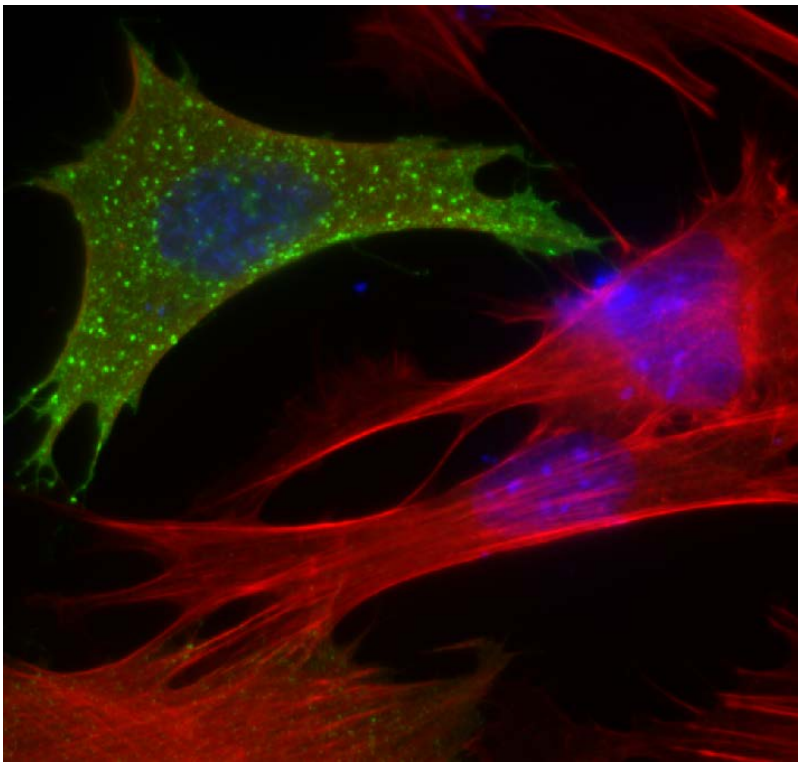
UPPSALA
UNIVERSITET

The role of RhoD and RhoD-binding proteins in the regulation of cancer cell adhesion and migration



Francisca Nunes de Almeida

Degree project in biology, Master of science (2 years), 2013
Examensarbete i biologi 45 hp till masterexamen, 2013
Biology Education Centre, Uppsala University, and Karolinska Institute, Department of Microbiology, Tumor and Cell Biology, Nobels vägen 16, 171 77 Stockholm, Sweden
Supervisors: Pontus Aspenström and Vishal Nehru
External opponent: Johan Lennartsson

Biology Education Centre, Uppsala University

Karolinska Institute, Department of Microbiology, Tumor and Cell Biology, Stockholm

THE ROLE OF RHOD AND RHOD-BINDING PROTEINS IN THE REGULATION OF CANCER CELL ADHESION AND MIGRATION

Francisca Nunes de Almeida

2013

ABSTRACT

Rho GTPases have been studied intensively during the past years, mainly due to their important role in the regulation of the actin cytoskeleton. Although most of the studies have been focused on RhoA, Rac1 and Cdc42, this master thesis brings into focus another member of this family, RhoD. Not much is known about this Rho GTPase. Recent experiments on RhoD show that this protein is involved in the regulation of the actin cytoskeleton, thus playing a central role in cell migration, cell adhesion and vesicular trafficking. The work provided in this master thesis tries to provide additional insight into the role of RhoD.

We start by testing three proteins as potential RhoD-binding partners: the nucleation-promoting factor junction-mediating and regulatory protein (JMY), the filamin-A interacting protein-like (FILIP1L) and the cortactin-binding protein 2 N-terminal like (CTTNBP2NL). Additionally, motivated by the characterisation of the interaction between RhoD and other proteins, such as the WASP homolog associated with actin membrane and microtubules (WHAMM) and the zipper-interacting protein kinase (ZIPK), we focuse our studies in increasing our knowledge on pathways downstream of RhoD. Firstly, we confirm the interaction between the RhoD-binding protein WHAMM and profilin. Secondly, we conclude that another RhoD-binding protein, ZIPK, reduces FAK activation, while RhoD counteracts this effect. In line with these findings, we show that both RhoD and FAK are involved in the regulation of the actin cytoskeleton dynamics. Lastly, we provide valuable information showing that RhoD in required for cell chemotaxis towards a PDGF-BB gradient and speculate a possible role of RhoD in the regulation of cell death.

Rho GTPases upregulation is found in several types of cancer. RhoD, as a regulator of both cell adhesion and cell migration, might play an important role in processes such as cell invasion and metastasis in cancer. Consequently, it will be of great future interest to study RhoD and the signalling pathways related to the cellular effects of this protein.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABP	Actin-binding protein
ADP	Adenosine-5’-diphosphate
ATP	Adenosine-5’-triphosphate
Arp2/3	Actin related protein 2/3
CC	Coiled-coil domain
CTTNBP2NL	Cortactin-binding protein 2 N-terminal like
DAPI	4’,6-diamidino-2-phenylindole
DAPK	death-associated protein kinase
ECM	Extracellular matrix
ER	Endoplasmic Reticulum
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FILIP1L	Filamin-A interacting protein-like
G-actin	Globular monomeric actin
GAP	GTPase-activating protein
GBD	GTPase-binding domain
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine-5’-diphosphate
GEF	Guanine nucleotide exchange factor

GTP	Guanosine-5’-triphosphate
JMY	Junction-mediating and regulatory protein
mDia	mouse diaphanous-related formin
MLC	Myosin light chain
NPF	Nucleation-promoting factor
N-WASP	Neural Wiskott-Aldrich syndrome protein
PBS	Phosphate-buffered saline
PDGF-BB	Platelet derived growth factor BB
Rab	Ras-like protein in brain
Ras	Rat sarcoma
Rho	Ras homologous
ROCK	Rho kinase
TRITC	Tetramethyl rhodamine isothiocyanate
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin-homologous protein
WHAMM	WASP homolog associated with actin membrane and microtubules
WH2	WASP Homology 2 domain
ZIPK	Zipper interacting protein kinase

INTRODUCTION

The human body requires that all our muscles, bones and ligaments work hand in glove, allowing a perfect harmonization between our movements and the environment. Analogously, cells possess three cytoskeletal filament systems, which are collectively named cytoskeleton, and give cells the capacity to develop a variety of functions, like the ability to move, to rearrange their shape and internal components, and ultimately to divide. The three types of fibres – actin filaments, microtubules and intermediate filaments – are responsible for numerous cellular processes, including the support of the plasma membrane, the control of the intracellular trafficking, the pulling of chromosomes during mitosis, cytokinesis, the contraction of muscle cells, the control of cell shape and cell movement[1]. However, these filament systems are ineffective by themselves. In order to function properly, the proteins that make up the filaments need to interact with hundreds of other proteins that regulate the three-dimensional organisation of the cytoskeleton.

This master thesis focuses on RhoD, a member of the Ras homologous (Rho) subfamily of GTPases, which are key regulators of the actin filament system. By binding to many effector proteins, Rho GTPases activate a variety of signal transduction pathways that regulate the above-mentioned cellular processes (*e.g.* intracellular trafficking, cell shape and cell migration). As a result, there is a close relation between the function of Rho GTPases and actin cytoskeleton dynamics, which will be further explored in this thesis.

The actin filament system

Actin is a globular protein found in nearly all eukaryotic cells [2]. With 42,000 Da of molecular weight, this is one of the most conserved proteins in a cell [3]. Actin exists in two different states: as a globular monomer (G-actin), or as a filamentous polymer (F-actin), each containing a Mg^{2+} ion that forms a complex either with ADP or ATP [3; 1]. X-ray crystallography studies by Kabsch and colleagues allowed them to propose a model for the three dimensional structure of G-actin in 1990, based on a

complex of the actin monomer with DNase I [4]. In this study it was also shown that the adenine nucleotide binds to actin in the cleft that exists between its two domains, in the middle of the protein [4; 5]. Further studies on the actin filament show that actin monomers assemble head-to tail, consequently giving a distinct polarity to the filament [5]. As a result, the end that exposes the nucleotide-binding cleft is called minus or pointed end, while the opposite end of the filament is named plus or barbed end [1; 3]. Each actin filament consists of two parallel protofilaments arranged in a right-handed double helix (Figure 1) [1; 5].

Since the ATP concentration in living cells is nearly ten times higher than the concentration of ADP, the subunits are added in the ATP-bound form to the actin filament [1]. After being added to the filament, the nucleotide in the actin monomer is hydrolysed and the free phosphate group is released while ADP remains bound [1]. In addition to this, the rate constant for subunit addition is faster for the plus end then for the minus end, due to structural differences between them [1]. Consequently, for certain concentrations of actin subunits, this rate of subunit addition will be faster than the hydrolysis of the nucleotide at the plus end, but not at the minus end. As a result, the plus end will have actin-ATP, while the minus end will change to actin-ADP. The hydrolysis of ATP to ADP reduces the binding affinity between the adjacent actin monomers [6]. Therefore, subunits continue to bind to the plus end with actin-ATP, while the minus end with actin-ADP is more vulnerable to disassembly (Figure 1c) [3]. This property is called treadmilling.

Based on *in vitro* studies, it has been shown that monomeric actin molecules can bind to each other spontaneously to initiate actin polymerisation, although this is a very unstable process and the molecules tend to disassemble quickly [1]. As a result, the initial step of actin polymerisation is very slow and requires that the actin subunits form a nucleus, consisting of at least three subunits that is stabilised by the contact among those monomers [1; 7]. This step is called nucleation (Figure 1a). Following nucleation, the nucleus rapidly starts to elongate by the constant addition of ATP bound actin monomers (Figure 1b) [1; 7; 6]. Finally, a steady state is approached when the rate of subunits addition equals the rate of subunits dissociation (Figure 1c) [1]. However, in vivo, cells have a variety of actin-binding proteins (ABPs) that catalyse the initial and time-consuming nucleation step, regulate elongation or induce cross-linking, thus regulating the dynamics of the actin cytoskeleton [6].

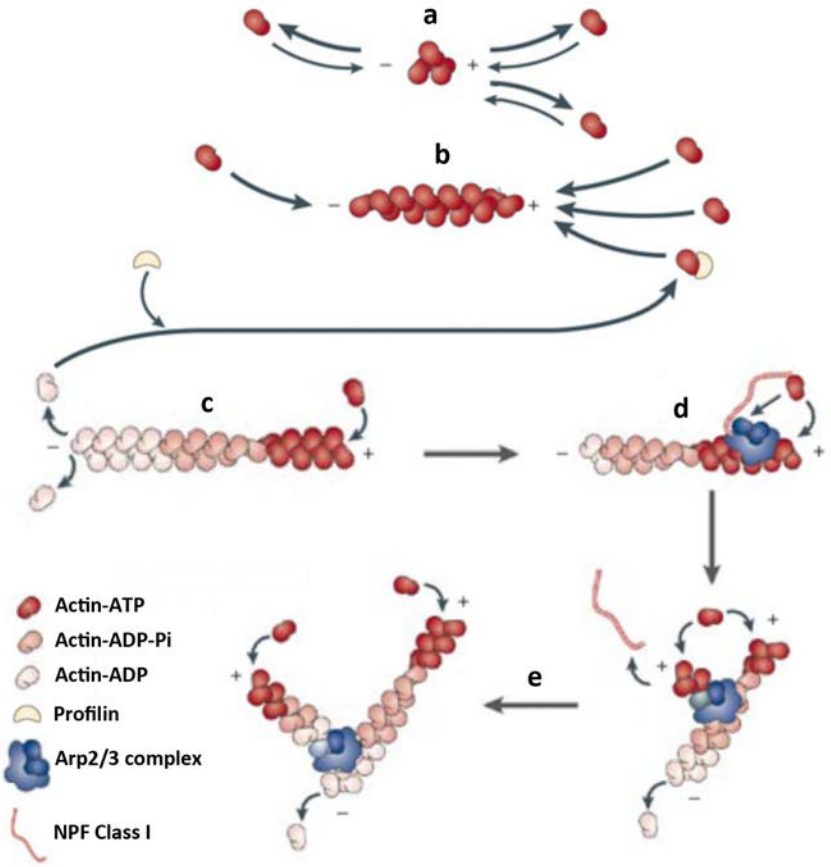


Figure 1 – Representation of the actin filament polymerisation. (a) Spontaneous actin nucleation with at least three actin subunits. (b) Elongation of the actin filament, until (c) it reaches a steady state and actin-ATP is added to the plus end, while actin-ADP disassembles from the minus end.. Actin-ADP undergoes nucleotide exchange, which is facilitated by profilin (d) A NPF Class I and the Arp2/3 complex promote the branching of the actin filament. (e) The NPF dissociates while actin polymerisation continues at the plus end of both mother and daughter filaments. Adapted from [8; 9], used with permission.

Actin binding proteins

There are many proteins that interact directly or indirectly with actin, regulating the different steps of actin polymerization. Some of them act by sequestering actin monomers (e.g. thymosin and profilin), others as nucleating proteins (e.g. formin and the Arp2/3 complex), or even as severing proteins (e.g. cofilin and gelsolin) or cross-linkers (e.g. filamin). Not all ABP will be analysed in this chapter, only the ones listed in Table 1.

Table 1 – List of proteins that bind to actin and their function.

Protein	Function
Profilin	Binds to G-actin promoting actin polymerization; binds to proteins with poly-L-proline motifs [10].
Arp2/3 complex	Increases actin nucleation and branching [11].
NPFs:	
- WHAMM	Induces Arp2/3-mediated actin polymerisation; associates with the microtubule cytoskeleton; associates with membranes of the *cis*-Golgi [11; 12].
- JMY	Associates with p300 in the nucleus, activating p53 transcription during stress response; induces Arp2/3-dependent and Arp2/3-independent actin nucleation [13; 14].
- Cortactin	Activates the Arp2/3 complex; interacts with F-actin; stabilizes Y-branches; interacts with N-WASP [15].

Profilin

Profilin is a 16 kDa protein that binds to actin monomers on the opposite side of the ATP/ADP-binding cleft [16]. At first, profilin was thought to keep actin in an unpolymerised form by sequestering G-actin [17; 18]. However, it was found later that when bound to ADP-actin, this protein catalyses the exchange of ADP for ATP increasing actin polymerisation [2; 19; 18]. The profilin-ATP-actin complex is then added to the free plus end of the actin filament, reducing the affinity of actin to profilin due to conformational changes [1; 18; 10]. Finally, profilin detaches and leaves the actin monomer bound to the actin filament being polymerised. The role of profilin in actin polymerization is largely regulated by inositol phospholipids (PPIs) [1]. Profilin is localised mainly at the cytosolic side of the plasma membrane, where it is inactivated by interaction with PPIs, mostly with phosphatidylinositol-3,4-bisphosphate (PIP_2) [10; 20]. This localisation allows profilin to rapidly increase actin polymerisation, producing actin-rich extensions that are involved in cell motility (e.g. filopodia and lamellipodia) upon extracellular signals that activate phospholipase C [1]. In addition to binding to actin and PPIs, profilin has been shown to bind poly-L-proline motifs in a variety of proteins, such as the actin regulators N-WASP, WAVE or formins [10].

Arp2/3 complex

The Arp2/3 complex was first discovered as a seven-protein 220 kDa complex that interacts with profilin [21; 15]. This complex uses two of its proteins (Arp2 and Arp3) to mimic actin, consequently inducing actin nucleation together with another actin monomer [11]. This new actin filament remains capped at the minus end by the Arp2/3 complex, but is free to elongate at the plus end [15]. Additionally, this protein complex can also bind to the side of polymerised actin filaments and creates a new branch with virtually 70º angle [15; 22]. As a result, the Arp2/3 complex plays a central role in nucleating and branching actin, and thus it is involved in processes such as cell motility and vesicular trafficking [22]. However, the Arp2/3 complex is rather inefficient by itself and it requires some additional factors to enhance its activity. The phosphorylation of Thr and Tyr residues in Arp2 and the binding of the complex to the actin filament are responsible for an increase in its activity [15]. Besides this, the Arp2/3 complex is also activated by a set of proteins commonly named nucleation-promoting factors (NPFs) [11; 15; 22].

Nucleation-promoting factors

Nucleation-promoting factors (NPFs) can be classified as type I NPFs or type II NPFs. The first ones are characterised by a WCA domain at the C-terminus, which consists of a WASP Homology 2 (WH2) motif followed by a Central and an Acidic region [23]. The WH2 motif binds to actin monomers, whereas the CA motif allows for the binding of the Arp2/3 complex (Figure 1d) [11; 15; 22; 23]. Therefore, NPFs type I include proteins with this WCA domain, such as the Wiskott-Aldrich syndrome protein (WASP), neural WASP (N-WASP), WASP family verprolin-homologous protein (WAVE or SCAR), WASP and SCAR homologue (WASH), WASP homolog associated with actin membrane and microtubules (WHAMM), and junction-mediating and regulatory protein (JMY).

