD positive vesicles to a more perinuclear distribution, and silencing of RhoD had a similar effect on Rabankyrin-5 positive vesicles. In addition, internalization of the platelet derived growth factor receptor β (PDGFRβ) after PDGF-BB stimulation was reduced after RhoD silencing in fibroblasts, together with a reduced PDGFRβ-downstream signaling [172].

II. AIMS OF THE THESIS

The vast majority of studies published on Rho GTPases has focused on the three classical members RhoA, Rac1 and Cdc42. The atypical Rho GTPases are generally less studied than the classical ones, but even among these, RhoD is still one of the most uncharacterized Rho GTPases. The overall aim of the thesis is to broaden the knowledge of the Rho GTPase RhoD.

Paper I:

We found that the endogenous RhoD, as well as its interaction partner WHAMM, localize to the Golgi apparatus. We therefore sought to examine how the two proteins affected the structure and function of this organelle.

- How does altered protein levels of RhoD and WHAMM affect Golgi homeostasis?
- How does altered protein levels of RhoD and WHAMM affect anterograde protein transport?

Paper II:

RhoD has been shown to modify the actin cytoskeleton in several cell lines. In this paper, we wanted to investigate the actin phenotype in cell types not previously studied. The cell types chosen had distinctively different actin phenotypes when non-transfected. We also studied cellular processes known to be strongly dependent upon actin dynamics in fibroblasts, a cell type we have previously used for RhoD studies.

- How does altered protein levels of RhoD affect the actin cytoskeleton in an enlarged cell type study?
- How does altered protein levels of RhoD affect cell migration and cell division?
- What happens to the actin phenotype when RhoD localization is defective?

Paper III:

One of the first publications of RhoD describes a function of RhoD at endosomes, but few additional studies on this matter have been published. Therefore, we investigated the dynamics of RhoD positive vesicles in fibroblast, where the localization to vesicles is distinct and relatively easy to monitor. Furthermore, the function of the unique N-terminal motif of RhoD was studied.

- Is there a difference in localization to vesicles between GTP-RhoD, GDP-RhoD and GTP/GDP-cycling RhoD?
- Is there a difference in vesicle size (due to altered fusion/fission) between GTP-RhoD, GDP-RhoD and GTP/GDP-cycling RhoD?
- Is the unique N-terminal of RhoD required for vesicle localization and vesicle fusion?
- Is the unique N-terminal of RhoD required to exert its effect on the actin cytoskeleton?

III. RESULTS AND DISCUSSION

This thesis focuses on the role of RhoD in three cellular dynamic processes; Golgi homeostasis, actin cytoskeleton dynamics and vesicle transport and fusion.

RhoD and Golgi (Paper I)

We found, using a commercial antibody that the endogenous RhoD localized to the Golgi apparatus, similar to its interaction partner WHAMM. We examined the co-localization of RhoD with several established Golgi markers, such as the cis-Golgi matrix protein 130 (GM130) or the trans-Golgi marker 1,4-galactosyltransferease (GalT). STED microscopy revealed that RhoD had a higher degree of co-localization with GalT than with GM130. Treatment with drugs disrupting the Golgi ribbon (Nocodazole) or the stacking of cisternae (brefeldin A) had no major effect on co-localization between WHAMM and GM130, as already reported [173]. However, RhoD could no longer be found together with GM130 after the same treatments. This suggests that WHAMM localizes to the cisterna membrane, while RhoD might be dependent upon a Golgi tethering protein to be associated with the Golgi membrane.

We further studied how the Golgi structure was affected by altered protein levels of RhoD and WHAMM, by analyzing the fraction of cells with Golgi "dispersion". We used this term in a broad sense, meaning both expansion of the Golgi apparatus and disruption of the Golgi ribbon structure into vesicles. Exogenous expression of RhoD in COS1 cells, independent of GTP/GTP-bound state, led to a dispersed Golgi phenotype in 40-55 % of the transfected cells, (compared to <10 % in control cells). In agreement with published data, overexpression of WHAMM led to disruption of the Golgi ribbon structure. Similarly, silencing of RhoD led to a dispersed Golgi apparatus in ~ 40 % of the cells whereas the corresponding number for silencing of WHAMM was ~30 % (compared to ~5 % in control cells).

