The role of Rap1 in cell adhesion and migration

Judith Heleen Raaijmakers

ISBN: 978-90-393-5052-2

Printed by Gildeprint Drukkerijen B.V., Enschede, the Netherlands

Financial support for the printing of this thesis from the Dutch Cancer Society, J.E. Jurriaanse stichting, Universiteit Utrecht, Universitair Medisch Centrum Utrecht and tebu-bio is gratefully acknowledged.

Coverdesign and photography: Carin Cruijsen and Judith Raaijmakers

The role of Rap1 in cell adhesion and migration

De rol van Rap1 in cel-adhesie en -migratie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 26 mei 2009 des ochtends te 10.30 uur

door

Judith Heleen Raaijmakers

geboren op 5 november 1981 te Nijmegen

Promotor: Prof. dr. J. L. Bos

The research described in this thesis was financially supported by the Dutch Cancer Society and the Centre for Biomedical Genetics

voor mama en papa

Look a little on the sunny side
Even when you feel you want to hide,
You gotta laugh, don't let your critics ever upset you,
'Cos for a while the cynics will all be out to get you.
You gotta be shrewd, you gotta be strong
You've gotta convince yourself that you are not wrong,
Whistle a tune and think of a catchy, happy, little song
And look a little on the sunny side.

It's very hard to please the people every single time,
But look a little on the sunny side.

The Kinks – Look a little on the sunny side

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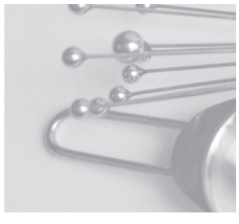

The regulation of cell migration

Cells adhere to their surroundings via interactions between integrins and the extracellular matrix. These interactions provide dynamic, bidirectional links to coordinate signaling pathways that control various cellular behaviors, such as cell migration. Cell migration plays an important role in many biological processes, including embryogenesis, wound healing, and the inflammatory response, but can also drive disease progression, *e.g.* in metastasis of cancer cells. Regulation of cell migration requires the temporal coordination and integration of processes occurring at different locations in the cell. Here, we describe the processes of cell adhesion and migration and give an overview of several of the signaling pathways involved in the control of cell migration.

Cell adhesion

The regulation of the adhesion of cells to their environment is important for the maintenance of epithelial tissue architecture and for various essential processes such as embryogenesis, tissue differentiation and the immune response. Cells adhere to each other, via cell-cell junctions, and to the extracellular matrix (ECM), using integrin-mediated adhesions. In addition, integrins serve as receptors for adhesive signals and regulate a variety of signaling pathways involved in growth signaling, differentiation, survival and migration (Danen and Sonnenberg, 2003).

Initiation of cell adhesion

Integrins are heterodimeric transmembrane receptors that bind components of the ECM or receptors on adjacent cells. There are currently 24 functional integrin heterodimers known in humans, consisting of different combinations of the 18 α- and 8 β-subunits. Both subunits have a large extracellular domain, a single-pass transmembrane domain and a short cytoplasmic domain (Hynes, 2002). All integrin heterodimers have specific binding preferences for different ECM components, such as fibronectin, collagen or laminin. Integrins also have the unique ability to regulate their adhesiveness dynamically, through inside-out signaling. In the inactive state, the integrin heterodimer is in a bent conformation due to interactions between the intracellular tails of the α- and β-chain. In this conformation, the extracellular headpiece has a low affinity for its ECM ligands. Inside-out signaling results in the binding of the head domain of the adaptor protein talin to the β-subunit cytoplasmic tail. This binding sterically disrupts the interaction between the α and β cytoplasmic domains, inducing a conformational change that is transmitted by the transmembrane domains to the extracellular domains, resulting in an open, extended conformation of the integrin. This open conformation has an increased binding affinity for its ligands (Ginsberg et al., 2005; Kim et al., 2003; Wegener et al., 2007). Adhesive strength can also be enhanced by clustering of multiple

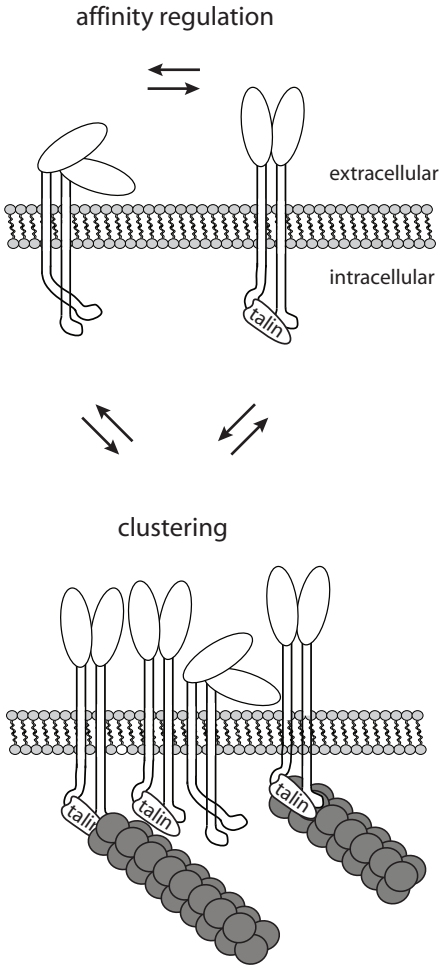

Figure 1. Integrin activation: affinity and avidity mechanisms
Inactive integrins are in a bent conformation with low affinity for their ligands. Binding of talin induces a conformational change, resulting in an open, extended integrin conformation with increased ligand binding affinity. A second way to increase adhesive strength is the induction of integrin clustering (avidity).

integrin dimers. This is called avidity regulation (Figure 1) (Kinbara et al., 2003; van Kooyk and Figdor, 2000).

Formation of adhesion complexes
As integrins span the membrane and interact with proteins on both sides of the plasma membrane, they do not only physically link cells to their environment, but also mediate bidirectional signaling (Geiger et al., 2001). Besides the modulation of integrin affinity and avidity through inside-out signaling, the activation and clustering of integrins also triggers outside-in signaling (Ginsberg et al., 2005; Kinbara et al., 2003). Upon integrin activation, various proteins are targeted to the site of adhesion to form adhesion complexes. Small G proteins of the Rho family (RhoA, Rac1 and CDC42) are important players in these signaling cascades. These three proteins all have distinct effectors that are involved in the regulation of the actin cytoskeleton, and in the regulation of the linkage between integrins and actin (Hall, 1998). The first small integrin complexes are called focal complexes (Geiger and Bershadsky, 2001). These are initiated in response to Rac (Hall, 1998). These focal complexes can turn over rapidly, or evolve to become a focal adhesion (FA) (Zaidel-Bar et al., 2004).

Maturation of adhesion complexes
The presence of contractile forces is one of the main requirements for FA formation; upon stimulation of contractility, diffusely distributed integrins are aggregated into FAs. On the other hand, if contractility is inhibited, integrins disperse from FAs and stress fibers and, consequently, FAs disassemble (Chrzanowska-

Wodnicka and Burridge, 1996). RhoA provides the driving force for focal adhesion assembly by activating myosin II and thereby increasing contractility (Burridge and Chrzanowska-Wodnicka, 1996). Downstream of Rho, the Rho effectors Rock (Rho kinase) and MLCK (MLC kinase) can both phosphorylate the myosin light chain (MLC) subunit of myosin II. Phosphorylation of myosin II promotes the assembly of myosin fibers and induces its interaction with actin. This activates the ATPase activity of myosin, leading to increased contractility and increased stress fiber formation, which is transmitted to the integrin (Ridley, 2001; Riento and Ridley, 2003). This induces the clustering of integrins and the recruitment of focal adhesion proteins and other signaling molecules (Chrzanowska-Wodnicka and Burridge, 1996). Rock can also affect myosin II activation in an indirect manner, by phosphorylating and inhibiting the myosin binding subunit (MBS) of MLC phosphatase (Figure 2) (Ridley et al., 2003). The importance of proper levels of contractility for FA formation during cell adhesion and spreading is illustrated by the tight regulation of Rho activity during adhesion. When cells adhere, Rho activity is initially decreased (in a FAK-dependent manner), whereas it returns to normal levels again after the initial phase of adhesion, when large focal adhesions are forming (Ren et al., 2000).

Stabilization or turnover?

Whether a focal complex becomes stabilized and matures into a FA or whether it is turned over directly, is tightly regulated (Webb et al., 2002). The exact composition of focal complexes and focal adhesions can differ between cell types, but phosphorylation of tyrosines is always essential, just as integrin activation and clustering are both a prerequisite for FA formation (Zaidel-Bar et al.,

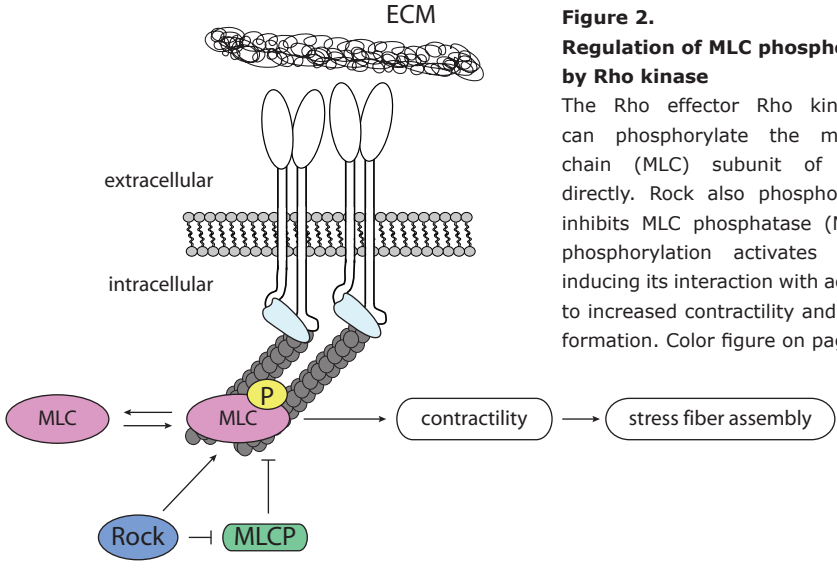

Figure 2.
Regulation of MLC phosphorylation by Rho kinase
The Rho effector Rho kinase (Rock) can phosphorylate the myosin light chain (MLC) subunit of myosin II directly. Rock also phosphorylates and inhibits MLC phosphatase (MLCP). MLC phosphorylation activates myosin II, inducing its interaction with actin, leading to increased contractility and stress fiber formation. Color figure on page 154.

2003). Once matured, FAs can turn over or grow further into a so-called fibrillar adhesion. Fibrillar adhesions are located in a more central region under the cell and are very stable compared to focal complexes and focal adhesions. Just like FA formation, the formation of fibrillar adhesions depends on actomyosin-based contractility. In contrast to FAs, the subsequent stability of fibrillar adhesions does not require contractility (Zaidel-Bar et al., 2004; Zamir and Geiger, 2001). As will be discussed below, proper regulation of adhesion and de-adhesion between cells and the ECM is essential for cell migration. Additionally, adhesion-dependent signaling can regulate cell survival and proliferation (Geiger et al., 2001).

Focal adhesion formation is not a random process

Although FAs are large protein complexes with >100 components, the entry of these proteins into the FA is a tightly regulated process. When FAs are assembled, individual proteins (or groups of proteins) are recruited in a regulated, sequential manner. The intracellular tail of integrins binds to the integrin-binding proteins talin, paxillin and kindlin. Other important proteins that are present in most, if not all, focal adhesions are focal adhesion kinase (FAK), that is important for signal integration, and proteins that link the actin cytoskeleton to the adhesion site, such as α-actinin and vinculin (Critchley, 2000; Larjava et al., 2008; Mitra et al., 2005; Zaidel-Bar et al., 2004; Zamir and Geiger, 2001).

Cell migration

Cell migration is essential for various important biological processes, such as embryogenesis, wound healing and the inflammatory response. Moreover, it also contributes to various pathological conditions, e.g. cancer, atherosclerosis and osteoporosis (Ridley et al., 2003). Efficient cell migration requires the continuous, coordinated formation and release of integrin-based adhesions. These processes are complex and require the regulated interaction of numerous molecules and the activation of specific signaling pathways (Webb et al., 2002).

