SIGNALING BEHIND BARS

A ROLE FOR BAR DOMAINS

Bart-Jan de Kreuk
Signaling Behind BARs: A role for BAR domains

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GENERAL INTRODUCTION

Mechanisms of molecular communication within and between cells are characterized by stunning complexity based on a limited set of common principles. Extracellular signals including soluble ligands as well as cell- or extracellular matrix-associated proteins and lipids engage cell surface receptors, initiating a chain of intracellular signaling events that culminate in an appropriate response. The intracellular transport of such signals can occur through chains of sequential protein modifications such as phosphorylation, through random diffusion of conformationally altered molecules and through the use of physical means such as tightly controlled transport on membrane domains or vesicles. In order to better understand molecular interactions and mechanisms that govern signal transduction within the cell’s interior, classical biochemical methods need to be combined with modern approaches such as high resolution imaging and the use of selective removal of proteins by RNA interference.

Here, we focus on the regulation of signal transduction and cellular responses by two classes of proteins that play distinct, complementary roles in molecular cell biology. These are the family of BAR-domain proteins, non-enzymatic adapters that bind to lipids as well as proteins, bridging intercellular vesicular structures with the cellular signaling machinery. Secondly, we focus on the family of small GTPases, molecular switches that play key roles in cytoskeletal dynamics, cell morphology and cell adhesion and migration. Below, we will briefly introduce these protein families and provide additional background on their role in two exemplary processes, i.e. growth factor signaling and the control of cell-cell contact.

The BAR Domain Family

BAR-domain proteins are capable of sensing membrane curvature, e.g. generated by formation of clathrin coated pits (Doherty and McMahon, 2009). In addition, by binding as banana-shaped dimers (Peter et al., 2004) to negatively charged lipids, such as phosphoinositides and phosphatidylserine, they can further promote membrane curvature, which eventually leads to either invagination or protrusion depending on the type of BAR domain (Suetsugu et al., 2010). The first BAR domain was characterized almost two decades ago in Amphiphysin (David et al., 1994). Since then, many proteins were found to harbor a BAR domain. Interestingly, several different BAR-like domains were identified based on sequence homology and structural similarity, leading to the expansion of the BAR-domain protein superfamily. Currently, this family (Table 1) comprises proteins encoding one of six classes of BAR-domains: the classical BAR domain, or the N-BAR, BAR-PH, PX-BAR, F-BAR, and I-BAR domains (Frost et al., 2009; Qualmann et al., 2011; Suetsugu et al., 2010). As most BAR domain proteins can form oligomers, and contain one or more protein-binding scaffolding/adaptor domains (e.g. SH3 domains), they can form a molecular link between processes such as membrane dynamics and actin reorganization (Kessels and Qualmann, 2004; Kessels and Qualmann, 2006). As a result, BAR-domain containing proteins have emerged as important regulators of intracellular signaling pathways.
Membrane Binding and Deformation

Two major mechanisms allow BAR proteins to sense and bind to membranes resulting in the induction of membrane curvature. The first, also called the scaffold mechanism, involves the binding of BAR-domain proteins to membranes as intrinsically-curved crescent-shaped dimers (Qualmann et al., 2011). These dimers are lined with positively charged amino acids that allow binding to the negatively charged surface of membranes. The localization of these positive residues determines the direction of curvature induced by BAR proteins. In most cases, the concave surface is lined with these positive residues, which results in the induction of invaginations as is the case for the Arfaptin BAR- or the CIP4 F-BAR domain (Qualmann et al., 2011). In contrast, in the I-BAR domains, such as in IRSp53, these positively charged residues line the convex surface of the dimer thereby inducing outward protrusions such as filopodia (Mattila et al., 2007). Alternatively, proteins of the N-BAR subclass partially embed hydrophobic or amphipathic domains into the membrane (Qualmann et al., 2011). By inserting an amphipathic α-helix, the N-BAR proteins Endophilin and Amphiphysin (Gallop et al., 2006; Peter et al., 2004), can sense and induce membrane curvature, which is aided by the concave-surface located positively charged amino acids. In addition to the N-BAR sub-class, one member of the F-BAR subfamily was shown to insert a hydrophobic domain into the membrane to induce curvature. In conjunction with the positively charged amino-acids at the concave surface of the F-BAR dimer, PACSIN proteins use hydrophobic “wedge” loops to sense and induce membrane curvature (Shimada et al., 2010; Wang et al., 2009), a mechanism similar to that used by amphipathic helices of the N-BAR sub-class.

Functions of BAR Domain Proteins

In general, BAR domain proteins can act as adaptor proteins linking various processes. Most BAR domain proteins comprise one or more protein-binding scaffolding/adaptor domains (e.g. SH3 domains) with which they associate to regulatory proteins including Rac1, N-WASP, Dynamin, and Synaptojanin (Chitu and Stanley, 2007; de Kreuk et al., 2011; Kessels and Qualmann, 2004). In addition to these protein-protein interaction domains, some BAR domain proteins use their membrane-binding BAR domain to mediate protein-protein interactions. This was shown for IRSp53 that utilizes its I-BAR domain for both Rac1 binding and formation of protrusions (by creating outward curvature) (Abou-Kheir et al., 2008; Miki et al., 2000). As mentioned above, BAR proteins are known to oligomerize (Kessels and Qualmann, 2004; Kessels and Qualmann, 2006) which allows for multiple interactions. Due to this feature, several BAR proteins were found to provide a link between membrane dynamics, e.g. through interactions with Dynamin, and the actin cytoskeleton, e.g. through interactions with N-WASP. During endocytosis, BAR proteins are translocated to the plasma membrane promoting formation of tubular invaginations mediated by their BAR domain followed by the targeting of Dynamin to these tubular invaginations to ensure proper vesicle scission. Subsequently, BAR proteins can target the actin-polymerization machinery to these sites, via interactions with N-WASP, to provide force needed for vesicle internalization. In line with their role in endocytosis and vesicle transport, BAR proteins are also implicated in traffic of growth factor receptors, regulating receptor activation and downstream signaling (see below). Finally, several BAR proteins were shown to regulate activity and function of GTPases. It was shown that the BAR protein Arfaptin is an effector of Arf family GTPases. Arfaptin interacts with Rac1 as well providing a link between the Arf and Rho family signalling pathways (Tarricone et al., 2001).
In general, it can be concluded that BAR domain proteins are important regulators of many processes by providing docking platforms for the targeting of proteins to specific sites or pathways, or by linking different processes to ensure proper signaling.

Rho-Family GTPases

RhoGTPases constitute a distinct subfamily within the superfamily of Ras-related small GTPases and are involved in the regulation of cell polarity and motility through their effects on the actin cytoskeleton and membrane traffic (Jaffe and Hall, 2005; Ridley, 2006). The subfamily of RhoGTPases consists of 22 members (Wennerberg and Der, 2004). Interestingly, although they share very high sequence homology, RhoGTPases induce unique biological effects (Bishop and Hall, 2000; Bosco et al., 2009). This is partially mediated by differential binding of effector proteins to activated GTPases. Although some effectors are known to bind to several activated RhoGTPases, often each GTPase interacts with a specific subset of effectors thereby regulating specific cellular responses (Bishop and Hall, 2000). Furthermore, the hypervariable C-terminal region of the RhoGTPases contributes to differential cellular signaling as well. In addition to its role in subcellular targeting and control of signaling (Michaelson et al., 2001; ten Klooster and Hordijk, 2007; van Henrik et al., 2003), the C-terminal hypervariable region of RhoGTPases was shown to mediate protein-protein interactions. Our group has extensively studied binding partners of the Rac1 C-terminus and found several proteins, such as PACSIN2, Caveolin1, and β-Pix, that interact specifically with the hypervariable region, independent of the activation status of Rac1 (de Kreuk et al., 2011; Nethe et al., 2010; ten Klooster et al., 2006). Thus, although RhoGTPases share high sequence homology, hardly any sequence homology is found in their C-terminal hypervariable domain (Fig. 1A) explaining the large variety in interaction partners and biological outcomes. However, this will not explain the wide variety of biological effects exerted by a single RhoGTPase. In this case, specific localized signaling is secured by so-called “spatio-temporal signaling modules” (Pertz, 2010) which allows RhoGTPases to interact with different regulators at different intracellular locations.

Regulation of RhoGTPase Activity

RhoGTPases act as molecular switches (Fig. 1B). They cycle between an inactive GDP-bound state and an active GTP-bound state (Hall, 1998). This transition is regulated by guanine-nucleotide-exchange factors (GEFs) that facilitate the exchange of GDP for GTP (Rossman et al., 2005) and by GTPase-activating proteins (GAPs) that stimulate the low intrinsic GTPase activity thereby hydrolizing GTP to GDP (Bernards and Settleman, 2004). An important feature in the activation cycle is the translocation of RhoGTPases between the cytosol and the plasma membrane. Whereas, inactive RhoGTPases reside in the cytosol, bound to RhoGDI (Rho guanine nucleotide dissociation inhibitor), most activated RhoGTPases are localized at the plasma membrane. This translocation suggests an important role for vesicular

Figure 1: Regulation of RhoGTPases. (A) This schematic representation of a RhoGTPase shows the N-terminal effector domain (ED) and the C-terminal hypervariable domain (HV). Sequences of the hypervariable domain of different RhoGTPases are indicated. (B) Activation cycle of RhoGTPases. In the cytosol, inactive GDP-bound RhoGTPases reside in complex with RhoGDI. Upon translocation to the plasma membrane, RhoGTPases can be activated by GEF proteins. Inactivation of RhoGTPases is achieved either by targeting to a degradation pathway or by GAP proteins.
RhoGTPases as Regulators of Cell Adhesion and Migration

Cell migration is an essential feature of physiological processes such as development and wound healing. The actin cytoskeleton regulates the capacity of cells to migrate by controlling cell polarity, organization of adhesive structures, and the generation of force (Ridley et al., 2003). RhoGTPases, and in particular Rac1, Cdc42, and RhoA, are important regulators of cytoskeletal remodeling and thereby regulate cell adhesion and migration. In order to migrate, cells acquire a polarized morphology. At the front, Arp2/3-mediated actin assembly drives formation of flat membrane protrusions, called lamellipodia, mediated by Rac1-driven actin polymerization as well as Cdc42-mediated formation of finger-like protrusions called filopodia (Jaffe and Hall, 2005; Le Clainche and Carlier, 2008). Upon formation of these membrane protrusions at the front, the cell forms mature adhesions that connect the actin cytoskeleton to the extracellular matrix. Whereas Rac1 and Cdc42 are generally considered to control actin remodeling at the front of the cell, RhoA acts primarily at the rear where it regulates actomyosin contractility and disassembly of adhesions (Jaffe and Hall, 2005; Le Clainche and Carlier, 2008), driving forward migration. Recent studies, however, have revealed that RhoA activation occurs in the leading edge as well (Pertz et al., 2006). This suggests that the regulation of the actin cytoskeleton by RhoGTPases is far more complex than initially thought. Furthermore, cytoskeletal remodeling is regulated in a temporal and spatial manner and does not rely on individual RhoGTPases but rather on a carefully balanced interplay between different GTPases. This idea is supported by the observation that altering signaling of one specific RhoGTPase affects the levels, activity and function of other GTPases as well (Boulter et al., 2010) further underscoring the complexity of RhoGTPase signaling.

Control of Epithelial Cell-Cell Contact by RhoGTPases

Cohesive sheets of epithelial cells which cover most, if not all, body surfaces are a fundamental feature of multicellular organisms. The epithelium forms a physical barrier that separates the internal milieu of the body from its external environment (Perez-Moreno et al., 2003; Watanabe et al., 2009). An important feature of the epithelium regulating this barrier function is the presence of tightly controlled cell-cell adhesions that are essential for tissue integrity.

Adhesion between epithelial cells is generally mediated by three types of junctions: desmosomes, tight junctions, and adherens junctions (Perez-Moreno et al., 2003). Here, I will mainly focus on tight- and adherens junctions, as the role of RhoGTPase signaling in desmosomes seems minimal. One major role of tight junctions is to act as anatomical fences separating the apical from the basolateral domains of the plasma membrane (Diamond, 1977). On the other hand, adherens junctions play key roles in tissue sorting during development (Stepniak et al., 2009). Furthermore, both tight- and adherens junctions are important focal sites for anchorage of the actin cytoskeleton. In addition, these junctions can function as signaling hubs by their local concentration of downstream effectors (Citi et al., 2011). Tight junctions harbor several different proteins such as occludin, claudin, and ZO-1 (Citi et al., 2011). Adherens junctions are characterized by the presence of cadherins, which are calcium-dependent transmembrane receptors. Intracellularly, the catenins (α, β, γ, and p120-catenin), form a complex with the cadherins (Perez-Moreno et al., 2003), linking the transmembrane cadherin to the actin cytoskeleton.

The actin cytoskeleton which is anchored to the junctional complexes, has a profound role in junction assembly, disassembly, and maturation (Mege et al., 2006). As RhoGTPases are important regulators of cytoskeletal remodeling, their role in epithelial junction remodeling has been extensively studied. By promoting the formation of lamellipodia, Rac1 was implicated in the initiation of epithelial cell-cell contacts (Citi et al., 2011; Yamada and Nelson, 2007). In contrast to Rac1, the contribution of which in junction remodeling is broadly accepted, the role of RhoA in cell-cell contact is less clear. Some studies indicate that RhoA signaling is important for junction disassembly, e.g. it was shown that during epithelial-mesenchymal transition, RhoA activity is important for cadherin-contacts disruption (Bhowmick et al., 2001). Similarly, Schlegel and coworkers showed that increased RhoA activity caused epithelial barrier breakdown. However, they also showed that RhoA activity was needed to maintain barrier function (Schlegel et al., 2011) indicating a dual function for RhoA in barrier regulation. Several other studies showed that RhoA is involved in stabilization of junctions as well (Baum and Georgiou, 2011; Braga et al., 1997). An elegant study from Yamada and Nelson,
which involved high-resolution live-cell imaging of RhoGTPase biosensors, showed the differential roles of Rac1 and RhoA in junction remodeling. Whereas Rac1 has a key role in initiating adhesive contacts, RhoA regulates expansion and completion of cell-cell contacts (Yamada and Nelson, 2007). Recently we identified a novel mechanism through which Rac1 regulates junctional integrity which is described in Chapter 6. Rac1 activity targets the E3 ligase Nedd4 to epithelial junctions thereby stabilizing the junctions. Here, Nedd4 regulates the ubiquitylation and subsequent degradation of the scaffold protein Dvl1, which was shown to be a negative regulator of epithelial cell-cell contacts (Elbert et al., 2006; Nethe et al., 2012).

Thus, RhoGTPases are key regulators of epithelial junction remodeling and a tightly controlled balance between the different RhoGTPases and their activities is required to maintain and modulate epithelial barrier integrity.

**Growth Factor Signaling**

Growth factor signaling, including through receptor tyrosine kinases, is key to fundamental processes such as proliferation, migration, and cell survival (Blume-Jensen and Hunter, 2001; van der Geer et al., 1994). Logically, in several human diseases such as cancer, aberrant expression and activation of growth factor receptors has been observed. Internalization and intracellular trafficking is central to growth factor signaling. One of the best studied growth factor receptor-mediated pathways is that induced by Epidermal Growth Factor (EGF). EGF binding to the extracellular domain of the EGF receptor leads to receptor autophosphorylation within the intracellular regions and activation. Subsequently, several downstream signaling pathways, such as the Erk and Akt pathways are activated (Gan et al., 2010). The level of EGF receptors on the cell surface is an important determinant for the outcome of downstream signaling, further underscoring the importance of endocytic traffic in the regulation of growth factor signaling.

**Endocytic Trafficking of the EGF Receptor**

In resting cells, the EGF receptor is constitutively internalized in the absence of ligand. Upon internalization, the EGF receptor traverses the endosomal compartment after which most of the receptor recycles to the plasma membrane (Fig. 2; left panel). As the EGF receptor internalization rate is much slower than its recycling rate, the majority of the EGF receptors are localized to the surface (Herbst et al., 1994). Upon ligand binding, the EGF receptor is rapidly internalized and targeted to early endosomes (Fig. 2; right panel). In addition to signaling from the plasma membrane, compartmentalized EGF receptor signaling from early endosomes occurs which is important for specific signaling pathways such as the Erk pathway (Sigismund et al., 2008). To limit continuous signaling, the EGF receptor is subsequently targeted either to lysosomes for degradation or it recycles (in its inactive conformation) back to the plasma membrane (Sorkin and Goh, 2008; Wiley, 2003). In both cases, signaling is terminated.

To avoid aberrant signaling, transport of the EGF receptor, as well as of other growth factor receptors, through the different endocytic compartments is under tight control. Through their role in regulating membrane dynamics, the BAR-domain family of proteins has been implicated in growth factor receptor transport trafficking. Some BAR domain proteins such as endophilin are involved in the internalization of the EGF receptor, controlling the initial steps of trafficking (Soubeyran et al., 2002). Other BAR-family proteins, such as CIP4 and the sorting nexins (SNX) 1 and 5 regulate late events in EGF receptor trafficking (Hu et al., 2009; Kurten et al., 1996; Liu et al., 2006). Instead of regulating EGF receptor internalization, they are involved in the sorting of the EGF receptor. Once internalized and targeted to early endosomes, these proteins regulate the fate of the EGF receptor by either targeting the receptor to lysosomes for degradation or to the recycling compartment.

Lack of control at any of these locations, e.g. through improper functioning of BAR proteins, could lead to delayed receptor degradation with increased and prolonged signaling as a result. In turn, this could lead to abnormal cell proliferation or migration which could possibly promote development of diseases such as cancer.
SCOPE OF THE THESIS

In this thesis, I wish to discuss the regulatory role of BAR-domain proteins in cellular control. In particular, I focused on the role of BAR-domain proteins in regulating RhoGTPase signaling and growth factor signaling. In addition, I focused on the Rac1-mediated regulation of epithelial cell-cell contacts.

In Chapter 2 we provide an overview of BAR-domain proteins involved in the regulation of RhoGTPases. BAR-domains are important modules functioning at the interface between the actin cytoskeleton and membrane dynamics. In this chapter, we discuss how membrane dynamics (e.g. endocytosis and vesicle traffic), regulated by BAR-domains, affects GTPase activation and function.

In Chapter 3 we identify the F-BAR domain protein PACSIN2 as an important negative regulator of Rac1 activation and signaling. We show that PACSIN2 regulates internalization of the small GTPase Rac1 thereby targeting Rac1 to intracellular sites for GAP-mediated inactivation. As a result, loss of PACSIN2 results in increased cell spreading and migration. This study identified a novel role for PACSIN2 in regulating Rac1 activation and signaling.

In Chapter 4 we characterize a novel role for the BAR-domain containing human minor histocompatibility antigen-1 (HMHA1) as a RhoGAP. Previously, HMHA1 was only studied in the context of its role in forming a histocompatibility barrier in transplantation. We show that HMHA1 regulates RhoGTPase activity and as a consequence affects the actin cytoskeleton and cell spreading and migration. In addition, biochemical studies suggest that HMHA1 is a genuine RhoGAP. This study identified a previously unknown role for HMHA1 in regulating RhoGTPase activation and function.

In Chapter 5 we identify a novel role for the F-BAR protein PACSIN2 in growth factor receptor activation and signaling. We demonstrate that PACSIN2 negatively regulates Epidermal Growth Factor (EGF) receptor activation and signaling by controlling receptor surface expression. Loss of PACSIN2 increases surface levels of the EGF receptor and, as a consequence, increases receptor activation and downstream signaling in response to EGF. Interestingly, we show that the role of PACSIN2 is not specific for the EGF receptor as signaling downstream of HGF in epithelial cells, but also in primary endothelial cells downstream of TNFα, is increased in PACSIN2 depleted cells as well.

In Chapter 6 we report a novel pathway by which Rac1 stimulates maturation of cell-cell contacts in epithelial cells. Rac1 triggers translocation of the HECT E3 ligase Nedd4 to cell-cell junctions. We show that Rac1 activity triggers Nedd4-mediated ubiquitylation of the scaffold protein Dvl1, a negative regulator of epithelial cell-cell contacts, and this could be prevented when Rac1 is inhibited. We also show that loss of Nedd4 results in decreased degradation of Dvl1 and as a consequence decreased junctional integrity. We demonstrate that Nedd4-mediated ubiquitylation is important as Nedd4 mutants, incapable of ubiquitylating, failed to induce Dvl1 degradation.

Moreover, Dvl1 mutants, insensitive to Nedd4-mediated ubiquitylation, decrease junctional integrity to an even greater extent than wild-type Dvl1. This study identified a novel pathway in which Rac1, in conjunction with Nedd4 and Dvl1, promotes maturation of cell-cell contacts.


BELANGEVALISATION OF THE THESIS
Chapter 1

Contains two motifs that control targeting and signaling specificity. J. Biol. Chem. 278, 39166-39175.


CONTROL OF RHOGTPASE FUNCTION BY BAR-DOMAINS

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ABSTRACT

Cytoskeletal dynamics are key to the establishment of cell polarity and the consequent coordination of protrusion and contraction that drives cell migration. During these events, the actin and microtubule cytoskeleton act in concert with the cellular machinery that controls endo- and exocytosis, thus regulating polarized traffic of membranes and membrane-associated proteins. Small GTPases of the Rho family orchestrate cytoskeletal dynamics. Rho GTPase signaling is tightly regulated and mislocalization or constitutive activation may lead to, for example, morphogenetic abnormalities, tumor cell metastasis or apoptosis. There is increasing evidence that traffic to and from the plasma membrane constitutes an important mechanism controlling Rho GTPase activation and signaling. This brief overview discusses a group of proteins that function at the interface between membrane dynamics and Rho GTPase signaling. These proteins all share a so-called BAR domain, which is a lipid- and protein binding region that also harbors membrane deforming activity. In the last 15 years, a growing number of BAR domain proteins have been identified and found to regulate Rho GTPase signaling. The studies discussed here define several modes of Rho GTPase regulation through BAR-domain containing proteins, identifying the BAR domain as an important regulatory unit bridging membrane traffic and cytoskeletal dynamics.

INTRODUCTION

Rho GTPases constitute a distinct subfamily within the superfamily of Ras-related small GTPases and are involved in the regulation of cell polarity and motility through their effects on the actin cytoskeleton, membrane traffic and cell adhesion (Jaffe and Hall, 2005; Ridley, 2006). Rho GTPases act as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. This transition is regulated by guanine-nucleotide-exchange factors (GEFs) that promote the exchange of GDP for GTP (Rossman et al., 2005) and by GTPase activating proteins (GAPs) that stimulate the low intrinsic GTPase activity (Bernards and Settleman, 2004). While activated Rho GTPases generally are localized at the plasma membrane, inactive Rho GTPases, with some exceptions, e.g. Rhob, associate with a cytosolic chaperone Rhoguanine nucleotide dissociation inhibitor (RhoGDI) (Garcia-Mata et al., 2011).

Increasing evidence indicates that traffic to and from the plasma membrane is an important event controlling Rho GTPase signaling. For example, active Rac1 resides in cholesterol-enriched membrane domains (del Pozo and Schwartz, 2007) and cell detachment can trigger internalization of these domains resulting in the inactivation of Rac1. Thus, internalization plays a key role in the regulation of Rac1 activity. In line with this, it was shown that the large GTPase Dynamin, which is involved in endocytosis, plays an indispensable role in Rac1 traffic. Dynamin inhibition results in an increase in Rac1 activity (Schlunck et al., 2004). This is accompanied by a relocation of active Rac1 to aberrant dorsal ruffles which results in inhibition of cell spreading and lamellipodia formation (Schlunck et al., 2004). Conversely, Rho GTPases control endocytosis and membrane dynamics. For example, Cdc42 regulates the uptake of GPI-anchored proteins and bacterial toxins via the CLIC/GEEC pathway which functions independently from clathrin or Caveolin-mediated internalization (Sabharanjak et al., 2002). Furthermore, constitutively active Rac1 and RhoA can inhibit clathrin-mediated endocytosis (Lamaze et al., 1996; Qualmann and Mellor, 2003). Thus, membrane traffic and its regulation are tightly linked to Rho GTPase activation and signaling.

In a recent study, we showed that the adapter protein PACSIN2 regulates the activity of Rac1. PACSIN2 is an F-BAR and SH3-domain-containing protein which is involved in membrane dynamics such as tubulation and internalization. Our findings suggest that PACSIN2 controls cell spreading and migration by targeting Rac1 to intracellular compartments for GAP-mediated inactivation (de Kreuk et al., 2011). PACSIN2 is part of the BAR-domain family of proteins that are important regulators of membrane dynamics. Currently, this family comprises proteins encoding one of six classes of BAR-domains: the archetypical BAR domain, or N-BAR, BAR-PH, PX-BAR, F-BAR, and l-BAR domains (Qualmann et al., 2011). BAR-domain proteins are capable of sensing membrane curvature and, by binding as banana-shaped dimers to phospholipids (the specificity of lipid binding depends on the type of BAR protein) they can further promote curvature, which eventually leads either to membrane...
invagination or protrusion depending on the type of BAR domain (Frost et al., 2009). As most BAR domain proteins can form dimers and contain one or more protein-binding scaffolding/adaptor domains, they link membrane dynamics to signaling proteins that control actin dynamics. As a result, many BAR-domain containing proteins are potentially important regulators of Rho GTPase-dependent signaling.

Here, we discuss the role of BAR-domain proteins in the regulation of Rho GTPases. So far, two classes of BAR-domain proteins have been characterized that affect Rho GTPase function: Proteins harboring a BAR domain that regulate Rho GTPase (Table 1) and proteins that, in addition to their BAR domain, encode a RhoGAP/GEF domain and regulate Rho GTPase activity (Table 2).

**REGULATION OF RHO GTPASE FUNCTION BY BAR DOMAIN PROTEINS THAT LACK A RHOGAP/GEF DOMAIN**

Over the past 15 years, several BAR-domain-containing proteins have been described that regulate the function of RhoGTPases (Table 1). These proteins are all structurally related and encode, next to the common BAR domain, one or more adapter- or scaffolding domains (Fig. 1). Recently, we have shown that the F-BAR domain protein PACSIN2 specifically interacts, through its SH3 domain, with the small GTPase Rac1. Via its F-BAR domain, PACSIN2 can bind to and induce invagination of the plasma membrane. We found that in HeLa cells, loss of PACSIN2 expression increases Rac1GTP levels and, as a consequence, promotes spreading and migration of cells. The effect of PACSIN2 on Rac1 activity depends on their association as well as on membrane binding, since a PACSIN2 BAR-domain mutant, deficient in membrane tubulation, fails to inactivate Rac1. Furthermore, we showed that inactivation of Rac1 by PACSIN2 is prevented when dynamin is inhibited. Our data therefore suggest a model in which PACSIN2, in conjunction with dynamin, promotes internalization of Rac1GTP, subsequently targeting it to intracellular sites for GAP-mediated inactivation (de Kreuk et al., 2011).

Another family of F-BAR domain-containing proteins that controls RhoGTPase function is the CIP4 family, consisting of CIP4 and Toca-1. Both CIP4 and Toca-1 interact with the small GTPase Cdc42 in fibroblasts (Aspenstrom, 1997; Ho et al., 2004), regulating Cdc42-dependent actin reorganization (Ho et al., 2004; Pichot et al., 2010). Activated Cdc42 interacts with Toca-1 and the N-WASP-WIP (WASP-Interacting Protein) complex which leads to activation of N-WASP and Arp2/3-mediated actin polymerization (Ho et al., 2004). Similar to Toca-1, CIP4 is an effector of activated Cdc42 (Aspenstrom, 1997). In addition, CIP4 promotes formation of invadopodia in breast cancer cells through the activation of N-WASP (Pichot et al., 2010). Both CIP4 and Toca-1 localize to membranes via their F-BAR domains where they act as scaffolding proteins for N-WASP and Cdc42. Whether the F-BAR domain is dispensable for this function. Several BAR-domain-containing proteins have been shown to regulate Rho GTPase function. These proteins encode, in addition to their common BAR domain, one or more adaptor- or scaffolding domains. Abbreviations for domains are as follows: CRIB, Cdc42/Rac1 interactive binding domain; F-BAR, Fes/CIP4 homology Bin/Amphiphysin/Rvs; I-BAR, inverted-Bin/Amphiphysin/Rvs; HR1, homology region 1 (Cdc42-binding domain); SH3, Src homology 3; WH2 (like), Wiskott-Aldrich homology 2 (like). Numbers indicate the number of amino acids. Drawings are not to scale.
protrusions through the interaction with and activation of Rac1 (both processes Zheng et al., 2010). MIM(B) binds and bundles actin filaments and induces membrane curvature), for Cdc42, the I-BAR domain mainly functions to create outward curvature. Unlike IRSp53, which mediates signals from both Rac1 and Cdc42, Abba and recruit Rac1 effectors that drive actin assembly (Bompard et al., 2005; Machesy and Johnston, 2007). Abba regulates plasma-membrane- and actin dynamics as well and interacts with Rac1 via its IMD/I-BAR domain, similar to MIM(B) (Saarkangas et al., 2008). Abba localizes with active Rac1 in membrane ruffles and was shown to bind to both wild-type and constitutively active Rac1 (Zheng et al., 2010). PDGF treatment enhanced the Abba-Rac1 interaction and an Abba mutant, deficient in Rac1 binding, prevented Rac1 activation and induction of membrane ruffling by PDGF (Zheng et al., 2010). These results reveal an important role for Abba in Rac1 signaling downstream of the PDGF receptor.

Thus, it is clear that BAR-domain proteins play key roles in regulating RhoGTPases and that the BAR domain itself is important for this function. Although BAR-domain proteins have similar structures, the mechanisms by which they regulate GTPases differ. Whereas some are targeted, via their BAR domain, to specific sites to control GTPase traffic (eg. PACSIN2), or act in concert with GTPases to ensure efficient activation of downstream signaling (eg. Toca-1), others form a physical link via their BAR domain between GTPases and their upstream activators (eg. Abba) or downstream effectors (eg. IRSp53). Moreover, some of the BAR-domain proteins (eg. Toca-1) act either as positive regulators or signal transducers, whereas others (eg. PACSIN2) serve to downregulate GTPase output.

REGULATION OF RHOGTPASE FUNCTION AND ACTIVATION BY BAR DOMAIN-CONTAINING GAPS OR GEFS

A large number of RhoGEF and RhoGAP proteins have been identified so far (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). More recently, several of these GAP/GEF proteins were shown to contain a BAR domain as well (Table 2) and to have important functions in controlling the activity and consequently the function of RhoGTPases. Similar to the BAR-domain proteins described in the previous section, these BAR-GAP/GEF proteins are structurally similar in that they all harbor a BAR domain, a GAP/GEF domain, and one or more scaffolding domains/regions (Fig. 2).

The Slit-Robo (sr)GAPs are critical for neuronal migration because of their inactivation of RhoGTPases. Four different family members (srGAP1-4) have been characterized (Carlson et al., 2011; Guerrier et al., 2009; Tribioli et al., 1996; Wong et al., 2001). Slit proteins are secreted, cell- or extracellular matrix-associated proteins that guide neuronal migration through binding to the transmembrane Robo receptors. Slit proteins increase the interaction between Robo1 and srGAP1 which results in the activation of srGAP1 and consequent inactivation of GTPases (Wong et al., 2001). Whereas srGAP1 regulates Cdc42, both srGAP2 and srGAP3 mediate their function through inactivation of Rac1. The srGAP2 F-BAR domain promotes formation of filopodia-like membrane protrusions and neurite outgrowth in cortical neurons.
(Guerrier et al., 2009). Thus the F-BAR and RhoGAP domain of srGAP2 cooperate to regulate neuronal cell migration. The third family member, srGAP3/WRP, is part of the WAVE1 complex (Soderling et al., 2002). WAVE1 induces actin polymerization downstream of activated Rac1 (Takenawa and Miki, 2001). As part of the WAVE1 complex, srGAP3/WRP functions as a signal-termination factor for Rac1 through its Rac1-GAP activity (Soderling et al., 2002). Furthermore, srGAP3/WRP regulates spine development through F-BAR domain-dependent formation of dendritic filopodia, and loss of srGAP3/WRP results in impaired long-term memory in mice (Carlson et al., 2011). Finally, the less-well characterized srGAP family member srGAP4/p115, is predominantly expressed in hematopoietic cells and was shown to stimulate the intrinsic GTPase activity of RhoA and to inhibit stress-fiber formation (Tribioli et al., 1996). Furthermore, srGAP4/p115 associates to MEKK1, thereby reducing MEKK1-induced signaling to the transcription factor AP-1 (Christerson et al., 2002). However, additional studies are necessary to understand the biological function of srGAP4/p115 and the role of the F-BAR domain in this process.

Similar to srGAPs, RICH1 (also called Nadrin), is a BAR domain-containing protein which also possesses a RhoGAP domain. RICH1 shows GAP activity towards Cdc42, Rac1, and RhoA (Harada et al., 2000; Richnau and Aspenstrom, 2001). 3T3 fibroblasts expressing full-length RICH1 or its isolated GAP domain were unable to form membrane ruffles after PDGF stimulation (Richnau and Aspenstrom, 2001). Furthermore, RICH1 localizes to tight- and adherens junctions in epithelial cells, mediated through its interaction with the adaptor protein Amot. RICH1 associates via its BAR domain with the ACCH domain of Amot (Heller et al., 2010; Wells et al., 2006). This interaction inhibits RICH1 function preventing RICH1 from properly downregulating activated Cdc42. In addition, Amot induces relocation of the polarity proteins Pals1 and Par-3. Thus, RICH1 in conjunction with Amot maintains the integrity of tight junctions through the regulation of Cdc42 activity and trafficking of polarity proteins (Wells et al., 2006). As an additional member of this family, RICH2, a RacGAP, was identified as a regulator of the actin cytoskeleton in epithelial cells. RICH2 and Ezrin interact with the integral membrane protein CD317 linking it to the actin cytoskeleton at the apical surface of polarized epithelial cells (Rollason et al., 2009). As RICH2 inhibits formation of Rac1-induced membrane ruffles (Richnau and Aspenstrom, 2001), its presence in this complex possibly ensures proper regulation of actin cytoskeleton remodeling at the apical side of polarized epithelial cells. The

Figure 2: BAR-Domain-containing RhoGAP/GEF proteins. Several BAR-domain proteins have been characterized that harbor in addition to their BAR domain also a RhoGAP/GEF domain. In addition, they encode of one or more scaffolding- or adaptor domains. Abbreviations for domains are as follows: BAR, Bin/Amphiphysin/ Rvs; C1, cysteine-rich phorbol ester binding; F-BAR, Fes/CIP4 homology Bin/Amphiphysin/Rvs; PH, pleckstrin homology; RhoGAP, Rho GTPase activating protein; RhoGEF, Rho guanine-nucleotide-exchange factors; SH3, Src homology 3. Numbers indicate the number of amino acids. Drawings are not to scale.