Golgi is a central organelle for protein trafficking. We therefore sought to investigate whether the effect of RhoD and WHAMM on Golgi homeostasis affected the anterograde protein transport by monitoring the transport of the GFP-tagged VSV-G protein. This protein is kept misfolded in the ER when cells are cultured at 40 °C. When lowering the temperature (to 32 °C), VSV-G is correctly folded and transported via the Golgi to the plasma membrane. By fixing transfected cells at various time points, we could follow the transport of VSV-G in

cells transiently transfected with RhoD or WHAMM constructs, as well as RhoD and WHAMM targeting siRNA. We could see that the transport between the Golgi apparatus and the plasma membrane was delayed when RhoD or WHAMM was silenced; however, the transport from ER-to-Golgi was indistinguishable from the control. Transient expression of RhoD or WHAMM gave a profound effect on the VSV-G transport all the way from the ER to the plasma membrane. Moreover, VSV-G was less protected against Endoglycosidase H cleavage in cells with altered RhoD and WHAMM protein levels, indicating a restricted Golgi function in these cells.

The question whether RhoD and WHAMM act through the same pathway cannot be unequivocally answered. Simultaneous silencing of RhoD and WHAMM gives an additive effect, indicating that RhoD and WHAMM act partly though different pathways. However, we could see that expression of RhoD wt, but not RhoD T31N, could rescue the Golgi dispersion caused by WHAMM silencing, indicating that RhoD helps to tether Golgi in absence of WHAMM. Moreover, since RhoD silencing affects the Golgi structure, the endogenous WHAMM localization is affected and vice versa. This could, however, be an indirect effect.

Exogenous expression can lead to many side effects due to sequestering of proteins and shift in equilibriums. The most significant finding of RhoD in this paper is therefore the effect on Golgi morphology after RhoD silencing. WHAMM silencing leads to an expansion of the Golgi, but the morphology is less altered. In addition to Golgi expansion, RhoD silencing results in loss of shape, and the Golgi starts to “unwind” (Fig 14). Golgi tethering factors keep the Golgi ribbon and stacks intact [174]. It is possible that RhoD is needed for the function or localization of such a protein, leading to an “unwound” Golgi in RhoD silenced cells. To date, the only known RhoD interaction partner at the Golgi is WHAMM [80]. However, WHAMM seems to reside in the trans-Golgi area and RhoD in the cis-Golgi area (Paper I and [173]), and disruption of the Golgi structure by Nocodazole and Brefeldin A treatment have different outcomes for the two proteins. This could indicate that the interaction between RhoD and WHAMM is transient. RhoD interacts with the N-terminal of WHAMM, which is needed for membrane localization [80, 175], suggesting that RhoD could interfere with the WHAMM localization.

With this article, we have shown that endogenous RhoD localizes to the Golgi apparatus, and that a proper balance of the protein is needed to maintain an intact Golgi structure and function.