The cell migration cycle

The process of migration occurs in a multistep cycle, in which the formation and release of focal adhesions is coordinated (Webb et al., 2002). In order for a cell to migrate in a certain direction, it must first become polarized so that it can extend protrusions in the desired direction. Next, two types of actin-based protrusions will form at the leading edge: lamellipodia, broad sheets of membrane, and filopodia, small spike-like protrusions. These protrusions are the sites where new adhesions to the ECM are made. After membrane protrusion has taken place, integrins are engaged and strong adhesions are formed underneath the protrusion's leading edge to attach the lamellipodium to the ECM. Then, the cell body can use the forces generated by the attachments to the ECM to translocate itself forward. At the same

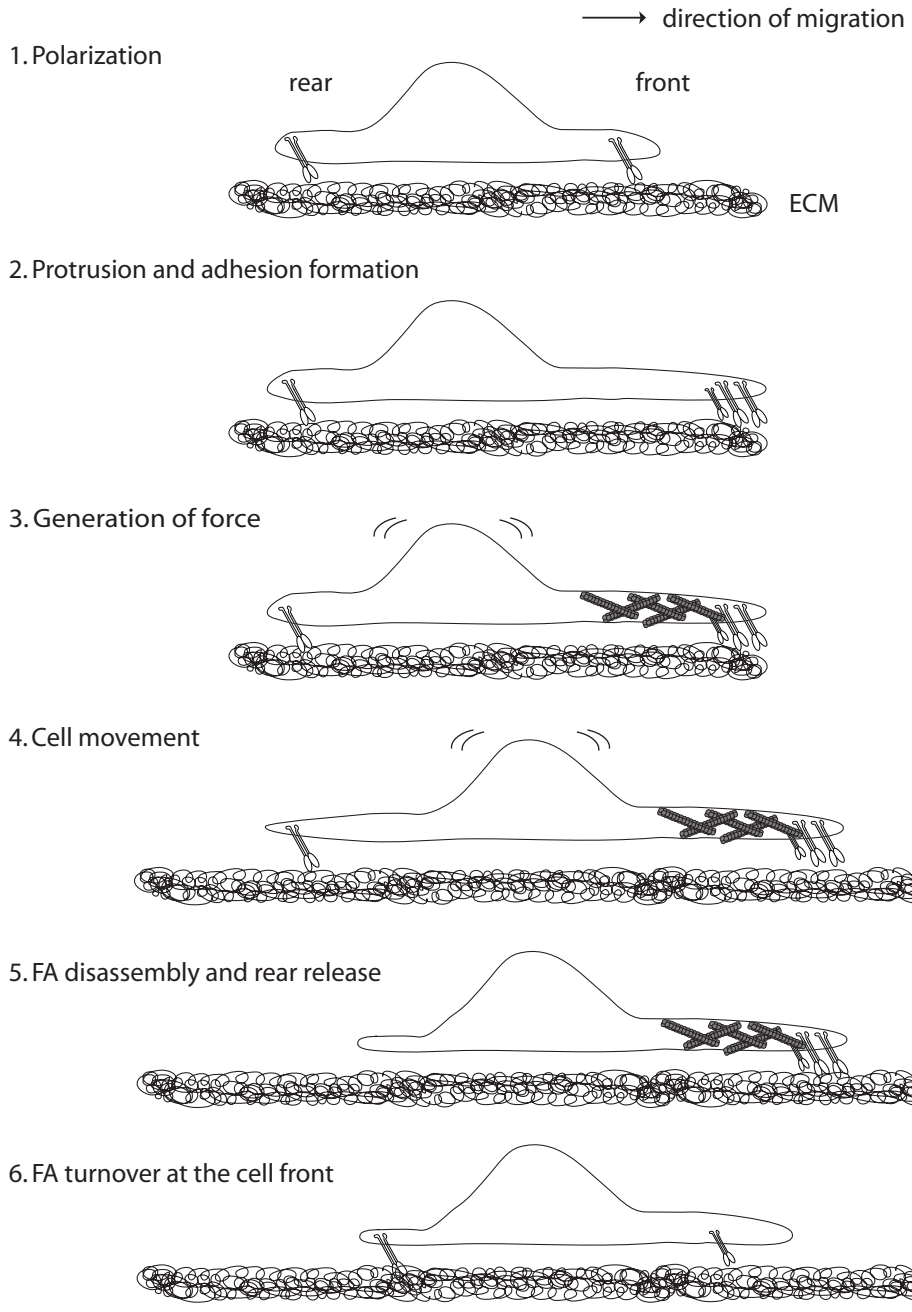

Figure 3. The migration cycle.
The process of migration occurs in a multistep cycle. 1) In order for a cell to migrate in a certain direction it must first become polarized. 2) Then, an actin-based protrusion is extended. New adhesions to the ECM are made to stabilize the protrusion. 3) Linkage to the actin cytoskeleton provides the force required for movement.

time, FAs at the rear are released and the cell rear can retract to allow the cell to move forward (Figure 3) (Ridley et al., 2003; Vicente-Manzanares et al., 2005; Webb et al., 2002). As they provide the attachments necessary to generate the contractile forces required for movement, FAs are key players in the regulation of cell migration (Burridge and Chrzanowska-Wodnicka, 1996). Their formation and disassembly are complex processes requiring the coordinated interaction of integrins, actin and actin-binding proteins, signaling molecules, adaptor proteins and microtubules (Figure 4). The major signaling pathways involved in the control of FA (dis)assembly will be discussed below.

Regulation of the actin network by Rho GTPases

After the establishment of polarization in the migrating cell (a process depending on the Rho GTPase CDC42, among others), the Rho GTPases are required to maintain tight spatiotemporal regulation of the actin network. This is required for a cell to properly adhere, spread and migrate. In a membrane protrusion, actin branching is induced by Rac and CDC42 and their effectors WASP and WAVE, to produce a lamellipodium (Miki et al., 1998; Pollard and Borisy, 2003). When, instead of branches, long parallel bundles of actin are formed in protrusions, it results in filopodia extension. Filopodia are induced by CDC42 through formins and VASP (Mattila and Lappalainen, 2008; Pollard and Borisy, 2003). At the ends of filopodia, FAs can form. Active Rho induces the stress fibers required for actomyosin-based contractility (Chrzanowska-Wodnicka and Burridge, 1996). The importance of these GTPases in maintaining cell polarity and regulating the actin cytoskeleton is illustrated by their tight spatiotemporal regulation. In migrating cells, the activity of Rac and CDC42 is restricted to the cell front, whereas RhoA operates at the rear of the cell (Ridley et al., 2003). This spatial segregation may be achieved by the GTPases themselves, as Rac and Rho can each suppress the other's activity (Evers et al., 2000).

Regulation of migration by adhesive strength

Effective cell migration depends on an optimal amount of adhesive strength to the ECM. Adhesion must be strong enough to provide the pulling forces required for cell movement, but weak enough to allow rapid detachment at the cell rear (Huttenlocher et al., 1996; Lauffenburger and Horwitz, 1996). Thus, intermediate amounts of adhesion strength provide the correct amount of tension for fast cell migration (Palecek et al., 1997). This dependence of migration velocity on the overall adhesive strength

Figure 3, continued

4) Next, the cell body can use the forces generated by the attachments to the ECM to translocate itself forward. 5) At the same time, FAs at the rear are released and the cell rear can retract to allow the cell to move forward. 6) At the front, adhesions are turned over to provide components for new adhesion at the leading edge.

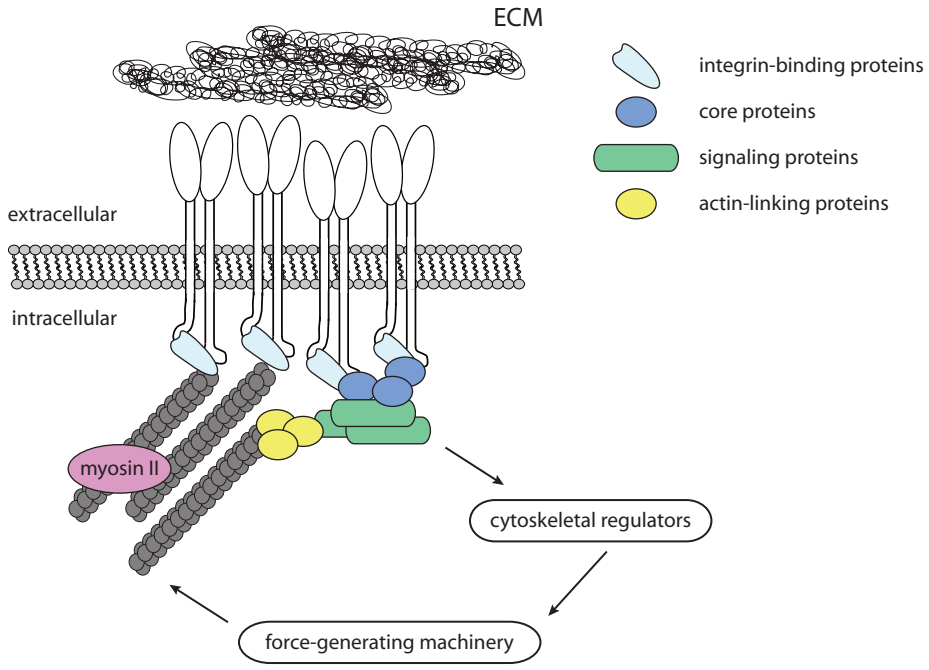

Figure 4. Focal adhesions: core proteins and linkage to the actin cytoskeleton.
The formation of focal adhesions is a complex process requiring the coordinated interaction of integrins, integrin-binding proteins, signaling proteins, actin regulators and -binding proteins and the contractile machinery. Color figure on page 154.

suggests one means by which the various molecules regulating adhesion complexes can effectively control the speed of cell migration (Schwartz and Horwitz, 2006). The Rac-induced focal complexes that form underneath the leading edge of a migrating cell are most important for rapid cell migration and turn over rather quickly (Parsons et al., 2000; Zaidel-Bar et al., 2003). Their turnover provides components for new nascent focal complexes forming under the extending lamellipodium. Upon activation of Rho and the induction of tension, these focal complexes (FCs) can mature into a more stable focal adhesion (Burridge and Chrzanowska-Wodnicka, 1996). Although these adhesions provide the strength to pull the cell body forward, they are less associated with rapid cell migration than FCs and tend to inhibit cell migration (Zaidel-Bar et al., 2003). Like FCs, FAs can turn over rapidly to provide components for new FAs. If they do not turn over, they become a so-called sliding focal adhesion (Wehrle-Haller and Imhof, 2003). These FAs are eventually disassembled at the cell rear to allow the release of the cell body to move forward. Turnover of FAs at the cell front and disassembly of FAs at the rear is not necessarily the same process. At the cell front, turnover accompanies the formation of new adhesions and protrusions. At the back, it is necessary for the

cell to move. As these two means of turnover serve different purposes, they use different mechanisms and are regulated differently (Webb et al., 2002). Importantly, both adhesion assembly and disassembly are dependent on myosin-generated tension (Gupton and Waterman-Storer, 2006).

Coordinated FA turnover is required for efficient cell migration

Although the mechanisms of adhesion assembly and disassembly are still rather poorly understood, it is clear that tight spatial and temporal regulation of these processes is required for efficient cell migration. The major signaling pathways involved in the control of FA (dis)assembly are: regulation of Rho-induced contractility, phosphorylation of FAK and paxillin, proteolysis of adhesion proteins by calpain-2 and microtubule dynamics (Burridge and Chrzanowska-Wodnicka, 1996; Ezratty et al., 2005; Franco and Huttenlocher, 2005; Webb et al., 2004; Zaidel-Bar et al., 2007).

The active FAK-Src complex is essential for adhesion disassembly

Focal adhesion kinase (FAK) is one of the first proteins to be recruited to a nascent FC (Kirchner et al., 2003; Zaidel-Bar et al., 2004). Normally, it is maintained in an inactive, auto-inhibited state, but upon recruitment to the integrin, it is autophosphorylated on tyrosine 397. This recruits Src family kinases, leading to phosphorylation of additional sites in FAK, thereby sustaining FAK and Src in their activated states (Schlaepfer et al., 2004). Phosphorylation of FAK creates binding sites for paxillin, p130Cas and other adaptor proteins, and links integrin activation to various downstream signaling pathways. For instance, activation of the FAK-Src complex results in the recruitment and phosphorylation of p130Cas, leading to binding of Crk and the RacGEF DOCK180 and ultimately to activation of Rac1. The adaptor protein paxillin is also a substrate of FAK and binds to Crk as well. Activation of Rac1 downstream of paxillin, through the adaptor PKL and RacGEF β-Pix, induces the activation of both Rac1 and CDC42 to coordinate cell spreading and motility. Disassembly of adhesions is also dependent on phosphorylation of tyrosine 397 and Src activation (Webb et al., 2004). One of the mechanisms by which FAK can regulate FA disassembly is by decreasing tension through the inhibition of RhoA and Rock (see below (Ren et al., 2000; Schober et al., 2007)). The importance of FAK and Src in regulating FA dynamics is illustrated by the fact that FAK-/- fibroblasts and cells lacking all three Src kinases (SYF-/-) have an increased number of larger FAs, defects in cell spreading and inhibited cell migration (Ilic et al., 1995; Klinghoffer et al., 1999). This was shown to be due to impaired FA disassembly (Ren et al., 2000; Webb et al., 2004). In addition to the regulation of Rho, regulation of calpain activity is yet another pathway through which the FAK-Src complex can enhance FA disassembly (Carragher et al., 2002).

Phosphorylation of paxillin is linked via several ways to dissociation of the focal adhesions

The regulation of paxillin phosphorylation is also involved in FA dynamics. Paxillin has no enzymatic activity, but it contains many binding sites for tyrosine kinases, serine/threonine kinases and GTPase activating proteins. Moreover, paxillin can bind to cytoskeletal proteins and other adaptor proteins that in turn recruit more proteins to form an extensive complex (Schaller, 2001). Paxillin is essential for FA targeting of FAK (Tachibana et al., 1995). In FCs and FAs, upon integrin activation, paxillin is phosphorylated at tyrosines 31 and 118 (Schaller, 2001; Turner, 2000). Tyrosine phosphorylation of paxillin has been suggested to be an important event in the decision between adhesion stability and disassembly. This is based on the observation that phosphorylated paxillin is present in higher concentrations in FCs than in FAs and absent from fibrillar adhesions (Zaidel-Bar et al., 2007). The binding affinity of paxillin for FAK is much lower when it is in an unphosphorylated state. Tyrosine phosphorylation of paxillin thus enhances the recruitment of FAK, which in turn stimulates adhesion turnover. When the balance between phosphorylated and unphosphorylated paxillin is shifted enough towards the unphosphorylated state, FAK can dissociate from FAs and this leads to stabilization of the complex (Zaidel-Bar et al., 2007). Interestingly, phosphorylation of paxillin has been shown to result in both an increase or a decrease in cell migration, depending on the cell type (Schaller, 2001; Zaidel-Bar et al., 2007). Moreover, FAK-/- cells have lower levels of these phosphorylated forms of paxillin compared to wild type cells (Webb et al., 2004). This points to a role for the interaction between the FAK-Src complex and phosphorylated paxillin in the regulation of adhesion disassembly.