Table 2: BAR-Domain-containing proteins that harbor a RhoGAP or RhoGEF domain. This table shows BAR-domain-containing proteins, harboring a GAP/GEF domain, involved in regulation of Rho GTPases. GAPase specificity, the type of BAR domain, presence of GAP/GEF domain, ARHGAP synonym, and the Uniprot KB accession number are indicated.
exact role of the BAR domain is not known in this process although it could well be that RICH2 is targeted to the membrane (where it interacts with CD317) via the lipid-binding properties of the BAR domain.

In addition to the srGAP family and the RICH family, one more family of BAR-domain containing RhoGAP proteins is expressed in mammalian cells. The GRAF (GTPase regulator associated with focal adhesion kinase-1) family consists of 4 members, GRAF-1 and Oligophrenin-1. GRAF proteins play a role in the clathrin-independent endocytosis pathway CLIC/GEEC (Doherty and Lundmark, 2009). GRAF1 exhibits GAP activity towards RhoA and Cdc42 and binds to Focal Adhesion Kinase (FAK) via its SH3 domain (Hildebrand et al., 1996). Moreover, GRAF1 regulates the uptake of, for example, GPI-anchored proteins and bacterial toxins via the CLIC/GEEC pathway and internalization via this pathway was shown to be dependent on Cdc42 activation (Lundmark et al., 2008; Sabharanjak et al., 2002). Through its BAR domain, GRAF1 localizes to tubular and vesicular membranes that define the CLIC/GEEC pathway. Here, GRAF1 regulates internalization of cargo by regulating the activity of Cdc42 via its GAP domain. Depletion of GRAF1, leading to impaired CLIC/GEEC function, reduces cell spreading and migration (Doherty et al., 2011) indicating the importance of well-coordinated membrane dynamics and protein traffic in the control of cell shape and motility.

A close relative of GRAF1, Oligophrenin-1, stimulates GTP hydrolysis of Cdc42, Rac1, and RhoA (Billuart et al., 1998). Through the regulation of GTPase activity and the interaction with endophilin A1, Oligophrenin-1 controls synaptic vesicle endocytosis (Nakano-Kobayashi et al., 2009). Oligophrenin-1 was also shown to be involved in cognitive impairment (Billuart et al., 1998). As malfunctions in synaptic vesicle recycling are linked to cognitive defects (Di et al., 2002) it could well be that Oligophrenin-1-associated cognitive impairment is caused by a defect in synaptic vesicle traffic due to improper Oligophrenin-1 signaling. A third GRAF family member, GRAF2, also known as PSGAP, has been shown to interact with PYK2 which is structurally related to FAK. PYK2 binds to the GRAF2 SH3 domain thereby inhibiting its RhoGAP function. This results in activation of Cdc42 and cytoskeletal reorganization (Ren et al., 2001). The exact role of the GRAF2 BAR domain needs further investigation but it could well be involved in targeting of GRAF2 to sites where GTPase regulation is required.

Finally, two more BAR-RhoGAP proteins have been characterized so far, GMIP and SH3BP1 (Aresta et al., 2002; Cicchetti et al., 1995). GMIP associates with the Ras-related protein Gem which is involved in regulating voltage-gated Ca2+ channels and cytoskeletal reorganization (Aresta et al., 2002; Beguin et al., 2001). Gem, which binds Ezrin at the plasma membrane, downregulates RhoA-dependent stress fibers via its interaction with GMIP which exhibits GAP activity towards RhoA but not Cdc42 and Rac1 (Aresta et al., 2002; Hatzoglou et al., 2007). The exact role of the GMIP BAR domain remains unclear. However, it was shown that the GMIP-Gem interaction is mediated via the GMIP N-terminal part which harbors the BAR domain (Aresta et al., 2002). Similar to IRSp53 (Miki et al., 2000), GMIP possibly uses its BAR domain for protein-protein interactions.

SH3BP1 exhibits GAP activity towards the Rac family GTPases and was shown to inhibit PDGF-induced membrane ruffling (Cicchetti et al., 1995). Furthermore, it was shown that SH3BP1 binds Exo84 and Sec8, both exocyst components, in a BAR domain-dependent fashion (Parrini et al., 2011). Together with the exocyst, SH3BP1 is targeted to the leading edge of polarized, motile cells. Here it mediates cell migration by regulating the activity of Rac1. Loss of SH3BP1 causes formation of disorganized instable protrusions (Parrini et al., 2011). Thus at the leading edge, in concert with GEF-mediated activation of Rac1, SH3BP1 ensures proper Rac1 inactivation to mediate efficient cell migration.

Whereas several RhoGAP proteins encode BAR domains, only one BAR-RhoGEF protein, called Tuba, has been described so far. Tuba has four N-terminal SH3 domains, a central DH domain followed by a BAR domain and two C-terminal SH3 domains (Fig. 2). Tuba was shown to exhibit GEF activity towards Cdc42 but not Rac1 and RhoA (Cestra et al., 2005; Salazar et al., 2003). As Tuba can bind both Dynamin and actin-regulatory proteins such as N-WASP and WAVE1 (Salazar et al., 2003), Tuba was proposed to be an important link between endocytosis, actin dynamics, and GTPase signaling (Salazar et al., 2003). Furthermore, Tuba activates Cdc42 and subsequently atypical PKC, thereby regulating polarized spindle orientation in epithelial cells (Qin et al., 2010). To be functional, RhoGEF proteins generally need a DH-PH motif. The DH domain forms the catalytic core while the PH domain can be involved in plasma membrane targeting and in protein-protein interactions (Rossman et al., 2005). In general, DH domains without adjacent PH domains are less active than those that are flanked by a PH domain (Rossman et al., 2005). Intriguingly, it was shown that the Tuba DH domain showed little activity compared to the DH-BAR fragment (Salazar et al., 2003). This suggests that the BAR domain of Tuba acts as a substitute for a PH domain.

It is clear that BAR-GAP/GEF proteins are important regulators of GTPase activation and consequent signaling. In general, the BAR domain is important for the targeting to membranes and to sites of actin dynamics where they can induce membrane curvature. In addition, the BAR domain can mediate protein-protein interactions. Thus, the BAR domain and GAP/GEF domain cooperate to regulate processes dependent on membrane traffic and actin remodeling including cell spreading, cell polarization and motility. It is perhaps not coincidental that apparently more RhoGAPs than RhoGEFs encode BAR domains. GTPase activation is generally associated with the translocation to the plasma membrane. Although it is not as firmly established that turning off GTPase signaling requires the reverse process, e.g. GTPase internalization, there is accumulating support for this notion, based on previous studies showing that e.g. dynamin, caveolin-1 and PACSIN2 are all required for proper Rac1 inactivation. The fact that also many RhoGAPs encode BAR domains therefore suggest a functional link between membrane traffic and termination of GTPase signaling.
CONCLUDING REMARKS

Over the past 15 years, a series of BAR domain-containing proteins have been characterized that are linked to Rho GTPase signaling pathways. The BAR domain itself, through its capacity to bind lipids as well as proteins, plays an important role in the regulation of Rho GTPase activity and output. BAR domains play important roles in the targeting of proteins to specific regions within the plasma membrane where actin remodeling is necessary (e.g. for formation of protrusions or stimulating endocytosis). At these sites, BAR-domain proteins can control Rho GTPase activity, either by regulating the activation status of Rho GTPases, as some of these proteins harbor a RhoGAP/GEF domain, or by linking Rho GTPases to their upstream activators (e.g. growth factor signaling) or to their downstream effectors (e.g. the actin machinery). Strikingly, the Rho GTPase-activating regulatory BAR domain proteins identified so far all harbor BAR, F-BAR, or I-BAR domains but not any of the other types of BAR domain. In conclusion, BAR-domain proteins are emerging as an important group of RhoGTPase regulators. As this field is relatively young and many previously identified proteins are now found to also include a BAR domain, it is very likely that in the near future much more BAR proteins that regulate Rho GTPase signaling will be identified. The challenge then lies in defining their contribution to the promotion or inhibition of localized GTPase signaling.

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REFERENCE LIST


THE F-BAR DOMAIN PROTEIN PACSIN2 ASSOCIATES WITH RAC1 AND REGULATES CELL SPREADING AND MIGRATION

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ABSTRACT
The Rac1 GTPase controls cytoskeletal dynamics and is a key regulator of cell spreading and migration mediated by signaling through effector proteins, such as the PAK kinases and the Scar and WAVE proteins. We previously identified a series of regulatory proteins that associate with Rac1 through its hypervariable C-terminal domain, including the Rac1 activator β-Pix (also known as Rho guanine nucleotide-exchange factor 7) and the membrane adapter caveolin-1. Here, we show that Rac1 associates, through its C-terminus, with the F-BAR domain protein PACSIN2, an inducer of membrane tubulation and a regulator of endocytosis. We show that Rac1 localizes with PACSIN2 at intracellular tubular structures and on early endosomes. Active Rac1 induces a loss of PACSIN2-positive tubular structures. By contrast, Rac1 inhibition results in an accumulation of PACSIN2-positive tubules. In addition, PACSIN2 appears to regulate Rac1 signaling; siRNA-mediated loss of PACSIN2 increases the levels of Rac1-GTP and promotes cell spreading and migration in a wound healing assay. Moreover, ectopic expression of PACSIN2 reduces Rac1GTP levels in a fashion which is dependent on the PACSIN2-Rac1 interaction, on the membrane-tubulating capacity of PACSIN2 and on dynamin. These data identify the BAR-domain protein PACSIN2 as a novel Rac1 interactor that regulates Rac1-mediated cell spreading and migration.

INTRODUCTION
Cell migration is an essential feature of physiological processes such as development, chemotaxis and wound healing. The capacity of cells to migrate is controlled by the actin cytoskeleton, which regulates cell polarity, the organization of adhesion structures and the generation of force. Such force is provided by actin polymerization at the front of polarized cells and actomyosin-based contraction at the rear (Ridley et al., 2003). In addition to localized actin polymerization, microtubule dynamics and vesicular transport are also important for cell migration. Intracellular vesicles regulate transport of signaling proteins to and from the leading edge and also control vesicular traffic, which is required for membrane protrusion at the leading edge. These events probably run in parallel, as many signaling molecules involved in cell motility can associate with the plasma membrane, as well as with intracellular vesicles. As a result, both endo- and exocytosis are increasingly recognized as important regulatory events, controlling cell motility in conjunction with the actin cytoskeleton (Fletcher and Rappoport, 2010; Scita and Di Fiore, 2010).

Cytoskeletal dynamics are controlled by small GTPases of the Rho family, in particular Rac1, Cdc42, and RhoA (Ridley et al., 2003). RhoGTPases cycle between an inactive GDP-bound state and an active GTP-bound state, and this transition is regulated by guanine-nucleotide-exchange factors (GEFs) that promote the exchange of GDP for GTP (Rossman et al., 2005) and by GTPase activating proteins (GAPs) that stimulate the low, intrinsic GTPase activity (Bernards and Settleman, 2004). Whereas most activated RhoGTPases are localized at the plasma membrane (del Pozo et al., 2000), inactive RhoGTPases associate with Rho guanine nucleotide dissociation inhibitor (RhoGDI), which is a cytosolic protein (del Pozo et al., 2002; Olofsson, 1999). Although there is increasing evidence that traffic to and from the plasma membrane constitutes an important aspect of RhoGTPase signaling, the underlying regulatory mechanisms are not well understood.

Among the family of Rho GTPases, Rac1 (Ras-related C3 botulinum toxin substrate 1) (Didsbury et al., 1989) is one of the most extensively studied members. Following activation, Rac1 can interact with a series of effector proteins, such as the PAK (p21 activated kinase) serine/threonine kinases, to trigger downstream signaling. Upon binding to activated Rac1, e.g. following integrin activation, PAK in turn becomes activated, regulating cytoskeletal dynamics, adhesion, and transcription (del Pozo et al., 2000; Price et al., 1998). In addition, Rac1 regulates formation of membrane ruffles and lamellipodia through members of the Scar and WAVE family of proteins (Tybulewicz and Henderson, 2009) that couple Rac1 to the Arp2/3 complex, which mediates actin polymerization (Chung et al., 2000).

In Rac1 proteins, the C-terminal hypervariable region is important for subcellular targeting and the regulation of signaling output (Michaelson et al., 2001; Nethe et al., 2010; Prieto-Sanchez and Bustelo, 2003; ten Klooster and Hordijk, 2007; van Hennik et al., 2003). In a proteomic screen for proteins associating with the C-terminus of Rac1,
we previously identified the Rac1 and Cdc42 GEF \( \beta \)-Pix (also known as Rho guanine-nucleotide-exchange factor 7) which recruits Rac1 to leading edge focal adhesions (FAs) and to the peripheral membrane (ten Klooster et al., 2006). In addition, we found a series of proteins that bind to the Rac1 C-terminus and translocate upon Rac1 activation. These include: the PP2A inhibitor SET, which translocates from the nucleus to the plasma membrane in response to Rac1 activity and cooperates with Rac1 in cell migration (ten Klooster et al., 2007), the adapter protein CD2-associated protein (CD2AP, also known as CMS), which translocates to cell-cell junctions following Rac1 activation and supports Rac1-dependent cell adhesion (van Duijn et al., 2010); and caveolin-1, which is recruited to focal adhesions by Rac1 activity and regulates the poly-ubiquitylation and degradation of Rac1 in an adhesion-dependent fashion (Nethe et al., 2010).

Here, we describe the identification of PACSIN2 (for ‘protein kinase C and casein kinase 2 substrate in neurons 2’) as a novel Rac1-binding protein that like CD2AP and caveolin-1, regulates endocytosis. PACSIN2, also known as Syndapin2, is a ubiquitously expressed, 486 amino acid membrane-associated adapter protein. Two additional PACSIN proteins exist in humans: PACSIN1, which is mainly expressed in brain tissues, and PACSIN3, which is expressed at high levels in skeletal muscle, heart and lung tissue (Modregger et al., 2000; Plomann et al., 1998; Ritter et al., 1999). PACSIN proteins form one branch of the Fer/CIP4 homology-BAR (F-BAR) domain protein family (Heath and Insall, 2008). They all share a highly conserved F-BAR domain at their N-terminus, which is a protein module that stabilizes and induces membrane curvature (Frost et al., 2009; Peter et al., 2004; Tsujita et al., 2006). The result of curvature induction by PACSIN proteins (and other F-BAR-domain-containing proteins) is the formation of invaginations and, subsequently, of vesicular-tubular structures that depend on the self-assembly of F-BAR modules into a helical coat (Frost et al., 2009).

In addition to the N-terminal F-BAR domain, PACSIN proteins harbor a central linker region, containing NPF motifs (in PACSIN1 and PACSIN2) or a proline-rich motif (PACSIN3), and a C-terminal Src Homology 3 (SH3) domain. Many proteins are known to bind the PACSIN SH3 domain, including dynamin, N-WASP and Saptonjan (Chitu and Stanley, 2007; Kessels and Qualmann, 2004). The F-BAR domain also mediates homo- and hetero-oligomerization of PACSIN proteins (Kessels and Qualmann, 2006), and this oligomerization is important for their capacity to act as adaptor proteins, linking the actin-regulatory proteins with the endocytic machinery (Kessels and Qualmann, 2004).

In this study, we show that the F-BAR protein PACSIN2 interacts, through its SH3 domain, with the C-terminus of Rac1. We show that reciprocal regulation affects both Rac1 and PACSIN2. Rac1 activity controls the subcellular distribution of PACSIN2, whereas PACSIN2 negatively regulates Rac1 activity, cell spreading and migration by promoting Rac1 inactivation. To our knowledge, this is the first report describing the regulation of Rac1 signaling output by an F-BAR-domain-containing protein.

**RESULTS**

PACSIN2 interacts via its Src homology 3 domain with the hypervariable domain of Rac1

In the course of a proteomic screen for proteins that bind to the Rac1 hypervariable C-terminus, we identified by mass spectrometry the F-BAR domain containing protein PACSIN2 as a novel Rac1 interactor. The interaction between Rac1 and endogenous PACSIN2 was confirmed by streptavidin-based pull-down assays using a bacterially expressed peptide encoding the C-terminus of Rac1 in lysates of COS7, HeLa and mouse embryonic fibroblast (MEF) cells (Fig. 1A). To test whether PACSIN2 also interacts with other Rho-family members, we performed a pull-down assay using bacterially expressed C-termini of related RhoGTPases. This experiment showed that PACSIN2 specifically interacts with the C-terminus of Rac1 (Fig. 1B). Using myc-tagged versions of the three PACSIN isoforms expressed in human (Modregger et al., 2000; Plomann et al., 1998; Ritter et al., 1999) we showed that the C-terminus of Rac1 can bind, with comparable efficiency, to PACSIN1, 2, and 3 (supplementary material Fig. S1A). To confirm that endogenous PACSIN2 also binds full-length Rac1 we performed pull-down assays with bacterially purified GST, GST-Rac1WT (wild-type protein), and GST-Rac1AC (which lacks the hypervariable domain) using cell lysates of Jurkat, COS7, and HeLa cells. These experiments showed that PACSIN2 interacts with the full-length Rac1 protein and that PACSIN2 binding to GST-Rac1AC is greatly reduced (Fig. 1C). This shows that the C-terminal domain of Rac1 is both necessary and sufficient for this interaction.

To study further the binding between PACSIN2 and Rac1 we performed pull-down experiments with bacterially purified GST and GST-PACSIN2 proteins using lysates of HeLa cells. These experiments showed that endogenous Rac1 interacts with GST-PACSIN2 (supplementary material Fig. S1B). Furthermore, using lysates of HeLa cells transfected with YFP-Rac1 WT, or the Rac1 Q61L, T17N, and V12 mutants, we showed that all constructs can bind to GST-PACSIN2, although the active mutants of Rac1 (Q61L and V12) bound more efficiently (Fig. 1D). To investigate further the relevance of the Rac1-PACSIN2 interaction we studied whether the endogenous proteins can interact. To test this, we isolated endogenous Rac1GTP with a bacterially expressed PAK1-CRIB domain (Price et al., 2003) and analyzed PACSIN2 binding to Rac1GTP. This experiment showed that endogenous PACSIN2 was found in complex with endogenous Rac1GTP (Fig. 1E). In addition, we studied whether Rac1 and PACSIN2 directly interact with each other. To study this, we used isolated GST and GST-PACSIN2 and performed a peptide pull-down assay using the bacterially expressed C-terminus of Rac1 and of several related RhoGTPases. This experiment showed that purified PACSIN2 interacts selectively with the Rac1 C-terminal peptide, indicating that this is a direct interaction (Fig. 1F).

The hypervariable C-terminus of Rac1 comprises two protein-binding motifs, a proline-stretch and a poly-basic region (Fig. 1G; upper panel) (van Hennik et al., 2003). Additional pull-down experiments using variant peptides of the Rac1 C-terminus...
(PPP>AAA; RKR>AAA) showed that binding of endogenous PACSIN2 is abolished when either the proline-stretch or the poly-basic region is mutated (Fig. 1G; lower panel). PACSIN2 does not interact with the 17-32 peptide (Fig. 1G; lower panel), which encodes part of the effector domain of Rac1 (Vastrik et al., 1999). Src homology 3 (SH3) domains are known to interact with proline-rich sequences (Li, 2005). To determine whether the PACSIN2 SH3 domain mediates the binding to Rac1 we performed pull-down assays with the Rac1 C-terminus as well as with GST-Rac1 using HeLa cells transfected with myc-tagged PACSIN2 WT or with an SH3 double mutant, PACSIN2-Y435E/P478L (Modregger et al., 2000). These mutations in PACSIN2 abolished the binding to the Rac1 C-terminus, as well as to full-length Rac1 (Fig. 1H; supplementary material Fig. S1C). Together, these data identify PACSIN2 as a novel interactor of the small GTPase Rac1.

Intracellular localization of PACSIN2

To study the intracellular localization of PACSIN2, we immunostained HeLa cells for endogenous PACSIN2 as well as for various markers and analyzed the distribution of these proteins by Confocal Laser Scanning Microscopy. The majority of PACSIN2 localized to perinuclear vesicles and to vesicular-tubular structures at the leading edge of polarized cells (Fig. 2A). Co-staining for F-actin showed no obvious colocalization of PACSIN2 with actin structures (Fig. 2A, upper panels). Similarly, co-staining for paxillin showed that PACSIN2 does not localize to focal adhesions (Fig. 2A, middle panels). Finally, co-staining for α-actinin, which localizes predominantly to membrane ruffles and cortical actin structures, showed that PACSIN2 in the leading edge localizes distal to, rather than in, membrane ruffles (Fig. 2A, bottom panels). In primary Human Umbilical Vein Endothelial Cells (HUVEC) and COS7 cells, endogenous PACSIN2 showed a similar subcellular distribution (supplementary material Fig. S2A,B). To confirm the specific detection of PACSIN2 in our immunostainings, we transfected HeLa cells with myc-tagged constructs of the three different PACSIN proteins. As expected, the antibody recognizes PACSIN2 and not PACSIN3 on Western blots (supplementary material Fig. S2C). The antibody cross-reacts to a limited extent with PACSIN1 (supplementary material Fig. S2C). However, PACSIN1 expression is

Figure 1: PACSIN2 interacts via its SH3 domain with the C-terminus of Rac1. (A) Pull-down (PD) experiments were performed using cell lysates from COS7, HeLa, and MEF with control peptide (Ctrl) or the Rac1 C-terminal peptide (Rac1), and associated PACSIN2 was detected by immunoblotting (IB). (B) Binding of PACSIN2 in HeLa cell lysates to the indicated GTPase C-terminal peptides shows specific binding to the Rac1-C-terminal peptide. β-Pix and SET (I2PP2A) were included as controls. (C) Full-length Rac1, but not Rac1 lacking the C-terminus, both fused to GST, associates with endogenous PACSIN2 in lysates from Jurkat, COS7, and HeLa cells. (D) Pull-down experiments using GST and GST-PACSIN2 (P2) were performed with lysates from HeLa cells transfected with YFP-Rac1 constructs as indicated. Association of YFP-Rac1 to GST-PACSIN2 was detected by immunoblotting. (E) To study the endogenous interaction, a CRIB-peptide pull-down for endogenous Rac1GTP was performed. Endogenous PACSIN2 in complex with Rac1GTP was detected by immunoblotting. (F) Peptide pull-down experiments with the indicated biotinylated GTPase C-termini were performed using purified GST or GST-PACSIN2 to study direct interactions. Association of purified GST-PACSIN2 to the peptides was detected by immunoblotting. (G) Use of modified peptides of the Rac1 C-terminus in pull-down experiments shows that both the proline-rich and poly-basic region in the Rac1 C-terminus mediate the interaction with endogenous PACSIN2. A Rac1 effector domain peptide (17-32) does not associate with PACSIN2. The Rac1 GEF β-Pix and the nuclear protein SET/I2PP2A, both known interactors of Rac1, were included as controls. (H) Pull-down experiments with the C-terminal peptide of Rac1 and of Rac2, as an additional control, were performed using cell lysates of HeLa cells transfected with the indicated myc-tagged PACSIN2 constructs. TCL, total cell lysates; PD, Pull-down; ED, Effector domain; HV, Hypervariable domain; PTD, protein transduction domain.
restricted to neuronal cells (Plomann et al., 1998) which is why we are confident that we
are only detecting PACSIN2 in the immunostainings. This was further confirmed by the
loss of immunostaining in cells transfected with the PACSIN2 siRNA (supplementary
material Fig. S2D).

We next studied PACSIN2 distribution in live cells. We transfected HeLa cells with
YFP- or mCherry-tagged PACSIN2 constructs and recorded their dynamics in real-
time. Similar to the distribution in fixed cells, PACSIN2 was found in the periphery,
at sites of membrane ruffling and in protrusions. Upon collapse of membrane ruffles,
PACSIN2 was internalized and co-localizes with newly formed elongated structures
that move towards the cell center and appear similar to the structures seen in fixed
(cells (Fig. 2B; supplementary material Movie 1). In addition, PACSIN2 was found
around perinuclear vesicles (supplementary material Fig. S2E). Together, these data
are in good agreement with published findings on the tubulating activity of PACSIN
proteins and the notion that PACSIN2 mediates endocytosis.

To determine whether these PACSIN2-containing tubular structures are indeed
generated by endocytosis, we incubated HeLa cells, expressing YFP-PACSIN2, with
various endocytic markers. In untransfected cells, internalized transferrin localized
to PACSIN2-positive perinuclear vesicles but not to the peripheral tubular structures
(Fig. 2C). Expression of PACSIN2 induced a reduction in internalized transferrin (data
not shown), in line with previous observations (Modregger et al., 2000; Qualmann
and Kelly, 2000). Furthermore, internalized Sulforhodamine101, which is a marker for
clathrin-independent fluid-phase endocytosis (Wubbolts et al., 1996), localized to
PACSIN2-negative tubular structures (supplementary material Fig. S2F). By contrast,
internalized Alexa594-labelled Cholera Toxin B (AF594-CtxB) which previously has
been used as a marker to label tubular-endocytic structures (Verma et al., 2010), co-
localized with PACSIN2 both in membrane patches and in peripheral tubular structures
(Fig. 2D). This supports the notion that the PACSIN2-positive tubules are generated
by endocytosis.

PACSIN2 colocalizes with Rac1 on early endosomes
To identify the PACSIN2-positive perinuclear vesicles (Fig. 2), HeLa cells were stained
for the early endosome marker (EEA1) (Mu et al., 1995) and for Rab5, which also
localizes to early endosomes (Christoforidis et al., 1999). These experiments showed
that the perinuclear, but not the peripheral pool of PACSIN2 resides on early endosomes

Figure 2: Intracellular localization of PACSIN2. (A) Intracellular localization of PACSIN2 was
studied by Confocal Laser Scanning Microscopy using HeLa cells. Endogenous PACSIN2 was
detected by immunostaining in combination with detection of F-actin, Paxillin, and α-Actinin.
A fraction of PACSIN2 localizes to vesicular-tubular structures that are most abundant in a
peripheral region, distal to the F-actin- and α-Actinin-rich membrane ruffles. PACSIN2 does not
localize to FAs. (B) Live-cell imaging of HeLa cells transfected with YFP-PACSIN2 shows that
internalized PACSIN2 is associated with vesicular-tubular structures that form and move inwards.

> upon collapse of membrane ruffles. Individual tubular structures are identified by the colored
arrowheads (see also supplementary material Movie 1). (C) Transferrin-Texas Red (10 µg/µl;
30 min.) labels PACSIN2-positive perinuclear vesicles. Endogenous PACSIN2 was visualized by
immunostaining. Peripheral tubular structures are indicated by arrowheads. The profile scan along
the indicated line is shown in the graph. (D) PACSIN2-positive peripheral tubules were labeled
with AF594-labelled CtxB (0.5 µg/µl; 10 min) in PACSIN2-transfected cells. Tubular structures are
indicated by arrowheads. Higher magnification images of the boxed area are included. Scale
bars, 10 μm.
This was further confirmed by transfecting HeLa cells with GFP-FYVE. FYVE domains have been shown to recognize PtdIns(3)P and to localize to early endosomes (Gillooly et al., 2003). In line with the data in Fig. 3A,B, PACSIN2 localizes to GFP-FYVE-positive early endosomes (supplementary material Fig. S2G). Together, these results show that the perinuclear, but not the peripheral pool of PACSIN2 localizes to early endosomes.

Given that PACSIN2 interacts with the small GTPase Rac1, it was important to establish if and where PACSIN2 and Rac1 colocalize. Immunostaining of HeLa cells for endogenous PACSIN2 and endogenous Rac1 showed that both proteins are present at perinuclear vesicles, which we had identified as early endosomes, as well as in peripheral membrane protrusions (Fig. 3C). To confirm that Rac1 in fact localizes to early endosomes, we immunostained HeLa cells transfected with GFP-FYVE for endogenous Rac1. In good agreement with the data in Fig. 3A-C, endogenous Rac1 was found to localize to FYVE-positive early endosomes (supplementary material Fig. S2H). In addition to the colocalization of endogenous Rac1 and PACSIN2 on early endosomes, we found that endogenous Rac1 localized to PACSIN2 tubules (supplementary material Fig. S2I, arrow) although this localization was less clear owing to the high levels of Rac1 in the cytoplasm. In line with this result, we found, in live-cell imaging experiments, that in cells transfected with mCherry-Rac1 WT and YFP-PACSIN2, both proteins colocalized on endocytic structures (supplementary material Movie 2).

PACSIN2 distribution is controlled by microtubules

Previously, it has been shown that PACSIN proteins can co-immunoprecipitate α- and γ-tubulin, and PACSINs have been suggested to function in microtubule (MT) assembly (Grimm-Gunter et al., 2008). Moreover, because the MT network is important in the control of vesicle transport, we tested to what extent PACSIN2 localization was controlled by MTs. We found that a fraction of peripheral PACSIN2 aligned with microtubules (Fig. 4A; supplementary material Fig. S3). In cells treated with nocodazole to depolymerize MTs, PACSIN2 distribution was altered in that the protein appeared restricted to vesicular structures that are evenly distributed throughout the cell. This was accompanied by a concomitant loss of the peripheral pool of PACSIN2 associated with vesicular-tubular structures (Fig. 4B; supplementary material Fig. S4A). Interestingly, whereas in control cells PACSIN2 only partially localized to early endosomes, after nocodazole treatment, all endogenous PACSIN2 was on early endosomes which were also dispersed (Fig. 4C and supplementary material Fig. S4B). Upon nocodazole treatment, the endogenous Rac1 distribution was dispersed similar to that of PACSIN2 (supplementary material Fig. S4C). The effect of nocodazole on PACSIN2 distribution was mimicked by expression of constitutively active RhoAV14 (supplementary material Fig. SSA) which is in line with published data showing that microtubule depolymerization activates RhoA (Enomoto, 1996). These data show that the microtubule network regulates the intracellular distribution of PACSIN2.

Rac1 signaling regulates PACSIN2 localization

Because PACSIN2 and Rac1 interact and co-localize, we next tested whether Rac1 controls PACSIN2 localization. Expression of a constitutively active Rac1 Q61L mutant drove endogenous PACSIN2 into the perinuclear pool, which was subsequently confirmed as comprising early endosomes, with a loss of peripheral PACSIN2 (Fig. 5A,B). To confirm this result, we treated HeLa cells with Cytotoxic Necrotizing Factor 1 (CNF1) to activate endogenous Rac1 (Lerm et al., 1999; Nethe et al., 2010). CNF1-mediated constitutive activation of Rac1, as with expression of Rac1 Q61L,
induced a loss of the peripheral pool of PACSIN2, concentrating PACSIN2 in the early endosomes (supplementary material Fig. S5B). Subsequent live-cell imaging studies showed that, whereas activated Rac1 induced a loss of peripheral PACSIN2 tubules, inhibiting Rac1 signaling by the expression of the inactive mutant Rac1 T17N induced an accumulation of PACSIN2-positive peripheral tubular structures (Fig. 6A, and Fig. 6B, top and middle panels). Interestingly, Rac1 WT as well as Rac1 T17N clearly colocalized with PACSIN2 on the tubular structures (Fig. 6A) in line with the results for endogenous Rac1 and PACSIN2 (supplementary material Fig. S2I). It is important to note that levels of expressed mCherry-Rac1 were similar to those of endogenous Rac1. Moreover, expression of Rac1 mutants did not affect the levels of endogenous Rac1 (Fig. 6B, bottom panel). In addition to the Rac1-T17N-mediated accumulation of PACSIN2-positive tubular structures, we found that, following treatment of

Figure 5: Rac1 signaling regulates PACSIN2 distribution. (A,B) Expression of myc-tagged Rac1Q61L concentrates endogenous PACSIN2 in a perinuclear region (A) at early endosomes as identified by co-transfection with GFP-FYVE (B). Higher magnification images of the boxed areas are also shown. Bars, 10 μm.

Figure 4: The microtubule network controls PACSIN2 intracellular distribution. (A) A fraction of PACSIN2 aligns with microtubules (MT, arrowheads). Endogenous PACSIN2 was detected by immunostaining in combination with α-Tubulin. (B,C) The MT network controls PACSIN2 and early endosome distribution. HeLa cells were treated with Nocodazole (10 μM, 30 min.) to depolymerize the MTs. Cells were allowed to reform microtubules after nocodazole washout for the indicated times, fixed and analyzed by Confocal Laser Scanning Microscopy. Endogenous PACSIN2, α-Tubulin, and EEA1 were detected by immunostaining. Following Nocodazole incubation, PACSIN2 (B) as well as early endosomes (C) become dispersed throughout the cell. This effect was reversible after 30-60 of washout. Loss of MTs was associated with a loss of PACSIN2-positive tubules. Higher magnification images of the boxed areas are shown in the bottom panels (Zoom). Bars, 10 μm.
YFP-PACSIN2-expressing HeLa cells with a pharmacological inhibitor of Rac1 (EHT1864) (Shutes et al., 2007), the number of PACSIN2 vesicular-tubular structures increased (supplementary material Fig. S5C). A similar effect was seen after siRNA mediated knock-down of Rac1 (Fig. 6C). Finally, we found that the SH3 domain mutant of PACSIN2, which cannot bind to Rac1, also promoted an accumulation of peripheral tubules, underscoring the notion that Rac1 activity, as well as Rac1 association with PACSIN2 is required to regulate the peripheral pool of PACSIN2 (supplementary material Fig. S5D). Together, these data show that Rac1 activity regulates the number of PACSIN2-positive tubular structures.