Similarly to all NPFs type I, WHAMM protein has a WCA domain at the C-terminus. Its WCA segment contains two WH2 domains, like N-WASP [15]. Therefore, WHAMM requires the presence of the Arp2/3 complex to nucleate actin. Besides inducing Arp2/3-mediated actin polymerisation, it has been shown that WHAMM associates with the microtubules with its central coiled-coil (CC) region, thus providing a crosstalk between the actin and microtubule filament systems [11].

Additionally, WHAMM co-localises to the *cis*-Golgi protein GM130 and to the Endoplasmic Reticulum (ER)-Golgi intermediate compartment (ERGIC), thereby regulating ER to Golgi transport [11; 12]. On the other hand, the WCA segment of the JMY protein contains three WH2 domains. This difference between the two proteins allows JMY, but not WHAMM, to nucleate actin in the absence of the Arp2/3 complex [15]. Despite having some clear structural differences, JMY and WHAMM are nearly 35 % identical and the main differences reside within their N-terminus [15]. JMY was first discovered in the nucleus as a p300 cofactor [14]. These two proteins form a complex that activates p53 transcription during stress response [14]. Only recently in 2009 it was discovered that JMY is an actin NPF that enhances cell motility, actually combining an Arp2/3-dependent and an Arp2/3-independent nucleation activity [13].

Type II NPFs also bind to the Arp2/3 through their acidic domain at the N-terminus, though they are considered to be weaker activators of this complex when compared to NPFs type I [15]. Moreover, they lack WH2 domains, harbouring repetitive sequences that interact with F-actin instead [15]. An example of an NPF type II is the protein cortactin. This protein also interacts with N-WASP via its C-terminal SH3 domains, which leads to an increase of N-WASP activity in the activation of the Arp2/3 complex [15; 24].

From all the data that has been published, it is possible to conclude that the actin cytoskeleton dynamics is tightly controlled via its many ABPs. The proteins mentioned previously are just the tip of the iceberg, and many other proteins contribute directly or indirectly to the regulation of this network of actin filaments. Actually, another set of proteins that plays a very distinct role in the regulation of the actin cytoskeleton is the Rho family of small GTPases.

Rho GTPases

Rho GTPases are one of the five major subfamilies comprising the Ras superfamily of small GTPases, together with Arf, Ran, Rab and Ras (Figure 2) [25]. Its twenty members can be found in all eukaryotic cells and are grouped into eight subfamilies: Rho, Rac, Cdc42, RhoD/Rif, Rnd, Wrch-1/Chp, RhoH and RhoBTB [26-28]. Rho GTPases can be further subdivided into two categories: classical and atypical Rho

GTPases. Members of the classical Rho GTPases (*i.e.* Rho, Rac, Cdc42 and RhoD/Rif subfamilies) are regulated based on a GTPase cycle, changing between an inactive GDP-bound state and an active GTP-bound conformation due to its intrinsic GTP-hydrolysing enzymatic activity [27]. On the other hand, atypical Rho GTPases (*i.e.* Rnd, Wrch-1/Chp, RhoH and RhoBTB subfamilies) usually do not follow this GTPase cycle, being regulated at their expression level, by proteasomal degradation or by protein-protein interactions instead [27].

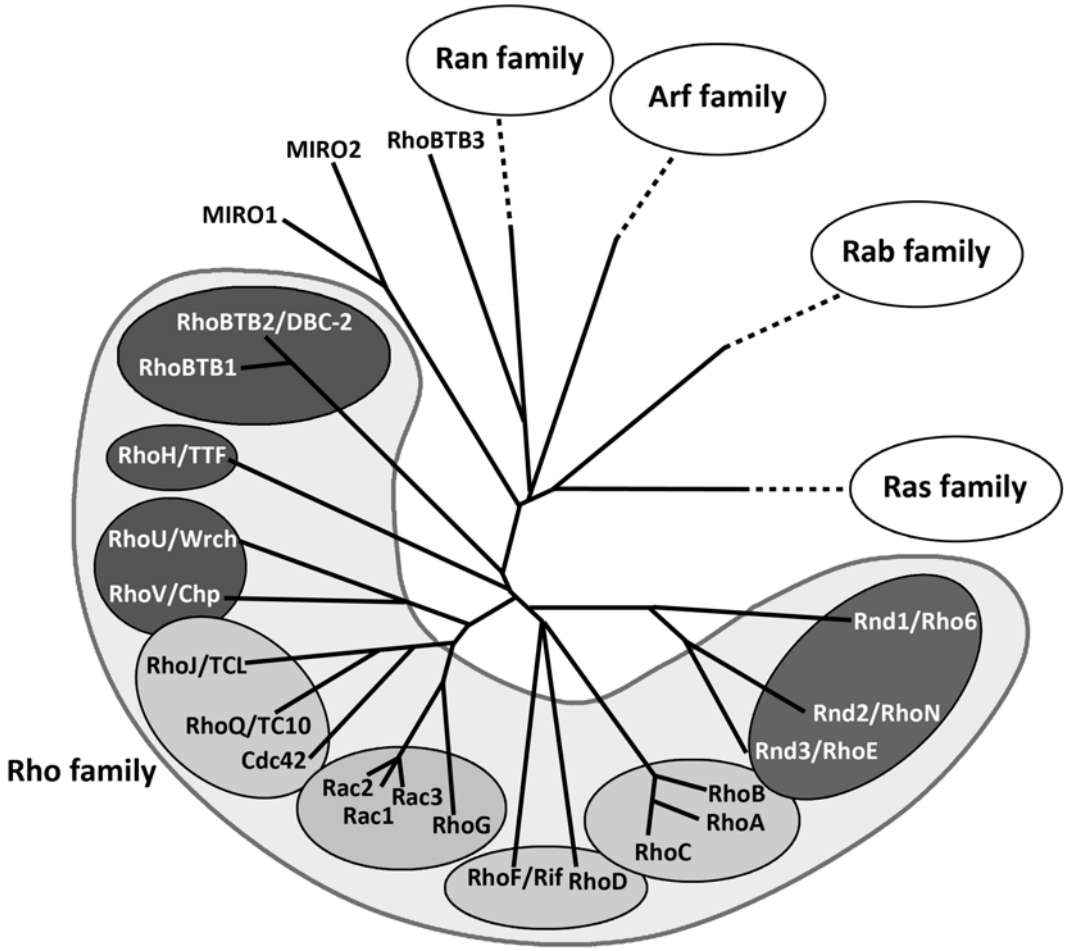


Figure 2 – Dendritic tree representation of the Ras superfamily of GTPases. The twenty members of the Rho GTPases family are grouped into eight subfamilies and divided into atypical (dark grey) and classical (light grey) Rho GTPases. Adapted from [26] with permission.

Regulation of Rho GTPases

As mentioned previously, classical Rho GTPases follow a GTPase cycle. Although these proteins have an intrinsic GTP-hydrolysing enzymatic activity, they do depend on external proteins that accelerate this process. The conversion of the GDP-bound to the GTP-bound state is accelerated by a set of proteins named guanine nucleotide exchange factors (GEFs) [26]. When GEFs bind to Rho GTPases, they decrease the affinity of Rho GTPases binding to GDP by deforming the phosphate-binding site [29].

As a consequence, GDP is released and, since the concentration of GTP is nearly ten times higher than GDP, GTP binds to the Rho GTPase instead [29]. However, the binding of this nucleotide decreases the affinity of GEF binding to the Rho GTPase, leading to the release of the GEF protein [29]. The reversed reaction, *i.e.* the conversion of the GTP-bound to the GDP-bound state, is catalysed by another set of proteins named GTPase-activating proteins (GAPs) [26]. These proteins accelerate the cleavage step of GTP hydrolysis into GDP, thus stimulating this process [29]. A third set of proteins regulates Rho GTPases activity and they are called guanine nucleotide dissociation inhibitors (GDIs) [30]. Rho GTPases are post-translationally modified at their C-terminus by prenylation [26]. This modification of Rho GTPases is required for the function and membrane localisation of the protein [31]. Rho GDIs act by binding to this C-terminal prenyl group and sequestering the GDP-bound form of Rho GTPases in the cytoplasm, thus preventing its binding to membranes [26]. In the GTP-bound conformation (*i.e.* in the active state), Rho GTPases are able to develop a variety of functions by interacting in a conformation-specific manner with effector proteins [28].

Functions of Rho GTPases

Although there are several studies on the 20 members of the Rho GTPase family, most of these studies still focus on the three members that were first isolated and characterized: Cdc42, RhoA and Rac1. Nevertheless, based on these studies, it is known that Rho GTPases are involved in the regulation of several cellular functions, like cell cycle progression, actin and microtubule dynamics, cell polarity, cell shape, cell migration or even vesicular trafficking [31].

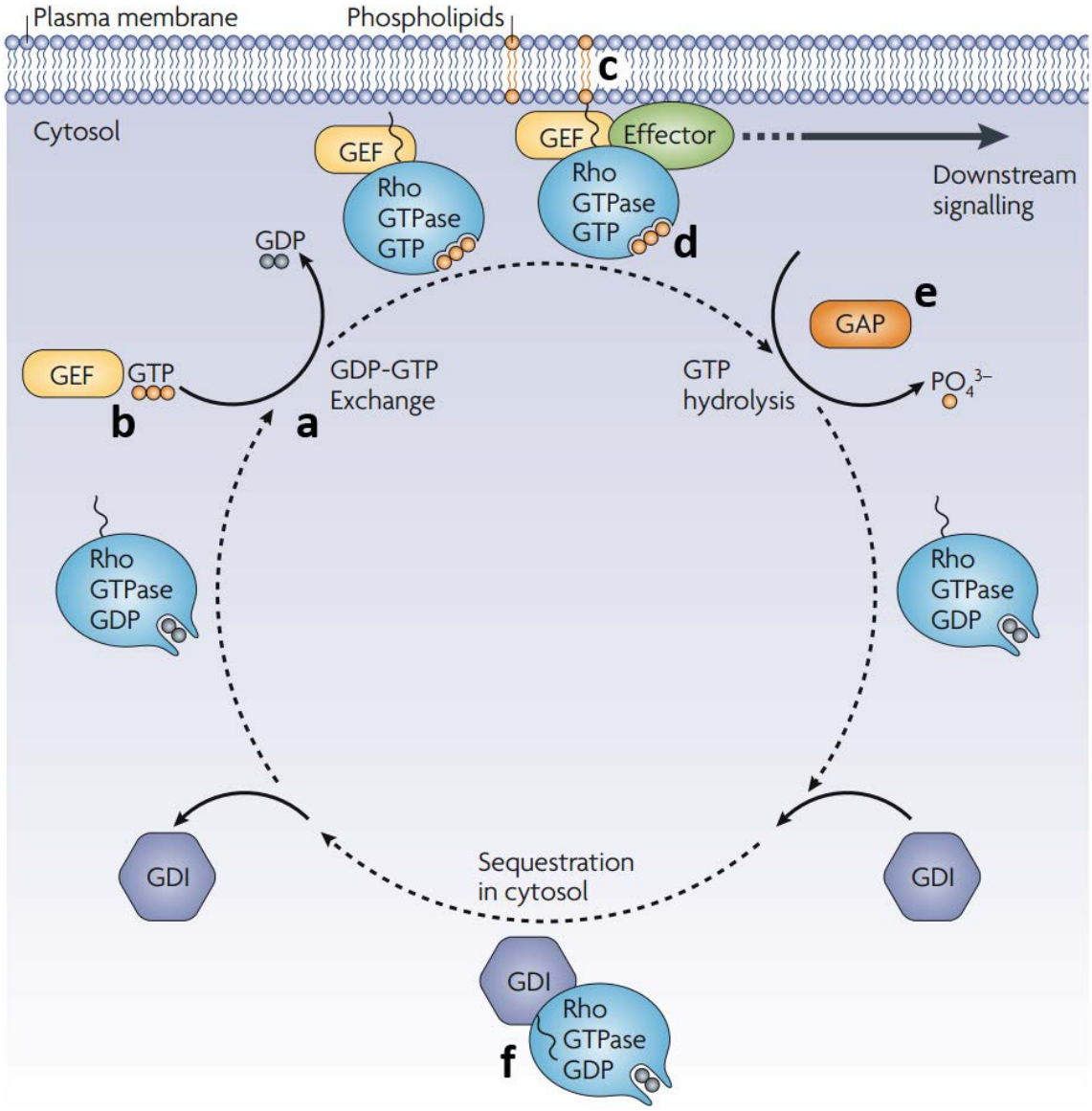


Figure 3 – Activity cycle and regulation of classical Rho GTPases. (a) The Rho GTPase is activated through exchange of GDP for GTP, (b) which is catalysed by GEFs. (c) The activated Rho GTPase is often anchored to the plasma membrane with its C-terminal prenyl group. (d) In the GTP-bound conformation, the protein can interact with effector proteins initiating downstream signalling. (e) Most Rho GTPases show a relatively low intrinsic GTP hydrolysis activity, which can be enhanced by binding of GAPs leading to Rho GTPases de-activation. (f) The inactive GDP-bound GTPases may be sequestered in the cytosol by GDIs, which prevents membrane binding, as well as the activation of Rho GTPases. Adapted from [32], used with permission.

Rho GTPases, and in particular Cdc42, have been shown to regulate cell polarity, which is of extreme importance for example during cell division in yeast [33]. In the same way, epithelial cells require a clear establishment of cell polarity, in order to distinguish the apical from the basolateral domains, and this process may involve Cdc42 and the Par6/aPKC/Par3 complex promoting the formation of tight junctions [28]. Both Cdc42 and Par proteins are also involved in the morphogenesis of neurons [28]. This particular Rho GTPase is recruited to the neurite that will become the axon and binds to the Par6/aPKC/Par3 complex, triggering the development of the axon [34]. Polarisation of both neuronal and epithelial cells is also induced by polarised vesicular trafficking, which has been shown to be regulated by Rho GTPases as well, via microtubule cytoskeleton re-organisation [28].

In addition to this, Rho GTPases also regulate both microtubule and actin cytoskeleton (the second will be described in more detail in the next section) [22; 23; 28; 35]. This regulation occurs via activation/deactivation of proteins that directly regulate the cytoskeleton dynamics, or by alterations in gene expression [28]. For example, the serum response factor (SRF) is a transcription factor regulated by Rho GTPases that act at the serum response element (SRE), which in turn is found at the promoters of genes encoding cytoskeleton components [28; 36].

Rho GTPases have also been shown to control steps in the cell cycle and cell division, by regulating cyclin-dependent kinases activity during G1 progression, by regulating actin and microtubules during M phase, and during cytokinesis. It is known that RhoA plays an important role in the assembly of the contractile ring during cytokinesis, by activation of the effector proteins citron kinase, Rho kinase (ROCK) and mDia formin, which increase actin polymerisation, alignment and bundling at the contractile ring [37]. Lastly, Rho GTPases are involved in the regulation of cell migration as well, due to their role in the regulation of the actin polymerisation and actin-myosin filament contraction, but this will be described in more detail in the following sections [28].

Actin cytoskeleton regulation by Rho GTPases

Nearly twenty years ago, several studies developed by Alan Hall showed that the activation of RhoA, Rac1 and Cdc42 induces the formation of actin-rich structures in

stress fibres, lamellipodia and filopodia, respectively [31; 38-41]. These findings show that these particular GTPases regulate specific signalling pathways that are responsible for both actin polymerisation and actin filaments bundling. Both Cdc42 and Rac1 induce actin polymerisation via indirect activation of the Arp2/3 complex through members of the WASP family [28]. Cdc42 can bind to NPFs WASP and N-WASP, which are inhibited by the intramolecular association of their WCA domain at the C-terminus and their GTPase-binding domain (GBD) [22]. When Cdc42 binds to the GBD it induces a conformational change leading to WASP/N-WASP activation [22]. The exposure of the WCA domain allows WASP/N-WASP to activate the Arp2/3 inducing actin polymerisation. In a similar way, Rac1 induces the formation of lamellipodia by activating WAVE, although this NPF is not auto-inhibited and does not have a GBD, thus not interacting directly with the GTPase [28; 42]. Actually, WAVE forms a complex with the proteins Abi1, Nap1 and PIR121, and this complex is recruited to lamellipodia upon Rac activation and its interaction with Abi1 [43]. Lastly, RhoA triggers stress fibre formation via the concerted action of ROCK and formins. Formins are dimeric proteins involved in both actin nucleation and elongation. At a first stage, each subunit of a formin protein binds to an actin monomer, which induces actin nucleation [1]. During actin elongation, the formin dimer remains bound to the plus end allowing the addition of more G-actin to the actin filament [1]. RhoA binds to the formin mDia1, which is auto-inhibited under normal circumstances, and activates this protein, allowing mDia1 to bind to the plus end of the actin filaments via its FH2 domain [28]. As previously mentioned, formins and in particular mDia1, also contain a poly-L-proline sequence that allows this protein to interact with the profilin-G-actin complex [10; 28]. Additionally, RhoA activates ROCK, which in turn phosphorylates and inhibits the myosin light chain (MLC) phosphatase [44]. This leads to increased phosphorylation of the MLC and actin-myosin interaction and bundling into stress fibres [6].