Down-regulation of Rho activity is associated with adhesion disassembly

The amount of FA turnover is inversely correlated with the level of Rho activity (Ridley, 2001; Schwartz and Shattil, 2000). When FA turnover is high, Rho activity is low, and when FA turnover is low, Rho activity is high. In normal cells, Rho activity is decreased in response to FAK phosphorylation during adhesion. FAK can inhibit Rho directly by binding several GAPs that negatively regulate Rho activity. A FAK-binding GAP for both RhoA and CDC42 is Graf. Additionally, p190RhoGAP is a common target for both Src and FAK (Schlaepfer and Mitra, 2004). The phosphorylation of p190RhoGAP is associated with decreased Rho activity and increased focal contact turnover (Schober et al., 2007). FAK-/- cells lack this level of regulation, leading to higher levels of RhoA activity which results in disturbed adhesion disassembly (Ren et al., 2000). The presence of phosphorylated paxillin in an FA can also inhibit Rho, via p120RasGAP (Zaidel-Bar et al., 2007).

Protease calpain degrades several focal adhesion components

The development of tension between adhesions at the rear and the retraction machinery, following myosin II activation, can be sufficient to open stretch-activated calcium channels that lead to the activation of calpain (Franco and Huttenlocher, 2005). There are several calpain isoforms, with ubiquitous or tissue-specific expression patterns (Franco and Huttenlocher, 2005). Studies using calpain inhibitors or calpain-/- cells have implicated the protease calpain2 in focal adhesion turnover and cell migration (Dourdin et al., 2001; Glading et al., 2000). Calpains are recruited to focal adhesions by FAK and cleave several components of focal adhesions like β-integrins, paxillin (Franco and Huttenlocher, 2005), FAK (Carragher et al., 2003) and talin (Franco et al., 2004). Talin is more readily cleaved by calpain2 than other substrates and its cleavage is sufficient to induce adhesion disassembly. Inhibition of talin cleavage by calpain2 results in inhibited turnover of talin and the FA components paxillin, vinculin and zyxin. These findings support the idea that cleavage of talin by calpain2 plays an important role in adhesion regulation (Franco et al., 2004).

Microtubule regrowth stimulates focal adhesion disassembly via a dynamin-FAK interaction

Although actin cables provide the protruding force for migration, and actomyosin-induced contractility is required for FA maturation and retraction of the cell body, the microtubule network is also involved in regulating cell migration. The observation that microtubules target focal adhesions and that there is a correlation between microtubule regrowth from the cell centre to the periphery and disassembly of focal adhesions, has pointed towards a possible role for microtubule growth in focal adhesion turnover. The targeting of FAs behind the leading edge or at the cell rear by microtubules induces their disassembly (Kaverina et al., 1999). Several mechanisms have been proposed to explain this. Firstly, microtubules (MTs) could facilitate disassembly by locally reducing the amount of tension, perhaps by inhibiting RhoA, or by inducing calpain-driven proteolysis of certain FA proteins (Broussard et al., 2008). However, in fibroblasts, the induction of focal adhesion disassembly was shown to be independent of the activity of Rho GTPases, paxillin, Src, Yes and Fyn (Ezratty et al., 2005). In contrast, FAK was essential for microtubule-induced disassembly, as shown by the inability of microtubule regrowth to induce disassembly in FAK-/- fibroblasts. The microtubule-FAK interaction was shown to be mediated by dynamin, a GTPase involved in endocytosis. FAK recruits dynamin to focal adhesions, where it might induce endocytosis of focal adhesion components, or be involved in the inhibition of the interaction between the actin cytoskeleton and integrins. Interestingly, dynamin does not seem to play a role in focal adhesion assembly, and regulates

disassembly specifically (Ezratty et al., 2005).

Epithelial - to - mesenchymal transition

During embryogenesis or wound healing, epithelial cells can undergo an epithelial - to - mesenchymal transition (EMT) to acquire the characteristics of mesenchymal cells and become more motile. This allows the cell to escape its surrounding epithelium and invade into other tissues. EMT therefore also contributes to a number of pathological processes such as cancer cell invasion and metastasis (Yang and Weinberg, 2008). EMT is loosely defined by three effects on the cellular phenotype. First, cells undergo morphological changes from an epithelial, cobblestone-like morphology with apico-basal polarity to single, spindle-shaped mesenchymal cells with migratory protrusions. Secondly, they start to express different differentiation markers. The third aspect involves the functional changes associated with the conversion of non-moving epithelial cells to motile cells that can invade into the ECM. Although not all three changes are consistently observed during an EMT, as cells can also undergo a partial EMT, the acquisition of migratory capacity is considered a functional hallmark of EMT (Thiery, 2002; Yang and Weinberg, 2008). Several extracellular signals are known to initiate EMT, including ECM proteins and soluble growth factors such as transforming growth factor β (TGFβ) or hepatocyte growth factor/ scatter factor (HGF/SF) (Birchmeier et al., 2003; Zavadil and Bottinger, 2005). Receptor-mediated signaling in response to these ligands triggers the activation of effector molecules, such as the Ras family GTPases. These effectors mediate the disassembly of adherens junctions and the cytoskeletal changes that occur during EMT. Transcriptional regulators are also activated, in order to regulate the changes in gene expression patterns underlying EMT (Moreno-Bueno et al., 2008; Thiery and Sleeman, 2006). In 2D cell culture, cells undergoing EMT obtain a more migratory, fibroblastic morphology, a loss of epithelial cell polarity and the expression of epithelial markers, such as E-cadherin, is reduced. This phenotype was first observed by stimulation of cells with HGF/SF and is also called 'scattering' (Weidner et al., 1993).

HGF signaling

Hepatocyte growth factor/ scatter factor signals through the transmembrane receptor tyrosine kinase c-Met that, in its oncogenic form, can strongly contribute to tumor cell invasiveness (Birchmeier et al., 2003). HGF/SF signals in a paracrine manner and is mainly synthesized by mesenchymal cells (Rosario and Birchmeier, 2003). Signaling downstream of Met is highly complex and integrates multiple signal transduction pathways. Essential for the activation of HGF signaling is the scaffolding protein Gab1. After activation of Met, Gab1 is recruited to the receptor and is phosphorylated. Subsequently, downstream signaling cascades involving Ras, Src, PI3K,

Rac and CDC42 are activated and this mediates effects of HGF on adhesion, motility, proliferation, survival, invasion and EMT (Birchmeier et al., 2003; Rosario and Birchmeier, 2003).

TGFβ signaling

The transforming growth factor β superfamily consists of many different signaling proteins, among which are the TGFβ isoforms (TGFβ1, -2, and -3) and bone morphogenetic proteins (BMPs) (Roberts and Sporn, 1993). TGFβ is a potent regulator of various processes, including cell growth, differentiation, apoptosis and migration (Massague et al., 2000; Siegel and Massague, 2003). Interestingly, TGFβ is known to stimulate both tumor suppression and tumor progression (Roberts and Wakefield, 2003). Suppression of tumors by TGFβ in epithelial cells occurs during the early stages of carcinogenesis, while during the later stages of carcinogenesis TGFβ is involved in tumor progression and promotes migration and metastasis (Bierie and Moses, 2006). EMT is induced by TGFβ in multiple cell lines *in vitro* (Kasai et al., 2005; Thiery, 2002; Zavadil and Bottinger, 2005). Although many studies have confirmed that TGFβ signaling is a primary inducer of EMT, the precise signaling pathways activated, may differ during various EMT events (Yang and Weinberg, 2008).

TGFβ binds to the type II TGFβ receptor (TβRII) on the cell surface. Once bound, the ligand-receptor complex has a high affinity for the type I TGFβ receptor (TβRI) and TβRI is phosphorylated by the constitutively active TβRII (Shi and Massague, 2003). TGFβ signaling downstream of the receptors can be divided into two pathways: the canonical, or Smad, pathway and the non-canonical, or non-Smad, pathways (Moustakas and Heldin, 2005; Shi and Massague, 2003). In canonical TGFβ signaling, the activation of the ligand-receptor complex leads to the phosphorylation of Smad2 or Smad3. Phospho-Smad2 or -3 then dimerizes with Smad4 and the heterodimer can enter the nucleus, bind to DNA and interact with other transcription factors to regulate transcription. In addition, there is a negative feedback loop mediated by inhibitory Smads. These Smads are induced by Smad signaling and block Smad2/3 phosphorylation by TβRI and heterodimerization of Smad2/3 and Smad4 (Shi and Massague, 2003). Smads are critical during EMT as they stimulate transcription of the Snail transcription factors that repress E-cadherin transcription (Batlle et al., 2000; Yang et al., 2003). A variety of other signaling pathways cooperate with TGFβ signaling to mediate EMT, including the ERK/MAPK cascade as well as Wnt, Notch and PI3K signaling (Moustakas and Heldin, 2005; Yang and Weinberg, 2008; Zavadil and Bottinger, 2005). Smad-independent TGFβ signaling has been suggested to regulate apoptosis, cell proliferation and EMT or cell migration (Moustakas and Heldin, 2005). In that case, the TGFβRII was found to phosphorylate the polarity protein Par6, leading to recruitment of the E3 ubiquitin ligase Smurf1 and subsequent degradation of RhoA, resulting in the weakening

of tight junctions (Ozdamar et al., 2005).

Both the HGF and the TGFβ signaling cascades result in changes in cell adhesion receptors and actin dynamics as part of their EMT program. For both growth factors, down-regulation of E-cadherin function is an important aspect of the induction of cell scattering (Boyer et al., 1997; Thiery and Sleeman, 2006). Interestingly, signaling via integrins can also contribute to EMT. For instance, the β1-integrin is essential for TGFβ-induced and collagen-stimulated EMT (Bhowmick et al., 2001; Valles et al., 1996). Furthermore, it was recently shown that HGF-induced scattering can also occur as a result of tensile forces generated by integrin-mediated adhesions, rather than by direct down-regulation of E-cadherin by HGF (de Rooij et al., 2005). Thus, for cells containing both cell-cell and cell-ECM adhesions, such as epithelial cells, crosstalk between and regulation of both types of adhesions is important for proper cell migration.

Rap1: an important regulator of adhesion processes

The small GTPase Rap1 is the closest homologue of Ras and initially attracted much attention because of its proposed antagonistic function to oncogenic Ras signaling (Kitayama et al., 1989). However, Rap1 has since been shown to be important in regulating adhesion-related processes in pathways independent from Ras (Bos et al., 2001). Most importantly, Rap1 regulates both cadherin-mediated cell junctions as well as integrin-mediated cell adhesions (Bos, 2005; Bos et al., 2003). Besides a role in these processes, many effectors of Rap1 are also implicated in the regulation of the actin cytoskeleton (Raaijmakers and Bos, 2008). Rap1 is activated by several extracellular signals and second messengers, one of them being cAMP. cAMP directly activates the Rap1GEF Epac (exchange protein directly activated by cAMP), leading to Rap1 activation (de Rooij et al., 1998). As cAMP is a widely used second messenger that also exerts many of its biological functions through activation of protein kinase A (PKA), an Epac-specific cAMP analogue was developed (Enserink et al., 2002). This analogue (8-CPT-2'OMe-cAMP, also termed 007) is now widely used to discriminate between the effects of cAMP on PKA and Epac.

Regulation of junctions

Intercellular adherens junctions are formed by the homotypic Ca^{2+}-dependent interaction of cadherins. In epithelial cells, the dominant cadherin at the cell junctions is E-cadherin. Intracellularly, cadherins are linked to the actin cytoskeleton through α- and β-catenin. The role of Rap1 in the regulation of adherens junctions was first discovered using the model organism *Drosophila melanogaster* (Knox and Brown, 2002). Loss of Rap1 resulted in an impairment of the even distribution of adherens junctions in *Drosophila* wings. Also in mammalian cells, Rap1 is required for the formation of E-cadherin-mediated cell junctions (Price et al., 2004).

The GEFs Dock4, C3G and PDZGEF2 have all been implicated in junction regulation upstream of Rap1 (Dube et al., 2008; Hogan et al., 2004; Yajnik et al., 2003). In endothelial cells, the main cadherin molecule involved is VE-cadherin. It is well established that junction formation is stimulated through the second messenger cAMP. Elevation of cAMP levels strengthens the junctions and reduces permeability of endothelium. Indeed, activation of Rap1 through Epac leads to the tightening of endothelial junctions (Cullere et al., 2005; Kooistra et al., 2005).

Regulation of integrins

A breakthrough in the understanding of Rap1 function came with the finding that Rap1 is a potent mediator of inside-out signaling to integrins. Over-expression of active Rap1 or activation of endogenous Rap1 is able to activate integrins whereas inhibition of Rap1 signaling inhibits integrin activation (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000). When integrin-mediated cell adhesion is induced by Mn^{2+} or by an integrin-activating antibody, this still requires Rap1 activation, suggesting that Rap1 signaling is required for the entire adhesion process (de Bruyn et al., 2002). Interestingly, Rap1 is able to regulate all integrins connected to the actin cytoskeleton (integrins of the β1, β2 and β3 family), but not those connected to intermediate filaments, such as integrin α6β4 (Enserink et al., 2004). Depending on the integrin and the cell type, Rap1 can regulate integrins both by affecting the binding of the integrin and its ligand (affinity) or by regulating the clustering of integrins on the cell surface (Bos, 2005; Kinbara et al., 2003). Although the mechanisms of how Rap1 induces adhesion in different cell lines remain elusive, several effectors have been identified to mediate Rap1 signaling. Several of these effector proteins have been proposed to function by activating integrins.