PACSIN2 is a negative regulator of Rac1 signaling

An important feature of cellular signaling pathways is the presence of feed-forward and feed-back loops. The data in Fig. 5 and 6 show that Rac1 signaling regulates PACSIN2 distribution. In a complementary set of experiments, we tested whether PACSIN2, in turn, regulates Rac1. Using siRNA-transfection, we found that loss of PACSIN2 induces an increase in GTP loading of endogenous Rac1 of between twofold and fourfold (Fig. 7A), suggesting that PACSIN2 negatively regulates Rac1 signaling. In line with these data, we found that loss of PACSIN2 promotes cell spreading on fibronectin, a Rac1-dependent response (del Pozo et al., 2000; Price et al., 1998) (Fig. 7B; upper panel). The three different siRNAs to PACSIN2 all induced a similar increase in cell spreading as analysed by electric cell substrate impedance sensing (ECIS) (Fig. 7B, lower panel), which is in good agreement with the increased Rac1GTP levels in these cells. Subsequent analysis of cell migration in a wound-healing assay (Lorenowicz et al., 2008) also showed that siRNA mediated knock-down of PACSIN2 increased migration of HeLa cells (Fig. 7C).

We also tested the effect of PACSIN2 overexpression on GTP loading of Rac1. In line with the above data suggesting that PACSIN2 is a negative regulator of Rac1, we found an 80% reduction in the levels of Rac1GTP in cells, transfected with PACSIN2 (Fig. 7D). Expression of PACSIN2-Y435E/P478L, the SH3 domain mutant that is deficient in Rac1 binding, reduced Rac1GTP levels only with approximately ~35% compared with that by WT PACSIN2. This partial effect might result from dimerization with endogenous PACSIN2, which requires part of the F-BAR-, rather than the SH3 domain (Fig. 7D). These data suggest that PACSIN2 association with Rac1 is important for efficient inactivation of Rac1. This effect was unrelated to caveolin-1-dependent Rac1 ubiquitylation and degradation that we recently identified (Nethe et al., 2010), as loss or overexpression of PACSIN2 or its mutants did not affect the expression or the ubiquitylation of endogenous Rac1 (data not shown).

Because PACSIN proteins regulate endocytosis and vesicle transport, it is attractive to suggest that PACSIN2 negatively regulates Rac1 function by controlling internalization of activated Rac1 from the cell periphery. Supplementary material Movie 1 shows that PACSIN2 is internalized in tubular structures. Expression of two separate F-BAR domain mutants of PACSIN2 (R50D and the double-mutant M124E/
M125E), which can no longer induce membrane tubulation but still do associate with Rac1 (supplementary material Fig. S6A,B) (Shimada et al., 2010; Wang et al., 2009), induced a more than fourfold increase in GTP loading of Rac1 (Fig. 7E). This finding indicates a link between the formation of vesicular-tubular structures by PACSIN2 and Rac1 inactivation.

Dynamin has an important role in endocytosis mediated by its role in scission of newly formed vesicles from the membrane (Hinshaw, 2000). We therefore investigated whether inhibition of Dynamin affects PACSIN2-associated regulation of Rac1. First, we studied the effects of Dynamin inhibition on PACSIN2 dynamics by live-cell imaging studies. Fig. 8A and supplementary material Movie 3 show that upon addition of Dynasore, which inhibits Dynamin by rapidly blocking coated vesicle formation (Macia et al., 2006), YFP-PACSIN2 accumulated on the plasma membrane and at peripheral vesicles. Furthermore, confocal studies showed that upon transfection of a dominant-negative mutant of Dynamin (K44), endogenous PACSIN2 accumulated on tubules (Fig. 8B) with a concomitant loss of PACSIN2 localization to a distinct perinuclear pool (supplementary material Fig. S7A). Similar results were obtained for HeLa cells treated with Dynasore (supplementary material Fig. S7B). Taken together, these data indicate that inhibition of Dynamin interferes with PACSIN2 localization and function. We therefore studied whether Dynamin regulates PACSIN2-mediated downregulation of Rac1-GTP. Ectopic expression of Dynamin K44 prevented the reduction in Rac1GTP levels, as induced by myc-tagged PACSIN2 (Fig. 8C). Furthermore, Dynasore transiently prevented PACSIN2-mediated downregulation of Rac1GTP (Fig. 8D) suggesting that PACSIN2 regulates Rac1 inactivation in a Dynamin-dependent fashion.

PACSIN2 could downregulate Rac1-GTP levels through two mechanisms. First, PACSIN2 could target Rac1 to specific sites to allow GAP-mediated inactivation. Alternatively, PACSIN2 could block Rac1 regulation by GEF proteins. To investigate this, we performed pull-down experiments with the biotinylated Pak1-CRIB domain using cell lysates of HeLa cells that had been transfected with either TrioD1 or Tiam1-C1199, GEF proteins known to activate Rac1 (Habets et al., 1994; van Buul et al., 2010), and studied whether endogenous Rac1 activation still occurred in the presence of PACSIN2. PACSIN2-mediated downregulation of Rac1GTP was found in the control situation as well as in the presence of GEF proteins. However, activation of Rac1 upon expression of the GEFs was detectable, even in the presence of PACSIN2 (Fig. 8E). These data show that PACSIN2 does not prevent Rac1 from being activated by GEF proteins and suggest that Rac1 inactivation occurs by its targeting to intracellular locations to allow GAP-stimulated GTP hydrolysis.

Collectively, these results suggest that PACSIN2 is an important regulator of the small GTPase Rac1 and that PACSIN2 limits Rac1GTP signaling by promoting Rac1 inactivation. This effect of PACSIN2 requires its Rac1-binding capacity, mediated by the SH3 domain, as well as its membrane-tubulating activity, residing in the F-BAR domain.
DISCUSSION

Rho family GTPase signaling requires proper intracellular targeting of the activating GEF, the GTPase, and effector proteins. For Rac1 GEFs, the relevant location is assumed to be at or near the plasma membrane, either in focal adhesions or in peripheral membrane ruffles. A series of different signaling events upstream of exchange factor recruitment, such as inositol lipid turnover or kinase activation, have previously been identified and as a result, this part of the Rac1 signaling pathway is relatively well established. By contrast, the mechanisms controlling the inactivation of Rac1, which needs to be equally well controlled in time and space, are less well understood. Here, we present data indicating that the F-BAR-protein PACSIN2, a regulator of endocytosis (Modregger et al., 2000), is part of a Rac1-inactivation pathway. Our data suggest that PACSIN2-regulated internalization of peripheral, Rac1-containing membrane protrusions downregulates Rac1 signaling, as reduction of PACSIN2 expression by siRNA promotes GTP loading of Rac1, cell spreading and migration. PACSIN2 does not localize to focal adhesions (FAs), which suggests that inactivation of Rac1 at these sites requires other mechanisms. These could involve caveolin-1, which is recruited to FAs by activated Rac1 and regulates Rac1 ubiquitylation and degradation (Nethe et al., 2010).

The interaction with PACSIN2 is specific for Rac1 and is the first described association of PACSIN2 with a member of the RhoGTPase-family. The PACSINs are not the only BAR-domain proteins with Rac1-binding capacity. Previously, arfaptin, an effector of the Arf GTPase, was found to interact with GDP-bound, as well as GTP-bound Rac1, through its BAR domain (Tarricone et al., 2001). The adapter protein IRSp53 (also known as brain-specific angiogenesis inhibitor 1-associated protein 2) interacts with activated Rac1 through its (inverted)-BAR domain linking Rac1 to WAVE, which stimulates actin polymerization by the Arp2/3 complex (Miki et al., 2000). Thus,
whereas the I-BAR protein IRSp53 might stimulate Rac1 signaling and protrusion, the F-BAR protein PACSIN2, which stimulates endocytosis, inhibits Rac1 signaling.

Through their F-BAR domain, PACSIN proteins bind membranes containing phosphatidyserine and phosphatidyl-inositol-(4,5)-bisphosphate and induce membrane curvature which results in the formation of invaginations and, subsequently, of vesicular-tubular structures which depend on the self-assembly of F-BAR modules into a helical coat (Frost et al., 2009). We show that upon collapse of membrane ruffles, PACSIN2 associates with these tubular structures, in line with the proposed role of PACSIN2 in endocytosis. In addition, PACSIN2 localizes to early endosomes. At tubules, as well as early endosomes, PACSIN2 co-localizes with Rac1. Our experiments using different markers for endocytosis indicate that PACSIN2 does not play a primary role downstream of clathrin-mediated endocytosis. The CtxB-labeling studies however, indicate that PACSIN2 co-localizes with internalized lipid rafts in the vesicular-tubular structures. Given that Rac1 associates with the PACSIN2-positive tubules, these data are in agreement with findings showing that Rac1 associates to cholesterol-rich membrane domains and that these domains are involved in internalization of active Rac1 (del Pozo et al., 2004).

We show that activation of endogenous Rac1 with CNF1 toxin or expression of a constitutively active mutant of Rac1 (Q61L) results in loss of PACSIN2-positive tubular structures, leaving PACSIN2 on early endosomes. Although the molecular basis for this is unknown, this finding may suggest that fission of the vesicular-tubular structures, for example, mediated by dynamin (Hinshaw, 2000), is stimulated by Rac1 activity. Previously, constitutively active Rac1 has been found to inhibit transferrin-receptor-mediated endocytosis (Lamaze et al., 1996). Similarly, our findings show that activated Rac1 reduces the number of PACSIN2-positive tubules, which could reflect a reduction in PACSIN2-mediated endocytosis. In line with the observation that active Rac1 promotes PACSIN2 distribution to the perinuclear area and reduces the number of PACSIN2-positive tubules, inhibition of Rac1 in a number of PACSIN2-tubular structures, as induced by a dominant-negative Rac1 mutant (T17N) and inhibition of endogenous Rac1 by a pharmacological inhibitor (EHT1864). Collectively, our data suggest that Rac1 activity regulates the localization of PACSIN2 and the abundance of PACSIN2-positive tubular structures.

We found that the differential PACSIN2 distribution into a peripheral and a perinuclear pool is regulated by MTs. In this respect, PACSIN2 behaves in a manner similar to amphiphysin-2, (Meunier et al., 2009), the distribution of which is also regulated by MTs. MT growth has been claimed to activate Rac1, but it is currently unclear to what extent this is directly linked to the regulation of Rac1 by PACSIN2. Our results indicate that, in epithelial cells, the microtubule network controls PACSIN2-mediated tubule formation and PACSIN2 distribution. PACSIN proteins are linked to the actin-regulating Arp2/3 complex through their binding to N-WASP (Qualmann and Kelly, 2000; Rohatgi et al., 1999). However, experiments using cytochalasin B to block actin polymerization were inconclusive, because cytochalasin B induced morphological changes in the cell, precluding proper analysis of PACSIN2 distribution (data not shown). Thus, the molecular mechanisms that underlie the Rac1-induced changes in the distribution of PACSIN2 remain to be established.

A key finding of the current study is that PACSIN2 is involved in the inactivation of Rac1 in a fashion that requires an intact F-BAR domain. As the F-BAR domain does not regulate the PACSIN2-Rac1 interaction, this suggests that the membrane-binding and/or tubulating activity of PACSIN2 is essential to inactivate Rac1. In addition, we show that inhibition of dynamin impairs PACSIN2 function and localization and prevents PACSIN2-mediated Rac1 inactivation. Together, these data suggest a model in which PACSIN2 regulates Rac1 by promoting its internalization. Interestingly, dynamin, which can associate with PACSIN2 (Kessels and Qualmann, 2004), was previously shown to regulate Rac1 (Schlu¨ck et al., 2004). The dominant-negative dynamin-K44 mutant was found to increase the formation of Rac1-positive tubular structures. Given that dynamin induces fission of tubules, generating intracellular vesicles such as early endosomes, dynamin might act downstream of PACSIN2 in a Rac1-inactivation pathway.

In contrast to caveolin-1 (Nethe et al., 2010), we found no evidence for PACSIN2 targeting Rac1 to an ubiquitylation and/or degradation pathway. Our finding that GEF proteins are still able to activate Rac1 in the presence of PACSIN2, may indicate that PACSIN2-associated Rac1 inactivation primarily relies on GAP proteins. Because the inactive Rac1 T17N mutant co-localizes to PACSIN2-positive tubules, it could well be that PACSIN2 contributes to targeting Rac1 to sites for GAP-mediated inactivation. One such GAP could be p50RhoGAP (also called Cdc42GAP and Rho GTPase-activating protein 1) which has GAP activity towards Rho, Cdc42, and Rac1 (Barford et al., 1993; Lancaster et al., 1994) and was found to localize to endosomal structures (Siromkany et al., 2006). Furthermore, several BAR-domain-containing RhoGAPs exist, such as RICH1, which has GAP activity for Cdc42 and Rac1. RICH1 binds the F-BAR-domain protein CIP4, which is structurally similar to the PACSIN proteins (Richnau et al., 2004; Richnau and Aspenstrom, 2001). We are currently pursuing this issue to determine the localization and relevance of selected RacGAPs, although these studies are complicated by the number of GAPs (Bernards, 2003) and the limited availability of sufficient, well characterized reagents.

We recently identified a series of other adapter proteins that interact with the C-terminal hypervariable domain of Rac1. These include CD2AP (CMS) and caveolin-1 (Nethe et al., 2010; van Duijn et al., 2010) that also, like PACSIN2, regulate endocytosis (Lynch et al., 2003; Parton and Richards, 2003). However, whereas CD2AP is recruited to cell-cell contacts upon Rac1 activation, caveolin-1 translocates to FAs. Thus, these proteins all reside at different intracellular locations and they all interact with Rac1 independently of each other (data not shown). These findings indicate that independent mechanisms regulate Rac1 signaling at different sites supporting the notion of parallel, but compartmentalized, Rac1 signaling. In these so-called `spatio-temporal signaling
modules’ (Pertz, 2010), GTPases can interact specifically with different regulators and effectors, securing localized signaling.

In conclusion, our previous and current data suggest the following model (Fig. 9). Following GEF-mediated activation and interaction with effectors at the plasma membrane, Rac1 signaling can be terminated through at least two independent pathways. First, we recently showed that caveolin-1 can target Rac1GTP for ubiquitylation and degradation in an adhesion-dependent fashion (Nethe et al., 2010). Second, we have shown that PACSIN2 promotes Rac1 inactivation, possibly by targeting Rac1 to sites where GAP proteins can stimulate GTP hydrolysis. This pathway is not associated with Rac1 degradation. Future experiments will be aimed at defining the subcellular locations to which PACSIN2 recruits Rac1 for its inactivation and the identification of the relevant RacGAPs.

**MATERIALS AND METHODS**

**Antibodies, Constructs, and Reagents**

Antibodies against the following proteins were used: Anti-PACSIN1 was described previously (Modregger et al., 2000). Anti-PACSIN2 (AP8088b) was from Abgent. Anti-GFP (632381) and anti-dsRed (8374-1) were from Clontech. Anti-HA (11867423001) was from Roche. Anti-Rac1 (610651), anti-Dynamin and anti-Paxillin (610620) were from Transduction Laboratories. Anti-Rac1 (05-389) was from Upstate Biotechnologies and anti-β-Pix (AB3829) was from Millipore. Anti-SET/I2PP2A (SC-25564) was from Santa Cruz. Anti-α-Actinin1 (A5044), anti-α-Tubulin (T6199), and anti-Actin (A3853) were from Sigma. F-Actin was detected using Texas-Red- or Alexa-633-labelled Phalloidin (Invitrogen). Secondary HRP-labelled antibodies for Western blotting were from Pierce. Secondary Alexa-labelled antibodies for immunofluorescence were from Invitrogen.

Dynasore (D7693, used at 80 μM at 37°C), Nocodazole (M1404, used at 10 μM at 37°C), and EHT1864 (E1657, used at 50 μM at 37°C) were purchased from Sigma. GST-CNF1 was isolated as previously described (Pop et al., 2004) and used at 300 ng/ml. AF-594-CtxB (C22842, used at 0.5 μg/ml at 37°C), transferrin Texas Red (T2875, used at 20 μg/ml at 37°C), and Sulforhodamine 101 (S359, used at 25 μM at 37°C) were purchased from Molecular Probes.

**Expression constructs**

To generate YFP-PACSIN2 and mCherry-PACSIN2, myc-PACSIN2 WT was used as a template for PCR using as a forward primer 5’-GAGATCGGTAC-CTCTGTCAC-CTACGATGACTCT-3’ and as a reverse primer 5’-GAGATCGGA-TCTCACTGGA-TAGCCTCGAC-3’. The product was cloned into pEYFP(C1) or pmCherry(C1) (Clontech Laboratories, Inc) using KpnI and BamHI restriction sites. Myc- and YFP-tagged PACSIN2 R50D and M124E/M125E were generated by Site Directed Mutagenesis using myc-PACSIN2 WT and pEYFP-PACSIN2 WT as templates for PCR. The R50D mutant was generated using as a forward primer 5’-GAGATCGCGGCGGACATCG-GAGAAGGCC-3’ and as a reverse primer 5’-CGCCTTCTCGATGTCCGCCCGCT-CATGC-3’. The M124E/M125E mutant was generated using as a forward primer 5’-CTTACAAAGCAGGAGGGCGCCCTCAAGGAGACCAAG-3’ and as a reverse primer 5’-CCTTGTCCTTGAGAAGCCGCCCCTCTCTGCTGTGAAG-3’. To generate GST-PACSIN2, myc-PACSIN2 WT was used as template for PCR using as a forward primer 5’-GAGATCGGTAC-CTCTGTCAC-CTACGATGACTCT-3’ and as a reverse primer 5’-GAGATCGCGGCGGACATCG-GAGAAGGCC-3’ and as a reverse primer 5’-GAGATCGGTAC-CTCTGTCAC-CTACGATGACTCT-3’ and as a reverse primer 5’-GAGATCGGTAC-CTCTGTCAC-CTACGATGACTCT-3’. The M124E/M125E mutant was generated using as a forward primer 5’-GAGATCGCGGCGGACATCG-GAGAAGGCC-3’ and as a reverse primer 5’-CGCCTTCTCGATGTCCGCCCGCT-CATGC-3’. The M124E/M125E mutant was generated using as a forward primer 5’-CTTACAAAGCAGGAGGGCGCCCTCAAGGAGACCAAG-3’ and as a reverse primer 5’-CCTTGTCCTTGAGAAGCCGCCCCTCTCTGCTGTGAAG-3’.

For siRNA-based knockdown: The sequence for control siRNA was: 5’-CGUACGCGGAAUACUUCGAtt-3’ (Eurogentec). The sequence for PACSIN2 siRNA-1 was: 5’-GGAGAAGCUGGCUAUCUCGAGAAtt-3’ (Eurogentec). PACSIN2 siRNA-2 (L-019666-02) was from Dharmacon. PACSIN2 siRNA-3 (SI02224292) was from Qiagen. Rac1 siRNA was as previously described (Nethe et al., 2010).
Cell culture and transfections
MEF, COS7, Jurkat, and HeLa cells were maintained at 37ºC under 5% CO₂ in Iscove’s Modified Dulbecco’s Medium (IMDM; Biowhittaker) containing 10% heat-inactivated Fetal Calf Serum (FCS; Life Technologies, Breda, The Netherlands), 300 μg/ml glutamine, 100 units/ml penicillin and streptomycin. Cells were passaged by trypsinization. Primary HUVECs were purchased from Lonza (Baltimore M.D) and cultured in EGM2 medium, supplemented with singlequots (Lonza Baltimore M.D).

HeLa cells were transiently transfected with FuGENE (Roche) or TransIT (Mirus) according to the manufacturers’ recommendations. Transfections of siRNA were performed with INTERFERin (Polyplus transfection) according to the manufacturers’ recommendations.

Pull-down assays
Peptide pull-downs were performed as described previously (ten Klooster et al., 2006). In short, cells were lysed in NP-40 lysis buffer (50mM TRIS/HCl pH 7.5, 100mM NaCl, 10mM MgCl₂, 10% glycerol and 1% NP40) supplemented with protease inhibitors (Complete mini EDTA, Roche, Almere, The Netherlands), centrifuged at 20.000g for 10 minutes at 4°C. The supernatant was then incubated with the indicated RhoGTPase C-terminal peptides (5 µg) in the presence of streptavidin-coated beads (Sigma) at 4°C for 1 hour with rotation. Protein association was assayed by Western blot analysis.

Isolation of proteins and mass spectometry analysis was performed as described (Kanters et al., 2008). GST-fusion proteins were purified from BL21 bacteria as described previously (ten Klooster et al., 2006). 50 µg of the indicated GST-fusion constructs was used for each pull-down. Protein association was assayed by Western blot analysis. For studying the direct interaction of Rac1 and PACSIN2, the GST-PACSIN2 fusion protein was eluted from the beads with a Glutathione buffer (10 mM glutathione and 50 mM Tris-HCl, pH 7.4) twice for 10 min (while rotating) at room temperature. Protein association was assayed by Western blot analysis. For the direct interaction of Rac1 and PACSIN2, the GST-PACSIN2 fusion protein was eluted from the beads with a Glutathione buffer (10 mM glutathione and 50 mM Tris-HCl, pH 7.4) twice for 10 min (while rotating) at room temperature. Protein association was assayed by Western blot analysis. Rac1 activation was assayed by a CRIB-peptide pull-down approach as described previously (Price et al., 2003); 30 µg of Pak1-CRIB peptide was used for each pull-down. Bound Rac1GTP was detected by Western blot analysis.

SDS-PAGE and Western blotting
Proteins were separated by SDS-PAGE (on 7.5, 10, or 12.5% poly-acrylamide gels depending on the size of the proteins of interest) and transferred onto Nitrocellulose transfer membrane (Whatman). Following blocking in 5% low fat milk in TBST (Tris-Buffered Saline Tween-20) the blots were incubated with the primary antibody overnight at 4°C. Next, the blots were washed 3 times for 10 minutes in TBST and subsequently incubated with HRP-coupled secondary antibodies (dilution 1:5000) in TBST for 1 hour at room temperature. Finally, blots were washed three times with TBST for 20 minutes each and subsequently developed by ECL (GE Healthcare).

Confocal Laser Scanning Microscopy
Cells were seeded onto fibronectin-coated glass coverslips, transfected with the indicated plasmids, and after 24 hours fixed with 3.7% formaldehyde (Merck) in PBS for 10 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Coverslips were then incubated for 15 minutes with 2% BSA at 37°C. Immunostainings were performed at room temperature for 1 hour with the indicated antibodies. Fluorescent imaging was performed with a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss Microimaging, Inc.) using a 63X/NA 1.40 or a 40X/NA 1.30 oil lens (Carl Zeiss Microimaging, Inc.). Image acquisition was performed with Zen 2008 software (Carl Zeiss Microimaging, Inc.). For Live-cell imaging, cells were seeded on fibronectin-coated glass coverslips, transfected with the indicated plasmids. After 24 hours, fluorescent imaging was performed.

Electric resistance measurements
For ECIS-based cell spreading experiments, gold ECIS electrodes (8W10E; Applied Biophysics) were treated with 10 μM L-cysteine for 15 minutes and subsequently coated with 10 µg/ml fibronectin in 0.9% NaCl for 1 hour at 37°C. Next, HeLa cells were treated as indicated, and seeded at a concentration of 100.000 cells per well in 400 μl IMDM with 10% FCS. Impedance was measured continuously at 45 kHz using ECIS model 9600. The increase in impedance, as a measure of cell spreading (Wegener et al., 2000), was recorded for 1 hour. When cells had formed a monolayer displaying stable transmonolayer resistance, electric wounding was performed (45 kHz and 4 V). The increase in resistance in the first hours after the wounding, as a measure of cell migration by wound healing, was subsequently recorded for up to 5 hours (Lorenowicz et al., 2008).

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REFERENCE LIST


Supplementary Figure S1: PACSIN2 interacts with Rac1. (A) Using peptide-based pull down assays, the Rac1 C-terminus was found to bind to the myc-tagged PACSIN1,2,3 isoforms, transfected into HeLa cells. (B) Pull-down experiments using GST and GST-PACSIN2 were performed with lysates from HeLa cells. Association to endogenous Rac1 was detected by immunoblotting (B). (C) The PACSIN2 SH3-domain mutant cannot interact with full-length GST-Rac1 using lysates of HeLa cells, transfected with indicated PACSIN2 constructs. Ponceau staining is included to confirm equal loading of GST-fusion proteins. TCL, total cell lysates; PD, Pull-down; ED, Effector domain; HV, Hypervariable domain; PTD, protein transduction domain.
Supplementary Figure S2: Localization of PACSIN2. (A,B) PACSIN2 distribution was studied in HUVEC and COS7 cells. Endogenous PACSIN2 was detected by immunostaining in combination with α-Tubulin. (C) To confirm the specific detection of PACSIN2 in our immunostainings, HeLa cells were transfected with myc-tagged constructs of PACSIN1, 2, and 3. Cell lysates were analyzed by Western blot. The PACSIN2 antibody recognized myc-PACSIN2 but not myc-PACSIN3. Weak staining of myc-PACSIN1 was observed. Total cell lysates were stained for myc to confirm expression of constructs. (D) Left panel: HeLa cells were transfected with control (siCtrl) and PACSIN2 (siP2-2) siRNA were analyzed by Confocal Laser Scanning Microscopy. Silencing of PACSIN2 results in a loss of PACSIN2 staining supporting the specificity of detection with this antibody. Right panel: Immunoblotting of HeLa cells transfected with PACSIN2 siRNA to indicate knock-down efficiency. (E) Using HeLa cells transfected with mCherry-PACSIN2, perinuclear vesicles (arrows) positive for PACSIN2 can be observed. (F) HeLa cells transfected with YFP-PACSIN2 were studied by live-cell imaging following incubation with Sulforhodamine101. Internalized Sulforhodamine101 localized to PACSIN2-negative tubular structures. (G) Endogenous PACSIN2 co-localizes with GFP-FYVE. (H) Endogenous Rac1 localizes to PACSIN2-positive tubular structures. Zooms are indicated by the boxes. Bars, 10 μm.

Supplementary Figure S3: PACSIN2-positive structures partially align with microtubules. (A) Using Zen Software (Carl Zeiss MicroImaging, Inc.) PACSIN2 co-localization with microtubules was assessed. In the co-localization plot, pixels from quadrant 3 are indicated in white and overlaid on the merged image to underscore the partial co-localization. (B) A fraction of PACSIN2 aligns with microtubules (See arrowheads). Endogenous PACSIN2 was detected by immunostaining in combination with α-Tubulin using HeLa cells or HUVEC. Zooms are indicated by the boxes. Bars, 10 μm.
Supplementary Figure S4: The microtubule network controls PACSIN2 distribution. (A, B) The MT network controls PACSIN2 and early endosome distribution. HeLa cells were treated with Nocodazole (10 μM; 30 min.) to depolymerize the MTs and subsequently cells were fixed and analyzed by Confocal Laser Scanning Microscopy. Endogenous PACSIN2, α-Tubulin and EEA1 were detected by immunostaining. Following Nocodazole incubation, PACSIN2 (A) as well as early endosomes (B) become dispersed throughout the cell. Loss of MTs was associated with a loss of PACSIN2-positive peripheral tubules. (C) Microtubule depolymerization resulted in dispersed localization of endogenous Rac1 and PACSIN2 as detected by immunostaining. Zooms are indicated by the boxes. Bars, 10 μm.
Supplementary Figure S5: Activation of Rac1, but not RhoA, induces a loss of peripheral PACSIN2. (A) HeLa cells, transfected with myc-tagged RhoAV14 were analyzed for RhoAV14 expression and endogenous PACSIN2 distribution. RhoAV14 mimics the nocodazole effect as PACSIN2 distribution is dispersed. Asterisks indicate non-transfected cells. (B) Rac1 activation by CNF1 induces perinuclear accumulation of PACSIN2. HeLa cells, treated or not with CNF1 (300 ng/ml) were analyzed for endogenous PACSIN2 and F-actin. In cells treated with CNF1, PACSIN2 distribution becomes limited to the perinuclear area. (C) Inhibition of Rac1 by EHT1864 inhibits turnover of PACSIN2-positive tubular structures. HeLa cells transfected with YFP-PACSIN2 were treated with the pharmacological Rac1 inhibitor EHT1864 (50 μM). Following addition of the inhibitor (at t = 2.5 min), PACSIN2 tubular structures (indicated with arrows) accumulate in the cell periphery. (D) HeLa cells were transfected with the YFP-PACSIN2 Y435E/P478L SH3 domain mutant and analyzed by live-cell imaging. Still images show an increase in PACSIN2-positive vesicular-tubular structures (indicated by arrows). Zooms are indicated by the boxes. Bars, 10 μm.

Supplementary Figure S6: YFP-PACSIN2 R50D and M124E/M125E localization and interaction with Rac1. (A) YFP-PACSIN2 R50D and M124E/M125E show a diffuse, cytoplasmic and non-tubular distribution. HeLa cells transfected with indicated constructs were studied by live-cell imaging. Stills are shown. Both mutants exhibit a cytoplasmic distribution and no tubular structures are observed. (B) YFP-PACSIN2 R50D and M124E/M125E can interact with the Rac1 C-terminus. Pull-down experiments were performed using cell lysates from HeLa transfected as indicated with the Rac1 C-terminal peptide (Rac1). Myc-tagged constructs were detected by Immunoblotting (IB). Total Cell Lysate (TCL); Pull-Down (PD). Bars, 10 μm.

Supplementary Figure S7: Dynamin inhibition affects PACSIN2 intracellular distribution. (A) Ectopic expression of Dynamin K44 induces a loss of perinuclear PACSIN2 (untransfected cells indicated by arrows). Endogenous PACSIN2 was detected by immunostaining. (B) HeLa cells treated with Dynasore were imaged by Confocal Laser Scanning Microscopy. Dynasore induces accumulation of PACSIN2-positive tubular structures. Endogenous PACSIN2 was detected by immunostaining. Bars, 10 μm.

SUPPLEMENTARY MOVIES

Supplementary Movie 1: Dynamics of YFP-PACSIN2. HeLa cells were transfected with YFP-PACSIN2 WT. Images were analyzed by time-lapse microscopy using a laser-scanning confocal microscope using a 63X/NA 1.40 oil lens (LSM510/Meta; Carl Zeiss Microimaging, Inc.). Frames were taken every 7 seconds for 5 minutes. Video runs at 10 frames/sec. Stills in Fig. 2B.

Supplementary Movie 2: Dynamics of YFP-PACSIN2 and mCherry-Rac1WT. HeLa cells were transfected with YFP-PACSIN2 WT (green) and mCherry-Rac1WT. HeLa cells were transfected with YFP-PACSIN2 WT (green) and mCherry-Rac1 WT (red). Images were analyzed by time-lapse microscopy using a laser-scanning confocal microscope using a 63X/NA 1.40 oil lens (LSM510/Meta; Carl Zeiss Microimaging, Inc.). Frames were taken every 10 seconds for 5 minutes. Video runs at 10 frames/sec.

Supplementary Movie 3: Effect of Dynasore on dynamics of YFP-PACSIN2. HeLa cells were transfected with YFP-PACSIN2 WT. After 2 minutes Dynasore was added (80 μM). Images were analyzed by time-lapse confocal microscopy using a laser-scanning confocal microscope using a 63X/NA 1.40 oil lens (LSM510/Meta; Carl Zeiss Microimaging, Inc.). Frames were taken every 10 seconds for 16 minutes. Video runs at 12 frames/sec. Stills images are in Fig. 8A.
THE HUMAN MINOR HISTOCOMPATIBILITY ANTIGEN-1 IS A RHOGAP AND REGULATES THE ACTIN CYTOSKELETON AND CELL SPREADING

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ABSTRACT
Rho-family GTPases such as Rac1, Cdc42, and RhoA are key regulators of the actin cytoskeleton and control essential processes such as cell spreading and migration. They regulate formation of protrusive structures such as filopodia and lamellipodia, as well as stress fiber formation and focal adhesions. RhoGTPase activity is under tight control as aberrant signaling can lead to various diseases such as cancer. Whereas Guanine nucleotide Exchange Factors (GEFs) mediate the exchange of GDP for GTP resulting in activation of RhoGTPases, GTPase Activating Proteins (GAPs) catalyze the low intrinsic GTPase activity of RhoGTPases thereby inactivating them.

Here we identify the physiological role of the Human Minor Histocompatibility Antigen-1 (HMHA1) protein, which encodes the minor H antigen-1 (HA-1), as a novel RhoGAP. In addition to its GAP domain, HMHA1 encodes a BAR domain, placing HMHA1 in a larger subclass of BAR-GAP proteins that includes GRAF1 and Oligophrenin-1. We show that HMHA1 constructs, lacking the N-terminal region, negatively affect the actin cytoskeleton as well as cell spreading. Furthermore, we show that HMHA1 regulates RhoGTPase activity both in vivo and in vitro and that the N-terminal BAR domain of HMHA1 autoinhibits its GAP function as mutants lacking this region failed to reduce GTP loading of RhoGTPases.

In conclusion, this study identifies the previously unknown physiological role for HMHA1 as a RhoGAP which regulates actin cytoskeleton remodeling and cell spreading.