Cell migration

Cell migration is of paramount importance not only in the development but also maintenance of many cellular processes in the body. At a first instance, it plays a central role during morphogenesis in embryonic development, where cells migrate in

order to create different tissues and organs [45]. At a later stage, cell migration continues to contribute to many other processes, like the renewal of skin and intestine epithelia, wound healing and immune response (*e.g.* macrophages and neutrophils migrate to sites of infection) [1; 45]. Cell migration is also often involved in several pathological conditions, like vascular diseases, chronic inflammatory diseases and cancer [45]. As a consequence, understanding the mechanism behind cell migration is one of the keys to develop therapeutic approaches to treat these diseases [45]. With the exception of sperm cells, almost all cells in animals migrate by crawling [1]. However, cell crawling is an extremely complex process that is also dependent on the cell type. The most commonly accepted model uses the actin cytoskeleton as the engine for crawling [46]. It is possible to divide cell migration into five sequential steps. At a first stage, actin-rich structures are generated at the leading edge (*i.e.* front) of the cell, creating a protrusion [46]. Secondly, this protrusion needs to adhere to the substrate, and thirdly, the cell body needs to move forward, which is named traction [46]. The two last steps are de-adhesion and retraction of the tail [46].

Cells create four different types of protrusions at the cell front: filopodia, lamellipodia, invadopodia and blebs [47]. Therefore, actin cytoskeleton and its regulators, and as a consequence proteins such as Rho GTPases that control these actin regulators, are tightly associated with the extension of these membrane protrusions [47]. Lamellipodia are thin sheet-like extensions of the plasma membrane, with active actin polymerisation and branching, which are mainly regulated by Rac1 [39; 47]. Filopodia are structures with parallel actin filaments used by the cell for guidance in order to explore the surroundings, and are mainly regulated by Cdc42 [40; 47]. Invadopodia are cell protrusions involved in the degradation of the extracellular matrix (ECM), thus requiring the delivery of vesicles with matrix-degrading proteases [47]. In a similar way to filopodia, this process is also mainly regulated by Cdc42. Blebs are balloon-like protrusions that can co-exist at the leading edge with lamellipodia [48]. However, while lamellipodia requires that the actin filaments interact with the plasma membrane, the opposite appears to happen for membrane blebbing [47]. RhoA/ROCK are possible regulators of membrane blebbing, but there are other MLC phosphorylation activators that can act independently of ROCK, such as the death-associated protein kinase (DAPK) [47; 48].

Another crucial aspect of cell migration is the ability of the cells to crawl towards a gradient. Cells need to be able to sense gradients and respond accordingly. This cell movement in response to a soluble factor gradient is known as chemotaxis [1]. It requires that the cell is able to decipher the external signals and to determine its polarity (*i.e.* what will be the leading edge and what will be the back of the cell). This process is also partially regulated by Rho GTPases, particularly by Cdc42, which is activated at the leading edge of the cell [45].

Rho GTPases and cancer progression

As mentioned in the previous sessions, Rho GTPases are key regulators of many physiological processes, such as cell polarity, cell migration and cell cycle progression. Consequently, impaired activity of these proteins is associated with several pathological conditions and particularly with cancer cell migration, invasion and metastasis. Contrary to what happens with Ras proteins, there are no point mutations in Rho GTPases that are associated with tumours [26]. Nevertheless, upregulation of these proteins or overexpression of an upstream/downstream protein in Rho GTPases signalling pathways is often detected in many types of human tumours [49]. Actually, it has been reported that RhoA, RhoC, Rac1-3, Cdc42, Wrch2 and RhoF are often upregulated in tumours, like breast cancer, melanomas and leukaemias among many others [26; 49]. The amount of information regarding how Rho GTPases work and how they contribute to cancer progression is increasing, but due to its complexity there is still an extensive territory to bring to light with promising therapeutical potential. Most of the studies have been focused on RhoA, Rac1 and Cdc42, thus little is known regarding the other members. However, it is known that they are involved in actin cytoskeleton regulation. Therefore, investigations need to be focused on these less studied members of the Rho family of small GTPases to clarify their role in cancer progression [26].

RhoD

RhoD is one of these less-characterised members of the Rho GTPases. Together with Rif (also known as RhoF), these proteins constitute a subgroup of the classical Rho

GTPases [50]. This protein was identified by PCR nearly 20 years ago and it is expressed in most tissues in mammals [51]. Its gene is located on chromosome 11, locus 11q14.3 [52]. Recent findings show that, unlike other Rho GTPases, both RhoD and Rif possess a faster nucleotide exchange than GTP hydrolysis [53]. This means that under resting conditions, these proteins exist mainly in the active GTP-bound state [53]. This raises several questions regarding their regulation and might mean that these proteins do not follow the classical switch mechanism of other classical Rho GTPases. Despite these differences, RhoD also contains a C-terminal CAAX box required for its membrane localisation, as well as a GTP-binding domain [52].

So far, it is known that overexpressed RhoD promotes the formation of filopodia. However, these filopodia are slightly different from the Cdc42-induced filopodia, since they are longer (20-40 μm compared to 8-15 μm) and thinner [52]. Additionally, it has been shown that RhoD binds to both WHAMM and Filamin A-binding protein (FILIP1), thus regulating cell adhesion and cell migration by a still unknown mechanism involving the re-organisation of the actin cytoskeleton [50]. Another protein, the zipper-interacting protein kinase (ZIPK), has been shown to bind to RhoD, inducing stress fibres and focal adhesion re-organisation [54]. Moreover, it was observed that RhoD also regulates membrane trafficking, which might provide a link between this process and the dynamics of the cytoskeleton [55; 56]. This is also supported by the clear phenotype of RhoD-overexpressing cells that show an evident localisation of RhoD in vesicular-like organelles.

The above information supports the fact that RhoD provides additional regulation of the actin cytoskeleton. However, the studies on the role of this protein are still in the very beginning. Consequently, more research is needed in order to unveil the signalling pathways involving this particular Rho GTPase.

AIMS

The aim of this thesis is to give additional insight into the biological functions of RhoD. As a result, the following topics are addressed throughout this thesis:

- Identification of additional RhoD-binding proteins, based on homology searches with previously discovered RhoD-binding partners;

- Characterisation of additional interactions between ABPs and their role in the actin cytoskeleton dynamics;

- Elucidation of the role of the interaction between RhoD and ZIPK regarding the phosphorylation of focal adhesion kinase (FAK);

- Clarification of the role of RhoD in cell chemotaxis.

MATERIALS AND METHODS

cDNA cloning and plasmid construction

JMY mouse mutants in pcDNA3-HA vector were a gift from Amanda Coutts, University of Oxford, UK. The full-length mutant was used to subclone additional mutants into a pRK5-FLAG vector (N. Tapon, MRC-LMCB, UCL, UK), using standard techniques and the following PCR primers: 5'-gcgagatctatgtcgttcgcgctgga-3' , 5'-gcggaattctagttctcccagtctgt-3', 5'-gcggaattcaccgctgccagtcagcat-3' and 5'-gcggaattcacggctcttcggggaaca-3' (Sigma-Aldrich). FLAG-tagged ZIPK constructs were a gift from Tim Haystead, Duke University, Durham, NC, USA. Additionally, the following constructs were used during the remaining experiments: pRK5-myc-RhoDwt, pRK5-myc-RhoD/G26V (active variant), pRK5-RhoD/T31N (dominant negative), pRK5-myc empty vector, pGW1-CMV-myc-CTTNBP2NL, EGFP-RhoDwt, EGFP-RhoD/G26V, EGFP-RhoD/T31N, EGFP empty vector, pRK5-FLAG vector, pRK5-FLAG-WHAMM, EGFP-Profilin, EGFP-Profilin/S133H, pcDNA3-FLAG-FILIP1L, myc-Rif/L77, pRK5-myc-RhoDwt/SAAX, pRK5-myc-RhoD/G26V/SAAX, myc-Rac1/L61, myc-RhoA/L63 and myc-Cdc42/V12.

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), mouse embryonic fibroblasts immortalized with the SV40 large T antigen (MEF), MEF cells deficient in focal adhesion kinase (MEF FAK-/-), MEF cells deficient in src, yes and fyn (MEF SYF-/-), and MEF SYF-/- cells with c-src reintroduced (MEF+Src) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin-streptomycin (HyClone, Thermo Scientific, Waltham, MA, USA). HEK293T cells were transfected with cDNA using standard calcium phosphate transfection protocol at pH 6.94 and were used 48h after transfection. The other cell-types were transfected with cDNA using JetPEI reagents (PolyPlus Transfection, Illkirch, France) according to the manufacturer's protocol and

were used for further experiments 24h after transfection. BJ human foreskin fibroblasts stably transfected with hTERT (BJhTERT) were cultured in Minimal Essential Medium GlutaMAX supplemented with 1 % (v/v) non essential aminoacids, 1 % (v/v) sodium pyruvate and 10 % (v/v) FBS (all from Life Technology). For starvation and migration experiments the same medium without FBS was used. Cells ($3x10^5$) were transfected by electroporation (Amaxa nucleofector I, Lonza) using 100 μL of Nucleofection Solution (Mirus) and 4 μL of RhoD siRNA (Ambion, 50 μM) or control siRNA (Ambion, 50 μM). Cells were used for experiments 48h after transfection. All cell lines were cultured at 37 ºC and 5 % CO_2.

Immunoblotting and co-immunoprecipitation

Cells (HEK293T or BJ/SV40T) were washed with phosphate-buffered saline (PBS) and 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 and 10 mM $MgCl_2$). The cell lysates were centrifuged at 13,000 rpm in a bench-top microcentrifuge for 15 min at 4 °C. The supernatant was collected and incubated with the primary antibody for 1 h at 4 °C. Following this, protein G-Sepharose (GE Healthcare) was added to the supernatant and incubated for 1 h at 4 °C. The beads were washed three times with Triton X-100 lysis buffer and then analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a nitrocellulose membrane (Hybond C, GE Healthcare), which was then incubated with the correspondent antibody followed by horseradish-peroxidase-conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare). The Western blots were revealed using Luminol immunoblotting reagent (Santa Cruz Biotechnology). The following antibodies were used: mouse anti-Myc (9E10) (Covance), mouse anti-Flag monoclonal (M2) (Santa Cruz Biotecnology), rabbit anti-HA polyclonal (Y-11) sc-805 (Santa Cruz Biotecnology), rabbit anti-GFP (Invitrogen), rabbit anti-Profilin (Alan Hall, MRC-LMCB, UCL, UK), rabbit anti-FILIP1L (Sigma-Aldrich), rabbit anti-FAK (c-20) sc-558 (Santa Cruz Biotechnology), rabbit anti-FAK phosphorylation site-specific antibody containing FAK[pY397] [pY407] [pY577] and [pY861] (Invitrogen), and mouse anti-β-actin AC-15 (Sigma-Aldrich). Densitometric analysis was performed using ImageJ software.

GST fusion protein purification and pull-down assay

Glutathione S-transferase (GST)-RhoDwt, GST-RhoD/G26V, GST-WHAMM (amino acid residues 559 to 809), GST-WASP (amino acid residues 442 to 502) and GST alone were expressed in *Escherichia coli* and purified using Glutathione Sepharose beads (GE Healthcare). For GST pull-down with GST-RhoD the same Triton X-100 lysis buffer was used as for the immunoprecipitation experiments in order to lyse the cells. GST-RhoDwt was pre-treated either with 100 mM NaCl, 5mM EDTA, 50 mM Tris-HCl pH 7.5, 10 μM GDP or GTPγs for 10 min at 30 ºC followed by the addition of 10 mM of $MgCl_2$ on ice. The cell lysates were centrifuged at 13,000 rpm in a bench-top microcentrifuge for 15 min at 4 °C. The supernatant was collected and incubated with the pre-treated beads for 30 min in the end-over-end at 4 ºC. The beads were washed three times with washing buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$ and 1% Triton X-100) and then analysed by SDS-PAGE. The same procedure described for the immunoblotting was followed. For the GST pull-down with GST-WHAMM and GST-WASP, four different lysis buffers were tested: lysis buffer 1 (10 mM Tris HCl pH 7.5, 0.5 % Triton X-100, 10 % Glycerol, 100 mM NaCl and 1% Aprotinin), lysis buffer 2 (10 mM Tris HCl pH 7.5, 0.5 % Triton X-100, 10 % Glycerol, 150 mM NaCl, 5 mM EDTA and 1 % Aprotinin), lysis buffer 3 (10 mM Tris HCl pH 7.5, 0.5 % Triton X-100, 10 % Glycerol, 150 mM NaCl, 2 mM EGTA and 1 % Aprotinin), and lysis buffer 4 (10 mM Tris HCl pH 7.5, 0.5 % Triton X-100, 10 % Glycerol, 150 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$ and 1 % Aprotinin). Also, the beads were only washed once with the corresponding buffer before adding to the lysates, thus not being pre-treated with GDP or GTPγs.

Immunocytochemistry

For immunocytochemistry, BJ/SV40T, MEF, MEF FAK -/-, MEF SYF -/- or MEF SYF+Src cells were fixed in 3 % paraformaldehyde in PBS for 25 min at 37 °C and washed with PBS three times. Then, they were permeabilised in 0.2 % Triton X-100 in PBS for 5 min at room temperature, washed again with PBS, blocked with 5 % FBS in PBS for 30 min at room temperature, and washed with PBS. The cells were incubated for 1 h with the primary antibody and then washed with PBS. Following this they were incubated for 1 h with the secondary antibody and washed with PBS. Finally, the

coverslips were mounted on microscopy slides with Fluoromount-G (Southern Biotechnology Associates), visualized using a Zeiss AxioCAM MRm digital camera connected to a Zeiss AxioVert 40 CFL microscope, and processed with the AxioVision software. The following antibodies were used for immunocytochemistry: rabbit anti-Myc (A-14) sc-789 (Santa Cruz Biotechnology), mouse anti-HA monoclonal (12CA5) (Roche), and Alexa Fluor 488-conjugated anti-rabbit (Molecular Probes); tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse (Jackson ImmunoResearch Laboratories), Alexa Fluor 488-conjugated phalloidin (Molecular Probes), TRITC-conjugated phalloidin (Sigma-Aldrich), and Alexa Fluor 350-conjugated phalloidin (Molecular Probes) were used in order to visualize F-actin; and 4',6-diamidino-2-phenylindole (DAPI) (Sigma) was used to visualize the nucleus.

Chemotaxis assay

Chemotaxis of BJhTERT cells in response to a stable gradient of platelet-derived growth factor (PDGF)-BB was tested using CellDirector Opal (Gradientech AB, Uppsala, Sweden). The chamber was coated with gelatin and the cells were injected into the chamber 24h after transfection ($2x10^5$ cells/mL). The cells were allowed to settle for additional 24h at 37 ºC, 5 % CO_2. Three hours before starting the experiment, the cells were starved. PDGF-BB gradient ranging from 0-20 ng/mL was generated in starvation medium and cell chemotaxis was tracked for 4 hours using Axiovert 200M (Zeiss). The cells were kept at 37 ºC during all the chemotaxis experiment. AxioVision software (Zeiss) was used for time-lapse imaging and Gradientech Tracking Tool software (Gradientech) was used for cell tracking.

qPCR

Transfected cells on a 3.5 cm dish were lysed at the same time of the chemotaxis experiment, using 350 µL of TRK Lysis Buffer (Omega) and 7 µL of β-mercaptoethanol. The lysed cells were stored in an eppendorf tube at -80 °C until all chemotaxis experiments were finished. RNA was purified using the Total RNA Kit I (Omega) according to manufacturer's instructions. Reverse transcription was preformed with iScript cDNA synthesis Kit (BioRad) and 500 ng of RNA according to

manufacturer's protocol; SsoFast EvaGreen Supermix (BioRad) was used for qPCR. The following primers were used: human RhoD (forward 5'-gagcggtacatggtcaacct-3' and reverse 5'-gtgacatcgaagcaaagcag-3'), and human β-actin (forward 5'-tctacaatgagctgcgtgtg-3' and reverse 5'-agcctggatagcaacgtaca-3').