Downstream effectors regulating adhesion

A number of effectors have been implicated in the control of integrins in hematopoietic cells. For instance in T-cells, Riam, RAPL and PKD, can contribute to the formation of an 'integrin activation complex' consisting of Rap1, one or more of these effectors, and perhaps further adaptor proteins, required to mediate integrin activation. This complex then translocates to the integrin upon Rap activation to induce cell adhesion (Menasche et al., 2007a). The Rap effector RAPL associates with the integrin αLβ2 (LFA1) in the presence of active Rap1 and stimulates lymphocyte adhesion to ICAM via increased affinity and clustering (Katagiri et al., 2003). However, RAPL expression is restricted to the hematopoietic cells, while Rap1 can induce cell adhesion in many other cell types.

In CHO cells reconstituted with the platelet integrin αIIbβ3 and talin, Riam, another effector of Rap1, was shown to regulate adhesion via integrin αIIbβ3. Here, the integrin-activation complex consists of active

Rap1, Riam and talin. Recruitment of talin to the integrin then leads to integrin activation and an increase in affinity (Han et al., 2006; Watanabe et al., 2008). In T-cells, Riam can regulate adhesion through integrins β1 and β2 via a complex containing the adaptor proteins ADAP (adhesion and degranulation-promoting adapter protein) and SKAP-55 (55-kDa Src kinase-associated phosphoprotein) (Menasche et al., 2007b).

Another effector in T-cells is the kinase PKD1. Again, upon Rap1 activation, a complex is assembled that translocates to the integrin to activate it. In the case of PKD1, integrin activation induced by Rap1 does not require its kinase activity, indicating a kinase-independent function for PKD in cell adhesion (Medeiros et al., 2005). These data have shed more light on the role of Rap1 in integrin-mediated adhesion in lymphocytes. However, it is not yet clear if these Rap1 effectors have identical functions in other cell types.

Rap in cell migration

Besides its role in cell adhesion, Rap1 has also been implicated in the regulation of cell polarity and migration. As has become clear in this chapter, adhesion, polarity and migration are all interlinked processes.

A role for Rap1 in regulating cell polarity was first identified in yeast. The Rap homologue in *S. cerevisiae*, Bud1p, determines the location of the bud site where the daughter cell will arise. Loss of Bud1 in yeast leads to randomized bud site localization (Chant and Herskowitz, 1991). A similar mechanism was found in neuronal cells. Here, Rap1B becomes restricted to the tip of one neurite. This event marks this neurite to become the future axon. Lack of Rap1 results in a loss of polarity and in the absence of an axon, whereas over-expression results in multiple axons per neuronal cell (Schwamborn and Puschel, 2004). In this context, Rap1 functions upstream of the Par polarity complex. Similarly, in lymphocytes the activation of Rap1 is required upstream of the Par complex for chemokine-induced polarization and chemotaxis (Gerard et al., 2007; Shimonaka et al., 2003).

In the unicellular amoeba *Dictyostelium discoideum*, Rap1 controls both polarity and migration. In these cells, spatial and temporal regulation of Rap1 is required for cell adhesion and the assembly of myosin during cell migration. Activated Rap1 is found at the leading edge of cells migrating in response to a chemotactic cue. Both cells expressing constitutively active Rap1 as well as cells over-expressing or lacking RapGAP1 exhibit defects in chemotaxis (Jeon et al., 2007a; Jeon et al., 2007b). Evidence of a role for Rap1 in migration in a multicellular organism was first described in *Drosophila*. In flies, Rap1 is an essential gene. Without Rap1, various cell types fail to migrate to their proper location, leading to severe defects in morphogenesis (Asha et al., 1999).

A role for Rap1 in migration in mammalian cells has mainly focused

on the process of chemotaxis using lymphocytes and vascular endothelial cells. When a chemotactic signal is sensed, circulating lymphocytes rapidly adhere to the endothelium. They next migrate across the endothelium to extravasate toward the inflamed region. Rap1 mediates all three steps, chemotaxis, adhesion and polarization, required for efficient lymphocyte function (Lorenowicz et al., 2006; Shimonaka et al., 2003; Tohyama et al., 2003). The requirement for Rap in chemotaxis was first shown in B cells, where the chemo-attractant SDF-1 resulted in activation of both Rap1 and Rap2 (McLeod et al., 2004). This activation was required for migration towards SDF-1 as well as for spontaneous migration of B cells. Others have shown that the activity of both Rap1 and RAPL is required for this polarization (Katagiri et al., 2004). Rap1 also affects endothelial cell migration. It localizes to the edge of migrating endothelial cells and regulates endothelial wound closure (Fujita et al., 2005). Also Epac was shown to accelerate endothelial cell migration. This then induces the formation of new cell-cell contacts to restore barrier function following endothelial damage (Lorenowicz et al., 2008). Endothelial cell migration is also crucial during angiogenesis. Angiogenesis is the main mechanism of vascular remodeling during development and wound healing. In mice, both Rap1A and Rap1B were shown to be required for angiogenesis and proangiogenic signaling in endothelial cells (Chrzanowska-Wodnicka et al., 2008; Yan et al., 2008). As in endothelial cell migration (Fujita et al., 2005), RAPL was proposed as the effector mediating Rap1 functions in angiogenesis (Carmona et al., 2009). Others have shown a role for the putative Rap1 effector Bmx in endothelial cell migration (Stoletov and Terman, 2004). In fibroblasts, most evidence points to a role for the GEF C3G in regulating adhesion and migration. Mouse embryonic fibroblasts (MEFs) lacking C3G are impaired in their adhesion and spreading and have an increased cell migration velocity. Over-expression of active Rap1 or another GEF can rescue this effect (Ohba et al., 2001). As knocking out C3G in the embryo is lethal, Voss et al. made mice with a hypomorphic C3G allele and found effects on cell migration in these MEFs as well. These cells display defects in adhesion and lack paxillin- and integrin β1-positive FAs. They also have an increased migration activity as measured in a wound healing-assay (Voss et al., 2003). It was later described that mouse embryos lacking C3G have defects in the migration of cortical neurons, leading to multipolar neurons (Voss et al., 2008). A role for C3G, as well as Rap1, in the regulation of cell migration of epithelial cells was described for the NBT-II carcinoma cell line. In these cells, over-expression of RapV12 down-regulated the complex formed by Crk, DOCK180 and paxillin, leading to a reduction in Rac1GTP levels resulting in an inhibition of cell migration velocity (Valles et al., 2004).

Outline of this thesis

As indicated above, adhesion, polarity and migration are all Rap-regulated processes that are intimately involved and interconnected in the regulation of cell migration. Therefore, we set out to investigate the role of Rap1 in growth factor-induced cell migration.

Chapter 2 describes how Rap1 and its close relative Ras are regulated and which effector proteins have been identified that mediate their downstream effects. The finding that activation of Rap1 can inhibit growth factor-induced cell migration is described in Chapter 3. We show that Rap1 inhibits cell migration in the absence of cell-cell junctions and that this inhibition correlates with a decrease in focal adhesion and protrusion dynamics. As an addendum to Chapter 3, we performed a gene expression profiling experiment on cells subject to this Rap1-induced migration block. We found no clear effects of 007 in gene expression that could explain long term Rap1 effects. Next, we sought to identify the mechanism responsible for the effect of Rap1 on focal adhesion dynamics. In Chapter 4, we investigated the effect of 007 on several regulators of FA dynamics and showed that these pathways were not affected by 007 stimulation. In Chapter 5, we used an siRNA approach to determine the molecular mechanism of Rap1-mediated regulation of focal adhesions. We used a small customized siRNA library to identify proteins involved in the effect of Rap1 activation of cell spreading and FA formation. In this screen, we show that these effects are mediated by Rap1A and identify a number of proteins that partially modulate the Rap1 effect. Chapter 6 describes the complex formation of the Rap1 effector Arap3 with the lipid phosphatase SHIP2, which is dependent on a direct interaction between the SAM domains of both proteins. Finally, the possible implications of the findings described in this thesis are discussed in Chapter 7.

References

Asha, H., de Ruiter, N.D., Wang, M.G. and Hariharan, I.K. (1999) The Rap1 GTPase functions as a regulator of morphogenesis in vivo. *Embo J*, **18**, 605-615.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J. and Garcia De Herreros, A. (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol*, **2**, 84-89.

Bhowmick, N.A., Zent, R., Ghiassi, M., McDonnell, M. and Moses, H.L. (2001) Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. *J Biol Chem*, **276**, 46707-46713.

Bierie, B. and Moses, H.L. (2006) TGF-beta and cancer. *Cytokine Growth Factor Rev*, **17**, 29-40.

Birchmeier, C., Birchmeier, W., Gherardi, E. and Vande Woude, G.F. (2003) Met, metastasis, motility and more. *Nat Rev Mol Cell Biol*, **4**, 915-925.

Bos, J.L. (2005) Linking Rap to cell adhesion. *Curr Opin Cell Biol*, **17**, 123-128.

Bos, J.L., de Bruyn, K., Enserink, J., Kuiperij, B., Rangarajan, S., Rehmann, H., Riedl, J., de Rooij, J., van Mansfeld, F. and Zwartkruis, F. (2003) The role of Rap1 in integrin-mediated cell adhesion. *Biochem Soc Trans*, **31**, 83-86.

Bos, J.L., de Rooij, J. and Reedquist, K.A. (2001) Rap1 signalling: adhering to new models. *Nat Rev Mol Cell Biol*, **2**, 369-377.

Boyer, B., Roche, S., Denoyelle, M. and Thiery, J.P. (1997) Src and Ras are involved in separate pathways in epithelial cell scattering. *Embo J*, **16**, 5904-5913.

Broussard, J.A., Webb, D.J. and Kaverina, I. (2008) Asymmetric focal adhesion disassembly in motile cells. *Curr Opin Cell Biol*, **20**, 85-90.

Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol*, **12**, 463-518.

Carmona, G., Gottig, S., Orlandi, A., Scheele, J., Bauerle, T., Jugold, M., Kiessling, F., Henschler, R., Zeiher, A.M., Dimmeler, S. and Chavakis, E. (2009) Role of the small GTPase Rap1 for integrin activity regulation in endothelial cells and angiogenesis. *Blood*, **113**, 488-497.

Caron, E., Self, A.J. and Hall, A. (2000) The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators. *Curr Biol*, **10**, 974-978.

Carragher, N.O., Westhoff, M.A., Fincham, V.J., Schaller, M.D. and Frame, M.C. (2003) A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. *Curr Biol*, **13**, 1442-1450.

Carragher, N.O., Westhoff, M.A., Riley, D., Potter, D.A., Dutt, P., Elce, J.S., Greer, P.A. and Frame, M.C. (2002) v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. *Mol Cell Biol*, **22**, 257-269.

Chant, J. and Herskowitz, I. (1991) Genetic control of bud site selection in yeast by a set of gene products that constitute a morphogenetic pathway. *Cell*, **65**, 1203-1212.

Chrzanowska-Wodnicka, M. and Burridge, K. (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol*, **133**, 1403-1415.

Chrzanowska-Wodnicka, M., Kraus, A.E., Gale, D., White, G.C., 2nd and Vansluys, J. (2008) Defective angiogenesis, endothelial migration, proliferation, and MAPK signaling in Rap1b-deficient mice. *Blood*, **111**, 2647-2656.

Critchley, D.R. (2000) Focal adhesions - the cytoskeletal connection. *Curr Opin Cell Biol*, **12**, 133-139.

Cullere, X., Shaw, S.K., Andersson, L., Hirahashi, J., Luscinskas, F.W. and Mayadas, T.N. (2005) Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. *Blood*, **105**, 1950-1955.

Danen, E.H. and Sonnenberg, A. (2003) Integrins in regulation of tissue development and function. *J Pathol*, **201**, 632-641.

de Bruyn, K.M., Rangarajan, S., Reedquist, K.A., Figdor, C.G. and Bos, J.L. (2002) The small GTPase Rap1 is required for Mn(2+)- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion. *J Biol Chem*, **277**, 29468-29476.

de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M.A. and Waterman-Storer, C.M. (2005) Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol*, **171**, 153-164.

de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A. and Bos, J.L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*, **396**, 474-477.

Dourdin, N., Bhatt, A.K., Dutt, P., Greer, P.A., Arthur, J.S., Elce, J.S. and Huttenlocher, A. (2001) Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J Biol Chem*, **276**, 48382-48388.

Dube, N., Kooistra, M.R., Pannekoek, W.J., Vliem, M.J., Oorschot, V., Klumperman, J., Rehmann, H. and Bos, J.L. (2008) The RapGEF PDZ-GEF2 is required for maturation of cell-cell junctions. *Cell Signal*, **20**, 1608-1615.

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Doskeland, S.O., Blank, J.L. and Bos, J.L. (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol*, **4**, 901-906.

Enserink, J.M., Price, L.S., Methi, T., Mahic, M., Sonnenberg, A., Bos, J.L. and Tasken, K. (2004) The cAMP-Epac-Rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the alpha3beta1

integrin but not the alpha6beta4 integrin. *J Biol Chem*, **279**, 44889-44896.

Evers, E.E., Zondag, G.C., Malliri, A., Price, L.S., ten Klooster, J.P., van der Kammen, R.A. and Collard, J.G. (2000) Rho family proteins in cell adhesion and cell migration. *Eur J Cancer*, **36**, 1269-1274.

Ezratty, E.J., Partridge, M.A. and Gundersen, G.G. (2005) Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol*, **7**, 581-590.

Franco, S.J. and Huttenlocher, A. (2005) Regulating cell migration: calpains make the cut. *J Cell Sci*, **118**, 3829-3838.

Franco, S.J., Rodgers, M.A., Perrin, B.J., Han, J., Bennin, D.A., Critchley, D.R. and Huttenlocher, A. (2004) Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol*, **6**, 977-983.