INTRODUCTION
Cell adhesion, spreading and migration require dynamic remodeling of the actin cytoskeleton. Key regulators of the actin cytoskeleton are the Rho-family GTPases and in particular Rac1, Cdc42, and RhoA (Ridley et al., 2003). These GTPases control cell morphology, polarity, cell adhesion and directional motility by regulating the formation of filopodia, lamellipodia, stress fibers, and focal adhesions in a tightly controlled fashion (Nobes and Hall, 1995a; Nobes and Hall, 1995b). RhoGTPases are so-called molecular switches that cycle between a GDP-bound inactive state, and a GTP-bound active state. When RhoGTPases are in their active state, downstream effectors, such as the p21-activated kinase (PAK) serine/threonine kinase for Rac1, or Rho-associated coiled-coil-containing protein kinase (ROCK) (Bishop and Hall, 2000) for RhoA, are activated to regulate downstream signaling.

Both activation and inactivation of RhoGTPases is under tight control. This is governed primarily by three distinct families of proteins. In response to extracellular stimuli, guanine nucleotide exchange factors (GEFs) control the exchange of GDP for GTP, thereby activating the GTPases (Rossman et al., 2005). On the other hand, GTPase activating proteins (GAPs) regulate the hydrolysis of GTP to GDP by catalyzing the low intrinsic GTPase activity of the GTPases, thereby inactivating the GTPases (Bernards and Settleman, 2004). In addition to GEFs and GAPs, Rho guanine nucleotide dissociation inhibitor (RhoGDI) is an important regulator of RhoGTPase activity. Whereas most activated RhoGTPases are associated to the inner leaflet of the plasma membrane, inactive RhoGTPases reside in the cytoplasm bound to RhoGDI (Garcia-Mata et al., 2011). Twenty-two human members of the Rho-family GTPases have been characterized so far (Wennerberg and Der, 2004). However, the number of RhoGEFs and RhoGAPs outnumber RhoGTPases by 3-4 fold (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). This suggests that cell-type specificity and selective subcellular localization are important aspects of regulating the (in)activation of a particular RhoGTPase. Furthermore, external stimuli can regulate RhoGTPase (in)activation by activating specific GEFs or GAPs (Tcherkezian and Lamarche-Vane, 2007).

The human minor Histocompatibility (H) antigens were originally detected as histocompatibility barriers in MHC-identical transplantation. Minor H antigens are peptides generated from specific intracellular proteins. Upon presentation of these peptides on the cell surface, cytotoxic T cells can be formed that target these epitopes (Goulmy, 1996). These T cells have been detected and isolated in patients after HLA-identical Stem Cell Transplantation (SCT) (Goulmy et al., 1983). HA-1 was the first autosomal minor H antigen identified (Goulmy et al., 1983). However, nowadays a series of minor H antigens have been characterized (Spiersings et al., 2004). As the minor H antigen HA-1 is encoded from the HMHA1 gene we will, from here forth, when discussing its cell biological role refer to the minor H antigen HA-1 as HMHA1. HMHA1 expression is restricted to the hematopoietic system (de Bueger et al., 1992).
Although absent in normal epithelial cells, HMHA1 gene expression is observed in many epithelial tumor cells (Klein et al., 2002) suggesting a possible role for HMHA1 in cancer. Indeed, in two humanized murine studies, we clearly demonstrated HMHA1-specific leukemia and solid tumor eradication (Hambach et al., 2006; Hambach et al., 2008). Sequence analysis of HMHA1 gene reveals a possible C-terminal RhoGAP domain as well as an N-terminal BAR domain. BAR domains are modules involved in membrane dynamics including endocytosis and vesicle transport (Frost et al., 2009; Qualmann et al., 2011). Many BAR domain-containing proteins have been shown to regulate RhoGTPase activity and function (De Kreuk and Hordijk, in press). One subclass of these proteins, including SH3BP1, Oligophrenin-1 (OPHN1), and GRAF1 (Cicchetti et al., 1995; Fauchereau et al., 2003; Hildebrand et al., 1996) bear a similar structure as HMHA1 in that they encode both a BAR-domain as well as a GAP domain. As the cellular role of HMHA1 is unknown we decided to investigate the biological function of HMHA1.

We show that HMHA1 mutants lacking the N-terminal BAR domain but encoding the GAP domain dramatically alter the F-actin cytoskeleton. Cells expressing these mutants show an overall loss of F-Actin as well as focal adhesions and, as a consequence, highly impaired cell adhesion/spreading. We also show that HMHA1 interacts and colocalizes with different RhoGTPases. Both in vitro and in vivo studies showed that HMHA1 regulates RhoGTPase activity. Similar to GRAF1 and OPHN1, the HMHA1 BAR domain auto-inhibits its GAP function. In summary, our data identify HMHA1 as a novel RhoGAP which regulates actin dynamics and cell spreading.

RESULTS
HMHA1 regulates the actin cytoskeleton and cell spreading
Sequence analysis of HMHA1 shows that it comprises an N-terminal Bin/Amphiphysin/Rvs (BAR) domain followed by a C1 domain and a RhoGAP domain. The C-terminal region consists of a proline-rich region as well as a PDZ-binding domain (Fig. 1A).

Figure 1: Localization and effects on F-actin of HMHA1 and selected mutants. (A) Schematic overview of the organization of HMHA1 and of the different constructs used in this study. (B) Intracellular localization of myc-tagged HMHA1 (and mutant constructs) was studied by Confocal Laser Scanning Microscopy following expression in HeLa cells. Myc-tagged HMHA1 was detected by immunostaining for the myc epitope in combination with detection of F-Actin with phalloidin. Full-length HMHA1 (FL) as well as HMHA1 N-term are partially localized at membrane ruffles as well as in the cytoplasm. For HMHA1 N-term localization at vesiculo-tubular structures is occasionally observed (arrows). Cells expressing FL or the N-term constructs are morphologically similar to control cells and no effects are seen on F-Actin (upper two rows). HMHA1 constructs lacking the C-terminal tail (GAP and C1-GAP) are partly mislocalized into protein aggregates. In cells expressing HMHA1 C1-GAP, C1-GAPtail, and GAPtail (marked with asterisks), F-Actin distribution is altered and cell morphology is dramatically changed. Higher magnification images of the boxed area are included. Scale bars, 10 μm.
GRAF1, have been identified that also encode a RhoGAP domain (De Kreuk and Hordijk, in press) and are involved in regulating the actin cytoskeleton. We therefore investigated the role of HMHA1 in RhoGTPase signaling and actin remodeling.

To study this, we generated immunotagged full-length and deletion constructs of HMHA1 (Fig. 1A). Because for several BAR-GAP proteins, including GRAF1 and OPHN1, it was shown that the BAR domain autoinhibits GAP function (Eberth et al., 2009), we generated both an N-terminal BAR-domain-containing construct as well as several C-terminal constructs that include the RhoGAP domain (Fig. 1A). Normally, expression of HMHA1 is restricted to the hematopoietic system (de Bueger et al., 1992; Klein et al., 2002). However, visualizing protein distribution and dynamics in leukocytes is challenging. Since HMHA1 gene expression was also shown in epithelial tumor cells (Klein et al., 2002), we decided to express the different constructs in HeLa cells, which do not express HMHA1, to assess protein distribution as well as potential effects on the actin cytoskeleton.

We found no effects of full-length HMHA1 (FL) or the N-terminal construct (N-term) on overall cell morphology of the HeLa cells compared to control cells (Supplementary Fig. S1; upper three rows). Interestingly, expression of constructs that lack the N-terminal BAR domain, with the exception of the construct encoding only the GAP domain (GAP), dramatically alter cell morphology. Cells expressing the C1-GAP, the C1-GAPtail and the GAPtail proteins show reduced membrane ruffling and extensive formation of cellular spines. Additionally, these cells show reduced cell spreading (Supplementary Fig. S1). Our results demonstrate that the GAP domain of HMHA1, in conjunction with flanking sequences induces a significant change in cell shape. The finding that FL HMHA1 does not induce this phenotype, is suggestive for a negative regulatory role of the BAR domain.

So far, the intracellular distribution of HMHA1 has not been investigated. Here we show that HMHA1 (FL) is localized to the cytoplasm as well as to membrane ruffles (Fig. 1B). A similar distribution was found for the N-terminal (N-term) construct, encoding the BAR domain. Interestingly, a fraction of HMHA1 (N-term) localizes to tubular structures (Fig. 1B) which are known to be formed by the membrane-deforming activity of BAR domains. The HMHA1 C1-GAPtail or GAPtail proteins show primarily a diffuse cytoplasmic distribution with a small fraction detectable on the plasma membrane (Fig. 1B). Interestingly, expression of HMHA1 C1-GAP and GAP results in formation of protein aggregates which suggests that the C-terminal tail-region is (partially) involved in proper localization of the HMHA1 protein (Fig. 1B).

Because the actin cytoskeleton is critical for regulating cell morphology and is under tight control of RhoGTPases, we examined F-actin organization in cells expressing the different HMHA1 mutants. Both full-length HMHA1 and the N-terminal construct show normal F-Actin distribution (Fig. 1B; upper two rows). However, constructs lacking the N-terminal BAR domain (C1-GAPtail, C1-GAP, GAPtail) show a dramatic loss of F-Actin compared to control cells (Fig. 1B; bottom 4 rows). To investigate whether HMHA1 influences the microtubule network the different HMHA1 constructs were

Figure 2: The HMHA1 N-terminal BAR domain prevents loss of focal adhesions. The effects of myc-tagged HMHA1 (and deletion constructs) on focal adhesion distribution was studied by Confocal Laser Scanning Microscopy following expression in HeLa cells. Similar to the effects on F-Actin distribution, cells expressing full-length HMHA1 (FL), N-term, or GAP (first, second and fifth rows) constructs show normal focal adhesion distribution as detected using Paxillin immunostainings. Expression of HMHA1 C1-GAP, C1-GAPtail, or GAPtail (marked with asterisks) induces loss of focal adhesions. Higher magnification images of the boxed area are included. Scale bars, 10 μm.
ectopically expressed and the distribution of the microtubule network analyzed. In contrast to what we observed for F-actin, the microtubule network is unaffected in cells expressing the N-terminal deletion constructs (Supplementary Fig. S2).

In addition to controlling cell-shape, the actin cytoskeleton regulates adhesion to the extracellular matrix via integrins and -associated adaptor proteins such as paxillin and talin, at focal adhesions (Vicente-Manzanares et al., 2009). As we observed dramatic effects on the actin cytoskeleton in cells expressing HMHA1 N-terminal deletion mutants, we subsequently analyzed possible effects on focal adhesion distribution. Focal adhesions were visualized by immunostaining for paxillin, a typical focal adhesion marker (Deakin and Turner, 2008). Similar to what we observed for cell morphology and the actin cytoskeleton, HMHA1 FL and N-term proteins did not affect the distribution of focal adhesions. In contrast, the C1-GAPtail, C1-GAP and GAPtail proteins induced a marked loss of focal adhesions compared to control cells (Fig. 2). Paxillin-positive structures were detected, but these were limited in number, located mainly at the periphery of the transfected HeLa cells and very small, suggestive for a defect in focal adhesion maturation. These experiments suggest an important role for HMHA1 in regulation of the actin cytoskeleton and, concomitantly, focal adhesion formation and distribution.

To confirm that cells expressing HMHA1 mutants lacking the N-terminal region are less adhesive, we analyzed cell spreading by ECIS (Electrical Cell-substrate Impedance Sensing; see Material and Methods). In these assays, cells are seeded on golden electrodes and the increase in impedance, a measure for cell spreading, is recorded in real-time (Wegener et al., 2000). Ectopic expression of HMHA1 FL (blue) or N-term (dark green) did not significantly reduce cell spreading compared to control cells (Fig. 3). However, cells expressing HMHA1 C1-GAPtail (black), C1-GAP (light green), and GAPtail (grey), showed a significant decrease in cell spreading. Similar to the effects on F-actin, HMHA1 GAP (magenta) did not significantly impair cell spreading (Fig. 3).

In summary, these data indicate that HMHA1 regulates cell spreading by regulating the F-actin cytoskeleton and focal adhesion formation and distribution. Mutants lacking the N-terminal BAR domain show loss of F-Actin and focal adhesions and impaired cell spreading. These data suggest an important auto-inhibitory role for the HMHA1 BAR domain similar to what has been observed for other BAR-GAP proteins such as OPN1 and GRAF1 (Eberth et al., 2009; Elvers et al., 2012). Finally, since the HMHA1 GAP protein does not affect cell morphology and F-actin distribution, in contrast to HMHA1 GAPtail or C1-GAP proteins, this suggests that either the C1 domain or the tail, which encodes a proline-rich region and a C-terminal PDZ-binding motif, is required for proper functioning of the HMHA1 RhoGAP domain.

**HMHA1 interacts and colocalizes with Rho-family GTPases**

The data in Figs. 1-3 show that HMHA1 can regulate the actin cytoskeleton. Because HMHA1 is putative RhoGAP and RhoGTPases, in particular Rac1, Cdc42, and RhoA, are important regulators of actin reorganization, we first analyzed whether HMHA1 co-localizes with RhoGTPases in HeLa cells. As HeLa cells do not express endogenous HMHA1, we transfected cells with myc-tagged FL HMHA1 and studied co-localization with endogenous Rac1. Interestingly, we found that HMHA1 co-localizes with Rac1 at sites with high actin dynamics such as peripheral membrane ruffles (Fig. 4A). As GAP proteins are known to preferably bind to active RhoGTPases, we cotransfected HMHA1 full-length with mCherryRac1Q61L, a constitutively active Rac1 mutant. Similar to endogenous Rac1, HMHA1 colocalizes with mCherryRac1Q61L in peripheral membrane ruffles (Fig. 4B). In addition, we analyzed co-localization of full-length HMHA1 with the constitutively active mutants of Cdc42 (G12V, Fig. 4C) or RhoA (V14, Fig. 4D). We observed partial co-localization of HMHA1 with Cdc42G12V and with RhoAV14 (Fig. 4C, D; arrows), although the extent of co-localization was less as compared to Rac1. As mentioned before, HMHA1 is a putative RhoGAP. One important criterion to define whether a protein is a genuine RhoGAP is the direct interaction with RhoGTPases, and in particular with the active, GTP-bound, conformation. To assess whether HMHA1 interacts with RhoGTPases, we performed pull-down experiments with GST-Rac1 (used as a model for RhoGTPases) in a cell-free system and determined the direct interaction of bacterially purified HMHA1 C1-GAPtail. Our data show that HMHA1 C1-GAPtail interacts with Rac1 preferably when Rac1 is loaded with GppNHp, a non-hydrolyzable analog of GTP (Supplementary Fig. S3A). Previously, we identified several proteins that regulate Rac1 activity, such as PACSIN2 and caveolin, that interact with the Rac1 C-terminal hypervariable domain (de Kreuk et al., 2011; Nethe et al., 2010). To assess whether purified HMHA1 requires the C-terminal hypervariable
domain for binding to Rac1, we performed pull-down experiments with GST-fusions of Rac1WT and Rac1ΔC, which lacks the hypervariable domain, loaded with GDP or GppNHp. In contrast to PACSIN2 and caveolin, HMHA1 C1-GAPtail binds to Rac1 independent of the C-terminal hypervariable domain (Supplementary Fig. S3B). To test the specificity of this interaction we performed pull-down experiments with GST-Rac1ΔC and GST-RhoAΔC loaded with either GDP or GppNHp. We found that purified HMHA1 directly interacts to a similar extent with both Rac1 and RhoA. In line with the above findings, HMHA1 preferably interacts with Rac1 and RhoA when they are in the active, GppNHp-bound, conformation (Supplementary Fig. S3C).

In summary, HMHA1 colocalizes with RhoGTPases at sites of high membrane dynamics. Furthermore, HMHA1 directly interacts with Rac1 and RhoA preferably in their active conformation. Together with previous experiments, which showed a role for HMHA1 in F-actin reorganization, these data indicate a possible role for HMHA1 as a RhoGAP.

Active Rac1 but not Cdc42 rescues the phenotype induced by HMHA1 C1-GAPtail

Next we investigated whether ectopic expression of constitutively active mutants of Rac1 (Rac1G12V and Q61L) or Cdc42 (G12V), which are unresponsive to GAP activity and therefore always in the active GTP-bound state, could rescue the dramatic phenotype induced by HMHA1 C1-GAPtail. Interestingly, both mCherry-Rac1Q61L and G12V were able to bypass the effect induced by HMHA1 C1-GAPtail (Fig. 5A) in that cells expressing both constitutively active Rac1 as well as HMHA1 C1-GAPtail show a more spread phenotype with less spine-like protrusions as compared to cells co-expressing an empty vector control (mCherry-EV) (Fig. 5A). In contrast to active Rac1 mutants, Cdc42G12V did not rescue cell morphology and spreading induced by the C1-GAPtail protein (Fig. 5B). Ideally, we would have liked to test whether constitutively active RhoA (V14) could also rescue cell morphology and spreading. Unfortunately, both active RhoA, due to an increase in contractility, and HMHA1 C1-GAPtail induce loosely spread cells. In the co-transfection experiments we could not detect any double-positive cells, most likely since these did not adhere properly and were washed off upon fixation and immunostaining. Therefore, whether activation of RhoA could rescue the dramatic phenotype induced by C1-GAPtail remains unclear.

Figure 4: HMHA1 colocalizes with RhoGTPases. (A-D) Colocalization of myc-tagged HMHA1 with endogenous Rac1 (A), Rac1 Q61L (B), Cdc42 G12V (C) and RhoA V14 (D) was studied by Confocal Laser Scanning Microscopy. Myc-tagged HMHA1 and HA-tagged Cdc42 and RhoA were detected by immunostaining in combination with detection of F-Actin. HMHA1 colocalized with endogenous Rac1 (A) and Rac1 Q61L (B) in membrane ruffles (arrows). A partial colocalization of HMHA1 with Cdc42 G12V (C) and RhoA V14 (D) was observed (arrows) although less clear than for Rac1. Higher magnification images of the boxed areas are included. Scale bars, 10 μm.
Figure 5: Constitutively active Rac1, but not Cdc42, rescues the altered cell morphology induced by HMHA1 C1-GAPtail. (A,B) Rescue experiments with constitutively active Rac1 Q61L and G12V (A) or Cdc42 G12V (B), co-expressed with the HMHA1 C1-GAPtail protein were performed in HeLa cells and analyzed by Confocal Laser Scanning Microscopy. Ectopically expressed proteins were visualized in combination with F-Actin. (A) Constitutively active Rac1 Q61L (middle panels) and G12V (bottom panels) were able to (partially) rescue the phenotype induced by C1-GAPtail. As a control, mCherry empty vector (EV; upper panels) was unable to rescue the phenotype. (B) Ectopic expression of constitutively active Cdc42 G12V did not rescue the phenotype induced by C1-GAPtail. Scale bars, 10 μm.

So far, we have shown that HMHA1 regulates the actin cytoskeleton as well as cell spreading. Furthermore, we showed that HMHA1 interacts and colocalizes with RhoGTPases, such as RhoA and Rac1. Also, the dramatic phenotype induced by HMHA1 C1-GAPtail could be rescued by constitutively active mutants of Rac1 that are unresponsive to GAPs.

HMHA1 is a RhoGAP in vitro and GAP function is inhibited by the BAR domain

Next we analyzed the homology of the HMHA1 predicted RhoGAP domain with p50RhoGAP, a prototypical RhoGAP. In addition, we included two well-known BAR-GAPs, GRAF1 and OPHN1, as HMHA1 also has a similar BAR-GAP architecture. Sequence alignment clearly shows that the RhoGAP domain of human HMHA1 shows high sequence homology, including a conserved Arg residue at position 797 (Fig. 6A; black bar), to different human RhoGAPs, such as p50RhoGAP, GRAF1, and OPHN1 (Fig. 6A). Next, we generated a homology model of the HMHA1 RhoGAP domain based on the structure of the human p50RhoGAP domain (Protein Data Bank (PDB) ID: 1tx4), which was the top-scoring model predicted by the Phyre protein structure prediction server (Kelley and Sternberg, 2009). Similar to other well characterized RhoGAPs, including BAR-RhoGAPs such as GRAF1 (in blue), the HMHA1 RhoGAP domain (in yellow) shows, with 9 α-helices, an exclusively helical structure (Fig. 6B). A hallmark of RhoGAPs and other GAPs is the formation of a high-affinity complex with the cognate inactive GDP-bound GTPase, only in the presence of aluminium fluoride AlFx. This mimics the transition state of the GTP hydrolysis (Rho⋅GDP⋅AlFx) (Rittinger et al., 1997). The position of the HMHA1 RhoGAP domain in the complex with human RhoA bound to GDP⋅AlFx (from RhoA⋅GDP⋅AlFx⋅p50RhoGAP; PDB ID: 1tx4), was defined through its superimposition onto the p50RhoGAP domain (Fig. 6B). As described for other RhoGAPs, the HMHA1 GAP domain interacts mainly with the P-loop and the switch regions of RhoA (in light-green, Fig. 6B). The invariant Arg797 of the HMHA1 RhoGAP domain which may represent the catalytic Arg residue (Arg finger) is orientated into the active site of RhoA, close to AlFx and the nucleotide phosphates (Fig. 6B). The catalytic Arg residue neutralizes the developing charge during the GTP hydrolysis and thus stabilizes the transition state (Jelen et al., 2009; Rittinger et al., 1997; Schaefer et al., 2011). The highly conserved Gln residue in the switch II region of Rho GTPases (Gln63 in RhoA, Gln61 in Rac1/Cdc42) is required for an efficient GAP-catalyzed GTP hydrolysis as well, since it coordinates the attacking water for the GTP cleavage (Jelen et al., 2009; Rittinger et al., 1997; Schaefer et al., 2011).

Our homology model indicates that HMHA1 contains the structural requirements to function as a GAP protein and to stimulate the GTP hydrolysis of Rho GTPases. To confirm this, we analyzed GAP activity of HMHA1 in vitro using purified proteins in a cell-free system (see Materials and Methods). As a control, we measured GAP activity...
activity of p50RhoGAP towards RhoA. As expected, when combining p50RhoGAP with RhoA, we observed an increase in the release of inorganic phosphate, generated upon GTP hydrolysis (Fig. 6C; red bars). To analyze HMHA1 GAP activity we used the purified C1-GAPtail protein. These experiments clearly show that HMHA1 C1-GAPtail catalyzes GTP hydrolysis mediated by Rac1, RhoA, and Cdc42 (Fig. 6C; purple versus yellow bars). As HMHA1 has a predicted RhoGAP domain, it should not show GAP activity towards Ras. As expected, HMHA1 C1-GAPtail did not catalyze GTP hydrolysis mediated by Ras (Fig. 6C, right bar). These data indicate that HMHA1 acts as a RhoGAP in vitro.

Several BAR-GAP proteins, including OPHN1 and GRAF1, are autoinhibited by their BAR domain (Eberth et al., 2009). In GAP assays, full-length GRAF1 and OPHN1 did not show GAP activity. However, mutants lacking the N-terminal BAR domain were capable of catalyzing GTP hydrolysis of RhoGTPases (Eberth et al., 2009). As HMHA1 is structurally similar to these proteins and dramatic effects on actin dynamics and cell spreading are observed upon expression of HMHA1 constructs lacking the BAR domain, we wondered whether the HMHA1 BAR domain auto-inhibits GAP function towards RhoGTPases. To analyze this we performed in vitro GAP assays using purified RhoA and HMHA1 FL, as well as the N-terminal construct including the BAR domain.
(N-term), and HMHA1 C1-GAPtail. Similar to what has been shown for GRAF1 and OPHN1, full-length HMHA1 as well as HMHA1 N-term showed little or no GAP activity compared to HMHA1 C1-GAPtail which lacks the N-terminal BAR domain (Fig. 6D; purple bars) suggesting that HMHA1 is both structurally as well as functionally similar to the BAR-GAPs GRAF1 and OPHN1.

In summary, these experiments show that HMHA1 is a genuine RhoGAP and its GAP function is inhibited by the N-terminal BAR domain.

**HMHA1 regulates GTPase activity in vivo**

Next we investigated whether HMHA1 regulates RhoGTPase activity in vivo using Rac1 as a model. We transfected HeLa cells with HMHA1 full-length (FL), the N-terminal region (N-term), and C-terminal constructs (C1-GAP and C1-GAPtail), that lack the BAR domain and measured Rac1GTP loading by a CRIB pull-down assay (see Materials and Methods). Similar to our in vitro results (Fig. 6C, D), full-length HMHA1 and the N-terminal region did not significantly affect Rac1GTP loading. However, both C1-GAP and C1-GAPtail drastically reduced Rac1GTP levels (Fig. 6E) indicating that HMHA1 functions in vivo similar as in vitro. As HMHA1 is not endogenously expressed in HeLa cells we could not analyze Rac1GTP loading when HMHA1 levels are reduced by short interfering RNAs. As expression of HMHA1 is, under normal conditions, restricted to the hematopoietic system, we performed a CRIB pull-down assay using Jurkat T cells that express endogenous HMHA1. HMHA1 expression was reduced via lentiviral shRNA to HMHA1. As expected, knock-down of HMHA1 in Jurkat cells significantly increased Rac1GTP loading (Fig. 6F). Whether HMHA1 regulates GTP loading of Cdc42 and RhoA in vivo as well remains to be investigated.

In summary, we show that HMHA1 regulates the organization of the actin cytoskeleton and cell spreading. We show that HMHA1 both interacts and colocalizes with RhoGTPases. Furthermore, HMHA1 shows GAP activity towards RhoGTPases both in vitro and in vivo and its GAP activity is inhibited by the N-terminal BAR domain. Together our data identify HMHA1 as a novel RhoGAP.

**DISCUSSION**

The human minor H antigen HA-1 has been widely studied in the setting of human Stem Cell Transplantation (SCT) (Hambach and Goulmy, 2005). The minor H antigen HA-1 is a nonameric peptide, encoded from the HMHA1 protein, which is presented to the immune system in an HLA-restricted fashion. The minor H antigen HA-1 is expressed on all cells of the hematopoietic system as well as on most solid epithelial tumors (de Bueger et al., 1992; Klein et al., 2002). Based on its extraordinary expression patterns, HA-1 is an ideal tumor target for Stem Cell based immunotherapy (Hambach and Goulmy, 2005). So much attention focused on the minor H antigen HA-1 in SCT, so few studies have addressed the cell biological role of its encoding gene, i.e. HMHA1. Challenge by the expression and immunological characteristics of the minor H antigen HA-1, we made a first attempt to disclose the function of the gene that encodes this H antigen, HMHA1. Sequence analysis of HMHA1 reveals that the protein encodes an N-terminal BAR domain followed by a C1 and a RhoGAP domain (Fig. 1A) suggesting a role for HMHA1 in the regulation of RhoGTPases (Spierings et al., 2004).

RhoGTPases are key regulators of the actin cytoskeleton and control fundamental processes such as cell spreading and migration (Le Clainche and Carlier, 2008). RhoGTPase signaling is tightly controlled as aberrant signaling has often been linked to malignancies. GAPs terminate RhoGTPase activity as they increase the low intrinsic hydrolysis rate of RhoGTPases (Bernards and Settleman, 2004). Thus, RhoGAPs act as brakes, limiting the duration and level of GTPase signaling output. In the present study, we identified HMHA1 as a novel RhoGAP. We found that HMHA1 has high sequence homology with known GAPs such as GRAF1 and p50RhoGAP including the critical arginine finger and surrounding residues in the catalytic domain. Furthermore, the model we generated of the HMHA1 RhoGAP domain with RhoA suggests that HMHA1 is a RhoGAP. Our in vitro studies further supported this by showing that HMHA1 has GAP activity towards the RhoGTPases, Rac1, Cdc42, and RhoA. Moreover, the N-terminal BAR domain of HMHA1 acts as an autoinhibitory module for GAP function as full-length HMHA1 showed little GAP activity in contrast to the purified C1-GAPtail construct. Based on these observations, HMHA1 can be positioned in a subfamily of structurally related proteins that comprise an N-terminal BAR domain followed by a RhoGAP domain (De Kreuk and Hordijk, in press).

Well-known proteins belonging to this subfamily of BAR-GAPs are RIC1H1, OPHN1, SH3BP1, and GRAF1 (Cicchetti et al., 1995; Fauchereau et al., 2003; Hildebrand et al., 1996; Richnau and Aspenstrom, 2001). Similar to what we observed for HMHA1, GAP function of these proteins is autoinhibited by their N-terminal BAR domain. For BAR-GAPs, such as OPHN1 and GRAF1, it was shown that the N-terminal BAR domain can interact with the GAP domain thereby inhibiting its function (Eberth et al., 2009). As HMHA1 is structurally similar to these BAR-GAPs, a similar mechanism of autoinhibition may apply for HMHA1. The mechanism by which the autoinhibition is released remains to be investigated. This most likely involves stimuli that either target these BAR-GAPs to specific sites within the cell or induce protein-protein interactions, unfolding the protein, thereby releasing the GAP domain from BAR-domain-mediated inhibition.

By regulating RhoGTPase activity, HMHA1 regulates the actin cytoskeleton. A C-terminal fragment of HMHA1, lacking the BAR domain, had dramatic effects on cell morphology. Similar effects were seen when mutants of SH3BP1 lacking its BAR domain were expressed (Cicchetti et al., 1995). Cells expressing the HMHA1 mutants lacking the N-terminus including the BAR domain show loss of F-Actin and focal adhesions. Moreover, these cells spread less compared to control cells. This phenotype is in line with our data showing that HMHA1 acts as a RhoGAP because RhoGTPase activity is needed for proper cell adhesion and migration. Although
we observe in vitro GAP activity towards Rac1, Cdc42, and RhoA, it could well be that in vivo, only a subset of these GTPases is subject to control by HMHA1. The dramatic phenotype induced by HMHA1 C1-GAPtail was rescued by co-expressing constitutively active Rac1 but not Cdc42 suggesting that HMHA1 primarily inactivates Rac1 in vivo. It is well established that the small GTPase RhoA regulates stress fiber formation and focal adhesion turnover (Gardel et al., 2010). Our data indicate that HMHA1 regulates RhoA in vitro and in vivo although rescue experiments have been inconclusive so far. Our Rac-activity assays indicate that HMHA1 regulates Rac1GTP loading in HeLa- and Jurkat cells, which further supports the notion that Rac1 is an in vivo target of HMHA1. Whether Cdc42 and RhoA are HMHA1 targets in vivo as well needs further investigation.

The epithelium forms a physical barrier separating the body from the external environment (Perez-Moreno et al., 2003). To maintain this barrier, epithelial cells form tight cell-cell adhesions with neighboring cells. In many epithelial cancers, a change in tissue architecture takes place called epithelial-mesenchymal transition (EMT) (Guarino, 2007; Guarino et al., 2007). This results in disruption of intercellular contacts as well enhanced cell motility which leads to release of single cells from the epithelial tissue (Guarino et al., 2007; Savagner, 2001) enabling these cells to invade the surrounding tissue. RhoGTPases, and in particular Rac1 and RhoA, regulate epithelial cell-cell adhesion (Citi et al., 2011; Yamada and Nelson, 2007). Thus, tightly controlled and balanced RhoGTPase signaling is essential for maintaining epithelial integrity and deregulation could therefore lead to weakening of cell-cell adhesions which is a first event driving EMT (Guarino et al., 2007; Savagner, 2001). Being a regulator of RhoGTPase output, abnormal HMHA1 expression in epithelial cells could result in EMT and induce tumor cell invasion and metastasis. Interestingly, although HMHA1 expression is restricted to the hematopoietic system under normal conditions (de Bueger et al., 1992), in many epithelial tumor cells HMHA1 gene expression was observed (Klein et al., 2002). Moreover, minor H antigen HA-1-specific cytotoxic T cells eradicate solid epithelial tumors in an in vivo animal model (Hambach et al., 2011). GST-Rac1ΔC and GST-RhoAΔC were a kind gift from A. Wittinghofer (Max-Planck Institute for Molecular Physiology, Dortmund, Germany).

MATERIALS AND METHODS

Antibodies, Reagents, and Expression constructs

**Antibodies:** Anti-Actin (A3853), anti-α-Tubulin (T6199), anti-HA (H3663), and anti-HMHA1 (HPA019816) were from Sigma. Anti-c-myc (13-2500) was from Invitrogen. Anti-Paxillin (610620) was from Transduction Laboratories. For immunofluorescence, anti-Rac1 (05-389) was from Millipore, and for Western blot anti-Rac1 (610651) was from Transduction Laboratories. Secondary HRP-labelled antibodies for Western blot were from Pierce. Secondary Alexa-labelled antibodies for immunofluorescence were from Invitrogen.

**Expression constructs:** To generate myc-tagged HMHA1 deletion constructs, pcDNA-2x-myc-HMHA1 was used as a template for PCR. The following primers were used: For myc-HMHA1 N-term, forward primer 5′-GAGATCGATATCAAGCTTTTCCAGGAAGAAACGAG-3′ and reverse primer 5′-GAGATCTCTAGAGGATCCGAGGGGC-3′. For myc-HMHA1 C1-GAPtail, forward primer, 5′-GAGATCGATATCAAGCTTTTTCGCCAGAGGGGC-3′ and reverse primer 5′-GAGATCTCTAGAGGATCCGAGGGGC-3′. For myc-HMHA1 GAP, forward primer 5′-GAGATCGATATCAAGCTTTTTCGCCAGAGGGGC-3′ and reverse primer 5′-GAGATCTCTAGAGGATCCGAGGGGC-3′. For myc-HMHA1 C1-GAPtail, forward primer 5′-GAGATCGATATCAAGCTTTTTCGCCAGAGGGGC-3′ and reverse primer 5′-GAGATCTCTAGAGGATCCGAGGGGC-3′.