Statistical analysis

Comparison of FAK tyrosine phosphorylation relative to the vector control was determined with a Student's *t*-test and data represents the mean of four experiments. The data from the chemotaxis assay was provided from four independent experiments and analysed with Mann-Whitney test. At least 50 cells were tracked each time. For all the experiments the error bars represent the standard error of the mean (SEM) and the values were considered statistically significant when $p<0.05$. GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA) was used for all the statistical analyses.

RESULTS

Homology searches identify three proteins closely related to the RhoD-binding proteins FILIP1 and WHAMM

Previous studies from the host group show that RhoD binds to WHAMM and FILIP1 in the regulation of cell adhesion and cell migration [50]. As a result, homology searches were performed with the aim of identifying new potential binding partners for RhoD (Figure 4). Based on these searches, four other proteins were identified with a similar coiled-coil region: JMY, FILIP1 like (FILIP1L), cortactin-binding protein 2 N-terminal like (CTTNBP2NL) and leucine zipper protein 1 (LUZP1). Especially JMY and FILIP1L show a very high similarity with WHAMM and FILIP1, respectively, with JMY being nearly 35 % identical to WHAMM [15].

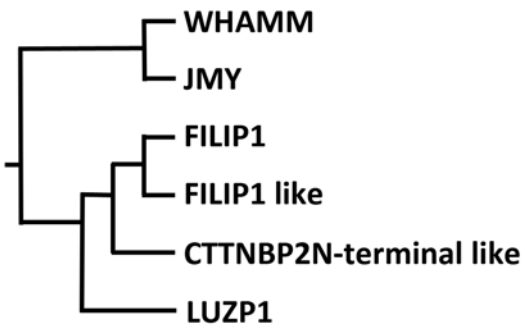


Figure 4 – Dendritic tree representation of closely related proteins to the RhoD-binding proteins WHAMM and FILIP1. The Clustal W algorithm was used to show the similarity between these two proteins, together with the additional similar proteins JMY, FILIP1L, CTTNBP2NL and LUZP1. Adapted from [50].

RhoD and JMY interaction

Based on the previous information we decided to test the interaction between RhoD and JMY. HEK293T cells were transfected with one of three different RhoD construct: RhoD *wild-type*, GTP-bound RhoD active variant (G26V), and GDP-bound RhoD dominant negative (T31N). A weak GTP-independent interaction was observed between RhoD and JMY full-length (Figure 5A). Moreover, when this interaction was

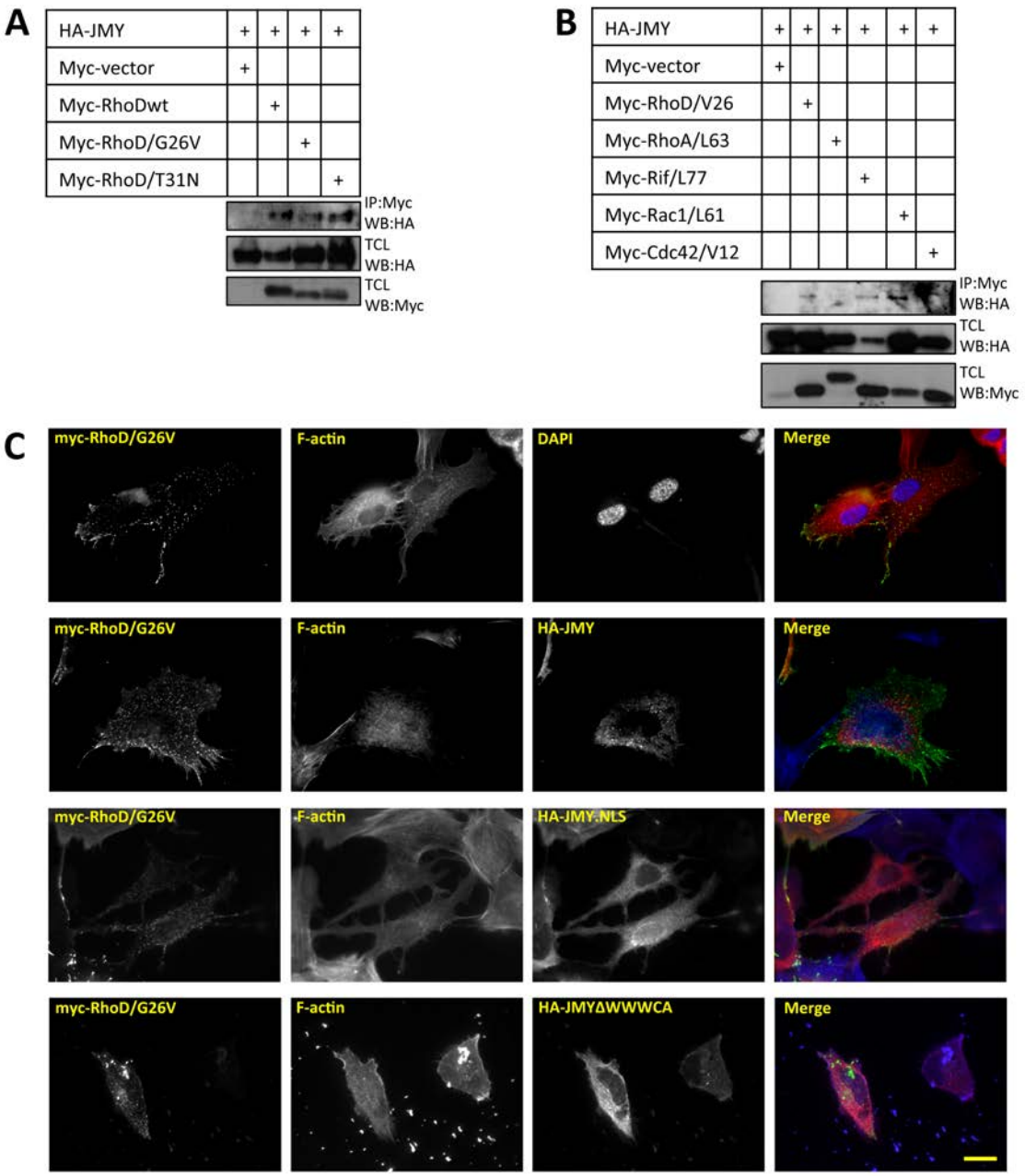


Figure 5 – RhoD and JMY interaction. (A) The interaction between HA-JMY and (A) Myc-RhoD (wt, active variant and dominant negative) (B) as well as with other GTPases was tested using transfected HEK293T cells and a co-immunoprecipitation assay. The presence of HA-JMY in the Myc precipitate was detected by a Western Blot analysis with an anti-HA antibody. (C) Transfected BJ/SV40T cells with Myc-RhoD/G26V and one of three HA-JMY constructs (HA-JMY, HA-JMY NLS and HA-JMYΔWWWCA) were visualised with rabbit anti-Myc followed by Alexa 488 anti-rabbit, mouse anti-HA followed by TRITC anti-mouse and Alexa 350 phalloidin. Scale bar: 20 µm.

tested with other GTPases, a positive result was observed with constitutively active mutants of Rif and Rac1 in addition to RhoD (Figure 5B). Additionally, BJ/SV40T cells were transfected with Myc-RhoD/G26V or Myc-RhoD/T31N and one of the following JMY constructs: HA-JMY full-length, HA-JMY NLS, *i.e.* with an additional NLS sequence at the N-terminus, and HA-JMYΔWWWCA, *i.e.* without the three actin-binding WH2 domains and the Arp2/3 complex-binding domains central and acidic (CA) (Figure 6A). Rabbit anti-Myc followed by Alexa 488 anti-rabbit was used to stain RhoD, mouse anti-HA followed by TRITC anti-mouse was used to stain JMY, Alexa 350 phalloidin was used to show F-actin and DAPI was used in cells untransfected with JMY to stain the nucleus (Figure 5C, Myc-RhoD/T31N not shown). No co-localisation was observed between RhoD and JMY. At a later stage, we decided to sub-clone fragments of JMY, in order to try to confirm and map the interaction between JMY and RhoD (Figure 6A). However, no interaction was observed (Figure 6B). Therefore, in order to try to clarify this situation, we purified GST-RhoD and proceeded with a GST pull-down assay (data not shown), again no interaction was observed.

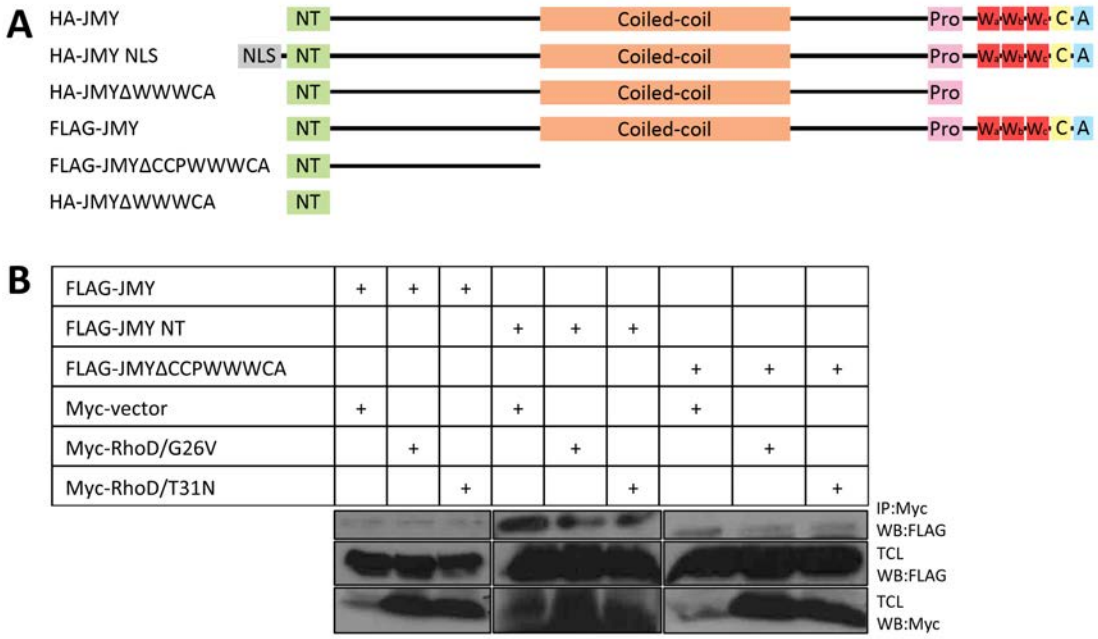


Figure 6 – Mapping of the RhoD-binding domain of JMY. (A) Domain organisation of the HA-JMY and FLAG-JMY constructs. (B) Mapping of the RhoD-binding site on JMY. The interaction was tested using transfected HEK293T cells followed by co-immunoprecipitation. The presence of FLAG-JMY in the Myc precipitate was detected by a Western Blot analysis with an anti-FLAG antibody.

WHAMM binds to the actin-binding protein Profilin

Although the interaction between JMY and RhoD was not clear, it is known that this protein, as well as its paralogous WHAMM, contain a proline rich sequence, which might be a profilin binding site, in similarity to WASP for example. To investigate the interaction between JMY/WHAMM and profilin we stably transfected HEK293T cells and tested by co-immunoprecipitation (data not shown). Although no interaction was observed, we also concluded that co-immunoprecipitation could not be considered the best approach. As a result, we decided to try another method, GST pull-down, to test this protein-protein interaction using GST-WHAMM and testing four different lysis buffers. A GST-WHAMM construct, including the proline rich sequence (GST-WHAMM 559-809), was used to test the interaction and a GST-WASP construct excluding the proline rich sequence (GST-WASP 442-502) was used as a negative control. The four tested lysis buffers are described in the Materials and Methods session. As shown in Figure 7, a clear and strong interaction is observed between WHAMM and profilin when the lysis buffers 1 and 2 were used (data not shown for lysis buffers 3 and 4).

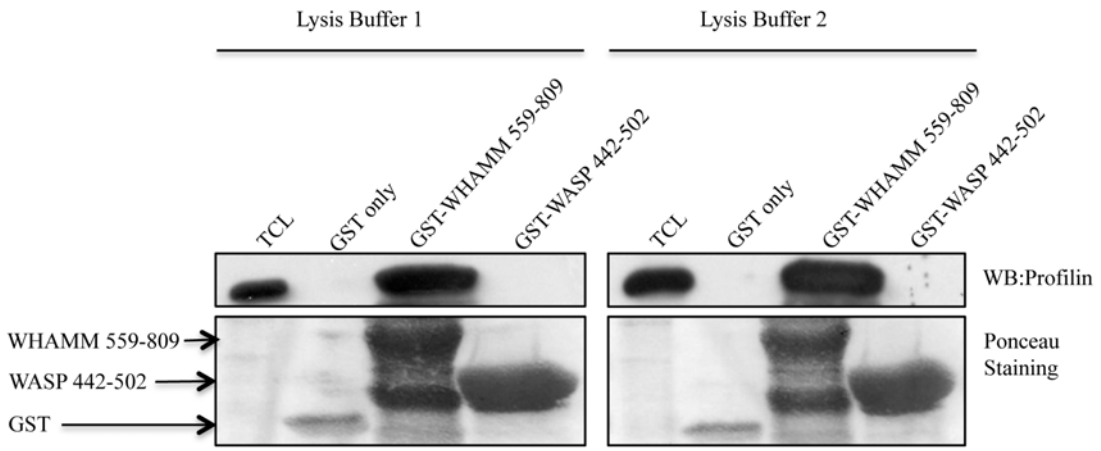


Figure 7 – WHAMM interaction with profilin. Overexpressed EGFP-Profilin from lysed HEK293T cells with Lysis Buffers 1 and 2 was precipitated with GST-WHAMM 550-809 and GST-WASP 442-502 in a GST pull-down assay. The presence of EGFP-profilin in the precipitate was confirmed by a Western Blot analysis with an anti-profilin antibody. GST only and GST-WASP 442-502 (*i.e.* excluding the proline sequence) were used as negative controls.

FILIP1L and CTTNBP2NL are not RhoD-binding proteins

In addition to JMY, two other proteins showed a high degree of similary with WHAMM and FILIP1. These proteins were FILIP1L and CTTNBP2NL. Therefore, the interaction between these proteins and RhoD was also tested. However, no interaction could be observed, either with CTTNBP2NL (Figure 8) or with FILIP1L (data not shown).

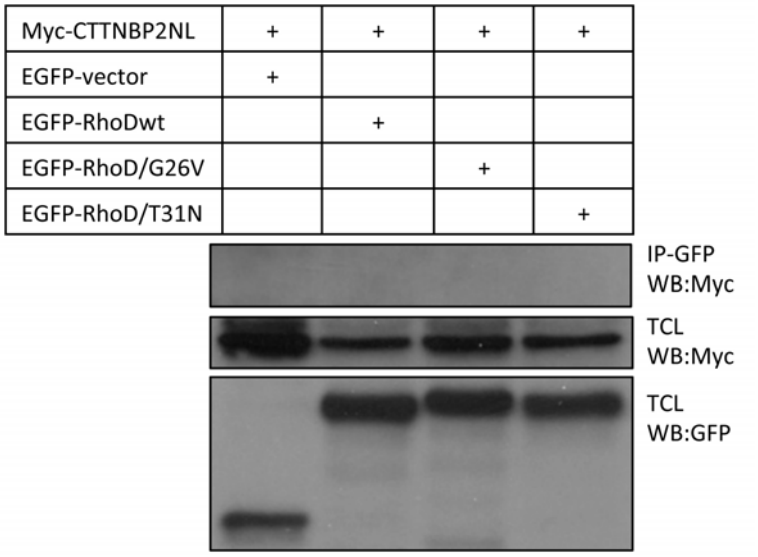


Figure 8 – Interaction between CTTNBP2NL and RhoD. HEK293T cells were stably transfected with Myc-CTTNBP2NL and EGFP-RhoD (wt, active variant or dominant negative). The presence of Myc-CTTNBP2NL in the GFP precipitates was confirmed by a Western Blot analysis using anti-myc antibody.

RhoD binds to ZIPK and supresses the ZIPK-induced decreased phosphorylation of FAK

This group has recently shown ZIPK as another RhoD-binding protein. Additionally, it was also shown that RhoD supresses the ZIPK-induced increase of focal adhesions size [54]. Since focal adhesion kinase (FAK) is involved in focal adhesion dynamics, we decided to clarify the effects of the overexpression of RhoD and ZIPK in FAK activation (*i.e.* FAK phosphorylated at the tyrosine residue 397). The results (Appendix I, Figure 4) show that both ZIPK and the ZIPK/D161A (*i.e.* the kinase deficient mutant) resulted in decreased phosphorylation of Y397, which means

decreased activation of FAK. This effect was not observed for the ZIPKΔLZ mutant (*i.e.* mutant without the leucine zipper domain). However, when RhoD was overexpressed together with ZIPK, it supressed the reduction of pY397. Contrarily to Y397, the phosphorylation of Y576 was not affected by overexpression of ZIPK or RhoD.