Fujita, H., Fukuhara, S., Sakurai, A., Yamagishi, A., Kamioka, Y., Nakaoka, Y., Masuda, M. and Mochizuki, N. (2005) Local activation of Rap1 contributes to directional vascular endothelial cell migration accompanied by extension of microtubules on which RAPL, a Rap1-associating molecule, localizes. *J Biol Chem*, **280**, 5022-5031.

Geiger, B. and Bershadsky, A. (2001) Assembly and mechanosensory function of focal contacts. *Curr Opin Cell Biol*, **13**, 584-592.

Geiger, B., Bershadsky, A., Pankov, R. and Yamada, K.M. (2001) Transmembrane extracellular matrix-cytoskeleton crosstalk. *Nat Rev Mol Cell Biol*, **2**, 793-805.

Gerard, A., Mertens, A.E., van der Kammen, R.A. and Collard, J.G. (2007) The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. *J Cell Biol*, **176**, 863-875.

Ginsberg, M.H., Partridge, A. and Shattil, S.J. (2005) Integrin regulation. *Curr Opin Cell Biol*, **17**, 509-516.

Glading, A., Chang, P., Lauffenburger, D.A. and Wells, A. (2000) Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem*, **275**, 2390-2398.

Gupton, S.L. and Waterman-Storer, C.M. (2006) Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell*, **125**, 1361-1374.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science*, **279**, 509-514.

Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J. and Ginsberg, M.H. (2006) Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol*, **16**, 1796-1806.

Hogan, C., Serpente, N., Cogram, P., Hosking, C.R., Bialucha, C.U., Feller, S.M., Braga, V.M., Birchmeier, W. and Fujita, Y. (2004) Rap1 regulates the formation of E-cadherin-based cell-cell contacts. *Mol. Cell. Biol.*, **24**, 6690-6700.

Huttenlocher, A., Ginsberg, M.H. and Horwitz, A.F. (1996) Modulation of cell migration by integrin-mediated cytoskeletal linkages and ligand-binding affinity. *J Cell Biol*, **134**, 1551-1562.

Hynes, R.O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell*, **110**, 673-687.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, **377**, 539-544.

Jeon, T.J., Lee, D.J., Lee, S., Weeks, G. and Firtel, R.A. (2007a) Regulation of Rap1 activity by RapGAP1 controls cell adhesion at the front of chemotaxing cells. *J Cell Biol*, **179**, 833-843.

Jeon, T.J., Lee, D.J., Merlot, S., Weeks, G. and Firtel, R.A. (2007b) Rap1 controls cell adhesion and cell motility through the regulation of myosin II. *J Cell Biol*, **176**, 1021-1033.

Kasai, H., Allen, J.T., Mason, R.M., Kamimura, T. and Zhang, Z. (2005) TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res*, **6**, 56.

Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatsu, K. and Kinashi, T. (2000) Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from

protein kinase C and phosphatidylinositol-3-OH kinase. *Mol Cell Biol*, **20**, 1956-1969.

Katagiri, K., Maeda, A., Shimonaka, M. and Kinashi, T. (2003) RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat Immunol*, **4**, 741-748.

Katagiri, K., Ohnishi, N., Kabashima, K., Iyoda, T., Takeda, N., Shinkai, Y., Inaba, K. and Kinashi, T. (2004) Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. *Nat Immunol*, **5**, 1045-1051.

Kaverina, I., Krylyshkina, O. and Small, J.V. (1999) Microtubule targeting of substrate contacts promotes their relaxation and dissociation. *J Cell Biol*, **146**, 1033-1044.

Kim, M., Carman, C.V. and Springer, T.A. (2003) Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science*, **301**, 1720-1725.

Kinbara, K., Goldfinger, L.E., Hansen, M., Chou, F.L. and Ginsberg, M.H. (2003) Ras GTPases: integrins' friends or foes? *Nat Rev Mol Cell Biol*, **4**, 767-776.

Kirchner, J., Kam, Z., Tzur, G., Bershadsky, A.D. and Geiger, B. (2003) Live-cell monitoring of tyrosine phosphorylation in focal adhesions following microtubule disruption. *J Cell Sci*, **116**, 975-986.

Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. and Noda, M. (1989) A ras-related gene with transformation suppressor activity. *Cell*, **56**, 77-84.

Klinghoffer, R.A., Sachsenmaier, C., Cooper, J.A. and Soriano, P. (1999) Src family kinases are required for integrin but not PDGFR signal transduction. *Embo J*, **18**, 2459-2471.

Knox, A.L. and Brown, N.H. (2002) Rap1 GTPase regulation of adherens junction positioning and cell adhesion. *Science*, **295**, 1285-1288.

Kooistra, M.R., Corada, M., Dejana, E. and Bos, J.L. (2005) Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. *FEBS Lett*, **579**, 4966-4972.

Larjava, H., Plow, E.F. and Wu, C. (2008) Kindlins: essential regulators of integrin signalling and cell-matrix adhesion. *EMBO Rep*, **9**, 1203-1208.

Lauffenburger, D.A. and Horwitz, A.F. (1996) Cell migration: a physically integrated molecular process. *Cell*, **84**, 359-369.

Lorenowicz, M.J., Fernandez-Borja, M., Kooistra, M.R., Bos, J.L. and Hordijk, P.L. (2008) PKA and Epac1 regulate endothelial integrity and migration through parallel and independent pathways. *Eur J Cell Biol*.

Lorenowicz, M.J., van Gils, J., de Boer, M., Hordijk, P.L. and Fernandez-Borja, M. (2006) Epac1-Rap1 signaling regulates monocyte adhesion and chemotaxis. *J Leukoc Biol*, **80**, 1542-1552.

Massague, J., Blain, S.W. and Lo, R.S. (2000) TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell*, **103**, 295-309.

Mattila, P.K. and Lappalainen, P. (2008) Filopodia: molecular architecture and cellular functions. *Nat Rev Mol Cell Biol*, **9**, 446-454.

McLeod, S.J., Shum, A.J., Lee, R.L., Takei, F. and Gold, M.R. (2004) The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *J Biol Chem*, **279**, 12009-12019.

Medeiros, R.B., Dickey, D.M., Chung, H., Quale, A.C., Nagarajan, L.R., Billadeau, D.D. and Shimizu, Y. (2005) Protein kinase D1 and the beta 1 integrin cytoplasmic domain control beta 1 integrin function via regulation of Rap1 activation. *Immunity*, **23**, 213-226.

Menasche, G., Kliche, S., Bezman, N. and Schraven, B. (2007a) Regulation of T-cell antigen receptor-mediated inside-out signaling by cytosolic adapter proteins and Rap1 effector molecules. *Immunol Rev*, **218**, 82-91.

Menasche, G., Kliche, S., Chen, E.J., Stradal, T.E., Schraven, B. and Koretzky, G. (2007b) RIAM links the ADAP/SKAP-55 signaling module to Rap1, facilitating T-cell-receptor-mediated integrin activation. *Mol Cell Biol*, **27**, 4070-4081.

Miki, H., Suetsugu, S. and Takenawa, T. (1998) WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *Embo J*, **17**, 6932-6941.

Mitra, S.K., Hanson, D.A. and Schlaepfer, D.D. (2005) Focal adhesion kinase: in command

and control of cell motility. *Nat Rev Mol Cell Biol*, **6**, 56-68.

Moreno-Bueno, G., Portillo, F. and Cano, A. (2008) Transcriptional regulation of cell polarity in EMT and cancer. *Oncogene*, **27**, 6958-6969.

Moustakas, A. and Heldin, C.H. (2005) Non-Smad TGF-beta signals. *J Cell Sci*, **118**, 3573-3584.

Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B.J., Maki, K., Miyazaki, J. and Matsuda, M. (2001) Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. *Embo J*, **20**, 3333-3341.

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.R., Zhang, Y. and Wrana, J.L. (2005) Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science*, **307**, 1603-1609.

Palecek, S.P., Loftus, J.C., Ginsberg, M.H., Lauffenburger, D.A. and Horwitz, A.F. (1997) Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature*, **385**, 537-540.

Parsons, J.T., Martin, K.H., Slack, J.K., Taylor, J.M. and Weed, S.A. (2000) Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene*, **19**, 5606-5613.

Pollard, T.D. and Borisy, G.G. (2003) Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, **112**, 453-465.

Price, L.S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F.J., Collard, J.G. and Bos, J.L. (2004) Rap1 regulates E-cadherin-mediated cell-cell adhesion. *J. Biol. Chem.*, **279**, 35127-35132.

Raaijmakers, J.H. and Bos, J.L. (2008) Specificity in Ras and Rap signaling. *J Biol Chem*.

Reedquist, K.A., Ross, E., Koop, E.A., Wolthuis, R.M., Zwartkruis, F.J., van Kooyk, Y., Salmon, M., Buckley, C.D. and Bos, J.L. (2000) The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *J Cell Biol*, **148**, 1151-1158.

Ren, X.D., Kiosses, W.B., Sieg, D.J., Otey, C.A., Schlaepfer, D.D. and Schwartz, M.A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci*, **113 (Pt 20)**, 3673-3678.

Ridley, A.J. (2001) Rho GTPases and cell migration. *J Cell Sci*, **114**, 2713-2722.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T. and Horwitz, A.R. (2003) Cell migration: integrating signals from front to back. *Science*, **302**, 1704-1709.

Riento, K. and Ridley, A.J. (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol*, **4**, 446-456.

Roberts, A.B. and Sporn, M.B. (1993) Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors*, **8**, 1-9.

Roberts, A.B. and Wakefield, L.M. (2003) The two faces of transforming growth factor beta in carcinogenesis. *Proc Natl Acad Sci U S A*, **100**, 8621-8623.

Rosario, M. and Birchmeier, W. (2003) How to make tubes: signaling by the Met receptor tyrosine kinase. *Trends Cell Biol*, **13**, 328-335.

Schaller, M.D. (2001) Paxillin: a focal adhesion-associated adaptor protein. *Oncogene*, **20**, 6459-6472.

Schlaepfer, D.D. and Mitra, S.K. (2004) Multiple connections link FAK to cell motility and invasion. *Curr Opin Genet Dev*, **14**, 92-101.

Schlaepfer, D.D., Mitra, S.K. and Ilic, D. (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim Biophys Acta*, **1692**, 77-102.

Schober, M., Raghavan, S., Nikolova, M., Polak, L., Pasolli, H.A., Beggs, H.E., Reichardt, L.F. and Fuchs, E. (2007) Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J Cell Biol*, **176**, 667-680.

Schwamborn, J.C. and Puschel, A.W. (2004) The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.*, **7**, 923-929.

Schwartz, M.A. and Horwitz, A.R. (2006) Integrating adhesion, protrusion, and contraction during cell migration. *Cell*, **125**, 1223-1225.

Schwartz, M.A. and Shattil, S.J. (2000) Signaling networks linking integrins and rho family

GTPases. *Trends Biochem Sci*, **25**, 388-391.

Shi, Y. and Massague, J. (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, **113**, 685-700.

Shimonaka, M., Katagiri, K., Nakayama, T., Fujita, N., Tsuruo, T., Yoshie, O. and Kinashi, T. (2003) Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow. *J Cell Biol*, **161**, 417-427.

Siegel, P.M. and Massague, J. (2003) Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer*, **3**, 807-821.

Stoletov, K.V. and Terman, B.I. (2004) Bmx is a downstream Rap1 effector in VEGF-induced endothelial cell activation. *Biochem Biophys Res Commun*, **320**, 70-75.

Tachibana, K., Sato, T., D'Avirro, N. and Morimoto, C. (1995) Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK. *J Exp Med*, **182**, 1089-1099.

Thiery, J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*, **2**, 442-454.

Thiery, J.P. and Sleeman, J.P. (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol*, **7**, 131-142.

Tohyama, Y., Katagiri, K., Pardi, R., Lu, C., Springer, T.A. and Kinashi, T. (2003) The critical cytoplasmic regions of the alphaL/beta2 integrin in Rap1-induced adhesion and migration. *Mol Biol Cell*, **14**, 2570-2582.

Turner, C.E. (2000) Paxillin and focal adhesion signalling. *Nat Cell Biol*, **2**, E231-236.

Valles, A.M., Beuvin, M. and Boyer, B. (2004) Activation of Rac1 by paxillin-Crk-DOCK180 signaling complex is antagonized by Rap1 in migrating NBT-II cells. *J Biol Chem*, **279**, 44490-44496.

Valles, A.M., Boyer, B., Tarone, G. and Thiery, J.P. (1996) Alpha 2 beta 1 integrin is required for the collagen and FGF-1 induced cell dispersion in a rat bladder carcinoma cell line. *Cell Adhes Commun*, **4**, 187-199.

van Kooyk, Y. and Figdor, C.G. (2000) Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr Opin Cell Biol*, **12**, 542-547.

Vicente-Manzanares, M., Webb, D.J. and Horwitz, A.R. (2005) Cell migration at a glance. *J Cell Sci*, **118**, 4917-4919.

Voss, A.K., Britto, J.M., Dixon, M.P., Sheikh, B.N., Collin, C., Tan, S.S. and Thomas, T. (2008) C3G regulates cortical neuron migration, preplate splitting and radial glial cell attachment. *Development*, **135**, 2139-2149.

Voss, A.K., Gruss, P. and Thomas, T. (2003) The guanine nucleotide exchange factor C3G is necessary for the formation of focal adhesions and vascular maturation. *Development*, **130**, 355-367.

Watanabe, N., Bodin, L., Pandey, M., Krause, M., Coughlin, S., Boussiotis, V.A., Ginsberg, M.H. and Shattil, S.J. (2008) Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin alphaIIbbeta3. *J Cell Biol*, **181**, 1211-1222.

Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T. and Horwitz, A.F. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol*, **6**, 154-161.

Webb, D.J., Parsons, J.T. and Horwitz, A.F. (2002) Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat Cell Biol*, **4**, E97-100.