In summary, in this study we identify HMHA1 as a novel RhoGAP that regulates the actin cytoskeleton and cell spreading. As endogenous HMHA1 expression is normally limited to the hematopoietic system, future studies should be aimed at defining the role of HMHA1 in leukocytes in the context of actin remodeling and cell migration. Also, further investigations are needed to determine the GTPases that are the in vivo targets of HMHA1. Furthermore, as HMHA1 expression is detected in several epithelial cancer cells, future studies should focus on how HMHA1 is involved in the transformation and invasive behaviour of these epithelial cells. Finally, HMHA1 might represent an excellent target for tumor therapy because healthy epithelium does not express HMHA1. The notion that HMHA1 indeed exerts GAP activity in vivo, supports further research in this area.
Sigma-Aldrich) was used as a negative control. Lentiviral particles expressing shRNA constructs were prepared using HEK293T cells and virus was transduced as described previously (Nethe et al., 2012).

SDS-PAGE and Western blot analysis
Proteins were separated on SDS-PAGE gels followed by transfer onto nitrocellulose transfer membrane using the iBlot Dry Blotting System (Invitrogen) according to the manufacturers’ recommendations. After blotting, membranes were blocked in 5% low fat milk in TBST (Tris-Buffered Saline Tween-20) for 30 minutes and subsequently the blots were incubated with the primary antibody overnight at 4°C. Next, the blots were washed 3 times for 30 minutes in TBST and subsequently incubated with HRP-conjugated secondary antibodies in TBST for 1 hour at RT. Finally, blots were washed 3 times with TBST for 30 minutes each. Blots were developed by ECL (GE Healthcare, Hoevelaken, The Netherlands).

Confocal Laser Scanning Microscopy
Twenty-four hours after cells were seeded on fibronectin-coated glass coverslips, the indicated plasmids were transfected. After 24 hours, cells were fixed by 3.7% formaldehyde (Merck) in PBS (10 minutes; RT) followed by permeabilization with 0.5% Triton X-100 in PBS (5 minutes; RT). Coverslips were then incubated for 15 minutes with 2% BSA in PBS at 37°C to prevent specific binding. Immunostainings were performed with the indicated antibodies (60 minutes; RT). Fluorescent imaging was performed with a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss MicroImaging, Inc.) using a 63X/NA 1.40 (Carl Zeiss MicroImaging, Inc.). Image acquisition was performed with Zen 2009 software (Carl Zeiss MicroImaging, Inc.).

Cell culture and transfections
Jurkat and HeLa cells were maintained at 37°C and 5% CO2 in Iscove’s Modified Dulbecco’s Medium (IMDM; Biowhittaker) supplemented with 10% heat-inactivated Fetal Calf Serum (Life Technologies, Breda, The Netherlands), 300 μg/ml glutamine, Dulbecco’s Medium (IMDM; Biowhittaker) supplemented with 10% heat-inactivated Fetal Calf Serum (Life Technologies, Breda, The Netherlands), 300 μg/ml glutamine, 2% BSA in PBS at 37°C to prevent specific binding. Immunostainings were performed with the indicated antibodies (60 minutes; RT). Fluorescent imaging was performed with a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss MicroImaging, Inc.) using a 63X/NA 1.40 (Carl Zeiss MicroImaging, Inc.). Image acquisition was performed with Zen 2009 software (Carl Zeiss MicroImaging, Inc.).

GST Pull-Down Assays
For studying the direct interaction of HMHA1 with Rac1 or RhoA, GST-fusion proteins were purified from BL21 bacteria as described previously (de Kreuk et al., 2011). GST-HMHA1 was cut with precision protease (GE Healthcare) overnight at 4°C while rotating. Next, supernatant, containing purified HMHA1 without GST-tag and beads, was harvested and used for the interaction studies or in vitro GAP assay. GST-Rac1 and RhoA were allowed to bind GDP or GppNHP overnight at 4°C while rotating. Binding of HMHA1 to the RhoGTPases was assayed by Western blot analysis using the anti-HMHA1 antibody.

RhoGTPase activity assays
Rac1 activation in HeLa or Jurkat cells, transfected/transduced as indicated, was analyzed by a CRIB-peptide pull-down approach as described previously (de Kreuk et al., 2011). Cells were lysed in NP-40 lysis buffer (50mM TRIS/HCl pH 7.5, 100mM NaCl, 10mM MgCl2, 10% glycerol and 1% NP40) supplemented with protease inhibitors (Complete mini EDTA, Roche). Subsequently, lysates were centrifuged at 20,000xg for 10 minutes at 4°C. The supernatant was then incubated with 30 μg of Pak1-CRIB peptide and incubated at 4°C for 1 hour while rotating. Bound Rac1GTP levels were detected by Western blot analysis. In vitro GAP activity of HMHA1 was measured using a RhoGAP Assay (BK105; Cytoskeleton) according to the manufacturers’ recommendations. In short, purified HMHA1 protein (see above) was incubated together with the small GTPases, Rac1, Cdc42, RhoA, and Ras in the presence of GTP (20 minutes; 37°C). Free inorganic phosphate (generated by the hydrolysis of GTP to GDP) was detected by CytoPhos and subsequently absorbance (650 nm) was measured. We used GTPase or GAP protein only as a negative control and as a measure for the intrinsic hydrolysis rate. p50RhoGAP was used as a positive control for the assay.

Electric resistance measurements
For ECIS-based cell spreading experiments, golden ECIS electrodes (BW10E; Applied Biophysics) were treated with 10 μM L-cysteine for 15 minutes. Subsequently electrodes were coated with 10 μg/ml fibronectin in 0.9% NaCl for 1 hour at 37°C. Next, HeLa cells, transfected as indicated, were seeded at a concentration of 100,000 cells per well in 400 μl IMDM with 10% FCS. Impedance was measured continuously at 45 kHz using ECIS model 9600. The increase in impedance, as a measure of cell spreading (Wegener et al., 2000), was recorded for one hour.

Homology Modeling
The homology model of the HA-1 RhoGAP domain was calculated by submitting the sequence of the human HMHA1 RhoGAP domain (residues 753-973) to the Phyre protein structure prediction server which includes sequence alignments with several RhoGAPs (Kelley and Sternberg, 2009). Superimpositions and figures were prepared with PyMOL (PyMOL Molecular Graphics System, Schoedinger, LLC).

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**Supplementary Figure S1:** HMHA1 induces morphological changes depending on the N-terminal BAR domain. Morphology of HeLa cells, transfected as indicated, was analyzed by phase contrast microscopy. Cells expressing HMHA1 full-length (FL), GAP, or N-term did not show any changes in morphology compared to control cells. HMHA1 C1-GAP, C1-GAPtail, and GAP-tail induce dramatic changes in cell morphology. In addition, these cells are less adhesive than control cells. Scale bars, 50 μm.

**Supplementary Figure S2:** HMHA1 is not involved in microtubule remodeling. The effects of myc-tagged HMHA1 (and deletion constructs) on microtubule distribution was studied by Confocal Laser Scanning Microscopy using HeLa cells. Myc-tagged HMHA1 and microtubules were detected by immunostaining. Although cell morphology is clearly affected, no major effects on microtubule distribution are observed in HeLa cells expressing the indicated HMHA1 constructs. Scale bars, 10 μm.
Supplementary Figure S3: HMHA1 directly interacts with RhoGTPase. (A) Pull-down experiments using GST-EV, and GST-Rac1 loaded with GDP or GppNHP, a GTP analog that cannot be hydrolyzed, show that HMHA1 C1-GAPtail directly interacts with Rac1 preferably when Rac1 is in the active conformation. Association of purified C1-GAPtail was detected by immunoblotting (IB). Ponceau staining indicates equal loading of GST input. (B) Pull-down experiments with GST-Rac1 FL or ΔC, both loaded with either GDP or GppNHP, show that HMHA1 C1-GAPtail directly interacts with active Rac1, independent of the Rac1 hypervariable C-terminus. Association of purified HMHA1 C1-GAPtail was detected by immunoblotting. (C) Pull-down experiments using GST-Rac1 or GST-RhoA, both loaded with either GDP or GppNHP show that purified full-length HMHA1 directly interacts with both active Rac1 and RhoA. Association of purified HMHA1 was detected by immunoblotting.
THE F-BAR PROTEIN PACSIN2 REGULATES GROWTH FACTOR RECEPTOR ACTIVATION AND SIGNALING

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ABSTRACT

Signaling via growth factor receptors, including the EGF receptor, is key to various cellular processes, such as proliferation, cell survival, and cell migration. In a variety of human diseases, such as cancer, aberrant expression and activation of growth factor receptors can lead to disturbed signaling. Intracellular trafficking is crucial for proper signaling of growth factor receptors. As a result, cell surface levels of growth factor receptors are an important determinant for the outcome of downstream signaling.

BAR-domain-containing proteins represent an important family of proteins that regulate membrane dynamics. In this study we identify a novel role for the F-BAR protein PACSIN2 in the regulation of growth factor receptor signaling. We show that internalized EGF as well as the (activated) EGF receptor are translocated to PACSIN2-positive endosomes. Furthermore, loss of PACSIN2 increased plasma membrane expression of the EGF receptor in resting cells and increased phosphorylation of the EGF receptor, as well as of Erk and Akt, in response to EGF compared to control cells. As a consequence, EGF-mediated cell proliferation was enhanced in PACSIN2-depleted cells.

In conclusion, this study identifies a novel role for the F-BAR-domain protein PACSIN2 in regulating growth factor receptor surface levels and downstream signaling.

INTRODUCTION

Signaling via receptor tyrosine kinases (RTKs) is essential for many cellular processes, such as proliferation, differentiation, cell survival, and cell migration (Blume-Jensen and Hunter, 2001; van der Geer et al., 1994). Aberrant expression and activation of RTKs is causally related to human diseases such as cancer, inflammation, and angiogenesis. One of the best studied members of RTKs is the Epidermal Growth Factor Receptor (EGF receptor, also known as ErbB1). The EGF receptor is ubiquitously expressed and increased expression of the receptor is often observed in cancer (Bublil and Yarden, 2007). Inactive EGF receptor resides on the cell surface. Binding of Epidermal Growth Factor (EGF) to the extracellular amino-terminal domain of the EGF receptor leads to receptor autophosphorylation and activation and subsequently to the activation of several downstream signaling pathways, such as Erk and Akt signaling (Gan et al., 2010).

Proper signaling of the EGF receptor, followed by its downregulation, depends on correct intracellular trafficking and localization to appropriate intracellular structures. The level of EGF receptor on the cell surface is an important determinant for the outcome of downstream signaling. In resting cells, approximately 2% of the EGF receptor is constitutively internalized in the absence of ligand and most of the internalized receptor recycles back to the plasma membrane (Herbst et al., 1994). Ligand binding induces rapid internalization of the EGF receptor. This ensures efficient termination of signaling by targeting the receptor for lysosomal degradation or recycling. Upon internalization of the EGF receptor, signaling can continue from early endosomes as well. This has been shown to be important for certain signaling pathways, including the Erk pathway, as inhibition of endocytosis was found to impair pathway activation (Sigismund et al., 2008; Vieira et al., 1996). Depending on the dose of EGF, the EGF receptor can be internalized via clathrin-mediated endocytosis, which has been implicated in recycling of the receptor, and clathrin-independent endocytosis, which has been linked to receptor degradation (Sigismund et al., 2005; Sigismund et al., 2008). It is now widely accepted that internalization and trafficking is an important mode to control EGF receptor signaling (Sorkin and Goh, 2008; Wiley, 2003).

BAR-domain-containing proteins represent an important family of proteins that regulate membrane dynamics. Via their conserved BAR domain, these proteins bind to, stabilize and induce membrane curvature. As a consequence, BAR domains induce invaginations of the plasma membrane and, subsequently, vesicular-tubular structures that are involved in membrane dynamics including receptor internalization (Frost et al., 2009; Tsuji et al., 2006). Several of these BAR-proteins have been implicated in controlling EGF receptor signaling. Cbl can form a complex with the adapter protein CIN85 and the BAR-protein endophilin that initiates EGF receptor internalization thereby controlling receptor signaling and downregulation. Preventing this complex formation inhibits EGF receptor internalization and delays receptor degradation resulting in increased signaling (Soubeyran et al., 2002). Furthermore, the F-BAR (Fer-CIP4 homology-BAR; a subclass of the BAR-domain family) protein CIP4 regulates
late events in EGF receptor trafficking from endosomes towards lysosomes which results in receptor degradation. Lack of CIP4 accumulated the EGF receptor on early endosomes with prolonged signaling as a result (Hu et al., 2009).

Here we describe an additional F-BAR family member, PACSIN2, that controls growth factor signaling. PACSIN2 associates to several proteins such as Rac1, Dynamin, N-WASP, and Synaptojanin via its C-terminal Src Homology 3 (SH3) domain (Chitu and Stanley, 2007; de Kreuk et al., 2011; Kessels and Qualmann, 2004). The PACSIN2 F-BAR domain, located in its N-terminal region, mediates membrane binding and is involved in homo- and hetero-oligomerization (Kessels and Qualmann, 2006). Due to this oligomerization, PACSIN2 can associate to multiple proteins at once, linking the actin-regulatory network with the endocytic machinery (Kessels and Qualmann, 2004).

The current study shows that PACSIN2 is a negative regulator of growth factor receptor activation and signaling. Initially we found that EGF is internalized to PACSIN2-positive vesicles and we could visualize accumulation of both total and activated EGF receptor on PACSIN2-positive endosomes upon EGF stimulation. We show that in PACSIN2 knock-down cells, as well as in cells expressing an SH3- or BAR-domain mutant of PACSIN2 (inhibiting protein-interactions and internalization respectively), EGF receptor surface levels were increased. Furthermore, EGF-mediated activation and phosphorylation of the EGF receptor as well as of its downstream targets, Erk and Akt, is enhanced in PACSIN2 knock-down cells. As a result, loss of PACSIN2 enhanced EGF-induced cell growth. Finally, we show that these effects are not specific for EGF because signaling by HGF, and in primary endothelial cells also by VEGF and TNFα, is similarly regulated by PACSIN2. In summary, these data show that PACSIN2 is a key regulator of growth factor signaling in epithelial and in endothelial cells, regulating growth factor receptor surface levels and downstream signaling.

RESULTS

The activated EGF receptor and internalized EGF localize to PACSIN2-positive early endosomes

In initial live-cell imaging experiments we found that a YFP-PACSIN2 fusion protein shows extensive internalization upon stimulation of the cells with EGF. Prior to stimulation, a fraction of PACSIN2 is constitutively internalized on vesicular-tubular structures that arise from membrane ruffles (de Kreuk et al., 2011). EGF stimulation induces PACSIN2 to accumulate on peripheral structures, both small tubules as well as vesicles, with a concomitant reduction of PACSIN2 localization at the peripheral membrane (Fig. 1A, Supplementary Movie 1). To analyze in more detail the effects of EGF on PACSIN2 distribution, we documented endogenous PACSIN2 localization by confocal microscopy. Similar to the data in Fig. 1A, PACSIN2 is in resting cells partially localized on vesiculo-tubular structures in the cell periphery as well as on perinuclear vesicles which we previously identified as early endosomes (de Kreuk et al., 2011) (Fig. 1B; upper panels). Upon EGF stimulation, PACSIN2-positive peri-nuclear vesicles become slightly enlarged (Fig. 1B; bottom panels and zoomed images). To test whether EGF localizes to the PACSIN2-positive compartment we analyzed the distribution of internalized Texas Red-labelled EGF (EGF-TR) by confocal microscopy. Five minutes after its addition, a fraction of internalized EGF-TR localized to PACSIN2-positive endosomes and this fraction was significantly increased (from 34 to 76%) after 10 or 15 minutes of EGF-TR internalization (Fig. 1C). At 30 minutes after addition, we could still find EGF-TR localizing to PACSIN2-positive endosomes, although less than in earlier time points suggesting that after 30 minutes, EGF-TR leaves the PACSIN2-compartment (Fig. 1C). This fits with the notion that internalized EGF traffics through the early endosomal compartment (where PACSIN2 is present) towards the recycling or late endosomal compartment (Haglund and Dikic, 2012; Sorkin and Goh, 2008).

To establish whether receptor activation results in translocation of the receptor to PACSIN2-positive endosomes, we stained unstimulated or EGF-TR-stimulated HeLa cells for endogenous PACSIN2. In addition, we visualized tyrosine-phosphorylated proteins using an antibody against phosphorytrosine (pTyr). Stimulation of cells with EGF-TR caused a distinct accumulation of tyrosine-phosphorylated proteins on PACSIN2-positive early endosomes (Supplementary Fig. S1). This suggests that the activated EGF receptor is targeted to PACSIN2-positive endosomes. To confirm this, we immunostained control or EGF-stimulated HeLa cells for the EGF receptor as well as specifically for the activated EGF receptor using a phospho-EGFR-specific (Tyr1068) antibody (Rush et al., 2012). We could not immunostain simultaneously for the endogenous EGF receptor and endogenous PACSIN2, since the antibodies for detection were from the same species. Because PACSIN2 colocalizes with Early Endosomal Antigen-1 (EEA1) on early endosomes (Fig. 2C; de Kreuk et al., 2011), we used EEA1 as an intermediate to analyze colocalization of the (activated) EGF receptor with PACSIN2. EGF stimulation induces accumulation of the (activated) EGF receptor on early endosomes (Fig. 2, A and B) where also PACSIN2 is localized (Fig. 2C). This data is in good agreement with what we showed for internalized EGF-TR (Fig. 1C).

Together, these data show that both internalized EGF-TR and the activated EGF receptor accumulate on PACSIN2-positive early endosomes upon EGF stimulation.

PACSIN2 regulates EGF receptor surface expression levels

As demonstrated above, both internalized EGF and the EGF receptor localize to a PACSIN2-positive endocytic compartment (Fig. 1 and 2). Since PACSIN2 is an important regulator of membrane dynamics, PACSIN2 may control endocytic traffic of the EGF receptor. We therefore questioned whether PACSIN2 can regulate surface levels of the EGF receptor. To study this, we performed surface biotinylation experiments (see Material and Methods). HeLa cells, treated with either siRNA or lentiviral shRNA constructs to reduce PACSIN2 expression, were incubated with sulfo-NHS-LC-biotin at 4°C to block internalization. Following streptavidin-based pull-downs of cell lysates we analyzed protein surface expression by Western blotting. To certify that
only surface proteins were isolated, we stained our blots for RhoGDI, known to be localized to the cytosol (Olofsson, 1999). As expected, RhoGDI was not detected in the pull-down fraction (Fig. 3). Interestingly, EGF receptor surface expression showed a marked increase in HeLa cells treated with either PACSIN2-specific siRNA (Fig. 3A) or lentiviral shRNA directed against PACSIN2 (Fig. 3B). Surface levels of β1-integrin were comparable in PACSIN2 knock-down cells versus control cells (Fig. 3, A and B) indicating that the loss of PACSIN2 does not in general affect surface proteins. These experiments indicate that, in the absence of EGF, PACSIN2 controls the surface levels of the EGF receptor.

We next questioned whether PACSIN2 can regulate EGF-mediated internalization of the EGF receptor. HeLa cells, treated with control and PACSIN2 siRNA oligos, were incubated with EGF for the indicated time points. We then performed a surface biotinylation experiment as described above and analyzed EGF receptor surface expression by Western blotting. As expected, EGF stimulation downregulated EGF receptor surface levels in control cells (Fig. 4A; upper and middle panel). Although we observed higher EGF receptor surface levels in PACSIN2 knock-down cells upon EGF stimulation compared to control cells, still an EGF-mediated downregulation of the receptor was observed (Fig. 4A; upper and middle panel). This could be explained by the fact that in resting cells, EGF receptor surface levels are already higher in PACSIN2 knock-down cells compared to control cells. These experiments indicate that, although PACSIN2 knock-down cells consistently show higher EGF receptor surface expression, EGF-mediated internalization of the receptor is not impaired.

We then questioned what the effects were on EGF receptor surface expression of the ectopic expression of wild-type PACSIN2 (P2-WT). In addition, we also expressed an SH3-domain mutant of PACSIN2 (P2-Y435E/P478L), which is impaired in binding to several proteins regulating membrane and cytoskeletal dynamics such as Dynamin and N-WASP (Chitu and Stanley, 2007; Kessels and Qualmann, 2004). Finally, we also expressed a BAR-domain mutant (P2-R50D), that, because of an arginine to aspartic acid exchange, is not able to form membrane-associating domains.

**Figure 1:** EGF stimulation alters PACSIN2 distribution and is internalized to PACSIN2-positive endosomes. (A) Live-cell imaging of HeLa cells, transfected with YFP-PACSIN2, shows that PACSIN2 is associated with vesicular-tubular structures upon constitutive internalization in unstimulated cells. EGF (100 ng/ml) stimulation transiently accumulated internalized PACSIN2 in peripheral structures, both small tubules as well as vesicles. (See also supplementary movie 1). (B) Intracellular localization of endogenous PACSIN2 in response to EGF (5 minutes; 100 ng/ml) was studied by confocal microscopy using HeLa cells. In resting cells, PACSIN2 is localized at membrane ruffles, partially on vesicular-tubular structures, and on perinuclear vesicles. Upon EGF stimulation, a reduction of peripheral tubules was observed together with enlarged PACSIN2-positive perinuclear vesicles. (C) EGF-TexasRed (100 ng/ml) transiently translocates upon internalization to PACSIN2-positive endosomes with a peak at 10-15 minutes. At 30 minutes, some EGF-TexasRed can still be observed on PACSIN2-positive vesicles. Colocalization images show the amount of EGF-TexasRed colocalized to PACSIN2 structures in white. Numbers depict mean colocalization ± SEM measured using Zen 2009 software. Scale bars, 10 μm. *** P<0.001 in comparison to 5 minute time point. # P<0.01 in comparison to 15 minute time point.
Figure 2: The activated EGF receptor accumulates on PACSIN2-positive early endosomes upon EGF stimulation. (A-C) Using confocal microscopy, colocalization of the EGF receptor (A), phosphorylated EGF receptor at Tyr1068 (B), and PACSIN2 (C) with Early Endosomal Antigen-1 (EEA1) was analyzed. Endogenous proteins were detected by immunostaining. EGF (5 minutes; 100 ng/ml) stimulation accumulates the (phosphorylated) EGF receptor on early endosomes (A-B) where PACSIN2 is also present (C). Colocalization plots show the amount of EGF receptor (A), phospho-EGF receptor (B), and PACSIN2 (C) localized to EEA1-positive endosomes in white. Bar diagrams show mean colocalization ± SEM. Scale bars, 10 μm. *** P<0.001.

Figure 3: Knock-down of PACSIN2 increases EGF receptor surface levels in resting cells. (A and B) Left panels: Surface biotinylation experiments, using HeLa cells, were performed to isolate all surface proteins. Endogenous PACSIN2, EGF receptor, and β1-integrin were detected by immunoblotting (IB). To certify that only surface proteins are isolated, RhoGDI, known to be localized to the cytosol, was detected by immunoblotting in conjunction with actin which, together with RhoGDI, served as a loading control for the PACSIN2 knock-down samples. EGF receptor surface expression showed a marked increase in HeLa cells treated with either PACSIN2-specific siRNA (A) or lentiviral shRNA directed against PACSIN2 (B). Surface levels of β1-integrin were comparable in PACSIN2 knock-down cells versus control cells. Right panels: Quantification of protein surface expression. Values are relative to control cells. Data are mean values ± SEM of three independent experiments. * P<0.05. TCL, Total Cell Lysate. PD, Pull-Down.
acid mutation at position 50, can no longer bind to membranes and induce vesicular-tubular structures required for proper PACSIN2-mediated membrane dynamics (Shimada et al., 2010; Wang et al., 2009). Compared to control cells, expression of wild-type PACSIN2 did not affect EGF receptor surface expression, neither in resting nor in EGF-stimulated cells (Fig. 4B; upper and middle panel). Interestingly, similar to the knock-down experiments, ectopic expression of both the SH3-domain and the BAR-domain mutants of PACSIN2, increased the EGF receptor surface levels in unstimulated cells. In addition, although higher surface levels of the EGF receptor were observed after EGF stimulation in cells expressing both PACSIN2 mutants, EGF still induced internalization of EGF receptor (Fig. 4B; upper and middle panel). Interestingly, we observed a slight increase in total EGF receptor levels as well (Fig. 4, A and B; bottom panels), suggesting that functional inhibition of PACSIN2 results in reduced receptor degradation. Moreover, we could (partially) prevent this in cells where proteosomal and lysosomal degradation was blocked using MG132 and Chloroquine respectively (data not shown).

Next, we analyzed internalization of the EGF receptor upon stimulation with EGF-TR by confocal microscopy. We allowed EGF-TR to internalize in HeLa cells, transfected with control or PACSIN2 siRNA, for several time points and analyzed colocalization of EGF-TR as well as the EGF receptor itself with early endosomes, visualized by EEA1 staining. After 15 minutes of internalization we did not find a statistically significant reduction in internalized EGF-TR and EGF receptor to early endosomes in PACSIN2 knock-down cells (Supplementary Fig. S2). In addition, after 30 or 90 minutes following addition of EGF-TR no major difference was observed either, with the exception of a small difference at t = 60 min, indicating that internalization of the EGF receptor upon stimulation with EGF-TR is not impaired in PACSIN2 knock-down cells (Supplementary Fig. S2).

Together, these data indicate that inhibiting PACSIN2 function, either by knockdown or by ectopic expression of dominant-negative mutants, resulted in increased EGF receptor expression at the plasma membrane. Under these conditions, ligand-induced loss of surface EGF receptor levels remain largely unaffected. Furthermore, internalization of EGF-TR is similar in control cells versus PACSIN2-depleted cells, suggesting that PACSIN2 is not required for ligand-induced EGF receptor internalization but may regulate ligand-independent receptor internalization, receptor degradation and/or recycling.

Knock-down of PACSIN2 increases EGF-mediated phosphorylation of the EGF receptor

To determine whether the increased surface levels of the EGF receptor upon loss of PACSIN2 affects cellular responses to EGF, we first examined EGF receptor phosphorylation in control or PACSIN2 knock-down cells. Initially, to visualize EGF receptor phosphorylation we used an antibody against tyrosine phosphorylated EGF receptor, which is seen as a band at around 170 kDa in control cells. In contrast, in PACSIN2 knock-down cells, this band is less prominent and a new band appears at around 100 kDa, which presumably represents phosphorylated EGF receptor.

Figure 4: Knock-down of PACSIN2 or expression of dominant-negative PACSIN2 mutants increases EGF receptor surface levels but not EGF-induced internalization. (A) Upper panel: Surface biotinylation experiments, using HeLa cells, were performed to isolate all surface proteins. Endogenous PACSIN2 and EGF receptor were detected by immunoblotting (IB). In non-stimulated cells, knock-down of PACSIN2 increased EGF receptor surface levels. In HeLa cells stimulated with EGF for the indicated time points, depletion of PACSIN2 increased EGF receptor surface levels compared to control cells. However, EGF-mediated internalization is not impaired. A slight increase in total EGF receptor expression was observed as well in PACSIN2 knock-down cells versus control cells. Middle and bottom panels: Quantification of EGF receptor surface (middle panel) and total EGF receptor (bottom panel) expression levels. Values are normalized to non-stimulated control cells. Data are mean values ± SEM of three independent experiments. (B) Upper panel: Surface biotinylation experiments, using HeLa cells transfected as indicated, were performed to isolate all surface proteins. Endogenous EGF receptor and myc-tagged constructs were detected by immunoblotting. Cells expressing wild-type PACSIN2 (P2-WT) did not show altered surface or total EGF receptor levels. However, a SH3-domain mutant (P2 Y435E/P478L) or a BAR-domain mutant (P2 R50D) increased EGF receptor surface levels compared to control cells (EV). In addition, EGF stimulation induced internalization of the EGF receptor albeit that levels of EGF receptor in cells expressing either mutant were still increased compared to control cells (EV). Middle and bottom panel: Quantification of EGF receptor surface (middle panel) and total EGF receptor (bottom panel) expression levels. Values are relative to non-stimulated control cells. Data are mean values ± SEM of three independent experiments.
proteins (pTyr) and focused on the 175 kD activated EGF receptor band. siRNA-mediated knock-down of PACSIN2 clearly increased EGF-mediated phosphorylation of the 175 kD band (Fig. 5A). Subsequently, we visualized EGF receptor phosphorylation with an antibody specific for phosphorylation on Tyr1068. Similar to what we observed in Fig. 5A, siRNA-mediated knock-down of PACSIN2 increased EGF receptor phosphorylation on Tyr1068 in response to EGF (Fig. 5B). To exclude off-target effects of the PACSIN2 siRNA, we examined EGF receptor phosphorylation in HeLa cells treated with a lentiviral shRNA targeting PACSIN2. In line with the results in Fig. 5B, lentiviral knock-down of PACSIN2 results in increased phosphorylation of the EGF receptor upon EGF stimulation (Fig. 5C). Interestingly, the effect of loss of PACSIN2 on EGF receptor phosphorylation mainly in the first 20 minutes because at later time points, EGF receptor phosphorylation declined to control levels (Fig. 5). In contrast, HeLa cells that ectopically express PACSIN2 show a slight reduction in EGF receptor phosphorylation in response to EGF (data not shown). However, this effect is limited, compared to the effects seen in the siRNA experiments, indicating that levels of endogenous PACSIN2 in HeLa cells are sufficient to regulate EGF receptor activation and phosphorylation.

Together, these data show that PACSIN2 acts as a negative regulator of EGF receptor activation and phosphorylation. The marked increase in EGF-mediated receptor phosphorylation observed in PACSIN2 knock-down cells reveals an important, previously unnoticed, role for PACSIN2 in regulating EGF receptor activation.

PACSIN2 negatively regulates signaling downstream of the EGF receptor

EGF engagement leads to activation of the EGF receptor and of several downstream intracellular signaling pathways, such as the Erk and Akt pathways (Grant et al., 2002; Wells, 1999). As we observed a marked increase in EGF receptor phosphorylation in PACSIN2 knock-down cells, we decided to study whether loss of PACSIN2 affects signaling downstream of the EGF receptor as well. Therefore, we analyzed EGF-mediated Erk phosphorylation in HeLa cells transfected with the PACSIN2 siRNA. Interestingly, in resting cells, loss of PACSIN2 results in a slight decrease in the levels of phosphorylated Erk1/2. In contrast, upon EGF stimulation we observed an increase in Erk phosphorylation in PACSIN2 knock-down cells compared to control cells (Supplementary Fig. S3, A and B). In combination, these changes result in enhanced Erk1/2 phosphorylation in response to EGF in PACSIN2 knock-down cells (Supplementary Fig. S3B; bottom panel).

To study Erk phosphorylation in a more quantitative manner, we used a NanoPro Assay (see Materials and Methods). Here, levels of (un)-phosphorylated Erk are quantitatively measured by separation of proteins based on their pI, followed by immunodetection. As shown in Fig. 6A, for each Erk isoform, both the unphosphorylated and phosphorylated species, a peak is generated and the size of the peak (measured by the area under the peak) represents the (phospho) protein levels. Using this approach, it is possible to study in a quantitative manner, the amount of Erk phosphorylation for both isoforms separately but in the same samples. Fig. 6A (upper panel) shows that in resting cells, the majority of Erk is in its unphosphorylated state. However, upon EGF stimulation, a shift towards the phosphorylated state can be clearly observed (Fig. 6A; bottom panel). Using this technique, we analyzed the effects of loss of PACSIN2 on EGF-mediated Erk phosphorylation.
phosphorylation. Similar to the Western blotting results, siRNA-mediated knock-down of PACSIN2 results in a significant increase in Erk phosphorylation in response to EGF compared to control cells (Fig. 6, B and C).

In addition to Erk phosphorylation, we analyzed whether loss of PACSIN2 affects EGF-induced Akt phosphorylation as well. Western blot analysis shows that upon knock-down of PACSIN2, levels of phosphorylated Akt are slightly decreased. However, upon stimulation with EGF, Akt phosphorylation is increased in PACSIN2 knock-down cells compared to control cells (Supplementary Fig. S4, A and B) resulting in enhanced EGF-mediated Akt phosphorylation in PACSIN2 knock-down cells. These findings are similar to what we observed for Erk phosphorylation. Next we analyzed Akt phosphorylation using the NanoPro Assay. In contrast to Erk, the peak pattern for Akt phosphorylation comprises a series of different Akt phospho-species. Therefore, we determined Akt phosphorylation based on the combined signal generated upon detection with a phospho-specific Akt antibody. The NanoPro 1000 system is capable of generating a Western blot-like representation of the data (Supplementary Fig. S4C; left panel) which gives an indication of the level of Akt phosphorylation. Similar to our Western blotting results, it is clear that loss of PACSIN2 results in increased Akt phosphorylation in response to EGF (Supplementary Fig. S4C; right panel). These experiments show that PACSIN2 is an important regulator of EGF-mediated signaling as knock-down of PACSIN2 results in enhanced phosphorylation of Erk and Akt in response to EGF.

EGF receptor signaling is involved in various processes including cell growth. Therefore, we analyzed the role of PACSIN2 in EGF-mediated cell growth. HeLa cells, treated with control or PACSIN2-specific siRNA oligos, were stimulated with EGF. 24 hours after addition of EGF, cell numbers were determined in the different conditions. Loss of PACSIN2 has no effect on proliferation of cells that were not stimulated with EGF. However, EGF stimulation did lead to a significant increase in cell number in PACSIN2 knock-down cells compared to control cells (Fig. 6D).

Together, these data show that PACSIN2 is a key player in regulating the activation and downstream signaling of the EGF receptor. EGF-mediated activation and phosphorylation of the EGF receptor as well as of its downstream targets, Erk and Akt, is enhanced in PACSIN2 knock-down cells. As a result, loss of PACSIN2 enhanced EGF-induced cell growth.