RhoD and FAK reduce the size of actin bundles in MEF cells

In line with the previous experiments, we decided to try to obtain a better understanding of the role of both RhoD and FAK in the dynamics of the actin cytoskeleton. In order to do this analysis we used four different cell lines: MEFwt, MEF FAK-/- (*i.e.* cells without FAK), MEF SYF-/- (*i.e.* cells without src, yes and fyn) and MEF+src (*i.e.* MEF SYF-/- with src reintroduced). These cells were transfected with Myc-RhoDwt, Myc-RhoD/G26V, Myc-RhoDwt/SAAX (*i.e.* with a SAAX motif instead of CAAX at the C-terminus), Myc-RhoD/G26V/SAXX and Myc-Rif/L77. As expected, it was possible to observe that RhoD localises mainly to vesicle-like structures. However, when the CAAX motif is substituted by a SAAX motif, RhoD loses this localisation, becoming mostly spread in the cytoplasm (Figure 9B and C). Still, the most striking observation was the effect of RhoD in the actin cytoskeleton (Figure 9A). We observed that RhoD reduced the size of actin bundles in all cell lines except MEF FAK-/- (Figure 9A, C and D; data not shown for MEF SYF-/- and MEF+src). Additionally, this effect turns out to be dependent on RhoD cellular localisation, since it was reversed when the cells were transfected with RhoD/SAAX (Figure 9C; data not shown for Myc-RhoDwt and Myc-RhoDwt/SAAX). Moreover, there did not seem to be a difference in the actin bundles when MEF FAK-/- cells were transfected with RhoD (Figure 9D). In addition to this, we also concluded that RhoD induces the formation of filopodia, regardless of the cell line or presence of a CAAX/SAAX motif at the C-terminus (data not shown).

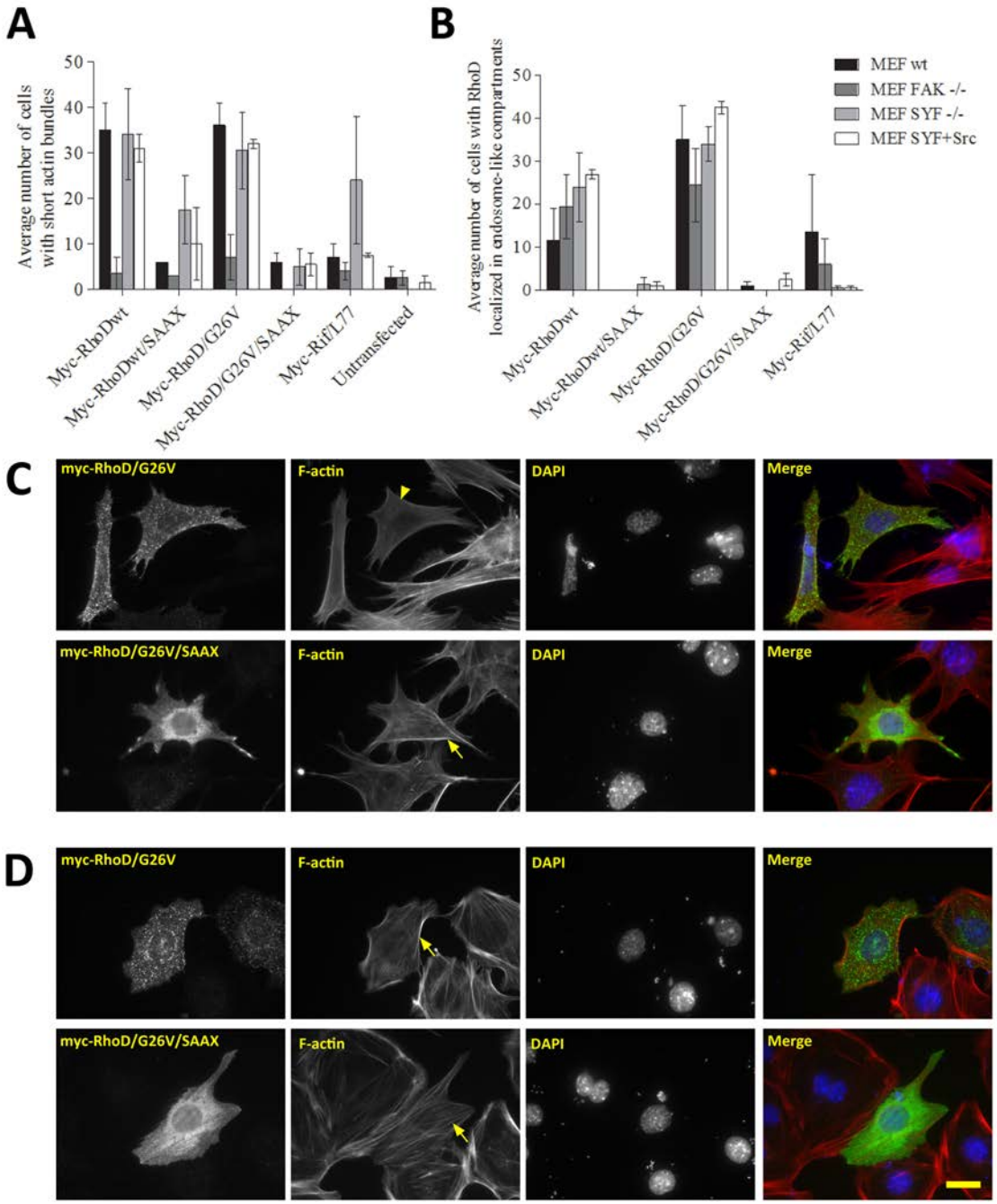


Figure 9 – (A) Quantification of the average number of cells with short actin bundles. (B) Quantification of the average number of cells with RhoD localised in vesicle-like structures. Fifty transfected cells were scored from two independent experiments, except MEF wt and MEF FAK-/- transfected with Myc-RhoDwt/SAAX, which are only from one experiment. Error bars represent SEM. Images of representative cells are shown in C and D. (C) Transfected MEF wt and (D) transfected MEF FAK-/- with Myc-RhoD/G26V or Myc-RhoD/G26V/SAAX were visualised with rabbit anti-Myc followed by Alexa 488 anti-rabbit, TRITC-phalloidin and DAPI. Scale bar: 20 μm.

RhoD is required for directional cell migration in the presence of a PDGF-BB gradient.

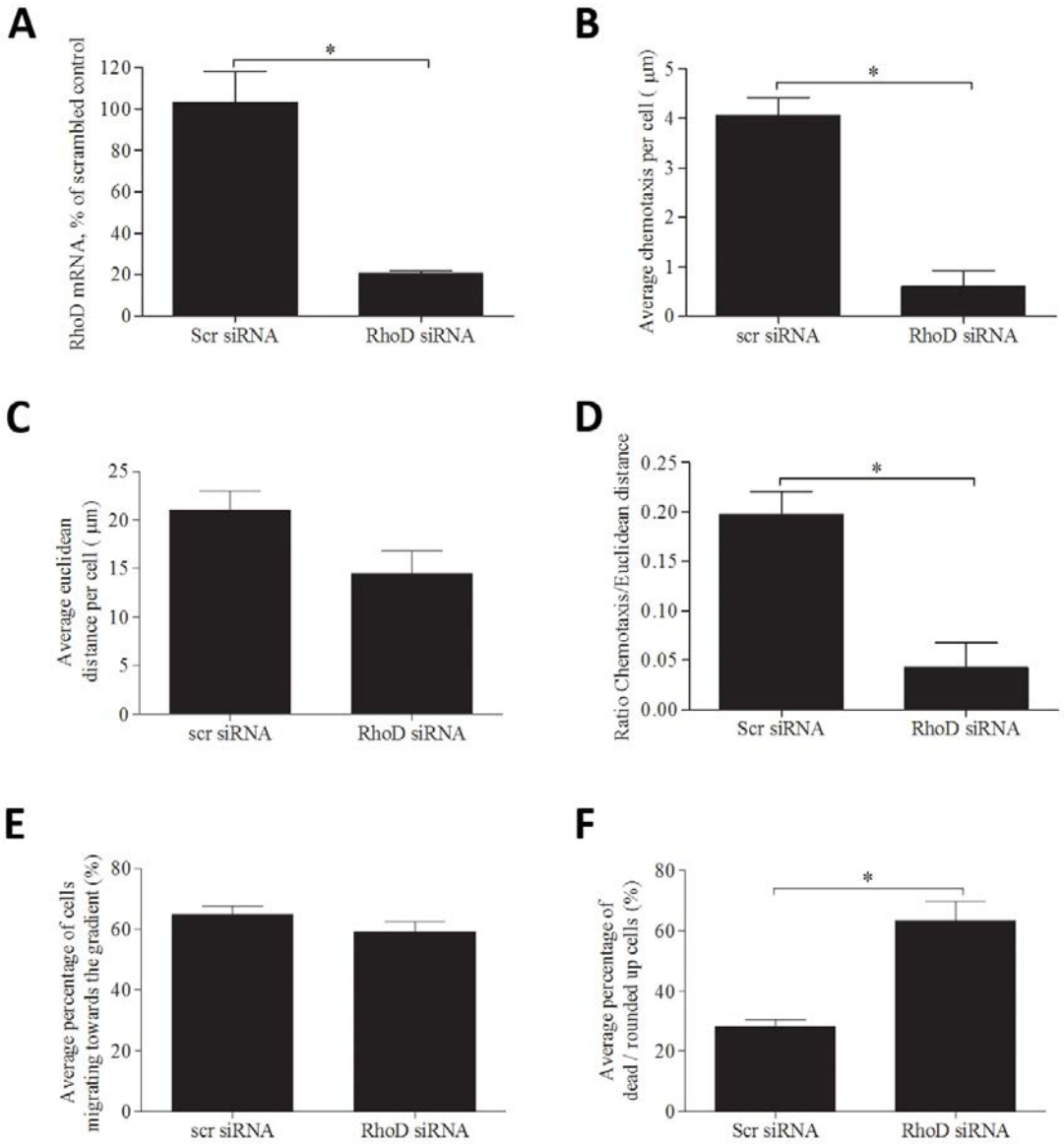


Figure 10 – RhoD is required for cell chemotaxis in the presence of a gradient of PDGF-BB. (A) Expression levels of RhoD mRNA 48h after transfection with scrambled (scr) or RhoD siRNA. (B-E) Cells were submitted to a PDGF-BB gradient ranging from 0-20 ng/mL and the migration was evaluated using a CellDirector Opal chamber and tracked with time-lapse microscopy 48h after transfection with siRNA. (F) Percentage of cells that rounded up during the tracking experiment in comparison to the total number of cells. Each bar from each graph shows the average results from four independent experiments ± standard error of the mean (SEM). P value was calculated with a Mann-Whitney test and * represents significant values with $P < 0.05$.

Previous experiments show that RhoD plays an important role in cell migration [50; 56]. In line with this, we decided to test how is BJhTERT cells migration affected by RhoD knockdown in the presence of a PDGF-BB gradient. Our results show that with a nearly 80% knockdown of RhoD, cells lose their ability to sense the PDGF-BB gradient (Figure 10A, B and D). While in the presence of RhoD the fibroblasts moved an average of 4 μm towards the gradient, a significantly reduced migration of less than 1 μm was observed in the absence of RhoD. However, this was not due to reduced cell migration as shown by Figure 10C, where it is possible to observe that the difference in the Euclidean distance between the cells treated with control (Scr) and RhoD siRNAs is not statistically significant. By Euclidean distance we mean distance between the starting position and the end position that one could measure with a ruler. Also, a similar percentage of cells migrated towards the gradient in both situations (Figure 10E) although this does not give any information regarding the distance migrated by each cell. Additionally, we noticed that an increased amount of cells were rounding up when knockdown for RhoD and this difference was shown to be statistically significant as well (Figure 10F). This could possibly mean that RhoD knockdown is inducing cell death.

DISCUSSION

After demonstrating that RhoD binds to both WHAMM and FILIP1, one of the approaches to obtain a better insight into the role of RhoD is to find additional binding partners for this protein. Consequently, based on the homology searches developed by the host group, we started by testing whether JMY is a RhoD-binding protein. Although our initial results seemed to be very favourable, when we repeated the experiments with the deletion mutants it was not possible to observe any interaction. Our results demonstrate that there seems to be a weak interaction between JMY and RhoD, however, this interaction might not be very stable resulting in these unclear data. One possible explanation is that the interaction between RhoD and JMY might be a transient interaction, meaning that the association between these two proteins is temporary, depending on certain conditions such as protein phosphorylation, protein localisation in certain compartments of the cell, etc. The approaches used to test this interaction (i.e. co-immunoprecipitation and GST pull-down) are based on physical methods that work better for stable and strong interactions. Therefore, further work is required based on other techniques to confirm the interaction between RhoD and JMY. On the other hand, there was no sign at all of interaction between RhoD and FILIP1L or CTTNBP2NL.

Our data provide compelling evidence that WHAMM binds to profilin. This is an expected observation since the structure of WHAMM shows the existence of a poly-proline domain and it is known that profilin binds to these proline-rich sequences. It is known that profilin binds to the poly-proline sequences of other NPFs, such as WASP and N-WASP, possibly having a role directly feeding actin monomers to the WH2 domain [57]. Our results suggest that profilin binds to WHAMM, therefore possibly developing an important role in the delivery of actin monomers to the WH2 domains of WHAMM. Future work is required in order to conclude if profilin also binds to JMY.

Further analysis on the interaction between RhoD and ZIPK provided important information regarding its role, particularly in focal adhesion dynamics. It was previously observed by other group members that ZIPK induces an increase in focal

adhesion size [54]. FAK localisation in focal adhesions resulted in an increased interest regarding the role of both ZIPK and RhoD in FAK activation. Our data shows that overexpression of ZIPK reduces the phosphorylation of the tyrosine residue 397 (Y397), and that this effect is reversed by combined overexpression of RhoD. Integrin-mediated activation of FAK releases its kinase domain from the auto-inhibitory conformation, leading to immediate phosphorylation of Y397 [58]. As a consequence, Y397 phosphorylation is directly associated with FAK activation. Consequently, our results show that ZIPK reduces FAK activation, while RhoD counteracts this effect. Previous studies show that ROCK1 phosphorylates and activates ZIPK [59]. Our study demonstrates an important link between RhoA and RhoD signalling pathways, where RhoA/ROCK1 activates ZIPK and RhoD inactivates it.

In line with the previous results, we tried to connect this signalling pathway including RhoD, ZIPK and FAK with Src. It is known that activated FAK forms a complex with Src kinase, triggering several signalling pathways, such as the Ras-Erk signalling pathway [58]. Our data suggests that RhoD overexpression reduces the size of actin bundles, and this effect seems to be dependent on FAK as well as on RhoD membrane-bound localisation. However, Src does not seem to be essential for this process. FAK has been associated with tumour progression and metastasis, and its overexpression has been found in many types of cancer [58]. Consequently, a better understanding of how RhoD regulates actin dynamics via FAK may provide valuable information for development of novel cancer therapies.

RhoD has also been shown to regulate cell migration. Previous studies show that fibroblasts and endothelial cells transfected with the active variant RhoD/G26V display reduced cell motility [56; 60]. In addition to this, previous results from this group demonstrate that RhoD and WHAMM are required in directed cell migration when a wound closure assay was preformed [50]. Our data strongly suggest that RhoD is required for BJhTERT fibroblasts chemotaxis towards a PDGF-BB gradient and that RhoD knockdown does not influence significantly cell motility. Additionally, we observed that in the absence of RhoD and significantly increased amount of cells started rounding up during the 4h period of the migration assay, when compared to the control. This most probably means that much more cells are dying when RhoD is absent. However, further studies with cell death assays are needed to confirm any possible involvement of RhoD with the regulation of cell death.

CONCLUSION

This study supports the involvement of RhoD in the regulation of the actin cytoskeleton, and hence that RhoD plays a central role in cell adhesion and cell migration. Our data demonstrates an important and novel link between ZIPK activating signalling pathways via RhoA/ROCK1 and deactivating signalling pathways via RhoD. Additionally, we provide compelling evidence that RhoD subcellular localisation is of paramount importance in the regulation of actin bundling via FAK. Lastly, we show that RhoD in required for cell chemotaxis.

Further studies are needed in order to provide a better understanding of the signalling pathways regulated by RhoD. As previously mentioned, Rho GTPases, as key regulator of a myriad of physiological processes, are often associated with several pathological conditions. RhoD, as a regulator of both cell adhesion and cell migration, might be involved in pathological processes such as cell invasion and metastasis in cancer. Consequently, it will be of great interest to study RhoD and the mechanisms underlying its physiological effects.