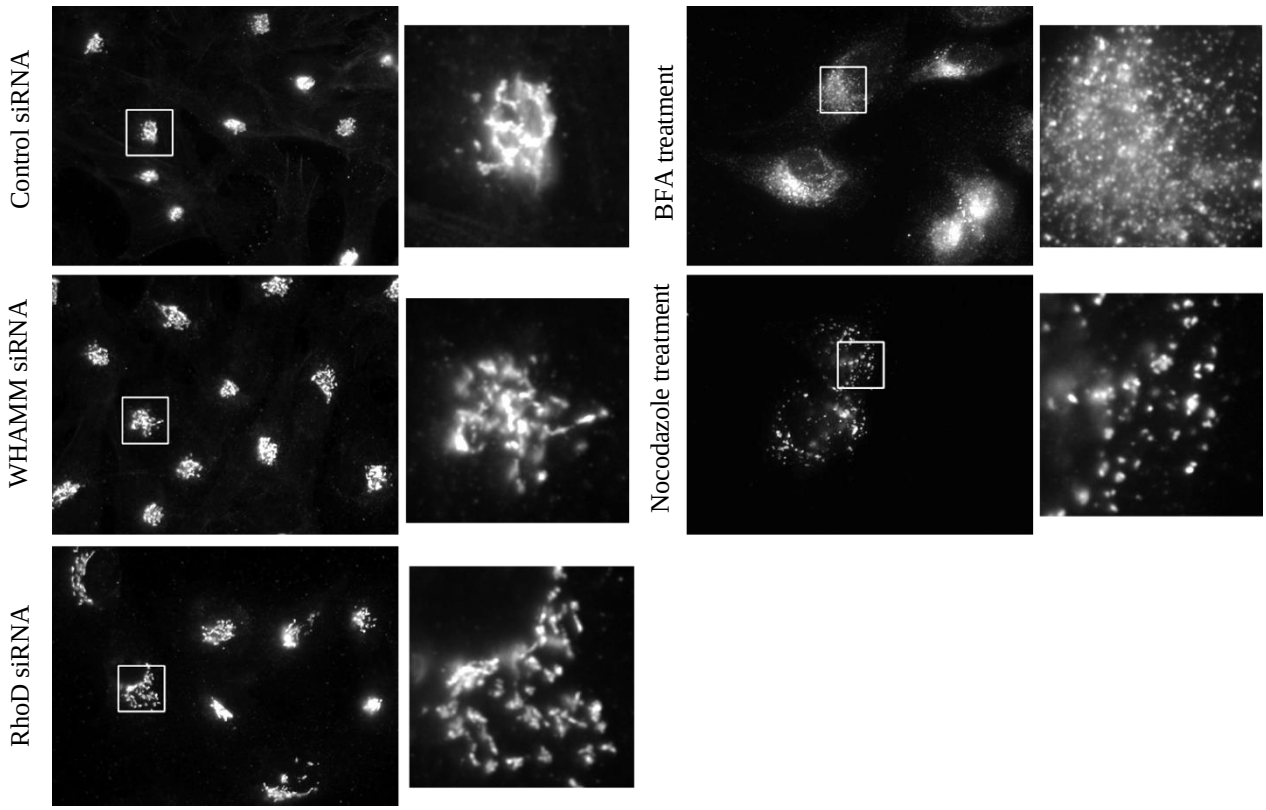

Figure 14. The Golgi marker GM130 in BJ fibroblasts treated with Ctrl siRNA, WHAMM siRNA, RhoD siRNA or the drugs Brefeldin A and Nocodazole.

RhoD and actin (Paper II)

We found that cell types with distinctly different actin cytoskeleton organization when non-transfected (HeLa, U2OS and U251MG), had a similar actin phenotype when overexpressing RhoD. This includes dissolution of stress fibers and the development of a less dynamic actin cytoskeleton, often accompanied by filopodia formation. In fact, also other F-actin structures, such as ruffles and cortical actin were dissolved and reorganized into thin actin bundles. Silencing of RhoD had an opposite effect, enhancing the actin organization present in each cell type, leading to more ruffles, cortical actin and stress fibers, depending on cell type. When RhoD was silenced in fibroblasts, we saw that actin-dependent cellular processes were affected, such as cell migration and cell proliferation.

For a fibroblast to migrate in a persistent way, the orientation and number of lamellipodia must be regulated. Live cell imaging of RhoD silenced fibroblasts showed that the cells had a decreased persistence of directed cell migration, since the total migration distance, but not the Euclidean distance, was longer compared to control cells, revealing a longer migration distance to reach the same goal. The same results were seen when adding a chemotactic cue

(PDGF-BB). Although cells lacking RhoD migrated roughly the same Euclidean distance compared to the control cells, the total migration distance was longer (but non-significant in these experiments). It has been reported that the PDGF downstream signaling is weaker in RhoD silenced fibroblasts [172], which could partly explain decreased sensitivity towards a PDGF gradient. However, complementary data from a scratch-wound assay reveals that the direct cell migration in absence of RhoD is suboptimal.

Considering the extensive regulation of actin reorganization during cell migration, it is not surprising that Rho GTPases are master regulators of directed cell migration [176]. Cells lacking RhoD showed no impairment of the general migratory machinery, as they migrated even longer distances than control cells over the same time period. Moreover, RhoD silenced cells still displayed a polarized cell body. In this study, we saw that overexpression of RhoD led to decreased actin dynamics in several cell types. It is not clear if the reciprocal situation, i.e. RhoD silencing, leads to a more dynamic actin cytoskeleton, but the increased number of ruffles in U251MG cells suggests that this is likely to be the case. Increased actin dynamics could interfere with the stability of a lamellipodia, thereby making it more difficult for the leading edge to steer the cell persistently in one direction. In addition, FA size is correlated with cell speed. Smaller or larger FAs than the optimum size leads to a decreased speed of migration [177]. RhoD knock down in fibroblast has previously been shown to increase the FA size, while overexpression leads to smaller FAs [80, 135].