PACSIN2 is a generic regulator of growth factor signaling

So far, we have shown that PACSIN2 is an important regulator of EGF receptor signaling. We questioned whether the regulatory role of PACSIN2 is specific for the EGF receptor or that PACSIN2 regulates growth factor receptor signaling in general. To study this we investigated whether PACSIN2 can regulate signaling downstream of the Hepatocyte Growth Factor (HGF) receptor, which is known to activate the MAPK pathway (Liu et al., 2002). HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with HGF. Using the NanoPro Assay, we analyzed Erk phosphorylation and we observed, similar to what we have shown for EGF, enhanced phosphorylation of Erk in response to HGF (Fig. 7A). Next we questioned whether the role for PACSIN2 is cell-type specific. Therefore we analyzed Erk phosphorylation...
in primary human endothelial cells in response to VEGF and TNFα, both known to activate the MAPK pathway (Mechtcheriakova et al., 2001). Human Umbilical Vein Endothelial Cells (HUVEC), treated with control or PACSIN2-specific lentiviral shRNA constructs, were stimulated with either TNFα or VEGF. Subsequently, Erk phosphorylation was measured using the NanoPro Assay. Phosphorylation of Erk, induced by TNFα (Fig. 7B), showed a marked increase in cells transduced with the PACSIN2 shRNA, compared to control cells. Moreover, although less prominent, loss of PACSIN2 also increased Erk activation downstream of VEGF (Fig. 7C).

Collectively, these data show that PACSIN2 regulates growth factor signaling. Loss of PACSIN2 results in an increase in Erk activation downstream of both EGF and HGF in epithelial cells and of TNFα and VEGF in endothelial cells indicating that PACSIN2 plays a key role in regulating growth factor receptor output, regulating various growth factors in different types of cell.

**DISCUSSION**

BAR-domains are found in proteins that are important regulators of membrane dynamics and vesicular traffic. Several BAR-domain proteins have been implicated in regulating growth factor signaling. The BAR-domain protein endophilin regulates, in conjunction with Cbl and CIN85, EGF receptor internalization. Inhibition of this complex, e.g. via Alix/AIP1, impairs proper receptor endocytosis thereby preventing signal termination with increased signaling as a result (Schmidt et al., 2004; Soubeyran et al., 2002). These studies underscored the importance of receptor internalization in controlling signaling output. In addition to internalization, receptor sorting towards the lysosomal pathway, with receptor degradation as a consequence, represents an alternative mechanism to terminate growth factor signaling. Several BAR-domain proteins have been shown to regulate the sorting of the EGF receptor. Sorting Nexins (SNXs), of which several comprise a BAR domain, have emerged as a group of proteins, associated with endosomal compartments, that regulate receptor trafficking (van Weering et al., 2010; Worby and Dixon, 2002). SNX1 and SNX5 associate with each other via their BAR domain. Interestingly, whereas SNX5 prevents EGF receptor degradation, SNX1 promotes degradation of the EGF receptor and attenuates the effect of SNX5 (Kurten et al., 1996; Liu et al., 2006).

To avoid aberrant growth factor signaling, with severe diseases as potential outcome, efficient signal termination is of great importance. Upon growth factor receptor activation, the receptor is internalized and subsequent post-endocytic traffic targets the receptor either for degradation or for recycling back to the plasma membrane. The increased surface EGF receptor levels in unstimulated, PACSIN2 knock-down cells could be due to decreased internalization or increased receptor recycling. Although we could not find clear evidence that PACSIN2 regulates ligand-dependent internalization of the EGF receptor, because EGF stimulation still reduced surface EGF receptor levels, it could well be that PACSIN2 is involved in ligand-independent internalization of the EGF receptor. Ligand-independent, constitutive receptor internalization is not well understood, as most studies focused on ligand-induced downregulation of growth factor receptors. However, it was shown that expression of dominant active Rab5 (Q79L) caused ligand-independent internalization of the EGF receptor thereby decreasing surface EGF receptor levels. As a result, upon EGF stimulation, less receptor activation and downstream signaling was observed (Dinneen and Ceresa, 2004). Interestingly, we have previously shown that PACSIN2 is localized to Rab5-positive endosomes (de Kreuk et al., 2011). Since Rab5Q79L accumulates PACSIN2 on these endosomes (unpublished data) it could well be that PACSIN2 functions in the same pathway regulating ligand-independent internalization of the EGF receptor. In accordance, we show that loss of PACSIN2 results in increased EGF receptor surface levels and subsequently increased EGF-mediated signaling.

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**Figure 7: PACSIN2 is a generic regulator of growth factor signaling.** (A–C) Using the NanoPro 1000 Assay, Erk phosphorylation was measured downstream of HGF in HeLa cells, and downstream of TNFα and VEGF in HUVECs. Upper and middle panels: Knock-down of PACSIN2 enhanced Erk1 (upper panels) and Erk2 (middle panels) phosphorylation downstream of hHGF (A), TNFα (B), and VEGF (C) compared to control cells. Bottom panels: Immunoblotting (IB) was performed to assess siRNA mediated knock-down of PACSIN2 in HeLa cells (A) and shRNA-mediated knock-down of PACSIN2 in HUVECs (B and C). Graphs are representative for three independent experiments. Data are normalized to basal phospho-Erk levels. Error bars indicate SEM.
Recent studies have revealed distinct internalization pathways depending on EGF concentrations. High (100 ng/ml) concentrations of EGF lead to clathrin-independent/lipid raft-dependent internalization of the EGF receptor while low (1 ng/ml) EGF concentrations lead to clathrin dependent internalization (Sigismund et al., 2005). Interestingly, some studies showed that this raft-mediated internalization is associated with EGF receptor degradation while clathrin-mediated internalization is associated with sustained signaling (Sigismund et al., 2008). We previously found evidence that PACSIN2 could function in raft-mediated endocytosis as we found Cholera Toxin B to be internalized via PACSIN2-positive tubular structures (de Kreuk et al., 2011) so it could well be that depletion of PACSIN2 shifts internalization of the EGF receptor to a clathrin-dependent pathway with increased signaling as a result. In line with this, we found no effect of PACSIN2 depletion on EGF receptor internalization when using low EGF concentrations (unpublished data). However, this topic requires further investigation as some studies have implicated PACSIN proteins in clathrin-mediated internalization as well (Modregger et al., 2000; Qualmann and Kelly, 2000).

In addition to internalization, an alternative mechanism that controls receptor surface levels is recycling. Similar to the F-BAR protein CIP4 (Hu et al., 2009), PACSIN2 could regulate receptor surface expression by regulating receptor sorting. Upon ligand-(in)dependent internalization, part of the receptor is targeted for degradation while the remainder recycles back to the plasma membrane (Sorkin and Goh, 2008; Wiley, 2003). Receptors destined for degradation travel via early endosomes to multivesicular endosomes/bodies (MVBs) and are eventually degraded in lysosomes. The ESCRT protein Tsg101 is involved in the formation of these MVBs (Razi and Futter, 2006). Upon depletion of Tsg101, EGF receptor degradation was impaired and consequently increased EGF-mediated receptor phosphorylation was observed (Rush et al., 2012). Similarly, preventing EGF receptor degradation by the pharmacological inhibitor monensin, which blocks acidification of early endosomes and thereby formation of lysosomes (King, 1984), resulted in accumulation of the EGF receptor on early endosomes and enhanced receptor phosphorylation (Rush et al., 2012). In line with these studies, we observe less degradation and increased MAP kinase activation when PACSIN2 is depleted. However, in these published studies, the EGF receptor is retained on early endosomes causing increased signaling. In contrast, we did not find clear evidence that PACSIN2 depletion retains the EGF receptor on early endosomes. Thus, both inhibition of ligand-independent internalization and a shift towards recycling would result in less receptor degradation and an increase in surface expression, which is what we observed in PACSIN2 knock-down cells or cells expressing dominant-negative PACSIN2 mutants.

An interesting additional finding of this study was that the regulatory role of PACSIN2 is not specific for the EGF receptor. Depletion of PACSIN2 enhanced Erk activation downstream of EGF and HGF in epithelial cells but also downstream of VEGF and TNFα in endothelial cells indicating a generic role for PACSIN2 in growth factor receptor signaling.

In conclusion, our data suggest the following model (Fig. 8). In control cells, constitutive internalization of the growth factor receptor takes place (a.). Subsequently, the receptor gets partially degraded (b.) but the main fraction recycles back to the plasma membrane (c.). When PACSIN2 is depleted, growth factor receptor at the surface accumulates, consequent to either inhibition of ligand-independent internalization (1.) or increased receptor recycling (2.), or both. As a result of increased surface receptor levels in PACSIN2-depleted cells, growth factor stimulation increases activation of the receptor and downstream signaling, possibly also from sustained endosomal signaling. In summary, this study identifies a novel regulatory role for the F-BAR-domain protein PACSIN2 in regulating growth factor receptor surface levels and signaling output.

Figure 8: PACSIN2 regulates growth factor receptor surface expression thereby controlling receptor output. In control cells, constitutive internalization (a.) of the growth factor receptor occurs. Upon internalization, a fraction is targeted for degradation (b.) while the remainder recycles back to the plasma membrane (c.). In PACSIN2 depleted cells, increased growth factor receptor surface levels are observed either by inhibiting ligand-independent endocytosis (1.) or by increased receptor recycling (2.). As a result of increased surface receptor levels (and possibly sustained endosomal signaling), in cells depleted for PACSIN2, growth factor stimulation enhanced receptor activation and downstream signaling (indicated by the blue arrows).
MATERIALS AND METHODS

Antibodies, Reagents, and Expression constructs

**Antibodies:** Anti-PACSIN2 (AP8088b) was from Abgent. Anti-Actin (A3853) was from Sigma. Anti-EEA1 (610457) was from BD Transduction Laboratories. Anti-pTyr (pY120, 03-7720), anti-Transferrin Receptor (13-6800), and anti-c-myc (13-2500) were from Invitrogen. Anti-β1-integrin (610468) and anti-RhoGDI (610255) were from BD Transduction Laboratories. Anti-EGFR (4267), anti-pEGFR-Y1068 (3777), anti-Akt (9272), and anti-p-Akt-S473 (37875) were from Cell Signaling. For Western blot, anti-Erk (SC-153) and anti-p-Erk (SC-7383) were from Santa Cruz. For the NanoPro assays, anti-Erk1/2 (06-182) was from Millipore and anti-p-Erk (9101) was from Cell Signaling. For detection in NanoPro, secondary HRP-labelled antibodies were from ProteinSimple. Secondary HRP-labelled antibodies for Western blot were from Pierce. Secondary Alexa-labelled antibodies for immunofluorescence were from Invitrogen. F-Akt was detected using Texas-Red- or Alexa-633-labelled Phalloidin (Invitrogen). Nuclei were stained with Hoechst (H-3569; Invitrogen).

**Reagents:** Recombinant Human Epidermal Growth Factor (hEGF; Cyt-217) and recombinant human Hepatocyte Growth Factor (hHGF; Cyt-244) were obtained from Prospeck and used at a concentration of 100 ng/ml for the indicated time points. EGF-Texas Red was obtained from Molecular Probes (E-3480) and used at a concentration of 100 ng/ml for the indicated time points. Recombinant human Vascular Endothelial Growth Factor (hVEGF) was obtained from R&D Systems (293-VE). Recombinant human Tumor Necrosis Factor-α (hTNF-α) was obtained from Peprotech (300-01A).

**Expression constructs:** pEYFP-PACSIN WT and myc-tagged PACSIN2 R50D were described previously (de Kreuk et al., 2011). Myc-tagged PACSIN2 WT and myc-tagged PACSIN2 Y435E/P478L were a kind gift from Markus Plomann (University of Cologne, Germany).

Cell culture and transfections

HeLa cells are maintained in a humidified atmosphere at 37°C and 5% CO2 in Iscove’s Modified Dulbecco’s Medium (IMDM; Biowhittaker) supplemented with 1% heat-inactivated Fetal Calf Serum (Life Technologies, Breda, The Netherlands), 300 μg/ml glutamine, 100 units/ml penicillin and streptomycin. Primary Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza (Baltimore M.D) and cultured in Modified Dulbecco's Medium (IMDM; Biowhittaker) supplemented with 10% heat-inactivated Fetal Calf Serum (Life Technologies, Breda, The Netherlands), 300 μg/ml glutamine, 100 units/ml penicillin and streptomycin. Primary Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza (Baltimore M.D) and cultured in EGM2 medium, containing singlequots (Lonza Baltimore M.D.). For ectopic expression, HeLa cells were transiently transfected with TransIT (Mirus) according to the manufacturers’ recommendations.

Lentiviral shRNAi and siRNA silencing

Lentiviral shRNA constructs for PACSIN2 from the TRC/Sigma Mission library were obtained from Sigma-Aldrich (St. Louis, MI, USA). The SHC002 scrambled shRNA construct (Sigma-Aldrich) was used as a negative control. All shRNA constructs were in the pLKO.1 vector backbone. shRNA-expressing lentiviral particles were prepared using HEK293T cells and virus was transduced as described previously (Nethe et al., 2010).

The sequence for control siRNA was: 5’- CGUAGCGGGAUACUUCGUATT-3’ (Eurogentec). PACSIN2 siRNA was described before (de Kreuk et al., 2011). Transfections of siRNA were performed with INTERFERIn (Polyplus transfection) according to the manufacturers’ recommendations.

SDS-PAGE and Western blot analysis

Proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose transfer membrane using the iBlot Dry Blotting System (Invitrogen) according to the manufacturers’ recommendations. Following blocking in 5% low fat milk in TBST (Tris-Buffered Saline Tween-20) for 30 minutes, the blots were incubated with the primary antibody overnight at 4°C. Next, the blots were washed 5 times for 10 minutes in TBST and subsequently incubated with HRP-conjugated secondary antibodies (dilution 1:5000) in TBST for 1 hour at RT. Finally, blots were washed 3 times with TBST for 30 minutes each and subsequently developed by ECL (GE Healthcare, Hoeveraken, The Netherlands).

Confocal Laser Scanning Microscopy

Cells, seeded on fibronectin-coated glass coverslips, were transfected with the indicated plasmids or siRNA, and after 24-48 hours fixed by 3.7% formaldehyde (Merck) in PBS for 10 minutes followed by permeabilization with 0.5% Triton X-100 in PBS (5 minutes; RT). Coverslips were then incubated for 15 minutes with 2% BSA in PBS at 37°C to block aspecific binding. Immunostainings were performed at RT for 1 hour with the indicated antibodies. Fluorescent imaging was performed with a confocal laser scanning microscope (LSM510/Meta; Carl Zeiss Microlmaging, Inc.) using a 63X/NA 1.40 (Carl Zeiss Microlmaging, Inc.). Image acquisition was performed with Zen 2009 software (Carl Zeiss Microlmaging, Inc.). For live-cell imaging, cells, seeded on fibronectin-coated glass coverslips, were transfected with the indicated plasmids. After 24 hours, fluorescent imaging was performed. Colocalization was analyzed using the Zen 2009 software (Carl Zeiss Microlmaging, Inc.).

Surface Biotinylation Pull-Down assay

Surface protein labeling studies were performed as follows. 24 Hours after seeding, cells were transfected with siRNA as indicated. 48 Hours after siRNA transfection, resting cells or cells, treated with hEGF for the indicated time periods were washed three times with cold PBS supplemented with 0.5 mM MgCl2 and 1 mM CaCl2. Cells were then incubated with 0.5 mg/mL sulfo-NHS-LC-biotin (21335, Thermo Scientific) in PBS for 30 minutes at 4°C. After biotinylation all unbound biotin was removed by quenching with PBS containing 100 mM glycine for 15 minutes at 4°C and subsequently washed three times with cold PBS supplemented with 0.5 mM MgCl2 and 1 mM CaCl2. Cells were then lysed in NP-40 lysis buffer (50mM TRIS/HCl pH 7.5, 100mM NaCl,
10mM MgCl$_2$, 10% glycerol and 1% NP40) supplemented with protease inhibitors (Complete mini EDTA, Roche, Almere, The Netherlands) and centrifuged at 20,000g for 10 minutes at 4°C. The supernatant, containing biotinylated surface proteins, was then incubated in the presence of streptavidin-coated beads (Sigma) at 4°C for 1 hour while rotating. Surface protein levels were assayed by Western blot analysis.

**NanoPro Assay**

Epk and Akt phosphorylation were measured by the NanoPro 1000 System (ProteinSimple) according to the manufacturers’ recommendations. In short, 48 hours after transfection with the indicated siRNA oligos, cells were stimulated with hEGF (100 ng/ml) and subsequently lysed in Bicine/CHAPS lysis buffer (ProteinSimple; 040-327) supplemented with 1x DMSO Inhibitor Mix (Protein Simple; 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple; 040-482). Lysates were centrifuged at 20,000g for 10 minutes at 4°C. Supernatant was loaded in small capillaries (ProteinSimple) together with Ampholyte premix G2 (ProteinSimple; 040-973) and 1x Standard Ladder 3 (ProteinSimple; 040-646). Isoelectric focusing of proteins was performed by applying 21,000 µW for 40 minutes. After focusing, UV light was used to cross-link proteins to the inner capillary wall. After that, the capillary was washed and immunoprobed for the indicated proteins followed by washing to remove unbound antibodies. Finally Luminol and peroxide were added to generate chemiluminescence which was captured by a CCD camera. Results were analyzed by software (Compass; ProteinSimple).

Peaks, generated using a total anti-Erk antibody, representing phospho-Erk isoforms were validated with phospho-specific antibodies against Erk. Percentage phosphorylation of Erk, using a total anti-Erk antibody, was measured by calculating the phospho-peak area as a percentage of total phospho- and non-phospho-peak areas.

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**REFERENCE LIST**


**SUPPLEMENTARY FIGURES**

Supplementary Figure S1: EGF stimulation induces translocation of tyrosine phosphorylated proteins to PACSIN2-positive early endosomes. Control HeLa cells, or HeLa cells stimulated with EGF-Texas-red (100 ng/ml; 5 min) were analyzed by confocal microscopy. PACSIN2 and tyrosine phosphorylated proteins (pTyr) were detected by immunostaining. In resting cells, hardly any tyrosine phosphorylated proteins are localized to PACSIN2-positive early endosomes (upper panels). In contrast, EGF-Texas-Red induces accumulation of tyrosine phosphorylated proteins to these PACSIN2-positive vesicles (bottom panels). Colocalization plots show the amount of tyrosine phosphorylated proteins localized to PACSIN2-positive endosomes in white. Bar diagram shows mean colocalization ± SEM. *** P<0.001. Scale bars, 10 μm.
Supplementary Figure S2: Internalization of EGF-Texas-Red or EGF receptor is similar in control cells versus PACSIN2-depleted cells. (A and B) HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with EGF-Texas-Red (100 ng/ml) for the indicated time points. Endogenous EEA1 and EGF receptor, in conjunction with EGF-Texas-Red, were visualized by immunostainings (Right panels). Colocalization of EGF-Texas-Red (A) or EGF receptor (B) with EEA1 was analyzed, using Zen 2009 software, as a measure for internalization (Left panels). In general, no significant differences were observed between control and PACSIN2-depleted cells. However, at 60 minutes of internalization, a small increase in early endosomal localization of EGF-Texas-Red was observed in PACSIN2-depleted cells. Bar diagram shows mean colocalization ± SEM. * P<0.05. Scale bars, 10 μm.

Supplementary Figure S3: PACSIN2 negatively regulates Erk phosphorylation downstream of EGF. (A) HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with EGF (100 ng/ml) for the indicated time points. Endogenous PACSIN2, Erk1/2, and phospho-Erk1/2 were detected by immunoblotting (IB). PACSIN2 depletion enhances EGF-mediated Erk1/2 phosphorylation compared to control cells within the initial 20 minutes. After 30-60 minutes, phospho-Erk1/2 levels declined to control levels. (B) Upper panel: Phospho-Erk1/2 levels relative to total Erk for each time point are shown. Data are normalized to basal levels of phospho-Erk1/2 in control cells. Mean values of three independent experiments are shown. Error bars indicate SEM. Bottom panel: EGF-mediated induction of Erk1/2 phosphorylation in control versus PACSIN2-depleted cells is shown. Data are relative to total Erk1/2 levels and normalized to basal levels of phospho-Erk1/2. Mean values of three independent experiments. Error bars indicate SEM.
SUPPLEMENTARY MOVIES

Supplementary Movie 1: EGF stimulation induces internalization of YFP-PACSIN2. HeLa cells were transfected with YFP-PACSIN2 WT. After 2 minutes EGF was added (100 ng/ml). Live-imaging analysis was performed with a laser-scanning confocal microscope using a 63X/NA 1.40 oil lens (LSM510/Meta, Carl Zeiss MicroImaging, Inc.). Frames were taken every 8 seconds for 20 minutes. Video runs at 7 frames/sec. Still images are in Fig. 1A.

Supplementary Figure S4: PACSIN2 negatively regulates Akt phosphorylation downstream of EGF. (A) HeLa cells, treated with control or PACSIN2-specific siRNA, were stimulated with EGF (100 ng/ml) for the indicated time points. Endogenous PACSIN2, Akt, and phospho-Akt (S473) were detected by immunoblotting (IB). Knock-down of PACSIN2 increased Akt phosphorylation downstream of EGF compared to control cells within the initial 20 minutes. After 30-60 minutes, phospho-Akt levels declined to control levels. (B) Left panel: Phospho-Akt (S473) levels relative to total Akt for each time point are shown. Data are normalized to basal levels of phospho-Akt in control cells. Mean values of two independent experiments are shown. Right panel: EGF-mediated induction of Akt phosphorylation in control versus PACSIN2-depleted cells is shown. Data are relative to total Akt levels and normalized to basal levels of phospho-Akt (S473). Mean values of two independent experiments are shown. (C) Akt phosphorylation measured by NanoPro Assay. Left panel: Control or PACSIN2 siRNA treated HeLa cells were analyzed in triplicate for Akt phosphorylation using the NanoPro Assay. Peaks generated are transformed in a Western blot-like representation of the data (Compass Software; ProteinSimple). All peaks are represented as bands and band intensity indicates phospho-Akt protein levels. PACSIN2 depletion decreased basal phospho-Akt levels. Upon EGF stimulation, enhanced Akt phosphorylation is observed in PACSIN2-depleted cells compared to control cells. Right panel: Quantification of Akt phosphorylation shows increased phospho-Akt levels in PACSIN2-depleted cells in response to EGF compared to control cells. Bar diagram shows levels of phospho-Akt normalized to levels in unstimulated cells. Mean values of Akt phosphorylation are shown. Error bars indicate SEM. *** P<0.001.
RAC1 ACTS IN CONJUNCTION WITH NEDD4 AND DISHEVELLED-1 TO PROMOTE MATURATION OF CELL-CELLCONTACTS

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ABSTRACT

The Rho-GTPase Rac1 promotes actin polymerization and membrane protrusion that mediate initial contact and subsequent maturation of cell-cell junctions. Here we report that Rac1 associates to the ubiquitin-protein ligase neural precursor cell expressed developmentally down-regulated 4 (Nedd4). This interaction requires the hypervariable C-terminal domain of Rac1 and the WW domains of Nedd4. Activated Rac1 co-localizes with endogenous Nedd4 at epithelial cell-cell contacts. Reduction of Nedd4 expression by shRNA results in reduced transepithelial electrical resistance (TER) and concomitant changes in the distribution of adherens and tight junction markers. Conversely, expression of Nedd4 promotes TER, suggesting that Nedd4 cooperates with Rac1 in the induction of junctional maturation.

We found that Nedd4, but not Nedd4-2, mediates the ubiquitination and degradation of the adapter protein dishevelled-1 (Dvl1), the expression of which negatively regulates cell-cell contact. Nedd4-mediated ubiquitination requires its binding to the C-terminal domain of Dvl1, comprising the DEP domain, and targets a N-terminal lysine-rich region upstream of the Dvl1 DIX domain. We found that endogenous Rac1 co-localizes with endogenous Dvl1 in intracellular puncta as well as on cell-cell junctions. Finally, activated Rac1 was found to stimulate Nedd4 activity, resulting in increased ubiquitination of Dvl1. Together, these data reveal a novel Rac1-dependent signalling pathway which, through Nedd4-mediated ubiquitination of Dvl1, stimulates the maturation of epithelial cell-cell contacts.

INTRODUCTION

Cell-cell adhesion is essential for tissue integrity and for the barrier function of epithelia. Cellular contacts are formed by homotypic interactions of specialized cell-adhesion molecules such as the cadherins. Such contacts are subject to tight regulation and can be very dynamic (Dejana, 2004; Hartsock and Nelson, 2008; Watanabe et al., 2009). In addition, cell-cell contacts play an important role in the spatial organization of signalling proteins and are key determinants of cell polarity (Iden and Collard, 2008; Mertens et al., 2005). Conversely, loss of cell-cell contact is required for cell division, tissue remodelling and efficient cell motility. The (dys)regulation of junctional integrity therefore represents a key feature of cellular transformation and of metastatic capacity of tumor cells (Cavallaro and Christofori, 2004).

The formation of cell-cell contact between neighbouring cells requires the initiation of nascent intercellular adhesions that subsequently mature to strong cell-cell junctions. This process is controlled by the microtubule (MT) and actin cytoskeleton which govern vesicular traffic and internalisation as well as the anchoring of cell-matrix- and cell-cell adhesion complexes (Harris and Tepass, 2010). A large number of regulatory proteins orchestrate MT and actin dynamics, of which the Rho-like GTPases are the most extensively studied.

Activation of RhoA, Rac1 and CDC42 can regulate loss as well as formation of cell-cell contacts and several downstream effectors such as Rho Kinase and IQGAP have been implicated in these events (Braga et al., 1997; Hordijk et al., 1997; Kuroda et al., 1997; Kuroda et al., 1998). The stimulation of actin polymerization by Rac1, through its activation of the Arp2/3 complex, is a key aspect of its capacity to initiate cell-cell contact as a result of membrane protrusion (Watanabe et al., 2009). However, specific molecular mechanisms that allow Rac1 to regulate intercellular adhesions have remained largely elusive.

In addition to post-translational modifications such as phosphorylation or acetylation, protein function and targeting are regulated by the conjugation of ubiquitin or ubiquitin-like proteins (Grabbe et al., 2011). Ubiquitination generally occurs at specific lysine residues within the target proteins, is reversible and therefore, like phosphorylation, a dynamic and tightly regulated modification. Addition of a single ubiquitin (mono-ubiquitilation) can affect protein localization (e.g. through internalization or through binding to other proteins), whereas addition of consecutive ubiquitins (poly-ubiquitilation) can act as a signal for proteasomal degradation (d’Azzo et al., 2005; Grabbe et al., 2011; Haglund et al., 2003; Pickart, 1997). Ras and RhoGTPases are subject to regulation by ubiquitination (Nethe and Hordijk, 2010). Importantly, localized RhoA ubiquitilation by Smurf promotes cell polarization and directional migration (Boyer et al., 2006; Sahai et al., 2007; Wang et al., 2003). Activated Rac1 is also subject to ubiquitination and degradation (Doye et al., 2002; Pop et al., 2004). Recently, our lab showed that poly-ubiquitilation of endogenous, active Rac1 is regulated by adhesion to fibronectin and by the Rac1-binding adapter protein caveolin-1 (Nethe et al., 2010).
In this study, we describe the identification of a newly identified Rac1-interacting protein, the ubiquitin ligase Nedd4 (neuronal precursor cell expressed and developmentally downregulated protein 4-1 (Kumar et al., 1992; Kumar et al., 1997). We found that loss of Nedd4 reduces epithelial cell-cell contact and transepithelial resistance. In addition, we could show that Nedd4, in conjunction with activated Rac1, promotes the ubiquitylation and degradation of the adapter protein dishevelled-1 (Dvl1), a negative regulator of epithelial cell-cell contact (Elbert et al., 2006). Together, our data identify a novel pathway downstream of Rac1, which enhances epithelial integrity through Nedd4 mediated degradation of Dvl1.

RESULTS
Nedd4 is a Rac1-associating protein

We and others previously showed that the hypervariable Rac1 C-terminal domain associates to protein and lipid kinases, as well as to the Rac1-GEF β-PIX and a series of adapter proteins (Modha et al., 2008; ten Klooster et al., 2006; ten Klooster et al., 2007; Tolia et al., 1998; van Duijn et al., 2010; van Hennik et al., 2003); (de Kreuk et al., 2011; Nethe et al., 2010). Here we report our analysis of a novel Rac1-interactor identified in the same proteomic screen, the HECT (Homologous to the E6-AP Carboxyl Terminus) ubiquitin ligase Nedd4 (Fig. 1A).

Following its initial identification, pull-down experiments using lysates from HeLa cells and peptides encoding the C-termini of a series of RhoGTPases showed that endogenous Nedd4 associated to the Rac1 C-terminus whereas binding to the C-termini of other RhoGTPases was either weak (e.g. Rac3) or undetectable (Fig. 1B). Use of peptides encoding mutations within the Rac1 C-terminal domain (van Hennik et al., 2003) subsequently showed that the polybasic region, rather than the proline-stretch, mediates Rac1 C-terminal binding to Nedd4 (Fig. 1C). Conversely, a GST-Rac1ΔC fusion protein, lacking the Rac1 C-terminal domain, showed reduced binding to Nedd4 as compared to full-length GST-Rac1 (Fig. 1D). This shows that the Rac1 hypervariable C-terminus is both necessary and sufficient for its association to Nedd4.

Confirmation of the association between endogenous Rac1 and endogenous Nedd4-1 by co-immunoprecipitation was unsuccessful, most likely due to low levels of the endogenous pools of either protein participating in the interaction and the fact that the Rac1 antibody recognizes the C-terminal hypervariable domain, which mediates the association to Nedd4. Nedd4 harbours a calcium- and lipid-binding C2 domain in its N-terminus that mediates membrane targeting (Plant et al., 1997; Wang et al., 2010). Pull-down assays using lysates from HeLa cells, expressing wild-type (WT) Nedd4 or a mutant lacking the C2 domain (Nedd4ΔC2; Fig. 1E), showed that both bound the Rac1 C-terminus, indicating that the C2 domain is dispensable for this interaction. Subsequent experiments using full-length GST-Rac1 showed that the central region of Nedd4, encoding the four WW domains, is sufficient for Rac1 binding (Fig. 1F).

Together, these data show that Nedd4 associates to Rac1 in a fashion that requires the Rac1 hypervariable C-terminal domain and the Nedd4 WW-domains.

Nedd4 localizes to junctions and promotes cell-cell contact

Immunoprecipitation of inactive (Rac1N17T) and active (Rac1Q61L) mutants, co-expressed in HeLa cells with HA-tagged Nedd4 showed that Nedd4 binds to both Rac1 mutants, albeit more efficiently to active Rac1 (Fig. 2A). Additional experiments in which Rac1 was expressed at levels below those of endogenous Rac1, confirmed these results, excluding artefactual binding due to Rac1 overexpression (Fig. S1A). We were not able, however, to show ubiquitylation of Rac1 by Nedd4 (data not shown).
suggesting that Rac1 is not a substrate for Nedd4 ubiquitin ligase activity. This result is in good agreement with two recent studies that identified HACE-1 as well as XIAP, but not Nedd4, as ubiquitin ligases for activated Rac1 (Oberoi et al., 2012; Torrino et al., 2011).

Subsequent analysis by confocal microscopy showed that endogenous Nedd4 co-localises with Rac1Q61L at cell-cell contacts and peripheral membrane ruffles (Fig. 2B). We could confirm such junctional co-localization also with WT-Rac1, but not with Rac1T17N (Fig. S1B). Activated Rac1 also localises to Focal Adhesions (FAs) (Nethe et al., 2010), but Nedd4 could not be detected at these sites (Fig. 2B). Interestingly, analysis of Nedd4 together with β-catenin showed that Nedd4 localized at mature, well-organized junctions but was absent from immature cell-cell contacts, which are characterised by a more diffuse band of β-catenin staining (Fig. 2C).

To test if Nedd4 contributes to the formation of epithelial cell-cell contacts, we used lentiviral shRNAs to deplete endogenous Nedd4 from HeLa cells and from H292 lung epithelial cells (Fig. 2D and data not shown). Reduction of Nedd4 expression impaired the formation of discrete cell-cell contacts in HeLa cells (Fig. 2E) as well as in H292 cells (Fig. 2F). In both epithelial cell types, proper formation of adherens and tight junctions, marked by β- and γ-catenin and by ZO-1, respectively, was impaired upon reduction of Nedd4 expression (Figs. 2E-G and data not shown). Similar effects on HeLa cell-cell contacts were found when cells were transfected with siRNA to Nedd4 (Figs. S2A,B). Conversely, re-expression of shRNA-resistant, murine Nedd4 rescued the formation of more mature junctions as detected by the linear distribution of β-catenin (Figs. 2H, S2C).