Wegener, K.L., Partridge, A.W., Han, J., Pickford, A.R., Liddington, R.C., Ginsberg, M.H. and Campbell, I.D. (2007) Structural basis of integrin activation by talin. *Cell*, **128**, 171-182.

Wehrle-Haller, B. and Imhof, B.A. (2003) Actin, microtubules and focal adhesion dynamics during cell migration. *Int J Biochem Cell Biol*, **35**, 39-50.

Weidner, K.M., Sachs, M. and Birchmeier, W. (1993) The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J Cell Biol*, **121**, 145-154.

Yajnik, V., Paulding, C., Sordella, R., McClatchey, A.I., Saito, M., Wahrer, D.C., Reynolds, P., Bell, D.W., Lake, R., van den Heuvel,

S., Settleman, J. and Haber, D.A. (2003) DOCK4, a GTPase activator, is disrupted during tumorigenesis. *Cell*, **112**, 673-684.

Yan, J., Li, F., Ingram, D.A. and Quilliam, L.A. (2008) Rap1a is a key regulator of fibroblast growth factor 2-induced angiogenesis and together with Rap1b controls human endothelial cell functions. *Mol Cell Biol*, **28**, 5803-5810.

Yang, J. and Weinberg, R.A. (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell*, **14**, 818-829.

Yang, Y.C., Piek, E., Zavadil, J., Liang, D., Xie, D., Heyer, J., Pavlidis, P., Kucherlapati, R., Roberts, A.B. and Bottinger, E.P. (2003) Hierarchical model of gene regulation by transforming growth factor beta. *Proc Natl Acad Sci U S A*, **100**, 10269-10274.

Zaidel-Bar, R., Ballestrem, C., Kam, Z. and Geiger, B. (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci*, **116**, 4605-4613.

Zaidel-Bar, R., Cohen, M., Addadi, L. and Geiger, B. (2004) Hierarchical assembly of cell-matrix adhesion complexes. *Biochem Soc Trans*, **32**, 416-420.

Zaidel-Bar, R., Milo, R., Kam, Z. and Geiger, B. (2007) A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *J Cell Sci*, **120**, 137-148.

Zamir, E. and Geiger, B. (2001) Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci*, **114**, 3583-3590.

Zavadil, J. and Bottinger, E.P. (2005) TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene*, **24**, 5764-5774.

chapter 2

Specificity in Ras and Rap signaling

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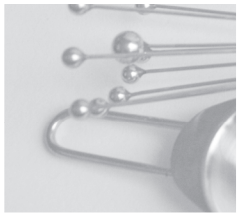
Journal of Biological Chemistry (in press)



Specificity in Ras and Rap signaling

Judith H. Raaijmakers and Johannes L. Bos

Department of Physiogical Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands

Abstract

Ras and Rap proteins are closely related small GTPases. Whereas Ras is known for its role in cell proliferation and survival, Rap1 is predominantly involved in cell adhesion and cell junction formation. Ras and Rap are regulated by different sets of guanine nucleotide exchange factors and GTPase activating proteins, determining one level of specificity. In addition, although the effector domains are highly similar, Rap and Ras interact with largely different sets of effectors, providing a second level of specificity. In this review we discuss the regulatory proteins and effectors of Ras and Rap with a focus on those of Rap.

Introduction

Ras-like small G-proteins are ubiquitously expressed, conserved molecular switches that couple extracellular signals to various cellular responses. Different signals can activate guanine nucleotide exchange factors (GEFs) that induce the small G-protein to switch from the inactive, GDP-bound state to the active, GTP-bound state. This induces a conformational change that allows downstream effector proteins to bind specifically to, and be activated by the GTP-bound protein, to mediate diverse biological responses. Small G-proteins are returned to the GDP-bound state by hydrolyzing GTP with the help of GTPase activating proteins (GAPs). Ras (H-Ras, K-Ras and N-Ras) and Rap proteins (Rap1A, 1B, 2A, 2B and 2C) have similar effector-binding regions that interact predominantly with Ras association (RA) domains or the structurally similar Ras binding domains (RBD), present in a variety of different proteins. Both protein families operate in different signaling networks. For instance, Ras is central in a network controlling cell proliferation and cell survival, whereas Rap1 predominantly controls cell adhesion, cell junction formation, secretion and cell polarity. These different functions are reflected in a largely different set of GEFs and GAPs. Also the downstream effector proteins operate in a selective manner in either one of the networks.

Guanine nucleotide exchange factors

GEFs for Ras and Rap proteins are usually multidomain proteins that contain a CDC25 homology domain mediating the exchange activity and a REM (Ras exchange motif) domain. GEFs for Ras include Sos1 and 2, RasGRF, RasGRP1 and RasGRP4. Rap can be activated by C3G, Epac1 and 2, RasGRP2, PDZ-GEF1 and 2 and PLCε (reviewed in (Bos et al., 2007)). Within the RasGRP family, RasGRP3 seems to be a more promiscuous GEF, affecting both Rap and Ras (Figure 1) (Yamashita et al., 2000). The general structural basis of nucleotide

exchange by CDC25 homology domains was revealed by the crystal structure of Sos in the presence of Ras (Boriack-Sjodin et al., 1998). When the catalytic helix of Sos is inserted into the guanine nucleotide-binding pocket of Ras, affinity for the bound nucleotide is decreased, resulting in its release. Because the concentration of GTP in a cell is higher than GDP, GTP will predominantly enter the empty nucleotide-binding pocket and, in its turn, displace the GEF. Recently, the crystal structure of Epac2 with Rap1 was determined, revealing a similar mechanism of nucleotide exchange (Rehmann et al., 2008). However, although the interfaces between Sos and Ras and Epac and Rap are both rather extensive, most residues at the interface are different in both GEFs. This shows that although the catalytic mechanism is conserved, the actual interactions are not, allowing the establishment of selectivity or, as in the case of RasGRP3, of promiscuity. The additional domains in the various GEFs are involved in regulating their activation or translocation. For instance, Sos activation involves translocation to tyrosine-phosphorylated proteins, release of auto-inhibition and allosteric regulation of catalytic activity by a distal Ras protein in a positive feedback loop (Margarit et al., 2003). Epac is activated by binding of cAMP, which induces a major conformational change to release its auto-inhibition (Rehmann et al., 2008). Thus, GEFs are well equipped to selectively regulate the activity of these small G-proteins in time and space in response to a large variety of different stimuli.

GTPase activating proteins

The hydrolysis of GTP in Ras and Rap1 is slow, but is accelerated several orders of magnitude by GAPs that insert an additional catalytic side chain into the nucleotide-binding pocket. GAPs acting on Ras include p120RasGAP, neurofibromin and GAP1. GTP hydrolysis on Rap is catalyzed by Rap1GAP and the Spa-1 family of GAPs (Spa-1, Spa-1-like and E6TP1) (Bos et al., 2007). The catalytic domains of Ras- and RapGAPs are structurally similar, yet the mode of stimulation of the GTPase reaction is different. RasGAPs use an arginine side chain as a catalytic group, whereas RapGAPs use an asparagine side chain. Catalysis by the arginine side chain involves a glutamine at position 61 of Ras. Indeed, many tumor mutations occur at position 61, to render Ras continuously in its active conformation. Rap proteins do not have a glutamine at position 61 and thus RapGAPs use a different mode of catalysis, which is provided by the asparagine side chain. There is also a group of GAPs that appears to have a dual specificity for both Ras and Rap, such as several GAP1 (RASAL, CAPRI, GAP^{IP4BP}) family members and synGAP (Kupzig et al., 2006; Pena et al., 2008). The isolated GAP domains of these GAPs have low activity towards Rap *in vitro,* but due to allosteric regulation by the additional C2 domain, they can function as RapGAPs *in vivo* (Pena et al., 2008). This indicates that there are ways around the selectivity of Ras-

and Rap-mediated GTP hydrolysis.

Specificity of Ras effectors

For Ras, the classic downstream effectors are the three Raf kinases (A-raf, B-raf and c-Raf), various PI 3-kinases and RalGDS members. Raf proteins mediate the Ras-induced activation of the ERK/MAPK cascade. They contain an RBD to which Ras binds with high affinity. Rap1 can also bind the Raf RBD *in vitro* (Rodriguez-Viciana et al., 2004) and was proposed to mediate cAMP-mediated effects on Raf kinases in a cell type-dependent manner (Stork and Schmitt, 2002). However, other studies could not reveal a direct connection between Rap1 and Raf kinase signaling (Bos et al., 2003) and thus this result remains controversial.

Another established Ras effector is phosphatidylinositol 3 kinase (PI3K). PI 3-kinases are heterodimeric proteins with a p110 catalytic and a p85 regulatory subunit. The p110 subunit has an RBD that binds to Ras. This interaction facilitates membrane translocation but in addition allosterically regulates the kinase activity (Pacold et al., 2000). Rap has also been suggested to regulate PI3K. For instance, Rap1 can modestly activate the p110α subunit of PI3K (Rodriguez-Viciana et al., 2004) and in B cells, Rap2 can bind PI3K, although in this system, this correlates with an inhibition of PI3K-mediated PKB activation (Christian et al., 2003).

RalGDS, Rgl and Rgl2 (Rlf) contain an RA domain and are GEFs for the small GTPase Ral, downstream of Ras. Their RA domains efficiently bind to both Ras and Rap1 *in vitro*. Interestingly, Rap1A has a higher affinity for the RalGDS RA domain than Ras and, indeed, also binds *in vivo* to RalGEFS (Rodriguez-Viciana et al., 2004). However, despite high affinity binding to Rap, evidence that endogenous Rap1 activation mediates RalGDS-Ral activation is lacking.

Effectors for Rap proteins

A large number of proteins have been identified as effectors of Rap proteins, particularly the adaptors RAPL, Riam, AF-6 and Krit1, the RacGEFs Tiam1 and Vav2 and the RhoGAPs RA-RhoGAP and Arap3.

AF-6/Afadin

AF-6 is an adaptor protein that localizes to cell-cell junctions and contains two RA domains. With its N-terminal RA domain, it binds both Ras and Rap1 with high affinity. Although initial studies showed that AF-6 may participate in Ras-induced junction breakdown (Yamamoto et al., 1997), others showed that AF-6 binds p120-catenin in a Rap-dependent manner to prevent internalization of E-cadherin (Hoshino et al., 2005). However, a longer isoform of AF-6 that regulates E-cadherin in a Rap-independent manner was recently described (Lorger and Moelling, 2006). Thus, a clear picture of the role of AF-6 as an effector of Ras and/ or Rap in junctions is still not present. In T-cells, AF-6 is a negative regulator of Rap-induced integrin-mediated cell adhesion (Zhang et al., 2005).

Krit1

Krev Interaction Trapped 1 (Krit1) is a Rap1-interacting protein that was originally identified in a yeast two-hybrid screen (Serebriiskii et al., 1997). This protein, also called CCM1, is found mutated in cerebral cavernous malformation, a disease associated with defects in brain vasculature. Krit1 has several ankyrin repeats and a FERM domain. This domain has a ubiquitin-like fold that is similar to RA domains and RBDs and can bind both Ras and Rap, although the affinity for Rap is higher than for Ras (Wohlgemuth et al., 2005). In agreement with this, recent evidence implicates Krit1 as a major effector of Rap1 in the control of endothelial cell-cell junctions (Glading et al., 2007). In these cells, Krit1 localizes to the junctions, where it associates with junctional proteins and is required for the Rap1-induced reduction in basal and cytokine-induced permeability of the junctions. Krit1 also associates with microtubules, two other CCM proteins (CCM2 and 3), and ICAP1 (a protein that binds to and negatively regulates the β1 chain of integrins) (Beraud-Dufour et al., 2007). The role of these proteins in Rap1/Krit1-mediated regulation of junction permeability is currently unclear.

Riam

Riam contains an N-terminal coiled-coil region, a central RA and PH domain and a proline-rich C-terminal region, with multiple FPPPP motifs capable of interacting with the EVH1 domains of the actin regulatory proteins Ena and VASP. Riam interacts with active Rap1 to stimulate the adhesion of Jurkat T-cells through β1 and β2 integrins (Lafuente et al., 2004). Ginsberg and coworkers showed that Riam is involved in stimulus-induced, Rap1-mediated recruitment of talin (Han et al., 2006). Talin subsequently binds to and activates the β chain of integrin αIIbβ3. In T-cells, Riam was found in a complex with ADAP (adhesion- and degranulation-promoting adapter protein) and SKAP-55 (55-kDa Src kinase-associated phosphoprotein), and this complex is required for Rap1 recruitment to the plasma membrane and T-cell receptor-induced, integrin-mediated cell adhesion to fibronectin and ICAM (Menasche et al., 2007).