RhoD silenced fibroblasts showed slower proliferation rate than control cells. We first suspected an impairment of the cytokinesis, since the cytokinesis is heavily dependent upon actin reorganization, and microinjection of RhoD G26V has been described to inhibit cytokinesis [135]. When measuring the time from pre-mitotic rounding up, to spreading after cytokines, we saw no difference in RhoD silenced cells compared to control cells, indicating that the prolonged cell cycle is caused by alterations during earlier phases of the cell-cycle. This is in agreement with the notion that a functional actin cytoskeleton and Rho GTPase regulation are essential also in the earlier stages of the cell-cycle [178].

Transient expression of several Rho GTPases, such as Rnd1 and Rnd3 has been reported to result in stress fiber dissolution. In addition, the expression of p190ARhoGAP leads to the inhibition of RhoA and thereby inhibition of stress fibers (Fig 15) [179]. Visualizing F-actin by the fluorescently labeled phalloidin shows that HeLa cells overexpressing Rnd1, Rnd3 and p190ARhoGAP lose their stress fibers. The dissolved stress fibers are not reorganized into other F-actin-containing structures, therefore, a weak actin signal is acquired. In contrast, in cells overexpressing RhoD, the F-actin signal remains strong. In these cells, the stress fibers

are dissolved, but instead, an intertwined weave of actin filaments is seen (Fig 15). This indicates that RhoD, in contrast to Rnd1 and Rnd3, rather stimulate a reorganization of the actin cytoskeleton, where thin actin bundles are induced at the expense of stress fibers, or cortical actin and ruffles in other cell types.

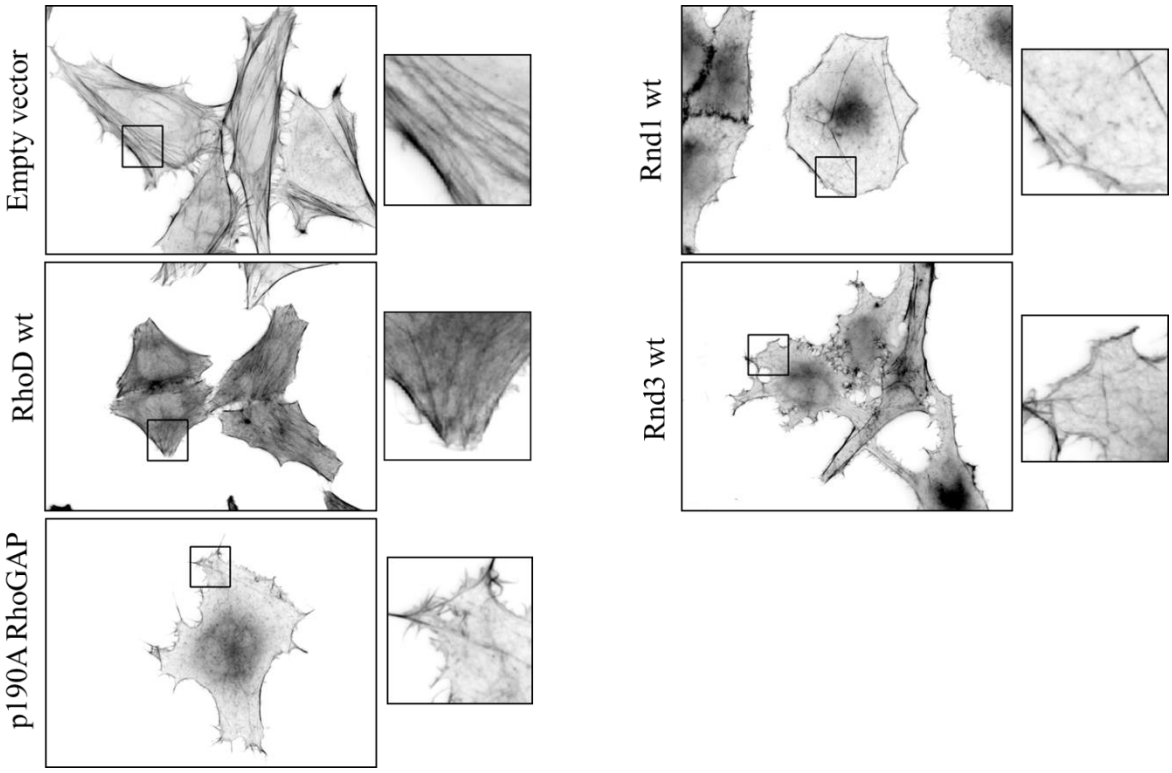

Figure 15. F-actin phenotype in HeLa cells overexpressing proteins known to dissolve stress fibers. Cells were stained with phalloidin. All acquired pictures were sub-saturated and later adjusted in an identical way, i.e. the stronger phalloidin signal in RhoD is not due to a different handling of the pictures.