Figure 2: Nedd4 promotes maturation of cell-cell junctions. (A) Immuno-precipitated, myc-tagged inactive (N17T) and active (Q61L) Rac1 were analyzed for binding to co-expressed HA-Nedd4 in HeLa cells. (B) HeLa cells, transfected with myc-Rac1Q61L, were fixed and immuno-stained for endogenous Nedd4 and myc. Z-stacks along the dashed line were analyzed for co-localization of Nedd4 with active Rac1. Arrow indicate co-localization of Nedd4 with active Rac1 at cell-cell junctions (left arrow in XZ image) and membrane ruffles. (Scale bar, 10μm). (C) HeLa cells were fixed and immuno-stained for endogenous Nedd4 and β-catenin. Arrows indicate the absence of Nedd4 at immature junctions (left in zoom) and its presence at mature junctions (Scale bar, 20 μm). (D) Western blot showing the reduction of endogenous Nedd4 protein upon transduction of HeLa cells with lentiviral shRNAs. (E) Confocal imaging for Nedd4 and β-catenin in HeLa cells transduced with the control or Nedd4 shRNA (Scale bar, 20 μm) (F) H292 lung epithelial cells were fixed 48 hrs following transfection with indicated shRNAs and immuno-stained for endogenous β-catenin and γ-catenin. XZ images were derived from Z-stack sections along the dashed lines. (Scale bar, 15 μm). (G) Similar as in (F), H292 cells were stained for endogenous ZO-1 as a marker for tight junctions (arrows in XZ image). XZ images were derived from Z-stack sections along the dashed lines. (Scale bar, 15 μm). (H) HeLa cells were transduced with control or Nedd4 shRNA and subsequently transfected with murine HA-Nedd4. Cells were analyzed for distribution of β-catenin and murine Nedd4 expression. Two exemplary images are shown; arrows indicate mature, linear cell-cell contacts. (Scale bar, 10 μm).
To confirm the relevance of Nedd4 for epithelial integrity, H292 lung epithelial cells, transfected with shRNA to reduce Nedd4 expression, were seeded on gold electrodes, and TransEpithelial Resistance (TER) was recorded by Electrical Cell-substrate Impedance Sensing (ECIS) (Lorenowicz et al., 2007). In this assay, cells are seeded in sufficient numbers to cover the electrode during cell spreading and TER is recorded continuously in a non-invasive fashion. The increase in TER in the first 2-10 hrs after seeding (depending on the cell type) reflects the speed and degree of cell spreading (Mitra et al., 1991). TER at later time-points represents junctional integrity. ShRNA-mediated loss of Nedd4 resulted in increased cell spreading, but TER in the second phase of the experiment was significantly lower as compared to the controls, indicative for reduced monolayer integrity (Fig. 3A), in line with the data in Fig. 2. Similarly, transfection of the cells with siRNA to Nedd4 also induced a significant reduction in TER (Fig. S2D). In a complementary experiment, we expressed full-length human GFP-Nedd4 in HeLa cells, FACS-sorted the GFP-positive cells and measured TER following cell seeding by ECIS. Expression of GFP-Nedd4 increased the TER of the monolayer relative to the controls, indicative for the induction of strong cell-cell contacts and concomitant junctional maturation (Fig. 3B).

To analyse the functional relevance of Nedd4 further, we used a calcium-switch assay. EGTA-mediated depletion of extracellular calcium resulted in a loss of stable cell-cell contacts in H292 lung epithelial cells, as concluded from a broad band of β-catenin staining, and a loss of Nedd4 from intercellular junctions (Fig. 3C). One hour after EGTA washout and re-addition of calcium, β-catenin but not Nedd4, concentrated at a subset of intercellular contacts. At 5 hours after calcium re-addition, junctions were reformed and both β-catenin and Nedd4 concentrated at intercellular junctions. Together with the ECIS data in Figs. 3A,B, these experiments suggest that Nedd4 concentrates at mature rather than at immature cell-cell contacts acting, in conjunction with activated Rac1, as part of a positive feedback loop that promotes junctional maturation.

**Nedd4 regulates ubiquitylation of Dishevelled-1**

Because Nedd4 is a ubiquitin ligase, we subsequently screened potential Nedd4 substrates, identified by a PPXY or RXXQE motif (Persaud et al., 2009), that could function downstream of Nedd4 in the control of epithelial cell-cell contacts. We here focus on the adapter protein dishevelled-1 (Dvl1), which negatively regulates cell-cell adhesion in epithelial MDCK cells (Elbert et al., 2006) and harbours a PPXY motif downstream of its DEP (dishevelled, Egl-10 and pleckstrin homology)-domain (Fig. 4A). To confirm the negative effects of Dvl1 expression on cell-cell contact in HeLa cells, we expressed a Dvl1-GFP fusion protein and analyzed the reformation of cell-cell contacts using a calcium-switch assay. Following loss of cell-cell contact by EGTA-mediated calcium depletion, restoration of junctional localization by the re-addition of calcium, marked by the localization of β-catenin, was impaired in cells that expressed Dvl1-GFP (Fig. 4B). This confirms the notion that Dvl1 expression negatively
regulated epithelial integrity. In line with these findings, siRNA-mediated reduction of Dvl1 expression showed a small but significant increase in TER (Fig. S3A,B).

To analyse the regulation of Dvl1 by Nedd4, we examined the levels of endogenous Dvl1 in GFP-Nedd4-transfected HeLa cells treated for 6 hrs with cycloheximide to block protein synthesis. Expression of Nedd4 reduced the levels of endogenous Dvl1, but not Dvl2 or Dvl3 (Fig. 4C). Conversely, cells depleted of Nedd4 showed an increase in endogenous Dvl1 without any effect on the levels of Dvl2 and Dvl3 (Fig. 4D). The shRNA-mediated loss of Nedd4 did not affect either the levels or nuclear translocation of β- and γ-catenin (Figs. 2E,F; 4D). This suggests that Nedd4-controlled stability of Dvl1 does not alter β-catenin signalling, as was shown for Dvl during canonical Wnt signalling (Gao and Chen, 2010).

Expression of Nedd4, but not of Nedd4-2 (Persaud et al., 2009), stimulated the ubiquitylation of Dvl1-HA (Fig. 4E). In contrast, ubiquitylation of Dvl1 remained unaffected upon inhibition of lysosomal degradation by chloroquine (Fig. 4E), a pathway that was recently implicated in the control Dvl2 degradation (Gao et al., 2010; Su et al., 2007). Together, these findings show that Nedd4 promotes the ubiquitylation and proteasome-dependent degradation of Dvl1.

Mapping the interaction between Dvl1 and Nedd4

To investigate the interaction between Nedd4 and Dvl1, we made GFP-tagged Nedd4 truncation constructs. GST-Dvl1, but not free GST, associated to the Nedd4 WW-region (amino acids 196-505; Nedd4WW), but not the C2 domain (amino acids 1-134; Nedd4C2) (Fig. 5A). In parallel, we analyzed the localization of these different Nedd4 domains (Fig. S4). Although the C2 domain is important for Nedd4 membrane-targeting, the isolated GFP-C2 domain concentrated mostly at perinuclear vesicles. The WW domains, but not the HECT domain, localised also to perinuclear vesicles whereas both constructs were also found at the peripheral membrane, similar to the full-length protein (Fig. S4). To further map the association of Dvl1 with Nedd4 and test the relevance of the interaction for Dvl1 ubiquitylation, we generated FLAG-tagged Dvl1 constructs lacking the DIX domain (ΔDIX) and the PDZ domain (ΔPDZ), and one lacking both the DEP domain and C-terminal region (ΔDEP-tail) and one lacking only the C-terminal region (Δ-tail). In addition, we generated two complementary Dvl1 protein fragments, the first comprising the C-terminus including the DEP domain (DEP-tail) and a second that extends from the PDZ domain to the C-terminus (PDZ-tail) (Fig. 5B).

We first analyzed the localization of these Dvl1 constructs by confocal imaging (Figs S5, S6A). In line with published data (Schwarz-Romond et al., 2005), full-length Dvl1 localised to puncta that were dispersed in the cytosol (Fig. S5). The different deletion and truncation constructs were found in various locations throughout the cell, including the perinuclear region, the nucleus, and to a varying extent associating with cytosolic puncta (Figs S5, S6A). Pull-down assays showed that GST-Nedd4 binds to full-length Dvl1, as well as the PDZ-tail and DEP-tail fragments of Dvl1 (Fig. 5B).
However, Nedd4 did not associate to a Dvl1 protein that lacked the DEP-domain and C-terminal region (ΔDEP-tail) (Fig. 5B) indicating that this region of Dvl1 mediates its association with Nedd4.

We next analyzed the ubiquitylation of Dvl1 by Nedd4. Dvl1 constructs lacking the DIX (ΔDIX) or PDZ (ΔPDZ) domains were ubiquitylated by Nedd4 (Fig. 5C). In contrast, removing the DEP domain and the adjacent C-terminal region (ΔDEP-tail) blocked ubiquitylation by Nedd4 (Fig. 5C). This is in line with our observation that this region is required for binding to Nedd4 (Fig. 5B). Analysis of the Dvl1Δtail protein, which does encode the DEP domain, but lacks the adjacent C-terminal portion as well as deletion of the N-terminal portion up to the PDZ domain all impaired Nedd4-mediated reduction of Dvl1 protein levels, indicative for reduced ubiquitylation and degradation. Expression of wtDvl and the Dvl1 3KR protein reduced TER, as measured by ECIS in HeLa cells, seeded on gold electrodes. Arrow indicates time point corresponding with quantification in the bar graph. (H) Schematic overview summarising the data on the Nedd4-Dvl1 interaction and ubiquitylation of Dvl1 indicating that the binding to Nedd4 is mediated by the Dvl1 DEP domain and PPXY motif and that ubiquitylation and degradation requires the three lysines in between the DIX and PDZ domains.

* p < 0.05 ** p < 0.01
(Dvl1-3KR). We found that, despite that fact that these Dvl1 proteins are expressed at different levels, Nedd4-induced loss in wt Dvl1 protein levels are not observed with any of these mutants (Fig. 5F). These data are in line with analysis of Nedd4-mediated ubiquitylation of these mutants (Fig. 5B). The three lysines appeared to be redundant as individual mutation of these residues did not prevent Nedd4-mediated ubiquitylation (data not shown).

To further underscore the biological relevance of these lysine residues, we transfected cells with wt Dvl1 and the Dvl1 3KR mutant and analyzed consequences for TER. The data in Fig. 5G show that wt Dvl1, but in particular the Dvl1 3KR mutant reduces junction maturation, in line with the data in Figs. 2-4 indicating that Nedd4-mediated degradation of Dvl1 serves to sustain and promote cell-cell adhesion.

In summary, these data show that Nedd4 binds, through its WW-domains, to the DEP domain of Dvl1 and subsequently ubiquitylates a lysine-rich region between the DIX and PDZ-domain, which results in Dvl1 degradation (Fig. 5G).

The C2 domain of Nedd4 regulates junctional localization and Dvl1 ubiquitylation

Expression of GFP-tagged Nedd4 together with Dvl1-HA showed that Dvl1 and Nedd4 co-localised in puncta that are detectable upon Dvl1 expression (Figs. 6A, S5) and that co-localise with β-catenin at cell-cell contacts (Fig. 6A). Junctional co-localization of Dvl1-HA and GFP-Nedd4 in areas lacking such puncta was also observed (Fig. S7A). Endogenous Dvl1 was, like Dvl1-HA, found in puncta and on cell-cell contacts that co-localised with GFP-Nedd4 (Figs. 6B, 7C). Thus, similar to the Drosophila Dvl1 homologue Dsh, a portion of human Dvl1 localizes at epithelial cell junctions (Bastock et al., 2003). Unfortunately, the available antibodies did not allow analysis of co-localization of endogenous Dvl1 with endogenous Nedd4. As an additional control, we also co-transfected GFP-Nedd4-2 with Dvl1-HA. However, GFP-Nedd4-2 did not localize to the Dvl1-HA-induced puncta, resulting in minimal co-localization, which is in agreement with the lack of Dvl1 ubiquitylation by Nedd4-2 (Figs. S7B, 4E).

To test whether junctional localization of Nedd4 is essential for Dvl1 ubiquitylation, we used the Dvl1 mutant lacking the C2 domain (∆C2-Nedd4) that does not localize to cell-cell contacts, even in the presence of activated Rac1Q61L (Figs. 6C, S7C) (Plant et al., 1997; Wang et al., 2010). The ∆C2-Nedd4 protein, similar to the catalytically inactive Nedd4C867S (Persaud et al., 2009), did not ubiquitylate Dvl1 (Fig. 6D). Additional studies showed that the ∆C2-Nedd4 protein did co-localise with Dvl1-positive puncta in the cytosol (Fig. S7D), indicating that it is the targeting of Dvl1 to the plasma membrane, rather than to cytosolic puncta, which is required for Dvl1 ubiquitylation. This is in good agreement with our finding that Dvl1 ubiquitylation by Nedd4 requires the DEP domain (Fig. 5C), as this region mediates the association of Dvl1 with the plasma membrane (Wong et al., 2000).
Rac1 activity promotes Nedd4 mediated ubiquitylation of Dvl1

Because Nedd4 and active Rac1 co-localize at cell-cell contacts (Fig. 2B), we tested whether Rac1 activity could stimulate Nedd4 activity. Co-expression of Rac1Q61L with GFP-Nedd4 promoted ubiquitylation of a series of endogenous proteins, the detection of which was further increased by blocking proteasomal degradation with MG132 (Fig. 7A). In addition, co-expression of an active (Rac1Q61L), but not an inactive (Rac1N17T) mutant of Rac1 with GFP-Nedd4, Dvl1-HA and His-tagged ubiquitin showed a clear increase in ubiquitylation of Dvl1-HA in the presence of GFP-Nedd4 (Fig. 7B). Conversely, the Rac1 inhibitor EHT1864 (Shutes et al., 2007) induces a reduction of Nedd4-mediated ubiquitylation of Dvl1 (Fig. S8A). Similar to endogenous Nedd4, endogenous Rac1 co-localised with endogenous Dvl1 at the peripheral membrane of single cells (Fig. S8B), at cell-cell junctions and at a fraction of Dvl1-positive puncta (Fig. 7C). Together, this data support a model in which active Rac1 stimulates Nedd4-mediated degradation of Dvl1 at intercellular junctions to promote epithelial cell-cell contact.

DISCUSSION

The current study describes a novel pathway by which Rac1 stimulates maturation of cell-cell contacts in epithelial cells. The pathway comprises Rac1-triggered translocation of the HECT ubiquitin ligase Nedd4 to cell-cell junction, and Nedd4-stimulated ubiquitylation and degradation of the scaffold protein Dvl1, a negative regulator of epithelial cell-cell contact (Elbert et al., 2006). These findings are summarised in a model, depicted in Fig. 8.

We and others previously identified a diverse array of proteins that bind to the hypervariable C-terminal domain in Rac1. These include the GEF β-PIX, adapter proteins such as Caveolin1, CD2AP, and PACSIN2, the nuclear oncogene SET/2PP2A, PIP-5-Kinase, the Rac1-effector PRK and most recently mTOR (de Kreuk et al., 2011; Modha et al., 2008; Nethe et al., 2010; Saci et al., 2011; ten Klooster et al., 2006; ten Klooster et al., 2007; Tolias et al., 1998; van Duijn et al., 2010; van Hennik et al., 2003). In contrast to effector proteins that only bind to GTP-bound Rac1, most of these interactions are nucleotide-independent. Yet, activated Rac1 recruits Caveolin1 to focal adhesions and CD2AP to cell-cell contacts (Nethe et al., 2010; van Duijn et al., 2010). Nedd4 appears to respond in a similar way, accumulating at mature cell-cell contacts in cells expressing activated Rac1. Our present results indicate that Nedd4 in fact co-operates with Rac1 in junctional maturation since loss of Nedd4 resulted in immature cell-cell contacts and reduced TER.

Loss of Nedd4 ligases does not appear to affect epithelial cell-cell contacts in mouse embryos. Deficiency of Nedd4 leads to elevated levels of thrombospondin, an inhibitor of angiogenesis, and consequent heart defects and embryonic lethality (Fouladkou et al., 2010). In addition, loss of Nedd4 results in impaired formation of the neuromuscular junction, a specialized type of heterotypic cell-cell contact (Liu et al., 2007).
Several mechanisms have been identified that regulate the activity of Nedd4 ligases. Nedd4 binds to adapter proteins, including Annexin XIIIb, Ndfip 1,2 and Grb10 that serve to recruit Nedd4 to specific membrane domains (e.g. lipid rafts), organelles (e.g. Golgi) or substrates (e.g. the IGF-1R) (Shearwin-Whyatt et al., 2006). In addition, Nedd4-2 can be serine phosphorylated, generating a binding site for 14-3-3 proteins that interfere with binding to ENaC, thereby regulating the expression of the sodium channel (Debonneville et al., 2001; Ichimura et al., 2005). The auto-inhibitory function of the C2 domain of Nedd4-2 is released upon binding to calcium, activating the ubiquitin ligase (Plant et al., 1997). The C2 domain of Nedd4 is required for function of the C2 domain of Nedd4-2 is released upon binding to calcium, activating the ubiquitin ligase Nedd4. We found that the C2 domain of Nedd4 deficiency that we report here becomes more apparent after birth or that Nedd4 and Nedd4-2 are functionally redundant in the regulation of epithelial junctions in vivo. The latter notion is supported by a recent study (Van Campenhout et al., 2011).

Although Dvl is an established regulator of cell polarity and cell-cell contact through its control of β-catenin-dependent, canonical Wnt signaling, there are several studies that link Dvl to cell-cell contact via additional signalling pathways (Schlessinger et al., 2007; Yamanaka and Nishida, 2007). Schlessinger et al. (Schlessinger et al., 2007) showed in rodent embryo fibroblasts that Dvl activity is downstream of the loss of cell-cell contact, induced following non-canonical Wnt5a-induced signalling. In epithelial MDCK cells, Dvl1 was found to negatively regulate cell-cell contact by reducing the association of E-cadherin with the actin cytoskeleton (Elbert et al., 2006). This effect was independent of canonical Wnt signalling. In our studies, Nedd4-mediated loss of Dvl1 did not correlate with any changes in expression levels of β-catenin, nor its translocation to the nucleus (Figs. 2E,F, 4D,E), suggesting that Nedd4 does not activate β-catenin-dependent Wnt signalling.

Although the notion that Rac1 activity enhances E-cadherin-based cell-cell adhesion dates back to work published by several groups already in 1997 (Braga et al., 1997; Hordijk et al., 1997; Takaishi et al., 1997), the underlying molecular mechanisms have not been addressed in much detail. The Rac1 effector IQGAP1 has been implicated as an important regulator, but its control of cell-cell adhesion is complicated as IQGAP1 can promote as well as inhibit E-cadherin function (Noritake et al., 2005). It is generally accepted that Rac1-stimulated, Arp2/3-mediated actin polymerization mediates Rac1- and E-cadherin dependent cell-cell contact (Noritake et al., 2005). In addition to actin, also MTs control formation and stabilization of cell-cell contact (Harris and Tepass, 2010). Dvl1 has previously been linked to the regulation of MT dynamics (Krylova et al., 2000) suggesting a potential functional connection. However, regulation of cell-cell contacts through the MT cytoskeleton is cell-type specific and this issue was not further addressed here. To which extent the actin cytoskeleton and MTs cooperate in the regulation of epithelial integrity downstream of the Rac1-Nedd4-1-Dvl1 pathway therefore remains to be established.
There is increasing evidence linking the cellular ubiquitylating machinery to both positive and negative control of cell-cell adhesion. The ubiquitin ligase Hakai is capable of triggering the ubiquitylation, internalization and degradation of E-cadherin, thereby weakening cell-cell contacts (Fujita et al., 2002). In mammary epithelial cells, the ubiquitin ligase Cbl regulates maintenance of adherens junctions by ubiquitylating EGF-R-stimulated Vav2 (Crose et al., 2009; Whitehead et al., 2009). In good agreement with our current findings, a recent study showed that siRNA-mediated loss of Nedd4 ubiquitin ligases in epithelial cells results in impaired cell-cell contacts and discontinuous tight junctions (Van Campenhout et al., 2011). Thus, findings from several groups using different cell types support our current findings that local ubiquitylation and degradation of proteins represents a key regulatory mechanism in the control of intercellular junctions.

MATERIALS AND METHODS

Cell culture
HeLa and H292 lung epithelial cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker) containing 10% heat inactivated FCS (Bodinco), 2mM L-glutamine and Penicillin/streptomycin (all purchased from PAA Cell Culture Company) 37°C and 5% CO₂. Cells were passaged by trypsinization.

Antibodies and inhibitors
The following antibodies were used: anti-Nedd4 (37-049; Millipore) for IF, anti-Nedd4 (CSF5; Cell Signaling) for WB, anti-Dvl1 (AB5970; Millipore) for WB, anti-Dvl1 (D3320; Sigma) for IF, anti-Dvl2 (AB5972; Millipore), anti-Dvl3 (AB5974; Millipore), anti-β-catenin (610154; BD Bioscience), anti-γ-catenin (SC-7900; SantaCruz), anti-ZO1 (610966; BD Bioscience), anti-Ubiquitin (MMs-257P; Covance), anti-GFP (JL-8; Clontech), anti-HA (H6908; Sigma) anti-c-myc (13-2500; Zymed). F-actin was stained with rhodamine-labeled phalloidin (Invitrogen). The Rac1 inhibitor EHT1864 (E1657) was from Sigma.

Cell transfection, DNA constructs and immunofluorescence microscopy
Cells were transiently transfected with FuGene (Roche) as described (Nethe et al., 2010). The following constructs were used: GST-Rac, GST-RacGD (a kind gift from R. Ahmadian, European Molecular and Cell Biology Laboratory, Heidelberg, Germany); GFP-Nedd4, originally from D. Rotin (The Hospital for Sick Children, Canada)(Pak et al., 2006), was kindly provided by J. Batt (University of Toronto, Canada); GFP-C675-Nedd4 was generated by site-directed mutagenesis (Stratagene); ΔC2-Nedd4, the C2 domain and WW1-4 region of Nedd4 were generated by PCR and subsequently cloned into pEGFP (C1)-tagged constructs, using Kpn1 and EcoR1 restriction sites; Nedd4-2-GFP was kindly provided by C. Thomas (Itani et al., 2005) (University of Iowa, USA); 6×His-myc-tagged ubiquitin expression plasmid, originally from R.R. Kopito (Ward et al., 1995) was kindly provided by J. Bertoglio (Inserm U749, France). GST-Dvl1 (Addgene); HA-tagged full-length Dvl1, ΔDIX, ΔPDZ and ΔDEP as previously described (Krylova et al., 2000), Flag-tagged Dvl1 constructs were as previously reported (Tauriello et al., 2010), Dvl1 was subsequently cloned in pE-phiyFP (C1)-tagged constructs.

For immuno-stainings, cells were washed with ice-cold PBS, fixed with 3.7% paraformaldehyde for 20 min at RT and subsequently permeabilised with 1% Triton, 10% glycerol in PBS for 3 min at RT. Cells were immuno-stained with indicated antibodies and confocal images were captured with a Zeiss 510 Meta laser-scanning confocal microscope. Z-stacks and X/Z-sectioning were generated and processed by means of LSM510 software. All data are representative for at least 3 or more experiments, unless indicated otherwise.

Lentiviral shRNAi and siRNA silencing
Lentiviral shRNA constructs from the TRC/Sigma Mission library were obtained from Sigma-Aldrich (St. Louis, MI, USA). The human Nedd4-specific constructs used were: TRCN000007550 (#a) and -7551 (#b). The SHC002 scrambled shRNA construct (Sigma-Aldrich) was used as a negative control. All shRNA constructs were in the pLKO.1 vector backbone. shRNA-expressing lentiviral particles were prepared using HEK293T cells and virus was transduced as described previously (Nethe et al., 2010). Dvl1 siRNA (EHU-060361) and Nedd4 siRNA (EHU-132581) were obtained from Sigma-Aldrich (St. Louis, MI, USA).

Reconstitution of cell junctions
Confluent H292 cells were washed 2 times with PBS and incubated for 3hrs with IMDM containing 2mEGTA. Cells were subsequently washed with PBS containing 1mM Ca²⁺ and incubated for indicated times with MDM containing 10% FCS, 2mM L-glutamine and 1mM Ca²⁺. Next, the cells were fixed, stained and examined by confocal microscopy.

Pull-down and ubiquitylation assays
Peptide pull-down assays were performed as described previously (ten Klooster et al., 2006). In short, each assay was performed with 5 μg of indicated biotin-labelled peptide, 25μl streptavidin-coated beads (Sigma-Aldrich) in NP-40 lysisbuffer (50mM Tris-HCl, 150mM NaCl, 10mM MgCl₂, 10% glycerol, 1% NP-40). All peptides were fused to a protein transduction domain sequence: YARAAARQARA, which by itself was included as the control in the pull-down experiments (van Hennik et al., 2003). GST-fusion proteins were purified from BL21 bacteria as described (Nethe et al., 2010) and 100 μg of the indicated GST-fusion protein was used per pull-down. Mass
spectroscopy analysis was performed as described (Kanters et al., 2008) and was used for the initial identification of Nedd4. Ubiquitylation of Dvl1 was assayed as described previously using HeLa cells, transfected with His-tagged ubiquitin (Nethe et al., 2010).

Peptide synthesis

Peptides were synthesized on a peptide synthesizer (Syro II) using Fmoc solid phase chemistry. Peptides encoded a biotinylated protein transduction domain (Biotin-YARAAARQRQAR) (Ho et al., 2001) followed by the 10 amino acids proceeding the CAAX domain for all used RhoGTPase peptides. The sequences of the Rac1 (P-A) and the Rac1 (PBQR) mutants are respectively: CAAAVKRKVK and CPPPVKKAAAK.

Electrical resistance measurements

ECIS-based cell spreading experiments were performed as previously described (ten Bragga et al., 2004). Cell spreading and monolayer formation were subsequently monitored by measuring the resistance at 4000 Hz.

REFERENCE LIST


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**SUPPLEMENTARY FIGURES**

**Figure S1: Rac1-Nedd4-1 interaction and localization.**

(A) Myc-tagged versions of Rac1T17N and Rac1Q61L (24 kD) were expressed in HeLa cells at levels far below that of endogenous Rac1 protein (22 kD). GFP-tagged Nedd4-1 was co-transfected and myc-Rac1 constructs were immunoprecipitated. Upper blot shows that a fraction of GFP-Nedd4-1 associates to myc-Rac1Q61L. (B) Myc-tagged versions of wild-type (WT) Rac1, Rac1T17N and Rac1Q61L were transfected in HeLa cells and cells were fixed and immunostained for endogenous Nedd4-1 and for myc. Arrows indicate Nedd4-1 on cell-cell contacts of Rac1WT and Rac1Q61L-transfected cells and lack of Nedd4-1 on cell-cell contacts of Rac1T17N-transfected cells. Boxes indicate regions corresponding to zoomed images. Nuclei are in blue. Scale bar, 10 μm.

**Figure S2: Nedd4 regulates cell-cell junction and transepithelial resistance.**

(A) Hela cells were transfected with siRNA to Nedd4 and cell lysates were analysed by SDS-PAGE. (B) Cells were transfected with siRNA to Nedd4 and analysed for localization of endogenous Nedd4 and b-catenin, as a marker for cell-cell contacts. Boxed areas correspond to zoomed images. Scale bar, 20 μm. (C) Cells were transduced with lentiviral shRNA to Nedd4, followed by transfection with mNedd4. Cells were subsequently fixed and stained for mNedd4 and b-catenin. Boxed areas correspond to zoomed images. Arrows indicate linear distribution of b-catenin, marking more mature cell-cell contacts. (D) Cells were transfected with siRNA to Nedd4 and seeded on gold electrodes for analysis of TER by ECIS. Curve shows reduced TER in cells transfected with the Nedd4 siRNA. Arrow marks the time point used for analysis, depicted in the bar graph on the right. *, p< 0.05.
Figure S3: siRNA to Dvl1 increases TER. (A) Cells were transfected with siRNA to Dvl1 and analysed for protein expression by Western blotting. (B) Cells, transfected with the Dbvl1 siRNA were seeded on gold electrodes and TER was measured using ECIS. Loss of Dvl1 expression induces a small, but statistically significant increase in TER. Arrow marks the time point used for analysis, depicted in the bar graph on the right. **, p < 0.01

Figure S4: Localization of Nedd4-1 constructs. Indicated Nedd4-1 constructs (see also Fig. 5A), tagged with GFP were transfected in HeLa cells and imaged by confocal microscopy. F-actin was detected using phalloidin. Nuclei are in blue. Boxes indicate regions corresponding to zoomed images. Two examples for each construct are shown. Scale bars, 10 μm.
Figure S5: Localization of Dvl1 constructs. HeLa cells were transfected with FLAG-tagged full-length (FL) Dvl1 and constructs encoding the Dvl1 ΔDEPtail and DEPtail constructs (see also Fig. 5B). Cells were immunostained and images were taken by confocal microscopy. F-actin was detected using phalloidin. Two examples for each construct are shown. FL-Dvl1 and Dvl1 ΔDEPtail localize to puncta, whereas the DEPtail protein is diffuse and partly co-localizing with F-actin. Boxes indicate regions corresponding to zoomed images. Nuclei are in blue. Scale bars, 10 μm.

Figure S6: Localization of Dvl1 constructs. (A) HeLa cells were transfected with the indicated FLAG-tagged Dvl1 ΔPDZ and the Dvl1 PDZtail constructs (see also Fig. 5B). Cells were immunostained and images were taken by confocal microscopy. F-actin was detected using phalloidin. Two examples for each construct are shown. The Dvl1 ΔPDZ appears in aggregates, seemingly distinct from Dvl-1 puncta, whereas the PDZtail protein is diffuse with some punctuate staining. Boxes indicate regions corresponding to zoomed images. Nuclei are in blue. Scale bars, 10 μm. (B) Mutation of lysine residues K218, K220, K225 to arginine in full-length Dvl1 (Dvl1-3KR) impaired Nedd4-mediated ubiquitylation in cells incubated with MG132. A Dvl1 truncation mutant (PDZ-tail), lacking the region comprising these three lysines, and full-length Dvl1 were included as controls.
**Chapter 6**

Figure S7: Localization of Dvl1, Nedd4 and Rac1.

(A) HA-tagged Dvl1 and GFP-Nedd4-1 were co-transfected in HeLa cells, fixed, immunostained and imaged by confocal microscopy. Boxed area corresponds to zoomed image. Arrows indicate co-localization on puncta as well as on intercellular junctions. (B) GFP-Nedd4-2 does not co-localize with HA-Dvl1. (Scale bar, 10 μm).

(C) Cells were transfected with GFP-Nedd4-1 constructs and analysed for localization at cell-cell contacts, marked by β-catenin (arrows). The GFP-ΔC2-Nedd4-1 protein does not localize at cell-cell contacts. (Scale bar, 10 μm). In the bottom panels, Rac1Q61L was co-transfected. Also here, GFP-ΔC2-Nedd4-1 protein did not localize to cell-cell contacts (arrow). (D) The GFP-ΔC2-Nedd4, but not GFP alone (top panels) colocalizes with Dvl1 in puncta (lower panels). Boxed areas correspond to zoomed images. (Scale bar, 20 μm).

Figure S8: Regulation of Dvl1 ubiquitylation by Rac1 activity.

(A) HeLa cells were transfected with HA-Dvl1, GFP-Nedd4-1 and His-tagged ubiquitin, treated with the Rac1 inhibitor EHT1864 (EHT; 50μM, 30 min.) and analysed for Dvl1 ubiquitylation. Inhibition of Rac1 by EHT reduces the levels of ubiquitylated Dvl1. (B) Endogenous Dvl1 and endogenous Rac1 were immunostained in single HeLa cells. Red arrows indicate co-localization at puncta and white arrow indicates co-localization at the peripheral membrane. Scale bar, 10 μm.
Signal transduction, i.e. the translation of external cues to intracellular responses, is essential for proper functioning of cells and thereby of tissues and organs. Disturbed signal transduction caused by, for example, abnormal activation or inactivation of specific signaling pathways, can cause development of diseases such as chronic inflammation as well as cancer. In both types of disorder, deregulated cell motility as well as changes in cell-cell contact between endothelial or epithelial cells plays an important role.

Rho-family GTPases contribute to many steps of cancer development and inflammation (Millan and Ridley, 2005; Sahai and Marshall, 2002). As key regulators of the actin cytoskeleton, RhoGTPases are important mediators of cell polarity, adhesion, and motility (Jaffe and Hall, 2005; Le Clainche and Carlier, 2008; Ridley et al., 2003) and are therefore essential for both tumor cell migration as well as leukocyte extravasation (Nourshargh et al., 2010; Sahai and Marshall, 2002). In addition to regulating cell motility, RhoGTPases contribute to cancer and inflammation by acting on endothelial and epithelial cell-cell contacts. As important regulators of epithelial cell-cell adhesion (Citi et al., 2011), RhoGTPases have been implicated in epithelial-mesenchymal transition (EMT) (Guarino et al., 2007; Savagner, 2001). EMT is characterized by weakening of cell-cell contacts, which may allow detachment of cells from the epithelium leading to tumor cell invasion (Guarino, 2007). In line with this, abnormal activation and/or expression of RhoGTPases has been observed in many types of cancer (Sahai and Marshall, 2002). In addition to regulating epithelial cell-cell adhesion, RhoGTPases are key regulators of endothelial cell-cell contacts (Beckers et al., 2010; Wojciak-Stothard et al., 2001; Wojciak-Stothard and Ridley, 2002). By regulating endothelial cell-cell adhesion, RhoGTPases control endothelial barrier function which is important as increased permeability can cause excessive leukocyte extravasation. Furthermore, RhoGTPases contribute to leukocyte extravasation by governing endothelial actin dynamics required for formation of so-called docking structures upon binding of the leukocyte to the endothelium (Carman et al., 2003; Carman and Springer, 2004). These docking structures are required for efficient leukocyte diapedesis. Thus, RhoGTPase activation and signaling is tightly controlled in order to prevent diseases such as chronic inflammation and cancer. Therefore, understanding of the mechanisms that drive these different processes is of great importance.