RAPL

RAPL (regulator of adhesion and cell polarity enriched in lymphoid tissues/ Nore1B/ Rassf5) is another regulator of Rap-induced, integrin-mediated adhesion in T-cells. *In vivo*, RAPL binds Rap1 after stimulation through the T-cell receptor or by chemokines. In the presence of active Rap1, RAPL binds to the α-chain of the αLβ2 integrin (LFA-1), resulting in its activation (Katagiri et al., 2003). Data from RAPL knock-out mice confirm its role in lymphocyte adhesion. Lymphocytes lacking RAPL are less adherent to ICAM and do not redistribute their integrins after cytokine stimulation. They are defective in cell migration and thus in homing to peripheral lymph nodes (Katagiri et al., 2004). Recently, the kinase Mst1 was identified as an effector of RAPL in T-cell adhesion. Activation of Rap1 promotes the binding of RAPL to Mst1

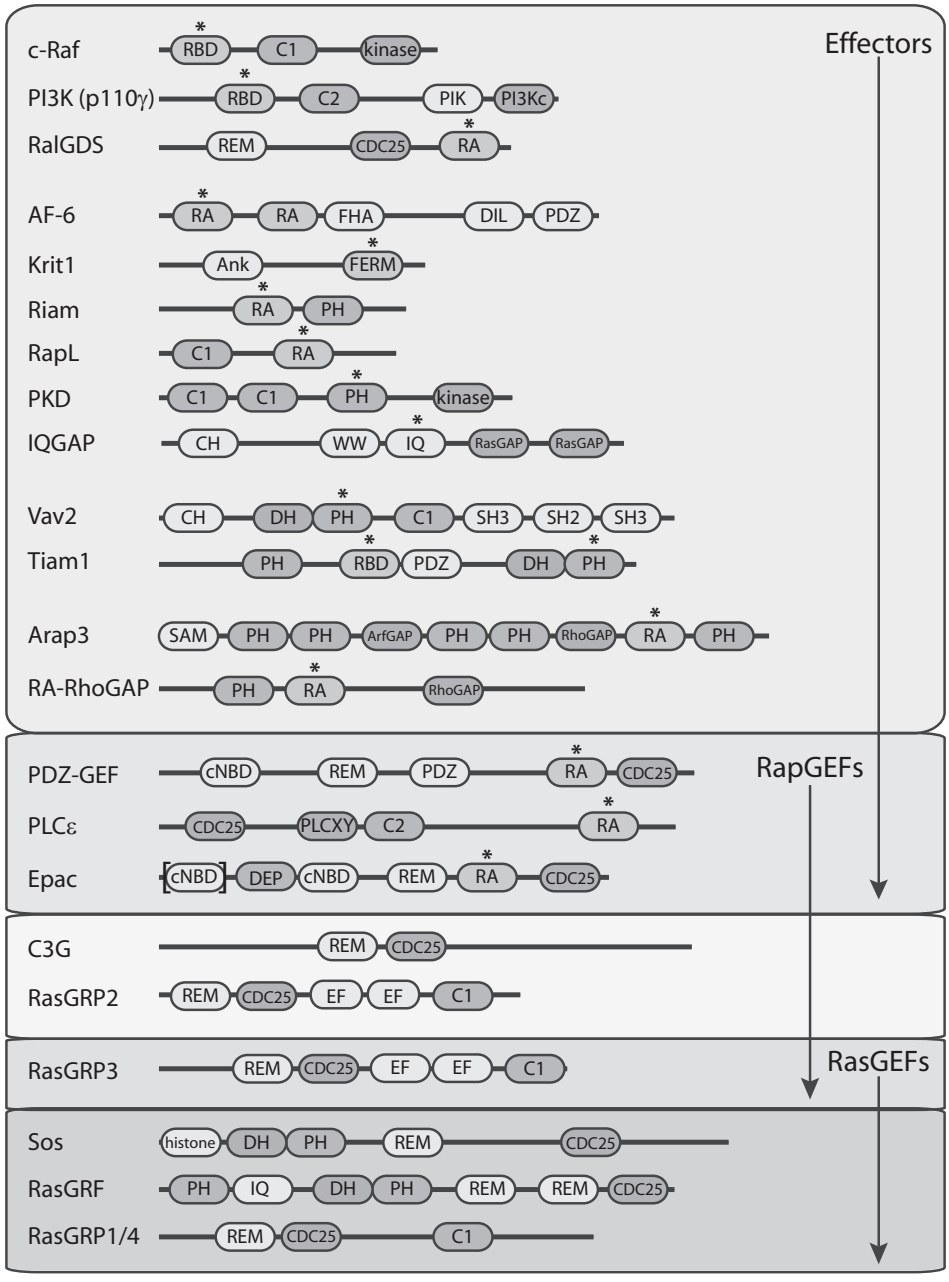


Figure 1. Ras and Rap effector proteins and GEFs

A schematic representation is shown of the domain structures of Ras- and RapGEFs and effector proteins discussed here. RA domains/RBDs are depicted in pink, catalytic domains in blue, lipid binding domains in green and other domains in yellow. Asterisks indicate domains required for Ras/Rap binding.

and their relocalization with LFA-1 to the leading edge to induce adhesion (Katagiri et al., 2006). Interestingly, whereas Rap1 and RAPL have been shown to regulate both LFA-1 affinity and clustering, Mst1 over-expression enhances only LFA-1 clustering. This suggests that LFA-1 clustering is critical for Rap1-induced T-cell adhesion and that there may also be Mst1-independent mechanisms by which Rap1 regulates LFA-1 affinity. Moreover, in a different T-cell line, RapL was found to interact with Rap2B, regulating random migration and not cell adhesion (Miertzschke et al., 2007).

PKD1

Also protein kinase D1 (PKD1) may regulate T-cell adhesion through Rap1. Rap1 binds to the PH domain of PKD1, and this interaction facilitates the activation of Rap1 as well as the recruitment of both proteins to the cytoplasmic tail of integrins in the immunological synapse (Medeiros et al., 2005). How the interaction with PKD1 induces Rap activation is currently unclear, but it does not depend on the kinase activity of PKD1, indicating that PKD1 functions as a scaffold here.

IQGAP1

IQGAP1 is a scaffold protein that interacts with actin and functions in a number of actin polymerization-driven processes. Recently, Rap1 was found to interact with IQGAP1 *in vitro* and to colocalize with IQGAP1 at the cell membrane (Jeong et al., 2007). The precise role for Rap1 in IQGAP1 function is currently unclear, as both GDP- and GTP-bound Rap1 proteins bind to IQGAP. Thus, the main function of both IQGAP1 and PKD1 appears to be the recruitment of Rap1 rather than being activated by GTP-bound Rap1 to induce biological effects downstream of Rap1.

Interconnectivity with Rac and Rho

In processes controlled by Rap1 signaling, such as junction formation and cell adhesion, Rho family proteins like Rac1, CDC42 and RhoA play a critical role. Rap proteins are directly linked to these proteins through the interaction with the RacGEFs Vav2 and Tiam1 and the RhoGAPs Arap3

Figure 1, continued

C1, protein kinase C conserved region 1; *C2*, Ca^{2+}-binding motif; *PIK*, PI3K accessory domain; *PI3Kc*, PI3K catalytic domain; *REM*, Ras exchange motif; *CDC25*, CDC25 homology; *FHA*, Forkhead-associated domain; *DIL*, dilute; *PDZ*, PSD-95, Dgl and ZO-1; *Ank*, ankyrin repeat; *FERM*, Band 4.1, Ezrin, Radixin, Moesin; *CH*, calponin homology; *PH*, pleckstrin homology; *DH*, Dbl homology; *SH2/3*, Src homology 2/3; *SAM*, sterile alpha motif; *cNBD*, cyclic nucleotide binding; *PLCXY*, phospholipase C catalytic regions X and Y; *DEP*, Dishevelled, Egl-10 and Pleckstrin; *EF*, Ef-hand; *histone*, histone domain.

Notes: 1) The interaction of Tiam1 with Ras has been described for the RBD; for Rap it was shown to bind to the DHPH domain. 2) The N-terminal cNBD is conserved in Epac2 alone and the RA domain-like sequence in Epac1 is not recognized as such by the SMART database (http://smart. embl-heidelberg. de). 3) Although described to be present in PLCε (Song et al., 2002; Wohlgemuth et al. 2005), a second RA domain in PLCε is not indicated in the SMART database. Color figure on page 155.

and RA-RhoGAP.

RacGEFs

In a search for proteins that could mediate Rap1-induced, Rac-dependent cell adhesion and spreading, Rap1 was found to interact with both Vav2 and Tiam1 (Arthur et al., 2004). Rap1 binds to their catalytic DH-PH domain but does not affect the catalytic activity of the two GEFs. Rather, Rap1 induces the translocation of Vav2 to localize Rac activity to sites of cell spreading. In T-cells, Rap1 binds to Tiam1 as well (Gerard et al., 2007). Here, Rap1 is proposed to recruit Tiam1 and the polarity complex to the future site of polarization. There, by a currently unknown mechanism, Rap1 activates CDC42, which then activates the polarity complex, which in its turn activates Rac through Tiam1. Rac then contributes to the induction of T-cell polarity. Since, in contrast to active Rap1, active Rac or CDC42 alone do not induce T-cell polarity, Rap1 may induce a parallel pathway for T-cell polarity, *e.g.* the Rap-RAPL and/or the Rap-Riam pathway. Also in neuronal cells, Rap1B through CDC42 and the polarity complex regulates polarity by defining which of the growing neurites becomes the future axon (Schwamborn and Puschel, 2004). Another connection between Rap1 and CDC42 was found in junction formation, where Rap1, together with Src, mediates nectin-induced activation of FRG, a GEF for CDC42 (Sato et al., 2005).

For Tiam1, the interaction of Rap1 with the DH-PH domain is surprising because this protein contains a genuine RBD, which mediates the interaction of Tiam1 with Ras. Indeed, Tiam1 mediates Ras-induced activation of Rac and thus is a genuine effector of Ras as well (Lambert et al., 2002). Rap1 was also reported to bind an ill-defined Tiam-STEF-SIF (TSS) homology domain in Tiam2 (STEF) to mediate cAMP-induced, Epac-mediated activation of Rac (Zaldua et al., 2007). Since Tiam1 and Tiam2 are rather homologous, these different binding sites are surprising and may require independent confirmation.

RhoGAPs

Arap3 is a dual GAP for both Arf6 and RhoA, with five PH domains and an RA domain that interacts with Rap, but not Ras. *In vitro*, Arap3 GAP activity towards Rho, but not Arf6, is stimulated by Rap1. *In vivo*, activation of Arap3 by Rap1 also requires PI3K activity. In cells, Arap3 seems to regulate growth factor-stimulated formation of lamellipodia. Both over-expression and knockdown of Arap3 in endothelial cells interfered with the normal ruffling response induced by PDGF (Krugmann et al., 2006).

RA-RhoGAP was identified in a yeast two-hybrid screen using a human brain cDNA library and active Rap1B as bait. This protein has an RA domain and a RhoGAP domain and, in addition, a PH domain and several annexin-like repeats. Active Rap1 binds to RA-RhoGAP and induces RhoGAP activity. Notably, a mutant lacking the RA domain fails to bind Rap1 but has a higher GAP activity for RhoA, strongly suggesting that the RA domain inhibits GAP activity,

which is relieved upon Rap1 binding. In undifferentiated neuronal cell lines, Rap1 further enhances RA-RhoGAP-induced neurite outgrowth, whereas small interfering RNA to RA-RhoGAP inhibits Rap-induced neurite outgrowth (Yamada et al., 2005).

Feedback control and interconnectivity

RapGEFs with an RA domain

PDZ-GEF1 and -2 are multidomain proteins that have, in addition to their catalytic CDC25 homology domains, a PDZ-domain, two cAMP-related binding domains, an RA domain and a C-terminal PDZ domain-binding motif. Both proteins interact with junction proteins of the Magi family, and indeed, PDZ-GEFs have been implicated in the control of cell-cell junctions (Dube et al., 2008; Sakurai et al., 2006). For PDZ-GEF1 it was shown that Rap1A and Rap2B are the preferred binding partners for its RA domain (Liao et al., 2001; Rebhun et al., 2000). This suggests that PDZ-GEF1 is subject to positive feedback by Rap1. PDZ-GEF2 can be activated by M-Ras to regulate Rap1-induced LFA-1 activation in response to TNFα (Yoshikawa et al., 2007). Epac2 is also a multidomain protein with a regulatory domain consisting of a DEP domain flanked by two cAMP binding sites and a catalytic region. An RA domain is located in between the REM and the CDC25 homology domains. This RA domain can interact with active H-Ras and may play a role in the translocation of Epac2 to the plasma membrane (Li et al., 2006). Also Epac1 has an RA-like sequence between the REM domain and the CDC25 homology domain, but for this RA domain no interacting proteins have been identified yet.

PLCε

Members of the phospholipase C family are key mediators of many extracellular signals. Upon activation by receptors, PLC converts phosphatidylinositol 4,5-bisphosphate into the protein kinase C-activating lipid diacylglycerol and the second messenger inositol 1,4,5-trisphosphate, which raises the cytosolic calcium concentration. PLCε has, in addition to its lipase catalytic domain, an N-terminal CDC25 homology domain and two C-terminally located RA domains (Song et al., 2001). As an effector, PLCε may be regulated by both Ras and Rap, as both proteins bind the C-terminal RA domain with high affinity (Song et al., 2001; Wohlgemuth et al., 2005). For instance, after PDGF stimulation, the rapid, initial PLCε activation was mediated by Ras, whereas prolonged activation was mediated by Rap1 (Song et al., 2002). Originally, the Ras effector function of PLCε was also supported by the observation that in PLCε knock-out mice, Ras-mediated skin tumor formation was reduced (Bai et al., 2004). However, more recent analysis indicates that this reduction is caused by a non-autonomous effect, *i.e.* PLCε deficiency resulted in a reduction in TPA-induced inflammation, suggesting that its role in tumor formation may be due to an increased inflammatory response (Ikuta et al., 2008). Recently, Epac and PLCε were shown to mediate β-

adrenergic receptor-induced calcium-induced calcium release (CICR) in cardiomyocytes, suggesting that Rap proteins activated by Epac induce PLCε activity (Oestreich et al., 2007). Also in 293 cells expressing the β-adrenergic receptor, Epac mediates the activation of PLCε through Rap2B (Schmidt et al., 2001). Thus, both Ras and Rap1 can activate PLCε, and differences in the reported selectivity may depend on the cell context.

As a GEF, PLCε has exchange activity towards Rap1 *in vitro* that is required for sustained PLCε activation upon PDGF stimulation, suggesting that as a GEF, it mediates its own prolonged activation through a positive feedback mechanism (Jin et al., 2001). Additionally, PLCε may mediate crosstalk between the Ras and Rap signaling networks.