Another striking sign that RhoD functions as a modulator of the actin cytoskeleton can be seen when the C-terminal CAAX-motif is mutated. This motif is found in many small GTPases and undergoes post-translational processing, leading to insertion of the small GTPase into lipid bilayers [1]. The mutation of the RhoD CAAX (which we named RhoD SAAX) prevented the prenylation (most likely a farnesylation) of RhoD and, as a consequence, the localization of RhoD is altered. RhoD SAAX still associated with the plasma membrane and induced filopodia, but it no longer localized to vesicles. Surprisingly, actin stress fibers were no longer suppressed, but instead strongly induced. This reveals the strong intrinsic ability of RhoD to regulate the actin cytoskeleton.

Taken together, this paper demonstrates that RhoD silencing has different effects on the actin cytoskeleton depending on cell type, but overexpression leads to induction of thin actin

bundles overriding all other actin-containing structures. Loss of RhoD in fibroblasts also results in effects on actin-dependent processes, such as cell migration and proliferation.

RhoD and vesicle dynamics (Paper III)

Exogenously RhoD has been reported to localize to vesicles in several cell types [52, 172]. To study the function of RhoD at vesicles, we used BJ fibroblast cells, as RhoD localization to vesicles in this cell line is distinct and thus relatively easy to monitor. We could see that both the GDP and GTP-bound RhoD were associated with vesicles. In contrast, only GTP-bound RhoD localized to the plasma membrane.

One of the initial publications on RhoD revealed a role in endosome fusion and trafficking [52]. We could see that RhoD wt and RhoD T31N positive vesicles fused and formed larger vesicles with visible lumina. However, the constitutively active RhoD G26V was associated with smaller vesicles without detectable lumina. These results are in line with earlier findings, where RhoD G26V inhibited Rab5-induced endosome fusion, but RhoD wt did not [52]. This suggests that RhoD must be inactivated to enable vesicle fusion. Furthermore, we saw a significant increase in number of vesicles per cell when expressing RhoD G26V compared to RhoD wt/T31N, but no difference between cells expressing RhoD wt and RhoD T31N, emphasizing the inability of RhoD G26V-postive vesicles to fuse.

When quantifying the localization of RhoD-positive vesicles in the cell, we saw that RhoD wt and T31N were mainly perinuclear, whereas the RhoD G26V vesicles were predominantly dispersed. This means that, in contrast to the localization of RhoD to vesicles, the subcellular localization of the vesicles is dependent upon the GTP/GDP-bound state of RhoD. Since RhoD G26V-postive vesicles were more dispersed, we expected a more pronounced localization to the plasma membrane, given that the vesicles eventually fuse with the plasma membrane. This could not be established, leaving the question open on how RhoD is inserted into the plasma membrane. Unfortunately, it is difficult to perform live-cell imaging on RhoD-vesicle dynamics, since fluorescent protein tags (GFP, mCherry, Tomato) strongly promote plasma membrane localization of RhoD, rendering the vesicles difficult to detect.

N-terminally to the GTPase domain, RhoD has a unique extension with unknown function. Deletion of these 12 amino acids, led to strikingly different vesicle phenotypes (however, not for the dominant negative RhoD). The ΔNT-RhoD wt and G26V-postive vesicles were often seen in clusters or as beads on a string along the plasma membrane border. They appeared

“sticky” and many cells were fully packed with vesicles. This observation raises the question if RhoD degradation is dependent upon the N-terminal motif. Just as its full length counterparts, the ΔNT-RhoD wt fused to form larger vesicles, whereas ΔNT-RhoD G26V did not.

We also investigated whether or not the N-terminal motif was required for RhoD to induce thin actin bundles in favor of stress fibers. We found that the deletion mutants had the same capability to dissolve stress fibers as the full length RhoD wt and G26V.

In this study, we showed that RhoD localization to the plasma membrane is GDP/GTP-dependent, whereas the vesicle localization is not. In addition, the size of the vesicle is dependent upon the GTP/GDP state of RhoD. The N-terminal motif of RhoD is not required for the RhoD-induced actin rearrangement, but its deletion causes altered localization and characteristics of the RhoD-postive vesicles.

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