In this thesis, we aimed to generate additional insights in modes of regulation that cells use to control RhoGTPase signaling. We describe several novel components such as the F-BAR protein PACSIN2 and HMHA1 that regulate RhoGTPase activity. In addition, we provide new insights into how Rac1, in conjunction with Nedd4 and Dvl1, regulates epithelial junction integrity. Finally, we identify a novel role for PACSIN2 in growth factor receptor activation and signaling.
Regulation of RhoGTPase activity and function

RhoGTPases act as molecular switches. Stimulation of cells by external cues allows guanine-nucleotide-exchange factors (GEFs) to catalyze the exchange of GDP for GTP thereby activating the GTPases (Rossman et al., 2005). Upon activation of RhoGTPases, specific downstream effectors, such as the p21-activated kinase (PAK) serine/threonine kinase for Rac1, or Rho-associated coiled-coil-containing protein kinase (ROCK) (Bishop and Hall, 2000) for RhoA, are in turn activated to further initiate downstream signaling. Inactivation of RhoGTPases is mediated by GTPase-activating proteins (GAPs) that stimulate the low intrinsic GTPase activity which controls hydrolysis of GTP to GDP (Bernards and Settleman, 2004). As human RhoGAPs and GEFs outnumber the 22 human members of the Rho-family GTPases (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007) it could be speculated that various factors such as activation upon external stimulation, selective intracellular localization, and cell-specific specific signaling and expression of GEFs and GAPs are important determinants in the regulation of RhoGTPase (in)activation.

An important feature in the activation of RhoGTPases is the translocation between the cytosol and the plasma membrane. In contrast to inactive GTPases that mainly reside in the cytosol in complex with RhoGDI (Rho Guanine Nucleotide Dissociation Inhibitor) (del Pozo et al., 2002; Olofsson, 1999), active RhoGTPases are localized at the plasma membrane. Furthermore, GTPase inactivation most likely occurs intracellularly, suggesting an important role for vesicular traffic in the regulation of RhoGTPase activity. In line with this, several studies have shown the importance of internalization for inactivation of the RhoGTPase Rac1. Loss of cell adhesion to the matrix induces internalization of Rac1 from cholesterol-rich membrane domains leading to the inactivation of Rac1 (del Pozo and Schwartz, 2007). Inhibition of internalization, e.g., by inhibition of Dynamin, results in increased Rac1 activity and aberrant downstream signaling (Schlunck et al., 2004). Thus, it is generally accepted that traffic plays a key role in regulating RhoGTPase activation and signaling.

Proteins of the BAR-domain superfamily are important regulators of membrane dynamics, including internalization and vesicular transport. So far, six subclasses of BAR-domains are represented in this family: the classical BAR domain, the N-BAR, BAR-Ph, PX-BAR, F-BAR, and I-BAR domains (Qualmann et al., 2011). As a result of sensing membrane curvature, BAR-domains associate as banana-shaped dimers with membranes and by doing so can further promote curvature which leads to formation of invaginations or protrusions depending on the type of BAR domain (Frost et al., 2009). Most BAR domain proteins contain one or more protein-binding scaffolding/adapter domains and are often capable of forming dimers (Chitu and Stanley, 2007; Kessels and Qualmann, 2004) allowing linkage of membrane dynamics to signaling pathways governing actin dynamics. Consequently, many BAR-domain proteins have been implicated in the regulation of RhoGTPase signaling. This topic has been reviewed in Chapter 2. BAR-domain proteins involved in RhoGTPase signaling can be divided in two major classes. First we have identified a class of BAR-domain proteins that regulate RhoGTPase function by mediating the internalization or targeting of specific GTPases, such as PACSIN2 (described in more detail in Chapter 3) or by forming a physical link between RhoGTPases and their up- or downstream activators/effectors, such as Abba and IRSp53. In addition, we identified a second class of RhoGTPase-regulating BAR-domain proteins that harbor, in addition to the BAR domain, also a RhoGAP/GEF domain and thereby control RhoGTPase function by regulating GTP binding or hydrolysis. Well-known members of this subclass are the BAR-RhoGEF, Tubα (Cestra et al., 2005) and the BAR-RhoGAPs, GRAF1, Oligophrenin-1, and SH3BP1 (Cicchetti et al., 1995; Fauchereau et al., 2003; Hildebrand et al., 1996). A common theme for many of these BAR-GAPs is the notion that their BAR domain is required for targeting to specific sites due to its membrane-binding capacity as well as for auto-inhibition. Specific stimuli, leading to, e.g., translocation or protein-protein interactions, can release this auto-inhibition by the BAR domain allowing localized downregulation of GTPase activity.

In Chapter 4 we identify a novel member of this BAR-RhoGAP family called Human Minor Histocompatibility Antigen-1 (HMHA1). Sequence alignment revealed high homology of HMHA1 with well-known RhoGAPs including p50RhoGAP and GRAF1. Furthermore, we generated a homology model of the HMHA1 GAP domain in association with RhoA which indicates that HMHA1 fulfills the structural requirements to function as a RhoGAP. In vitro GAP assays with purified proteins confirmed that HMHA1 is a genuine RhoGAP. Interestingly, in contrast to the HMHA1 C1-GAPtail construct, which lacks the BAR domain, full-length HMHA1 shows little GAP activity towards RhoGTPases suggesting that the N-terminal BAR domain acts as an inhibitory module for GAP function. These observations place HMHA1 in a larger subfamily of RhoGAPs that comprise both a BAR- and a RhoGAP domain (De Kreuk and Hordijk, in press) that are auto-inhibited by their N-terminal BAR domain (Eberth et al., 2009). As a regulator of RhoGTPase activation, HMHA1 controls actin dynamics. Cells expressing HMHA1 mutants that lack the N-terminal BAR domain, show altered F-Actin and focal adhesion distribution and are less adhesive. This correlates well with the notion that inactivation of RhoGTPases affects cell adhesion and migration. Although in vitro HMHA1 shows GAP activity towards Rac1, Cdc42, and RhoA, the in vivo targets remain to be investigated. Our data suggest that Rac1 and RhoA but not Cdc42 are in vivo targets of HMHA1 but further studies are needed to confirm this. In normal conditions, HMHA1 expression is restricted to the hematopoietic system (de Bueger et al., 1992) indicating that in other cell types different GAPs are involved in the regulation of RhoGTPases. This further underscores the notion that cell-type specific expression of RhoGAPs is an important aspect in the regulation of GTPase (in)activation.

As described in Chapter 2, many BAR-family proteins have been described to regulate RhoGTPase activation and signaling. In Chapter 3 we identify the F-BAR protein PACSIN2 as a novel regulator of Rac1 activation and signaling. Although
several BAR proteins, such as the I-BAR protein IRSp53 and the BAR protein Arfaptin (Miki et al., 2000; Tarricone et al., 2001), were previously described to regulate Rac1, no studies have focused on PACSIN proteins and their role in regulating RhoGTPases. In this study we show that PACSIN2 interacts with the hypervariable C-terminal region of Rac1. Thus, PACSIN2 is part of a larger group of proteins we identified earlier to interact with the Rac1 C-terminus and regulate its function. These include caveolin1, the Rac1 GEF β-Pix, and the nuclear proto-oncogene SET/12P22 (Nethe et al., 2010; ten Klooster et al., 2006; ten Klooster et al., 2007). Although these proteins all interact with the same region in Rac1, they act independently of each other and reside in different intracellular compartments supporting the notion of compartmentalized, parallel signaling in so-called ‘spatio-temporal signaling modules’ (Pertz, 2010). In addition, we showed that PACSIN2 regulates Rac1 activity, and as a consequence cell spreading and migration, by promoting its internalization. Furthermore, PACSIN2-mediated downregulation of Rac1GTP levels could be prevented when Dynamin function was inhibited. These data are in line with other studies that showed the importance of Dynamin-mediated internalization in regulation of Rac1 activity (Schlunk et al., 2004). Further support comes from studies that show that PACSIN2 associates with Dynamin (Chitu and Stanley, 2007; Kessels and Qualmann, 2004).

**RhoGTPases in the regulation of cell-cell contacts**

Via anchorage to junctional complexes, the actin cytoskeleton is involved in regulating junction assembly, disassembly, and maturation (Mege et al., 2006). RhoGTPases, and in particular Rac1 and RhoA, have been implicated in the regulation of cell-cell adhesion (Citi et al., 2011; Wojciak-Stothard and Ridley, 2002). Moreover, RhoGTPases are important regulators of epithelial-mesenchymal transition (EMT) which is caused by weakening of cell-cell contacts resulting in the dissociation of single cells from the epithelium (Guarino et al., 2007; Savagner, 2001). In line with this, aberrant expression of RhoGTPases has been observed in many cancers (Sahai and Marshall, 2002).

Although some studies found opposing effects of RhoGTPases on junction stability, it is clear that tightly balanced RhoGTPase signaling is key to junction stabilization and the integrity of the epithelium (Citi et al., 2011; Yamada and Nelson, 2007). Although the regulatory role of RhoGTPases in junction stability has been widely studied, we identified in Chapter 6 a novel mechanism through which Rac1 stabilizes epithelial cell-cell junctions. Upon Rac1 activation, the HECT E3 ligase Nedd4 is translocated to cell-cell junctions where it colocalizes with the scaffold protein Dvl1, which is a negative regulator of epithelial cell-cell contact (Elbert et al., 2006). Our data suggest that here, Nedd4 mediates the ubiquitylation of Dvl1 leading to degradation of Dvl1 and as a consequence junctional integrity is increased. When Nedd4 expression was reduced by RNA interference, Dvl1 levels increased and as a result, junctional integrity was impaired. As mentioned before, RhoGTPases regulate junction integrity by controlling actin dynamics. However, in this study we found no effects of either Nedd4 or Dvl1 on the actin cytoskeleton. Interestingly, in unpublished data we did see clear effects on the microtubule network in that depletion of Nedd4 increased the acetylation of microtubules. It has been well established that the microtubule network is important in the regulation of cell-cell junctions by allowing transport of specific proteins such as N-Cadherin to and from junctions (Mary et al., 2002). Also, due to different affinities for microtubule-linked motor proteins, acetylated microtubules show altered directionality of protein traffic along microtubules (Reed et al., 2006). Interestingly, Dvl1 also increases acetylation of microtubules (Krylova et al., 2000) suggesting that decreased Dvl1 degradation in Nedd4-depleted cells results in increased microtubule acetylation. As microtubule acetylation affects the stability of, and traffic along microtubules, it could well be that the Rac1-Nedd4-Dvl1 axis mediates junctional integrity by controlling polarized traffic along the microtubule network. Indeed, experiments with TSA, a pharmacological inhibitor of histone deacetylases (HDACs), or Tubastatin, an inhibitor of HDAC6, increased microtubule acetylation and resulted in decreased junctional integrity similar to what we observed for Nedd4-depleted cells (unpublished data). In addition to the regulation of epithelial cell-cell junctions by actin dynamics, we thus revealed a novel microtubule-dependent mechanism through which Rac1 stabilizes cell-cell junctions.

Abnormal expression or activation of GAPs and GEFs, important regulators of GTPase activity (Bernards and Settleman, 2004; Rossman et al., 2005) is also frequently associated with cancer development and progression (Vigil et al., 2010). In Chapter 4 we identified HMHA1 as a novel RhoGAP regulating the actin cytoskeleton. Although in normal conditions, HMHA1 expression is restricted to the hematopoietic system (de Bueger et al., 1992), HMHA1 expression was observed in many epithelial tumors as well (Klein et al., 2002). Thus, aberrant expression of the RhoGAP HMHA1, and as a consequence disturbed RhoGTPase signaling, could be key to the transforming and invasive character of these epithelial tumor cells, although further studies are needed to firmly proof this.

Signaling via growth factor receptors is essential for many cellular processes such as proliferation, cell survival, and cell migration (Blume-Jensen and Hunter, 2001; van der Geer et al., 1994). Activation of growth factor receptors leads to activation of many downstream signaling pathways including activation of RhoGTPases (Itoh et al., 2008; Samson et al., 2010) and of the Erk and Akt pathways (van der Geer et al., 1994). Growth factor stimulation also triggers rapid internalization of the growth factor receptor. Although compartmentalized signaling from endosomes does occur, internalization generally results in the termination of signaling either by targeting the receptor for degradation or by recycling inactive receptors back to the plasma membrane (Sorkin and Goh, 2008; Wiley, 2003). Moreover, the amount of receptor on the cell surface is an important determinant for the magnitude of signaling output. Thus, it is clear that traffic of growth factor receptors is important in modulating growth factor signaling. Through their role in regulating membrane dynamics, the BAR-domain superfamily has often been implicated in regulating growth factor signaling.
In Chapter 5 we show that PACSIN2 is a novel regulator of Epidermal Growth Factor (EGF) receptor activation and signaling. Similar to BAR-family proteins such as endophilin and CIP4 (Hu et al., 2009; Soubeyran et al., 2002), PACSIN2 regulates traffic of growth factor receptors and thereby controls signaling output. We show that PACSIN2 depletion results in increased surface levels of the EGF receptor in resting cells. Upon EGF stimulation, we still observed increased surface expression of the EGF receptor compared to control cells. However, EGF-mediated internalization of the receptor still occurred normally suggesting that PACSIN2 does not regulate ligand-dependent internalization of the EGF receptor. Despite normal internalization, EGF stimulation increased receptor activation as well as downstream signaling in the absence of PACSIN2. In line with the notion that receptor surface levels are an important determinant for signaling output, it could well be that in PACSIN2-depleted cells, either through regulating ligand-independent internalization or by promoting recycling, the increased EGF receptor surface levels allow for increased signaling upon stimulation. Interestingly, it was previously shown that dominant active Rab5 (Q79L) GTPase caused ligand-independent internalization of the EGF receptor resulting in decreased surface EGF receptor levels. Subsequently, upon EGF stimulation, less receptor activation and downstream signaling was observed (Dinneen and Ceresa, 2004). These observations, together with our data that show that PACSIN2 localizes to Rab5-positive endosomes (de Kreuk et al., 2011) and that dominant-active Rab5Q79L accumulates PACSIN2 on these endosomes (unpublished data) suggest that PACSIN2 and Rab5 function in a similar pathway regulating traffic of the EGF receptor independent of the ligand. However, both for PACSIN2 and Rab5 a role in recycling of the (in)active receptor cannot be excluded. Therefore, further studies are needed to determine the exact mechanisms via which PACSIN2 regulates EGF receptor signaling. The regulatory role of PACSIN2 appears not specific for the EGF receptor as increased signaling, following loss of PACSIN2 expression, was also observed downstream of Hepatocyte Growth Factor (HGF) or Tumor Necrosis Factor-α (TNFα), suggesting a more general regulatory role for PACSIN2 in growth factor receptor signaling. As we have shown that PACSIN2 depletion results in increased Rac1 activity (Chapter 3) as well as increased EGF receptor activation and signaling (Chapter 5) it could well be that PACSIN2 links EGF signaling to RhoGTPase activation. However, although we found that the PACSIN2-Rac1 interaction was increased upon EGF stimulation (unpublished data) we could find no biochemical evidence for such a link, suggesting that PACSIN2 regulates both processes through independent mechanisms, but further studies are needed to confirm this.

In conclusion, in this thesis we describe several novel components of growth factor receptor and RhoGTPase activation and signaling. We have demonstrated that the F-BAR protein PACSIN2 is an important regulator of Rac1 output and, as a consequence, cell spreading and migration. This study further established the importance of traffic in the regulation of RhoGTPase function. In addition, we identified HMHA1 as a novel hematopoietic cell-restricted RhoGAP that regulates the actin cytoskeleton as well as cell spreading. Because expression of the HMHA1 has been observed in epithelial tumor cells as well, future studies should define the mechanisms through which HMHA1 regulates the transformation and invasiveness of these tumor cells. Furthermore, owing to its role as a minor histocompatibility antigen, T cells are generated against epitopes of HMHA1. As the healthy epithelium does not express HMHA1, it might prove an excellent target for tumor therapy. In addition, we describe a novel mechanism through which Rac1 regulates epithelial junction stability independent of the actin cytoskeleton. Although many studies focused on the regulatory role of RhoGTPase in junction remodeling in the context of actin dynamics, our study revealed that additional mechanisms through which RhoGTPases affect cell-cell junctions exist, further underscoring the complexity of RhoGTPase signaling in junction remodeling. Finally, although many pathways that regulate growth factor receptor traffic have already been described, we demonstrate in this thesis a novel component, PACSIN2, that acts as an important regulator of growth factor receptor activation and signaling.
REFERENCE LIST


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Het actine skelet van een cel zorgt, net zoals het skelet van een mens, voor vorm en stevigheid. Maar waar het menselijk skelet een rigide structuur is, is het actine skelet van een cel zeer dynamisch omdat het voortdurend wordt afgebroken en opgebouwd. Het actine skelet speelt een belangrijke rol in de beweging en hechting van cellen aan de uitwendige matrix en de hechting tussen verschillende cellen. Deze contact punten tussen cellen onderling worden ook wel juncties genoemd. Weefsels en organen bestaan niet uit geïsoleerde cellen maar uit groepen cellen die samen structuren en barrières vormen. Ook al lijken deze cellen stevig aan elkaar vast te zitten, toch zijn deze barrières dynamische structuren die continu open gaan en dan weer sluiten. Dit is van groot belang op verschillende plaatsen in het lichaam bijvoorbeeld bij het endotheel, een enkele laag cellen die de binnenkant van onze bloedvaten bekleedt. Tijdens infecties zullen witte bloedcellen deze endotheel barrière oversteken om de infectie in de onderliggende weefsels op te ruimen. Hiervoor gaat het endotheel tijdelijk open om, zodra de cellen gepasseerd zijn, weer te sluiten. Het is dan ook vanzelfsprekend dat, wanneer dit niet goed gereguleerd is, witte bloedcellen te weinig of juist te veel door deze laag endotheelcellen zullen bewegen wat kan leiden tot chronische ontsteking.

Daarnaast is het behouden van deze barrière ook erg belangrijk voor het epitheel. Dit zijn cellen in o.a. de huid, de darm en de longen die “het binnen” van onze lichaam beschermen van “het buiten”. Ook hier is het dus belangrijk dat deze barrière goed gereguleerd is en alleen open gaat wanneer dat nodig is. Ontregeling van het actine skelet kan leiden tot het loslaten van cellen van het epitheel. Deze cellen kunnen zich vervolgens makkelijker gaan delen of zich verplaatsen naar andere plekken in het lichaam wat kenmerkend is voor het uitzetten van kankercellen. Naast het loslaten van de omliggende cellen door verlies van cel-cel contact, is voor het uitzetten ook de beweeglijkheid van cellen erg belangrijk. Bij celbeweging, ook wel cel-migratie genoemd, worden aan de voorkant van een cel uitstulpingen gevormd terwijl aan de achterkant samentrekking plaats vindt. Door dit herhaaldelijk uit te voeren, beweegt een cel vooruit. Cellbeweging is nodig voor het normaal functioneren van het lichaam terwijl bijvoorbeeld abnormaal verhoogde migratie kan ook weer leiden tot chronische ontsteking of uitzetting van kankercellen.
Bij al deze processen speelt het actine skelet een essentiële rol. Belangrijke regulatoren van het actine skelet zijn eiwitten van de Rho-familie van kleine GTPases en in het bijzonder Rac1, RhoA, en Cdc42. Deze eiwitten zijn enzymen en "moleculaire schakels" die zich in het bijzonder kenmerken doordat zij zowel in een "aan" als "uit" stand kunnen staan. Om verstoorde signalering te voorkomen is het van groot belang dat het "aan" en "uit" zetten van deze GTPase eiwitten zeer goed gereguleerd wordt. Het is dan ook niet vreemd dat in verschillende ziektes deze eiwitten vaak in verhoogde aantallen of in een verhoogde staat van activiteit (de "aan" stand) worden aangetroffen. Omdat onregeling van signalering via kleine GTPases vaak leidt tot ernstige ziektes zoals uitzetting van kanker cellen en chronische ontsteking is het dus van groot belang dat de regulatie van deze eiwitten goed beoordeeld wordt zodat duidelijk wordt wat er in normale situaties gaande is en nog belangrijker, wat in geval van ziekte fout gaat.

In dit proefschrift proberen we nieuwe inzichten te verschaffen in hoe cellen signaleren via deze GTPase eiwitten reguleren. We hebben twee eiwitten, PACSIN2 en HMHA1, bestudeerd die erg belangrijk zijn voor het reguleren van de activiteit van het GTPase eiwit Rac1 en daardoor cel-hechting en -beweging reguleren. Verder laten wij een nieuw mechanisme zien waarbij welk Rac1 de hechting tussen verschillende cellen beïnvloedt. Daarnaast laten wij ook zien hoe PACSIN2 groeifactoer signalering, wat onder andere belangrijk is voor celgroei en -beweging, reguleert.

Zoals eerder genoemd functioneren RhoGTPases als moleculaire schakels. Externe signalen leiden tot activatie van RhoGTPases wat wordt geregeld door een groep speciale eiwitten, de GEFs (Guanine nucleotide Exchange Factors). Deze activatie leidt tot een keten aan intracellulaire reacties. Natuurlijk moet deze signalering ook op tijd weer gestopt worden. Hiervoor is een andere groep eiwitten, de GAPs (GTPase Activating Proteins), belangrijk. Deze zorgen dat de RhoGTPases weer in het cytoplasma, de vloeistof waarmee de cel gevuld is, terecht komen. In het algemeen kunnen wij zeggen dat geactiveerde RhoGTPases zijn die de RhoGTPase activiteit in cellen te controleren. Deze studie heeft dus laten zien dat eiwit-transport een zeer belangrijke manier is om RhoGTPase activiteit in cellen te controleren.

**Hoofdstuk 3** beschrijven wij hoe het F-BAR eiwit PACSIN2 de activiteit, en mede daardoor de functie, van het RhoGTPase Rac1 reguleert. Wij laten zien hoe PACSIN2 aan Rac1 bindt en dat PACSIN2 de activiteit van Rac1 beïnvloedt door het reguleren van het transport van Rac1. Actief Rac1, dat zich aan het celmembraan bevindt, wordt door PACSIN2 gebracht naar intracellulaire locaties waar de GAP eiwit Rac1 kunnen inactiveren ("uit" zetten). Door middel van mutanten van PACSIN2 laten wij zien dat het BAR domein tussen PACSIN2 en Rac1 alsmede de functie van PACSIN2 in eiwit-transport belangrijk is om Rac1 activiteit te verlagen. Extra bewijs dat eiwit-transport inderdaad een belangrijke rol speelt komt van experimenten die laten zien dat PACSIN2 Rac1 niet meer kan inactiveren wanneer wij met behulp van remmers het eiwit-transport van het membraan naar de intracellulaire structuren stoppen. Deze studie heeft dus laten zien dat eiwit-transport een zeer belangrijke manier is op RhoGTPase activiteit in cellen te controleren.

**Hoofdstuk 4** identificeren wij HMHA1 (Human Minor Histocompatibility Antigen-1) als een nog niet eerder beschreven RhoGAP. De sequentie van HMHA1 laat zien dat naast het GAP domein er zich ook een BAR domein bevindt in HMHA1. Hierdoor kan HMHA1 geplaatst worden in een grotere groep van GAP eiwitten, namelijk de BAR-GAPs welke ook in **Hoofdstuk 2** beschreven zijn. Onderzoek aan de aminozuurvolgorde van HMHA1 laat zien dat het GAP domein van HMHA1 zeer homoloog is aan dat van al bekende GAP eiwitten zoals p50RhoGAP en GRAF1. Onze studie laat zien dat HMHA1 in een celvrij systeem, dat wil zeggen met geïsoleerde eiwitten in een reageerbuis, zowel Rac1, RhoA, en Cdc42 inactiveren. Interessant aan deze studie is dat het BAR domein van HMHA1 auto-inhibitor is tegenover het volledige HMHA1 eiwit weinig GAP activiteit heeft in tegenstelling tot een mutant die dit BAR domein mist. Dit fenomeen is kenmerkend voor de subklasse van BAR-GAPs waartoe, zoals eerder vermeld, eiwitten zoals GRAF1 en SH3BP1 ook behoren. Als consequentie van het direct reguleren van de activiteit van RhoGTPases is HMHA1 logischerwijs ook betrokken bij de regulatie van het actine skelet en cel-hechting. In het celvrij systeem laten wij zien dat HMHA1 in staat is Rac1, RhoA, en Cdc42 te inactiveren. Dit hoeft echter niet in levende cellen niet voor alle drie het geval te zijn. Bepaalde signalen zullen HMHA1 activeren en daardoor misschien selectief een bepaalde RhoGTPase beïnvloeden. Onze resultaten suggereren dat in levende cellen HMHA1 in ieder geval Rac1 activatie reguleert en mogelijk ook RhoA. Cdc42 lijkt geen doelwit voor HMHA1. Hoe dan ook, om definitieve conclusies te kunnen trekken zullen we dit in nog meer detail moeten bestuderen.

In deze studie hebben wij dus een nieuwe rol voor HMHA1 geidentificeerd als regulator van RhoGTPase activiteit. Deze vindt kan grote implicaties hebben aangezien in epitheliale kanker cellen HMHA1 expressie is aangetrokken terwijl in

**Hoofdstuk 2** beschrijven wij hoe het BAR-GAP eiwit PACSIN2 de activiteit, en mede daardoor de functie, van het RhoGTPase Rac1 reguleert. Wij laten zien hoe PACSIN2 aan Rac1 bindt en dat PACSIN2 de activiteit van Rac1 beïnvloedt door het reguleren van het transport van Rac1. Actief Rac1, dat zich aan het celmembraan bevindt, wordt door PACSIN2 gebracht naar intracellulaire locaties waar de GAP eiwit Rac1 kunnen inactiveren ("uit" zetten). Door middel van mutanten van PACSIN2 laten wij zien dat het BAR domein tussen PACSIN2 en Rac1 alsmede de functie van PACSIN2 in eiwit-transport belangrijk is om Rac1 activiteit te verlagen. Extra bewijs dat eiwit-transport inderdaad een belangrijke rol speelt komt van experimenten die laten zien dat PACSIN2 Rac1 niet meer kan inactiveren wanneer wij met behulp van remmers het eiwit-transport van het membraan naar de intracellulaire structuren stoppen. Deze studie heeft dus laten zien dat eiwit-transport een zeer belangrijke manier is om RhoGTPase activiteit in cellen te controleren.
gezonde epitheel cellen HMHA1 afwezig is. Zoals eerder al vermeld kan verstoord RhogTPase signalering leiden tot het uitzetten en kanker cellen. Dit suggereert dat de veranderde hoeveelheden van HMHA1, en daardoor dus de verstoorde RhogTPase signalering, het vermogen tot uitzetten van deze cellen beïnvloedt. Verdere studies zijn nodig om dit idee te bewijzen.

Zoals hierboven besproken is de regulatie van contacten tussen cellen zeer belangrijk en kan verstoord regulatie hiervan leiden tot ziektes. Doordat het actine skelet verankerd zit aan cel-cel contacten speelt het een belangrijke rol bij de regulatie van de stabiliteit van deze contacten. Voor RhogTPases, regulator van het actine skelet, is het dan ook meer dan logisch dat zij belangrijk zijn voor de regulatie van cel-cel contacten. Veel studies hebben gekeken naar de specifieke bijdragen van verschillende RhogTPases in de regulatie van cel-cel contacten maar toch laten deze studies soms tegenovergestelde resultaten zien. Dit geeft aan dat de regulatie van cel-cel contacten door RhogTPases zeer complex is en dat het vooral om de balans tussen de activiteit van de verschillende RhogTPases gaat.

In Hoofdstuk 6 beschrijven wij een nieuw mechanisme van welk het RhogTPase Rac1 cel-cel contacten stabiliseert, onafhankelijk van het actine skelet. Rac1 zorgt ervoor dat het eiwit Nedd4 zich verplaatst naar cel-cel contacten. Nedd4 is een speciaal type eiwit dat andere eiwitten kan “merken” zodat deze worden herkend door het systeem dat de afbraak van eiwitten verzorgt. Wij vonden dat Nedd4, in samenwerking met Rac1, het eiwit Dvl1 (dishevelled1) “merkt” welke daardoor daarna wordt afgebroken. Dit resulteert in verminderde hoeveelheden van het Dvl1 eiwit. Deze bevinding is erg interessant want eerdere studies door anderen hebben aangetoond dat Dvl1 een negatief effect heeft op de stabiliteit van cel-cel contacten. Als wij Nedd4 expressie verlagen zien we minder afbraak en dus verhoogde niveaus van Dvl1, en verzakte cel-cel contacten. Dvl1 staat ook bekend om het feit dat het de acetylering van micro-tubuli (een andere vorm van het cel-skelet) reguleert. Deze acetylering heeft tot gevolg dat de stabiliteit van de micro-tubuli en ook de richting van transport van eiwitten over deze micro-tubuli verandert. Het is aangetoond dat transport van bepaalde eiwitten over deze micro-tubuli belangrijk is voor de stabiliteit van cel-cel contacten. Dit is erg interessant aangezien wij in cellen waar Nedd4 expressie verminderd is, meer acetylatie zien van deze micro-tubuli (en zoals eerder vermeld ook verhoogde niveaus van het Dvl1 eiwit).

Dit suggereert dat Nedd4, gestimuleerd door Rac1 activiteit, het eiwit Dvl1 afbrekt en daardoor de niveaus van geacetyleerde micro-tubuli verlaagt, wat als gevolg heeft dat cel-cel contacten worden versterkt. Deze studie heeft dus een vernieuwende signaleringsroute laten zien volgens welke RhogTPases de stabiliteit van contacten tussen cellen kan beïnvloeden.

Signalering via groefactoren is erg belangrijk voor verschillende processen zoals cel deling en -beweging. Daarnaast kunnen groefactoren de cellen stimuleren langer te blijven leven. Aangezien kancercellen als kenmerken hebben dat ze vaker delen, langer leven, en sneller bewegen, is het niet vreemd dat in veel kankers abnormale signalering door groefactoren waargenomen is. Daarom staat groefactor signalering onder strenge controle van de cel. Transport van groefactoren en hun receptoren speelt hierbij een belangrijke rol omdat de cel op deze manier de signalering op tijd kan stoppen. De receptoren voor groefactoren zitten in het plasma membraan, de buitenwand van de cel. Aan de buikvlank kunnen deze receptoren de juiste groefactor binden wat leidt tot activatie van de receptor en het aanzetten van een serie signaleringspaden in de cel. Het transporteren van de geactiveerde receptoren naar binnen in de cel leidt tot inactivatie van deze receptoren of zelfs tot afbraak. In beide gevallen wordt de signalering beëindigd.

Zoals eerder al vermeld, spelen BAR eiwitten een belangrijke rol bij transport van eiwitten in de cel. In Hoofdstuk 5 laten wij zien hoe PACSIN2 signalering van een specifieke groefactor receptor, de EGF receptor, reguleert. Door de expressie van de EGF receptor op het plasma membraan te reguleren, bepaalt PACSIN2 de signalering via EGF. Zo laten wij zien dat in cellen waarin PACSIN2 niveaus verlaagd zijn, er meer EGF receptor op het oppervlak zit. Als gevolg van dit zien wij na stimulatie met EGF een verhoogde signalering via intracellulaire signaleringspaden zoals via Erk en Akt. Daarnaast zien wij ook in deze cellen verhoogde celdeling na EGF stimulatie wat kenmerkend is voor de verhoogde signalering via Erk en Akt. Wij laten ook zien dat niet alleen signalering door EGF wordt gereguleerd door PACSIN2 maar ook signalering door andere groefactoren zoals HGF (Hepatocyte Growth factor) en TNFα (Tumor Necrosis Factor).

De resultaten in dit proefschrift laten zien dat BAR domein eiwitten weer zeer belangrijke regulator zijn van verschillende cellulair processen zoals het reguleren van contacten. Daarnaast beschrijven wij een nog niet eerder gevonden manier van welke Rac1 de cel-cel contacten van epitheelcellen reguleert. Door de expressie van de specifieke groefactor receptor, de EGF receptor, reguleert. Door de expressie van de EGF receptor op het oppervlak van de cel-cel contacten van epitheelcellen reguleert. Doordat de expressie van de groefactor receptor wordt gereguleerd door PACSIN2 leidt tot activering van de receptor en het aanzetten van een serie signaleringspaden in de cel. Het transporteren van de geactiveerde receptoren naar binnen in de cel leidt tot inactivatie van deze receptoren of zelfs tot afbraak. In beide gevallen wordt de signalering beëindigd.
CURRICULUM VITAE

Bart-Jan de Kreuk was born on the 4th of November 1982 in Bevenwijk, the Netherlands. From 1995 to 2001 he attended the Bonhoeffer college high school in Castricum. In 2002, he started his study Biomedical Sciences at the Free University (VU) in Amsterdam. Here, he obtained his bachelor degree in 2005. As part of his Master Biomedical Sciences (Free University, Amsterdam), he went in 2005 on an ISEP scholarship to San Diego State University where he spent two semesters. Upon return, he did his first internship at the Department of Microbiology and Immunology at the VU medical center in Amsterdam. In March 2008, after obtaining his Master degree, he started as a PhD student at Sanquin Research in the Department of Molecular Cell Biology, under supervision of Prof. Dr. Peter Hordijk. The results of this research are described in this thesis.

After obtaining his PhD degree, Bart-Jan will start working as a post-doctoral researcher in the Department of Prof. Dr. Mark H. Ginsberg at the University of California at San Diego (UCSD).

LIST OF PUBLICATIONS

4. De Kreuk BJ, Anthony EC, Geerts D, Hordijk PL. The F-BAR Protein PACSIN2 regulates growth factor receptor activation and signaling. Submitted
DANKWOORD


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development of the new train layout and of course San Diego-London seems like a long distance but hey, a direct flight is not so bad! We look forward to your visits to San Diego so we can have a nice lunch (read: hamburger with champagne) at Seaport Village in the sun.

And then all at the end, not because you are the last in line, but because sometimes the last one called is actually the number 1. Mari-Liis, thank you for all your support during these years. Who would have guessed 7 years ago that you would be moving to the Netherlands to live with that weird boy with his colorful shorts! Not even to mention that special day in September last year. Best choice in my life! Now a couple of months to go and we will start our adventure. Funny that we will be moving back to the place where everything started. I know we will have an amazing time there! Again, thank you for everything! Ma armastan sind!