ACKNOWLEDGMENTS

I am truly thankful to my supervisor, Pontus Aspenström, for receiving me in his group, introducing me to the wonderful field of Rho GTPases. His guidance and patience were essential to the accomplishment of this one-year journey.

I am also sincerely grateful to my co-supervisor, Vishal Nehru, for patiently teaching me the practical skills I needed to develop this work.

I would like to show my gratitude to all members of Aspentröm's group at the Microbiology, Tumor and Cell Biology Department of Karolinska Institute: Annica, Katarina, Magdalena, Fabien, Alexandra, Despoina, Laura, Flo, Daniel and Kerstin.

Many thanks to Johan Kreuguer, for receiving me in his lab for a short period. I am also truly thankful to Johan Heldin for kindly teaching me during that period.

Also, I want to thank to my friends in Uppsala, Marina, André, Daniel, Xana and Leonor for their constant support and friendship.

Last but not least, I could not have done this work without the support from my family, especially from my parents and my brother. Although being a particularly tough year, it is good to feel that even being 3,500 km apart we are always together no matter what.

REFERENCES

1. Alberts B., Johnson A., Lewis J., Raff M., Roberts K. and Walter P. (2008) "Molecular biology of the cell" 5th ed., Garland Science, New York, Abingdon.
2. Bearer E. L. (1993) "Role of actin polymerization in cell locomotion: molecules and models" Am J Respir Cell Mol Biol, 8, 582-591.
3. Lodish H. F., Berk A., Matsudaira P., Kaiser C. A., Krieger M., Scott M. P., *et al.* (2003) "Molecular cell biology" 5th ed., W. H. Freeman, New York.
4. Kabsch W., Mannherz H. G., Suck D., Pai E. F. and Holmes K. C. (1990) "Atomic structure of the actin:DNase I complex" Nature, 347, 37-44.
5. Bremer A. and Aebi U. (1992) "The structure of the F-actin filament and the actin molecule" Curr Opin Cell Biol, 4, 20-26.
6. Prasain N. and Stevens T. (2009) "The actin cytoskeleton in endothelial cell phenotypes" Microvasc Res, 77, 53-63.
7. Stossel T. P. (1993) "On the crawling of animal cells" Science, 260, 1086-1094.
8. Goley E. D. and Welch M. D. (2006) "The ARP2/3 complex: an actin nucleator comes of age" Nat Rev Mol Cell Biol, 7, 713-726.
9. Nurnberg A., Kitzing T. and Grosse R. (2011) "Nucleating actin for invasion" Nat Rev Cancer, 11, 177-187.
10. Ding Z., Bae Y. H. and Roy P. (2012) "Molecular insights on context-specific role of profilin-1 in cell migration" Cell Adh Migr, 6, 442-449.
11. Rottner K., Hanisch J. and Campellone K. G. (2010) "WASH, WHAMM and JMY: regulation of Arp2/3 complex and beyond" Trends Cell Biol, 20, 650-661.
12. Campellone K. G., Webb N. J., Znameroski E. A. and Welch M. D. (2008) "WHAMM is an Arp2/3 complex activator that binds microtubules and functions in ER to Golgi transport" Cell, 134, 148-161.
13. Zuchero J. B., Coutts A. S., Quinlan M. E., Thangue N. B. L. and Mullins R. D. (2009) "p53-cofactor JMY is a multifunctional actin nucleation factor" Nat Cell Biol, 11, 451-459.

14. Shikama N., Lee C. W., France S., Delavaine L., Lyon J., Krstic-Demonacos M., *et al.* (1999) "A novel cofactor for p300 that regulates the p53 response" Mol Cell, 4, 365-376.
15. Campellone K. G. and Welch M. D. (2010) "A nucleator arms race: cellular control of actin assembly" Nat Rev Microbiol, 11, 237-251.
16. Carlsson L., Nystrom L. E., Sundkvist I., Markey F. and Lindberg U. (1977) "Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells" J Mol Biol, 115, 465-483.
17. Carlsson L., Nystrom L. E., Lindberg U., Kannan K. K., Cid-Dresdner H. and Lovgren S. (1976) "Crystallization of a non-muscle actin" J Mol Biol, 105, 353-366.
18. Yarmola E. G. and Bubb M. R. (2009) "How depolymerization can promote polymerization: the case of actin and profilin" BioEssays, 31, 1150-1160.
19. Mockrin S. C. and Korn E. D. (1980) "Acanthamoeba profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine 5'-triphosphate" Biochemistry, 19, 5359-5362.
20. Ostrander D. B., Gorman J. A. and Carman G. M. (1995) "Regulation of profilin localization in Saccharomyces cerevisiae by phosphoinositide metabolism" J Biol Chem, 270, 27045-27050.
21. Machesky L. M., Atkinson S. J., Ampe C., Vandekerckhove J. and Pollard T. D. (1994) "Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose" J Cell Biol, 127, 107-115.
22. Rotty J. D., Wu C. Y. and Bear J. E. (2013) "New insights into the regulation and cellular functions of the ARP2/3 complex" Nat Rev Mol Cell Biol, 14, 7-12.
23. Veltman D. M. and Insall R. H. (2010) "WASP Family Proteins: Their Evolution and Its Physiological Implications" Mol Biol Cell, 21, 2880-2893.
24. Ammer A. G. and Weed S. A. (2008) "Cortactin branches out: roles in regulating protrusive actin dynamics" Cell Motil Cytoskeleton, 65, 687-707.
25. Rojas A. M., Fuentes G., Rausell A. and Valencia A. (2012) "The Ras protein superfamily: evolutionary tree and role of conserved amino acids" J Cell Biol, 196, 189-201.

26. Vega F. M. and Ridley A. J. (2008) "Rho GTPases in cancer cell biology" FEBS Letters, 582, 2093-2101.
27. Aspenstrom P., Ruusala A. and Pacholsky D. (2007) "Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases" Exp Cell Res, 313, 3673-3679.
28. Jaffe A. B. and Hall A. (2005) "Rho GTPases: biochemistry and biology" Annu Rev Cell Dev Biol, 21, 247-269.
29. Bos J. L., Rehmann H. and Wittinghofer A. (2007) "GEFs and GAPs: critical elements in the control of small G proteins" Cell, 129, 865-877.
30. Rossman K. L., Der C. J. and Sondek J. (2005) "GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors" Nat Rev Mol Cell Biol, 6, 167-180.
31. Etienne-Manneville S. and Hall A. (2002) "Rho GTPases in cell biology" Nature, 420, 629-635.
32. Iden S. and Collard J. G. (2008) "Crosstalk between small GTPases and polarity proteins in cell polarization" Nat Rev Mol Cell Biol, 9, 846-859.
33. Pruyne D. and Bretscher A. (2000) "Polarization of cell growth in yeast. I. Establishment and maintenance of polarity states" J Cell Sci, 113 (Pt 3), 365-375.
34. Schwamborn J. C. and Puschel A. W. (2004) "The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity" Nat Neurosci, 7, 923-929.
35. Daub H., Gevaert K., Vandekerckhove J., Sobel A. and Hall A. (2001) "Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16" J Biol Chem, 276, 1677-1680.
36. Miralles F., Posern G., Zaromytidou A. I. and Treisman R. (2003) "Actin dynamics control SRF activity by regulation of its coactivator MAL" Cell, 113, 329-342.
37. Glotzer M. (2001) "Animal cell cytokinesis" Annu Rev Cell Dev Biol, 17, 351-386.
38. Ridley A. J. and Hall A. (1992) "The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors" Cell, 70, 389-399.

39. Ridley A. J., Paterson H. F., Johnston C. L., Diekmann D. and Hall A. (1992) "The small GTP-binding protein rac regulates growth factor-induced membrane ruffling" Cell, 70, 401-410.
40. Nobes C. D. and Hall A. (1995) "Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia" Cell, 81, 53-62.
41. Hall A. (1998) "Rho GTPases and the Actin Cytoskeleton" Science, 279, 509-514.
42. Eden S., Rohatgi R., Podtelejnikov A. V., Mann M. and Kirschner M. W. (2002) "Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck" Nature, 418, 790-793.
43. Innocenti M., Zucconi A., Disanza A., Frittoli E., Areces L. B., Steffen A., *et al.* (2004) "Abi1 is essential for the formation and activation of a WAVE2 signalling complex" Nat Cell Biol, 6, 319-327.
44. Morgan-Fisher M., Wewer U. M. and Yoneda A. (2013) "Regulation of ROCK activity in cancer" J Histochem Cytochem, 61, 185-198.
45. Ridley A. J., Schwartz M. A., Burridge K., Firtel R. A., Ginsberg M. H., Borisy G., *et al.* (2003) "Cell migration: integrating signals from front to back" Science, 302, 1704-1709.
46. Mitchison T. J. and Cramer L. P. (1996) "Actin-based cell motility and cell locomotion" Cell, 84, 371-379.
47. Ridley A. J. (2011) "Life at the leading edge" Cell, 145, 1012-1022.
48. Bovellan M., Fritzsche M., Stevens C. and Charras G. (2010) "Death-associated protein kinase (DAPK) and signal transduction: blebbing in programmed cell death" FEBS J, 277, 58-65.
49. Gomez del Pulgar T., Benitah S. A., Valeron P. F., Espina C. and Lacal J. C. (2005) "Rho GTPase expression in tumourigenesis: evidence for a significant link" BioEssays, 27, 602-613.
50. Gad A. K., Nehru V., Ruusala A. and Aspenstrom P. (2012) "RhoD regulates cytoskeletal dynamics via the actin nucleation-promoting factor WASp homologue associated with actin Golgi membranes and microtubules" Mol Biol Cell, 23, 4807-4819.

51. Chavrier P., Simons K. and Zerial M. (1992) "The complexity of the Rab and Rho GTP-binding protein subfamilies revealed by a PCR cloning approach" Gene, 112, 261-264.
52. Gad A. K. B. and Aspenström P. (2010) "Rif proteins take to the RhoD: Rho GTPases at the crossroads of actin dynamics and membrane trafficking" Cellular Signalling, 22, 183-189.
53. Jaiswal M., Fansa E. K., Dvorsky R. and Ahmadian M. R. (2013) "New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm" Biol Chem, 394, 89-95.
54. Nehru V., Almeida F. N. and Aspenstrom P. (2013) "Interaction of RhoD and ZIP kinase modulates actin filament assembly and focal adhesion dynamics" Biochem Biophys Res Commun, 433, 163-169.
55. Murphy C., Saffrich R., Grummt M., Gournier H., Rybin V., Rubino M., *et al.* (1996) "Endosome dynamics regulated by a Rho protein" Nature, 384, 427-432.
56. Murphy C., Saffrich R., Olivo-Marin J. C., Giner A., Ansorge W., Fotsis T., *et al.* (2001) "Dual function of rhoD in vesicular movement and cell motility" Eur J Cell Biol, 80, 391-398.
57. Chereau D., Kerff F., Graceffa P., Grabarek Z., Langsetmo K. and Dominguez R. (2005) "Actin-bound structures of Wiskott-Aldrich syndrome protein (WASP)-homology domain 2 and the implications for filament assembly" Proc Natl Acad Sci U S A, 102, 16644-16649.
58. Zhao J. and Guan J. L. (2009) "Signal transduction by focal adhesion kinase in cancer" Cancer Metastasis Rev, 28, 35-49.
59. Hagerty L., Weitzel D. H., Chambers J., Fortner C. N., Brush M. H., Loiselle D., *et al.* (2007) "ROCK1 phosphorylates and activates zipper-interacting protein kinase" J Biol Chem, 282, 4884-4893.
60. Tsubakimoto K., Matsumoto K., Abe H., Ishii J., Amano M., Kaibuchi K., *et al.* (1999) "Small GTPase RhoD suppresses cell migration and cytokinesis" Oncogene, 18, 2431-2440.

APPENDIX I

Nehru V., **Almeida F. N**., Aspenström P. (2013) “Interaction of RhoD and ZIP kinase modulates actin filament assembly and focal adhesion dynamics” Biochemical and Biophysical Research Communications, 433, 163-169.

Used with permission.

Biochemical and Biophysical Research Communications 433 (2013) 163–169

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Vishal Nehru, Francisca Nunes Almeida, Pontus Aspenström *

Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, P.O. Box 280, Nobels väg 16, SE-171 77 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 13 February 2013
Available online 26 February 2013

Keywords:
Rho GTPases
Actin
RhoD
ZIP kinase
Focal adhesion

ABSTRACT

RhoD is a member of the classical Rho GTPases and it has an essential role in the regulation of actin dynamics. Furthermore, RhoD also localizes to early endosomes and recycling endosomes, indicating additional roles in the regulation of endosome trafficking. A yeast two-hybrid screen identified Zipper-Interacting Protein Kinase (ZIPK) as a RhoD target. We found that RhoD interacts with ZIP kinase in a GTP dependent manner and modulates actin and focal adhesion reorganization. Interestingly, while ectopic expression of ZIPK in fibroblasts induces actin reorganization and actomyosin contraction seen as stress fiber bundling and membrane blebbing, the concomitant expression of active RhoD suppressed this phenotype. Previously, RhoD has been associated with focal adhesion regulation, and in line with this notion, we observed that ZIPK resulted in reorganization of focal adhesion and increased adhesion size. Importantly, the RhoD activity suppressed ZIPK-dependent effects on FAK activity, indicating a functional interplay between RhoD and FAK in the focal adhesion dynamics.

1. Introduction

Rho GTPases are key regulators in cellular pathways that control essential cellular functions, such as cell morphology, intracellular transport and cell migration [1]. Historically, there has been a major focus on the three classical Rho members RhoA, Rac1 and Cdc42. However, there is an increasing interest in the less studied Rho GTPases members, since there is an emerging awareness they are important signaling molecules [2–4]. We have focused on RhoD, since this Rho member harbors some unique cellular functions [5–9]. Similar to the rest of the classical Rho GTPases, RhoD binds and hydrolyses guanine nucleotides. However, in comparison to classical Rho members, RhoD has a much higher intrinsic nucleotide exchange activity [10]. This property is something RhoD shares with Rif, Wrch-1 and the tumor-associated Rac1 splice variant Rac1b [10,11]. Since the intracellular concentration of GTP exceeds the concentration of GDP by a factor of approximately 10 times, this means that RhoD is predominantly in a GTP-loaded conformation in resting cells. Till date, RhoD has not been found to be regulated by any guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs), which are the conventional positive and negative regulators of Rho GTPases [2]. Intriguingly, the fact that RhoD is likely to function in a constitutively active state, suggests that its activity is under regulatory regime that is distinct from the classical GEFs and GAPs. This regime could involve regulation at the transcriptional level or by posttranslational modifications, similar to what has been found for the Rnd subfamily of Rho GTPases [12].

Several observations support the notion that the RhoD activity has a negative influence on cell migration. For instance, a study performed by Tsubakimoto et al., which employed a phagokinetic track assay, observed a decrease in cell migration in fibroblasts upon ectopically expressing the constitutively active variant RhoD/G26V [13]. Furthermore, Murphy et al., found that RhoD/G26V-expressing endothelial cells were effectively immotile, both in the presence and absence of a chemoattractant (basic fibroblast growth factor) [8]. In line with this concept, we observed that knock down of RhoD or its binding-partner WHAMM resulted in a decreased directed migration of human foreskin fibroblasts in a wound closure assay [14]. This indicated that over-activity, as well as under-activity of RhoD-dependent pathways, can affect cell migration in a negative manner. The RhoD-dependent effects on cell migration, is most likely linked to its profound effects on the organization of the actin filament system. RhoD was shown to trigger the formation of peripheral protrusions in several cell-types, including baby hamster kidney (BHK), HeLa, NIH3T3 and porcine aortic endothelial (PAE) cells [2,6]. The RhoD-related Rif also promotes the formation of long protrusions that emerge from the periphery or from the dorsal side of the cells [15]. Although, RhoD and Rif induce the same type of filopodia, there is a clear difference between the two Rho members: RhoD, but not Rif, localizes to early endosomes and has a role in endosome motility [6,8,16]. This

* Corresponding author. Fax: +46 8 330498.
E-mail address: pontus.aspenstrom@ki.se (P. Aspenström).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.02.046

clearly suggests that RhoD is a working at the interface between actin reorganization and membrane trafficking [9].

In order to gain insight into the mechanisms underlying the RhoD-dependent cellular effects, we sought to identify RhoD-binding partners that could provide clues to this regulation. The yeast two-hybrid system is a powerful tool for the study of protein: protein interactions and, using this technique, we identified Zipper-interacting protein kinase (ZIPK) as a candidate RhoD-binding protein. ZIPK, also known as death-associated protein kinase-3 (DAPK3) or DAP-like kinase (Dlk), is a member of the DAPK, serine/threonine protein kinase family, which also include DAPK, DAPK-related protein 1 (DRP-1), DAPK-related apoptosis-inducing protein kinases-1 and -2 (DRAK-1, and DRAK-2) [17–19]. Activation of these kinases is linked to death-associated cellular changes, such as membrane blebbing, cell rounding and the formation of autophagic vesicles [19]. ZIPK is considered to function as a tumor suppressor and mutations in the ZIPK gene has been found in tumors [20,21]. Interestingly, the DAPK proteins have previously not been associated with Rho GTPase signaling. In this article, we describe a novel role for RhoD in modulating ZIPK-dependent stress fibers bundling, membrane blebbing and focal adhesion dynamics.

2. Materials and methods

2.1. Antibodies and DNA work

The following antibodies were used: mouse anti-Myc (9E10) (Convance, Princeton, NJ, USA); monoclonal mouse anti-Flag (M2) (Sigma–Aldrich, St. Louis, MO, USA); rabbit anti-Myc, mouse anti-phospho-tyrosine (PY99) and rabbit anti-FAK (Santa Cruz Biotechnology, Santa Barbara, CA, USA); rabbit anti-FAK [pY397] and anti-FAK[pY576] (BioSource-Invitrogen, Carlsbad, CA, USA); TRITC-conjugated anti-mouse, and aminomethylcoumarin acetate (AMCA)-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Alexa Fluor 488-conjugated phalloidin (Molecular Probes-Invitrogen, Carlsbad, CA, USA) was used to visualize filamentous actin. The yeast two-hybrid screen has been described before [14]. FLAG-tagged cDNA constructs encoding human ZIPK were generous gifts from Tim Haystead, Duke University, Durham, NC, USA. The construction of pRK5Myc encoding the different mutants of Murine RhoD, has been described before [14].

2.2. Cell culture, transfection and immunoprecipitation

HEK293T cells and human foreskin BJ fibroblasts stably transfected with hTERT and SV40 Large T antigen (BJ/SV40 cells) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin. (HyClone, Thermo Scientific, Waltham, MA, USA). The cells were cultured at 37 °C in an atmosphere of 5% CO_2. The cells were transfected using JetPEI reagents (PolyPlus Transfection, Illkirch, France) according to the protocol provided by the manufacturer.

For the immunoprecipitation, the transiently transfected cells were lysed on ice in Triton X-100 buffer (20 mM HEPES, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1% aprotinin) 24 h post transfection. The lysed cells were collected in microcentrifuge tubes and centrifuged for 15 min at 4 °C. The supernatants were incubated together with the primary antibodies for 1 h, after which the immunoprecipitates were collected on protein G-Sepharose (GE Healthcare, Uppsala, Sweden) for 1 h at 4 °C. The beads were washed three times with Triton X-100 buffer and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE); subsequently they were transferred to Nitrocellulose (Hybond C, GE Healthcare, Uppsala, Sweden). Immunoblotting analyses were performed with the antibodies as specified in the figure legends, followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, Uppsala, Sweden). The Western blots were revealed using the Luminol immunoblotting reagent (Santa Cruz Biotechnology, Carlsbad, CA, USA).

2.3. Immunocytochemistry

The BJ/SV40T cells were seeded on coverslips and transfected using JetPEI. The cells were fixed 20–24 h post transfection in 3% paraformaldehyde in phosphate buffered saline (PBS) for 25 min at 37 °C, and washed with PBS. The cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min, washed again in PBS, and then 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 intervals of 1 h, followed by washing in PBS. The coverslips were mounted on microscopy slides using of Fluoromount-G (Southern Biotechnology Associates), and the cells were photographed using a Zeiss AxioVert 40 CFL microscope attached to a Zeiss AxioCAM MRm digital camera, and the AxioVision software. The cellular effects induced by ectopic expression were determined by microscopy analysis. At least 100 cells were scored for each transfection condition. The statistical analyses using Student's *t*-test throughout the study were based on experiments that had been repeated at least three times. Quantification of the mean focal adhesion size was made using the ImageJ software. Fifteen randomly selected fields of view from each condition were photographed and used for the image analysis. The experiment was repeated three times.

3. Results and discussion

3.1. ZIPK is a RhoD binding partner

Previously, we performed a yeast two-hybrid system screen with the constitutively active RhoD/G26V mutant fused to the DNA-binding domain of GAL4 as the bait, to screen a human mammary gland cDNA library fused to the GAL4 activation domain. In addition to the already described binding partner FILIP1 [14], we identified ZIPK as a potential RhoD binding-protein (Fig. 1A). ZIPK consists of a kinase domain at the N-terminus, putative nuclear localization signal (NLS) motives and a leucine-zipper (LZ) type of dimerisation domain and at the C-terminus (Fig. 1A). ZIPK been shown to regulate actin dynamics, primarily through phosphorylation of the Myosin regulatory light chain [22]. Since Rho GTPases have regulatory roles in actin dynamics, these findings make ZIPK an attractive candidate to regulate cytoskeletal reorganization downstream of RhoD. We performed an immunoprecipitation assay to study the RhoD binding-capacity. We transiently transfected FLAG-tagged ZIPK together with constitutively active (G26V) and dominant negative (T31N) mutants of RhoD in HEK293T cells. We found that ZIPK binds in a GTP-dependent manner to RhoD, since interacted with the active mutant of the GTPase and not to the dominant negative variant of RhoD (Fig. 1B). Furthermore, we tested the interaction between RhoD and a kinase-inactive mutant of ZIPK (D161A) and a mutant lacking the leucine-zipper domain (ΔLZ). We found that both mutants bound RhoD but ZIPK/ΔLZ had lost the GTP-dependency of the interaction (Fig. 1C).

3.2. RhoD activity can suppress the ZIPK-induces cell contraction and stress fiber bundling

We next wanted to study the role of ZIPK- and RhoD-induced effects on the reorganization of the actin filament system. To this

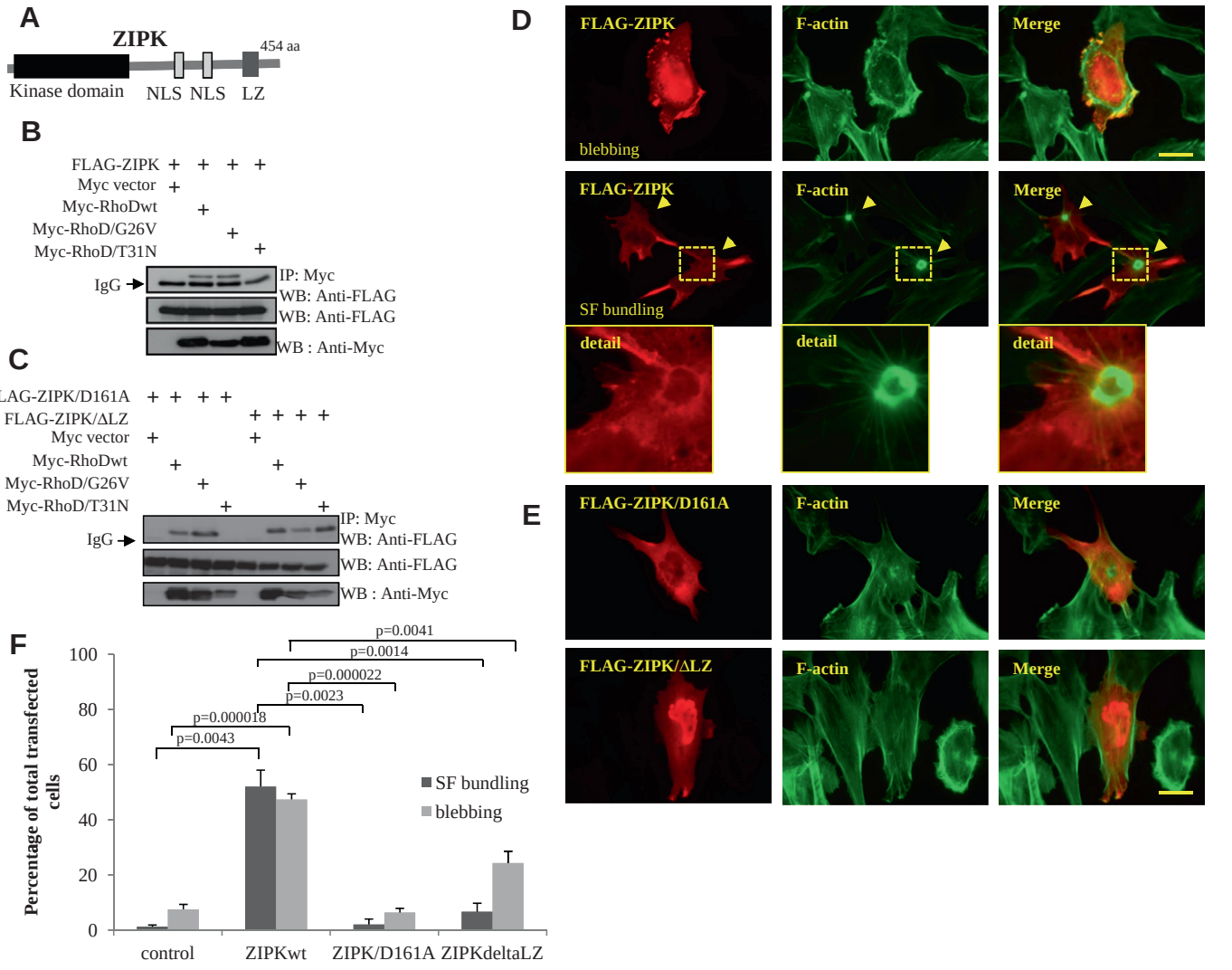


Fig. 1. ZIPK is a RhoD binding protein. (A) The domain organization of ZIPK. NLS = nuclear localization signal. (B) The interactions between constitutively active and dominant negative mutants of RhoD and ZIPK was analysed by immunoprecipitation in transiently transfected HEK293T cells. The presence of FLAG-ZIPK in the Myc precipitates was revealed by Western blotting, using anti-FLAG antibodies. (C) The interaction between the RhoD mutants and mutants of ZIPK was analysed by immunoprecipitation followed by Western Blotting to detect the ZIPK mutants in the RhoD immunoprecipitates. (D) FLAG-ZIPK transiently transfected in BJ/SV40T cells was visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Scale bar, 20 μm. Magnified images show detail of the star-shaped contracted actin bundles (arrowheads). (E) FLAG-ZIPK/D161A and ZIPK/ΔLZ were transiently transfected in BJ/SV40T cells and visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Scale bar, 20 μm. (F) Quantification of the cellular effects induced by ZIPK; bundling of stress fibers (SF) and membrane blebbing. At least 100 cells per experiment from three independent experiments were scored. The error bars represent standard deviation.

end, we transfected FLAG-tagged ZIPK in BJ/SV40T cells and stained the cells for filamentous actin. We found that ZIPK induced a dramatic reorganization of the actin filament system. This was seen as a condensation of stress fibers into thick bundles, often localized in a doughnut-shaped assembly with stress fiber bundles emanating from the ring in a star-like fashion (Fig. 1D quantification in F), similar to what has been reported in HeLa cells [22]. Ectopic expression of ZIPK also induced membrane blebbing, however, we rarely noticed membrane blebbing and stress fiber bundling in the same cells, instead the phenomena appeared mutually exclusive (Fig. 1D). In addition, the membrane blebbing was not associated with loss of cell adhesion. This unique actin-bundling activity was clearly dependent on the ZIPK kinase activity, since a kinase deficient mutant (ZIPK/D161) was unable to induce actin stress fiber bundling (Fig. 1E and F). In addition, the ZIPK mutant lacking the leucine zipper domain (ΔLZ) did not induce stress fiber bundling, however the membrane blebbing activity was less affected (Fig. 1F). In addition, we noticed that the wild-type and kinase-inactive variants of ZIPK were localized to the cytoplasm, in contrast to the ΔLZ mutant, which was predominantly localized in the cell nucleus (Fig. 1D and E). This indicates that the LZ domain is important for the regulation of the subcellular localization of ZIPK.

Interestingly, wild-type or the constitutively active mutant RhoD cotransfected with ZIPK effectively suppressed the ZIPK-induced stress fiber bundling (Fig. 2A and C). The constitutively active RhoD/G26V also suppressed membrane blebbing, effectively reverting the phenotype to normal fibroblast cells, however, the wild-type RhoD did not suppress the ZIPK-induced blebbing. In contrast, the dominant negative mutant RhoD/T31N was unable to suppress the ZIPK-dependent stress fiber bundling as well as membrane blebbing (Fig. 2B and C), demonstrating that the effect is dependent on the GTP-bound status of RhoD. Similarly, RhoD harboring a mutation in the membrane targeting CAAX motif of

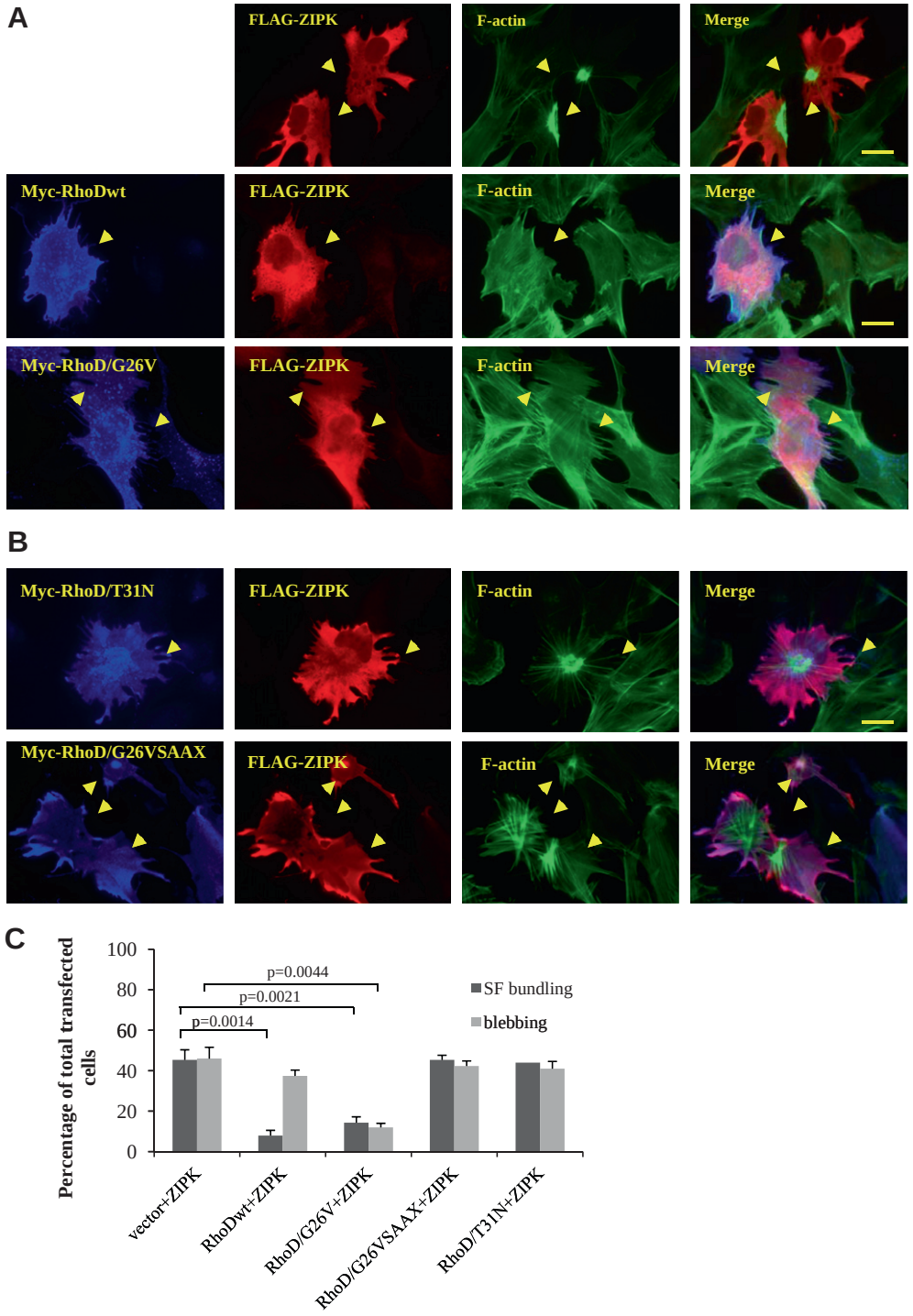


Fig. 2. RhoD suppresses the ZIPK-induced stress fiber dissolution. (A) FLAG-ZIPK was transiently transfected alone or together with RhoDwt or RhoD/G26V in BJ/SV40T cells. FLAG-ZIPK was visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Myc-tagged RhoD was visualized using rabbit anti-Myc antibodies followed by AMCA-conjugated anti-rabbit antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Arrowheads denote transfected cells. Scale bar, 20 μm. (B) FLAG-ZIPK was transiently transfected together with RhoD/T31N or RhoD/G26VSAAX in BJ/SV40T cells. FLAG-ZIPK was visualized using mouse anti-FLAG antibodies followed by TRITC-conjugated anti-mouse antibodies. Myc-tagged RhoD was visualized using rabbit anti-Myc antibodies followed by AMCA-conjugated anti-rabbit antibodies. Filamentous actin was visualized using Alexa Fluor488-conjugated phalloidin. Scale bar, 20 μm. Arrowheads denote transfected cells. (C) Quantification of the cellular effects induced by ZIPK; bundling of stress fibers (SF) and membrane blebbing. At least 100 cells per experiment from three independent experiments were scored. The error bars represent standard deviation.

RhoD (RhoD/SAAX) was unable to suppress the ZIPK phenotype, implicating membrane targeting of RhoD as an additional critical parameter (Fig. 2B and C). We could not find that ZIPK significantly affected the RhoD-dependent filopdia formation or RhoD localization to endosomes (Fig. 2A). ZIPK was not found in the RhoD-positive early endosomes, suggesting that ZIPK interacts with RhoD in other subcellular compartments, most likely at the plasma membrane.

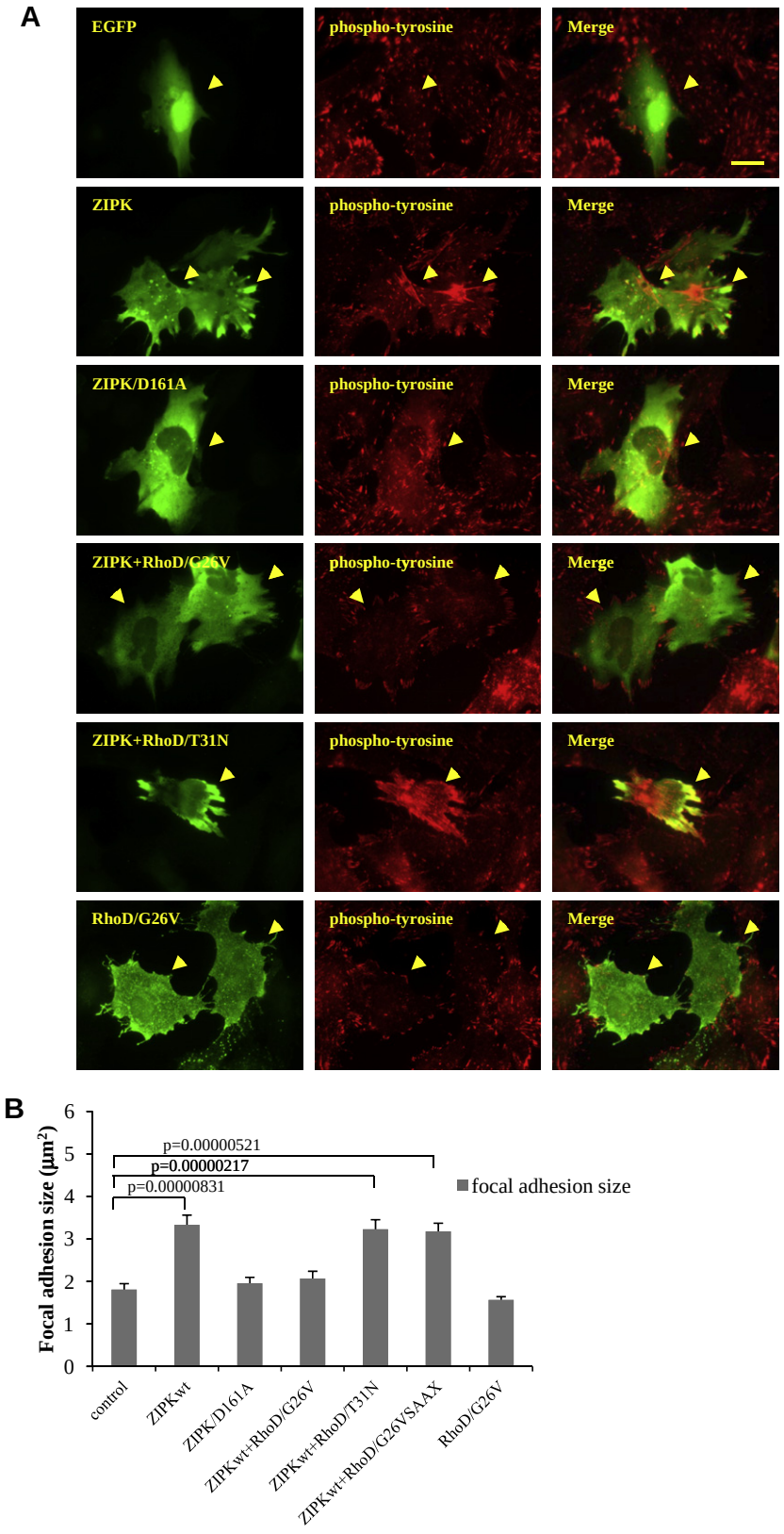


Fig. 3. ZIPK-induced focal adhesion dissolution requires an intact kinase activity. (A) EGFP (control), Myc-RhoD/G26V, FLAG-ZIPK or FLAG-ZIPK/D161A was transiently transfected in BJ/SV40T cells, or FLAG-ZIPK was transiently transfected together with Myc-RhoD/G26V or Myc-RhoD/G26DSAAX in BJ/SV40T cells. Transfected cells were visualized using rabbit anti-FLAG antibodies followed by Alexa Fluor488-conjugated anti-rabbit antibodies. Focal adhesion proteins were visualized with phospho-tyrosine-specific mouse antibodies followed by TRITC-conjugated anti-mouse antibodies. Scale bar, 20 μm. FLAG-ZIPK was visualized by rabbit anti-FLAG antibodies followed by Alexa Fluor488-conjugated anti-mouse antibodies. Focal adhesion proteins were visualized with a phospho-tyrosine-specific mouse antibody followed by TRITC-conjugated anti-mouse antibodies. Scale bar, 20 μm. (B) Quantification of the mean focal adhesion size, using the ImageJ software. Fifteen randomly selected fields of view from each condition were photographed and used for the image analysis. The experiment was repeated three times. The error bars represent standard error of the mean.

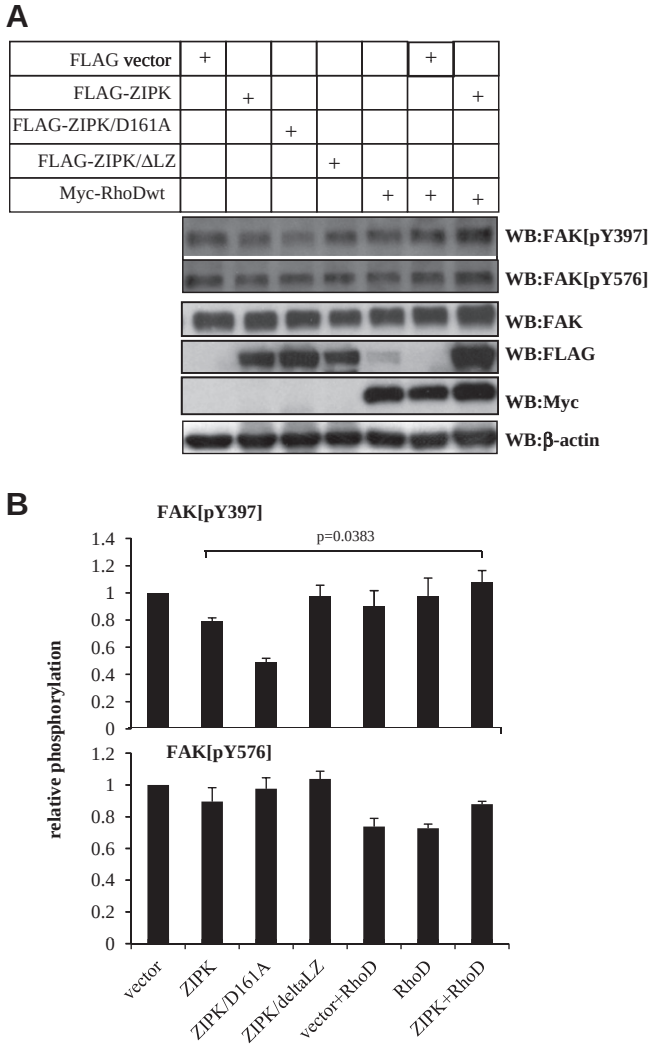


Fig. 4. ZIPK- and RhoD-dependent effects on FAK tyrosine phosphorylation. (A) The DNAs depicted in the figure were transiently transfected into BJ/SV40T cells. FAK phosphorylation at amino-acid residue Y397 or Y576 was determined by Western blotting using rabbit phosphospecific antibodies. (B) The alteration in phosphorylation relative to the vector control was determined by densitometry analysis using the ImageJ software. The data represent the mean of four experiments. The error bars represent standard error of the mean.

3.3. RhoD can modulate the ZIPK-induced focal adhesion dissolution

Since ZIPK had such a profound effect on stress fiber organization, we decided to analyze the effect on focal adhesion organization. To this end, we analyzed transiently transfected BJ/SV40T cells for focal adhesion assembly. ZIPK induced in a reorganization of focal adhesion components resulting in a dramatic increase in the focal adhesions size (from 1.8 μm^2 to 3.3 μm^2). This effect was dependent on an intact kinase activity, since the kinase inactive mutant ZIPK/D161A did not increase the focal adhesion size (Fig. 3A and B). Again, the simultaneous expression of constitutively active RhoD/G26V suppressed the ZIPK-dependent increase in focal adhesion size. This effect was dependent on the GTP-bound status and membrane targeting of RhoD, since neither the RhoD/T31N nor the RhoD/G26VSAAX mutants were unable to suppress ZIPK-induced focal adhesion size (Fig. 3A and B).

Focal adhesions dynamics is clearly associated with the activity of focal adhesion kinase (FAK). FAK is activated by integrin through disruption of an auto-inhibited conformation and FAK activation is positively correlated with the phosphorylated status of tyrosine residue 397 (Y397) [23,24]. ZIPK-expression in BJ/SV40T resulted in a marked decrease phospho-Y397; notably, the kinase deficient ZIPK/D161A also resulted in decreased phospho-Y397 (Fig. 4A and B). In contrast, the ZIPKΔLZ mutant did not result in decreased of phospho-Y397. Expression of RhoD alone, did not result in any significant alteration in Y397 phosphorylation, importantly, it suppressed the ZIPK-dependent decrease in phospho-Y397 (Fig. 4A and B). In contrast to phosphorylation on Y397, phosphorylation on tyrosine residue 576 was not significantly affected by ZIPK or RhoD expression (Fig. 4A and B).

4. Conclusions

We provide data demonstrating that RhoD interacts with ZIPK in a GTP-dependent manner and modulates stress fiber and focal adhesion reorganization. Rho GTPases have not previously been identified as binding partners for the DAPK proteins. However, there are indications that ZIPK can be regulated by Rho-dependent pathways via phosphorylation and activation by the RhoA-target Rho kinase 1 (ROCK1) [25]. Thus, our data suggest the existence of an intricate cross-talk between ZIPK activating signaling cues via RhoA/ROCK1 and inactivating signalling cues via RhoD. It will be of great future interest to study if additional DAPK members bind small GTPases, and to learn more about the signalling networks intercalating the activities of Rho GTPases and DAPK family kinases.

Acknowledgments

This study was supported by Grants to PA from Karolinska Institutet, Swedish Cancer Society and the Swedish Research Council.

References

[1] A.B. Jaffe, A. Hall, Rho GTPases: biochemistry and biology, Annu. Rev. Cell Dev. Biol. 21 (2005) 247–269.
[2] P. Aspenström, A. Fransson, J. Saras, The Rho GTPases have diverse effects on the organization of the actin filament system, Biochem. J. 377 (2004) 327–337.
[3] A. Boureux, E. Vignal, S. Faure, P. Fort, Evolution of the Rho family of ras-like GTPases in eukaryotes, Mol. Biol. Evol. 24 (2007) 203–226.
[4] S.J. Heasman, A.J. Ridley, Mammalian Rho GTPases: new insights into their functions from in vivo studies, Nat. Rev. Mol. Cell Biol. 9 (2008) 690–701.
[5] P. Chavrier, K. Simons, M. Zerial, The complexity of the Rab and Rho GTP-binding protein subfamilies revealed by a PCR cloning approach, Gene 112 (1992) 261–264.
[6] C. Murphy, R. Saffrich, M. Grummt, H. Gournier, V. Rybin, M. Rubino, P. Auvinen, A. Lütcke, R.G. Parton, M. Zerial, Endosome dynamics regulated by a Rho protein, Nature 384 (1996) 427–432.
[7] F. Shimizu, T.K. Watanabe, S. Okuno, Y. Omori, T. Fujiwara, E. Takahashi, Y. Nakamura, Isolation of a novel human cDNA (rhoHP1) homologous to rho genes, Biochim. Biophys. Acta 1351 (1997) 13–16.
[8] C. Murphy, R. Saffrich, J.C. Olivo-Marin, A. Giner, W. Ansorge, T. Fotsis, M. Zerial, Dual function of RhoD in vesicular movement and cell motility, Eur. J. Cell Biol. 80 (2001) 391–398.
[9] A.K.B. Gad, P. Aspenström, Rif proteins take to the RhoD: Rho GTPases at the crossroads of actin dynamics and membrane trafficking, Cell. Signal. 22 (2010) 183–189.
[10] M. Jaiswal, E. Kalawy Fansa, R. Dvorsky, R.M. Ahmadian, New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm, Biol. Chem. 394 (2012) 89–95.
[11] A. Shutes, A.C. Berzat, A.D. Cox, C.J. Der, Atypical mechanism of regulation of the Wrch-1 Rho family small GTPase, Curr. Biol. 14 (2004) 2052–2056.
[12] P. Chardin, Function and regulation of Rnd proteins, Nat. Rev. Mol. Cell Biol. 7 (2006) 54–62.
[13] K. Tsubikamoto, K. Matsumoto, H. Abe, J. Ishii, M. Amano, K. Kaibuchi, T. Endo, Small GTPase RhoD suppresses cell migration and cytokinesis, Oncogene 18 (1999) 2431–2440.
[14] A.K.B. Gad, V. Nehru, A. Ruusala, P. Aspenström, RhoD regulates cytoskeletal dynamics via the actin-nucleation-promoting factor WHAMM, Mol. Biol. Cell. 23 (2012) 4807–4819.
[15] S. Ellis, H. Mellor, The novel Rho-family GTPase rif regulates coordinated actin-based membrane rearrangements, Curr. Biol. 10 (2000) 1387–1390.

[16] S. Gasman, S. Kalaidzidis, M. Zerial, RhoD regulates endosome dynamics through diaphanous-related formin and Src kinase, Nat. Cell Biol. 5 (2003) 195–204.
[17] T. Kawai, M. Matsumoto, K. Takeda, H. Sanjo, S. Akira, ZIP kinase, a novel serine/threonine kinase which mediates apoptosis, Mol. Cell. Biol. 18 (1998) 1642–1651.
[18] D. Kögel, O. Plöttner, G. Landsberg, S. Christian, K.H. Scheidtmann, Cloning and characterization of Dlk, a novel serine/threonine kinase that is tightly associated with chromatin and phosphorylates core histones, Oncogene 17 (1998) 2645–2654.
[19] S. Bailik, A. Kimchi, The death-associated protein kinases: structure, function, and beyond, Annu. Rev. Biochem. 75 (2006) 189–210.
[20] D. Gozuacik, A. Kimchi, DAPK protein family and cancer, Autophagy 2 (2006) 74–79.
[21] J. Brognard, Y.-W. Zhang, L.A. Puto, T. Hunter, Cancer-associated loss-of-function mutations implicate DAPK3 as a tumor-suppressing kinase, Cancer Res. 71 (2011) 3152–3161.
[22] M. Murata-Hori, Y. Fukata, K. Ueda, T. Iwasaki, H. Hosoya, HeLa ZIP kinase induces diphosphorylation of myosinII regulatory light chain and reorganization of actin filaments in nonmuscle cells, Oncogene 20 (2001) 8175–8183.
[23] J. Zhao, J.-L. Guan, Signal transduction by focal adhesion kinase in cancer, Cancer Metastasis Rev. 28 (2009) 35–49.
[24] X. Peng, J.-L. Guan, Focal adhesion kinase: from in vitro studies to functional analyses in vivo, Curr. Protein Pep. Sci. 12 (2011) 52–67.
[25] L. Hagerty, D.H. Weitzel, J. Chambers, C.N. Fortner, M.H. Brush, D. Loiselle, H. Hosoya, T.A. Haystead, ROCK1 phosphorylates and activates zipper-interacting protein kinase, J. Biol. Chem. 282 (2007) 4884–4893.