Concluding remarks

Ras and Rap are highly homologous proteins, each functioning in different but interconnected signaling networks. This specificity of Ras and Rap is achieved by upstream regulatory proteins and downstream effectors (Figure 1). Most GEFs for Ras or Rap contain a CDC25 homology and a REM domain for catalysis and the mechanism of nucleotide exchange is comparable between RasGEFs and RapGEFs. However, the interaction interfaces between the GEF and its GTPase differ between the different pairs, generally resulting in their tight specificity for each other. In contrast, RasGAPs and RapGAPs do not use the same catalytic mechanism. Whereas RasGAPs provide an arginine side chain as the catalytic group, RapGAPs insert an asparagine side chain in the GTP-binding pocket for catalysis. In addition, GEFs and GAPs are multidomain proteins that regulate these GTPases in time and space, and thus, localization and timing are also important elements in the specificity.

A second way of ensuring specificity in signaling is through effector proteins. Most effectors bind Ras and Rap proteins via an RA domain or an RBD. *In vitro*, many of these domains bind to both Ras and Rap, albeit with different affinity. However, *in vivo,* the various effectors are rather specific. In part, this difference in selectivity for RA domain/RBD may be determined by flanking sequences or by differences in subcellular localization. However, some effectors may be used by both Ras and Rap proteins, *e.g.* AF-6 and Tiam1. The conclusion that the specificity of Ras and Rap is determined at least at two levels, by both upstream regulators and downstream effectors, implies that results obtained using over-expression of mutant GTPases that are constitutively active lack at least one level of this specificity control. Results obtained with such mutants should therefore be interpreted with care.

For Rap, the list of effectors is rapidly expanding and contains proteins both with and without catalytic activity, which are mostly involved in all aspects of cell adhesion and modulation of the actin cytoskeleton. A number of effectors have been implicated in the control of integrins. For instance, in T-cells, Riam, RAPL

and PKD have all been described as effectors. They may form an 'integrin activation complex' consisting of Rap1 and several effectors and perhaps further adaptor proteins, required to mediate integrin activation. This complex is then translocated to the integrin upon Rap activation to induce cell adhesion. Also a number of effectors have been identified that regulate the actin cytoskeleton, in particular the GEFs Vav2 and Tiam1 for Rac proteins and GAPs Arap3 and RA-RhoGAP for Rho proteins. These effectors apparently determine the balance between Rac and Rho signaling and, as such, regulate the dynamics of the actin cytoskeleton. Finally, an interesting aspect in the control of Ras family proteins is the presence of RA domains in GEFs, such as Epac proteins, PDZ-GEFs and PLCε. These RA domains may be responsible for feedback control or for the connection between signaling networks.

Thus, in the last couple of years, it is truly appreciated that Rap proteins serve in signaling networks that are largely different from Ras signaling networks. However, as with all signaling networks, there is interconnectivity. It is the challenge for the future to understand both networks and the interconnectivity between these and other networks in full detail.

Acknowledgements

We thank Holger Rehmann and Fried Zwartkruis for comments on this manuscript. This work was supported by the Dutch Cancer Society (to J.H.R.) and by the Netherlands Genomics Initiative through the Cancer Genomics Centre (to J.L.B.).

References

Arthur, W.T., Quilliam, L.A. and Cooper, J.A. (2004) Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. *J. Cell. Biol.*, **167**, 111-122.

Bai, Y., Edamatsu, H., Maeda, S., Saito, H., Suzuki, N., Satoh, T. and Kataoka, T. (2004) Crucial role of phospholipase Cepsilon in chemical carcinogen-induced skin tumor development. *Cancer Res*, **64**, 8808-8810.

Beraud-Dufour, S., Gautier, R., Albiges-Rizo, C., Chardin, P. and Faurobert, E. (2007) Krit 1 interactions with microtubules and membranes are regulated by Rap1 and integrin cytoplasmic domain associated protein-1. *Febs J*, **274**, 5518-5532.

Boriack-Sjodin, P.A., Margarit, S.M., Bar-Sagi, D. and Kuriyan, J. (1998) The structural basis of the activation of Ras by Sos. *Nature*, **394**, 337-343.

Bos, J.L., de Bruyn, K., Enserink, J., Kuiperij, B., Rangarajan, S., Rehmann, H., Riedl, J., de Rooij, J., van Mansfeld, F. and Zwartkruis, F. (2003) The role of Rap1 in integrin-mediated cell adhesion. *Biochem Soc Trans*, **31**, 83-86.

Bos, J.L., Rehmann, H. and Wittinghofer, A. (2007) GEFs and GAPs: critical elements in the control of small G proteins. *Cell*, **129**, 865-877.

Christian, S.L., Lee, R.L., McLeod, S.J., Burgess, A.E., Li, A.H., Dang-Lawson, M., Lin, K.B. and Gold, M.R. (2003) Activation of the Rap GTPases in B lymphocytes modulates B cell antigen receptor-induced activation of Akt but has no effect on MAPK activation. *J Biol Chem*, **278**, 41756-41767.

Dube, N., Kooistra, M.R., Pannekoek, W.J., Vliem, M.J., Oorschot, V., Klumperman, J., Rehmann, H. and Bos, J.L. (2008) The RapGEF PDZ-GEF2 is required for maturation of cell-cell junctions. *Cell Signal*, **20**, 1608-1615.

Gerard, A., Mertens, A.E., van der Kammen, R.A. and Collard, J.G. (2007) The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. *J Cell Biol*, **176**,

863-875.

Glading, A., Han, J., Stockton, R.A. and Ginsberg, M.H. (2007) KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. *J Cell Biol*, **179**, 247-254.

Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J. and Ginsberg, M.H. (2006) Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol*, **16**, 1796-1806.

Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T. and Takai, Y. (2005) Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. *J Biol Chem*, **280**, 24095-24103.

Ikuta, S., Edamatsu, H., Li, M., Hu, L. and Kataoka, T. (2008) Crucial role of phospholipase C epsilon in skin inflammation induced by tumor-promoting phorbol ester. *Cancer Res*, **68**, 64-72.

Jeong, H.W., Li, Z., Brown, M.D. and Sacks, D.B. (2007) IQGAP1 binds Rap1 and modulates its activity. *J Biol Chem*, **282**, 20752-20762.

Jin, T.G., Satoh, T., Liao, Y., Song, C., Gao, X., Kariya, K., Hu, C.D. and Kataoka, T. (2001) Role of the CDC25 homology domain of phospholipase Cepsilon in amplification of Rap1-dependent signaling. *J Biol Chem*, **276**, 30301-30307.

Katagiri, K., Imamura, M. and Kinashi, T. (2006) Spatiotemporal regulation of the kinase Mst1 by binding protein RAPL is critical for lymphocyte polarity and adhesion. *Nat Immunol*, **7**, 919-928.

Katagiri, K., Maeda, A., Shimonaka, M. and Kinashi, T. (2003) RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat Immunol*, **4**, 741-748.

Katagiri, K., Ohnishi, N., Kabashima, K., Iyoda, T., Takeda, N., Shinkai, Y., Inaba, K. and Kinashi, T. (2004) Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. *Nat Immunol*, **5**, 1045-1051.

Krugmann, S., Andrews, S., Stephens, L. and Hawkins, P.T. (2006) ARAP3 is essential for formation of lamellipodia after growth factor stimulation. *J Cell Sci*, **119**, 425-432.

Kupzig, S., Deaconescu, D., Bouyoucef, D., Walker, S.A., Liu, Q., Polte, C.L., Daumke, O., Ishizaki, T., Lockyer, P.J., Wittinghofer, A. and Cullen, P.J. (2006) GAP1 family members constitute bifunctional Ras and Rap GTPase-activating proteins. *J Biol Chem*, **281**, 9891-9900.

Lafuente, E.M., van Puijenbroek, A.A., Krause, M., Carman, C.V., Freeman, G.J., Berezovskaya, A., Constantine, E., Springer, T.A., Gertler, F.B. and Boussiotis, V.A. (2004) RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. *Dev Cell*, **7**, 585-595.

Lambert, J.M., Lambert, Q.T., Reuther, G.W., Malliri, A., Siderovski, D.P., Sondek, J., Collard, J.G. and Der, C.J. (2002) Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism. *Nat Cell Biol*, **4**, 621-625.

Li, Y., Asuri, S., Rebhun, J.F., Castro, A.F., Paranavitana, N.C. and Quilliam, L.A. (2006) The RAP1 guanine nucleotide exchange factor Epac2 couples cyclic AMP and Ras signals at the plasma membrane. *J Biol Chem*, **281**, 2506-2514.

Liao, Y., Satoh, T., Gao, X., Jin, T.G., Hu, C.D. and Kataoka, T. (2001) RA-GEF-1, a guanine nucleotide exchange factor for Rap1, is activated by translocation induced by association with Rap1*GTP and enhances Rap1-dependent B-Raf activation. *J Biol Chem*, **276**, 28478-28483.

Lorger, M. and Moelling, K. (2006) Regulation of epithelial wound closure and intercellular adhesion by interaction of AF6 with actin cytoskeleton. *J Cell Sci*, **119**, 3385-3398.

Margarit, S.M., Sondermann, H., Hall, B.E., Nagar, B., Hoelz, A., Pirruccello, M., Bar-Sagi, D. and Kuriyan, J. (2003) Structural evidence for feedback activation by Ras. GTP of the Ras-specific nucleotide exchange factor SOS. *Cell*, **112**, 685-695.

Medeiros, R.B., Dickey, D.M., Chung, H., Quale, A.C., Nagarajan, L.R., Billadeau, D.D. and Shimizu, Y. (2005) Protein kinase D1 and the beta 1 integrin cytoplasmic domain control beta 1 integrin function via regulation of Rap1 activation. *Immunity*, **23**, 213-226.

Menasche, G., Kliche, S., Chen, E.J., Stradal,

T.E., Schraven, B. and Koretzky, G. (2007) RIAM links the ADAP/SKAP-55 signaling module to Rap1, facilitating T-cell-receptor-mediated integrin activation. *Mol Cell Biol*, **27**, 4070-4081.

Miertzschke, M., Stanley, P., Bunney, T.D., Rodrigues-Lima, F., Hogg, N. and Katan, M. (2007) Characterization of interactions of adapter protein RAPL/Nore1B with RAP GTPases and their role in T cell migration. *J Biol Chem*, **282**, 30629-30642.

Oestreich, E.A., Wang, H., Malik, S., Kaproth-Joslin, K.A., Blaxall, B.C., Kelley, G.G., Dirksen, R.T. and Smrcka, A.V. (2007) Epac-mediated activation of phospholipase C(epsilon) plays a critical role in beta-adrenergic receptor-dependent enhancement of Ca2+ mobilization in cardiac myocytes. *J Biol Chem*, **282**, 5488-5495.

Pacold, M.E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C.T., Walker, E.H., Hawkins, P.T., Stephens, L., Eccleston, J.F. and Williams, R.L. (2000) Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. *Cell*, **103**, 931-943.

Pena, V., Hothorn, M., Eberth, A., Kaschau, N., Parret, A., Gremer, L., Bonneau, F., Ahmadian, M.R. and Scheffzek, K. (2008) The C2 domain of SynGAP is essential for stimulation of the Rap GTPase reaction. *EMBO Rep*, **9**, 350-355.

Rebhun, J.F., Castro, A.F. and Quilliam, L.A. (2000) Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction. *J Biol Chem*, **275**, 34901-34908.

Rehmann, H., Arias-Palomo, E., Hadders, M.A., Schwede, F., Llorca, O. and Bos, J.L. (2008) Structure of Epac2 in complex with a cyclic AMP analogue and RAP1B. *Nature*, **455**, 124-127.

Rodriguez-Viciana, P., Sabatier, C. and McCormick, F. (2004) Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. *Mol Cell Biol*, **24**, 4943-4954.

Sakurai, A., Fukuhara, S., Yamagishi, A., Sako, K., Kamioka, Y., Masuda, M., Nakaoka, Y. and Mochizuki, N. (2006) MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. *Mol Biol Cell*, **17**, 966-976.

Sato, T., Irie, K., Okamoto, R., Ooshio, T., Fujita, N. and Takai, Y. (2005) Common signaling pathway is used by the trans-interaction of Necl-5/Tage4/PVR/CD155 and nectin, and of nectin and nectin during the formation of cell-cell adhesion. *Cancer Sci*, **96**, 578-589.

Schmidt, M., Evellin, S., Weernink, P.A., von Dorp, F., Rehmann, H., Lomasney, J.W. and Jakobs, K.H. (2001) A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nat Cell Biol*, **3**, 1020-1024.

Schwamborn, J.C. and Puschel, A.W. (2004) The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.*, **7**, 923-929.

Serebriiskii, I., Estojak, J., Sonoda, G., Testa, J.R. and Golemis, E.A. (1997) Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22. *Oncogene*, **15**, 1043-1049.

Song, C., Hu, C.D., Masago, M., Kariyai, K., Yamawaki-Kataoka, Y., Shibatohge, M., Wu, D., Satoh, T. and Kataoka, T. (2001) Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. *J Biol Chem*, **276**, 2752-2757.

Song, C., Satoh, T., Edamatsu, H., Wu, D., Tadano, M., Gao, X. and Kataoka, T. (2002) Differential roles of Ras and Rap1 in growth factor-dependent activation of phospholipase C epsilon. *Oncogene*, **21**, 8105-8113.

Stork, P.J. and Schmitt, J.M. (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol*, **12**, 258-266.

Wohlgemuth, S., Kiel, C., Kramer, A., Serrano, L., Wittinghofer, F. and Herrmann, C. (2005) Recognizing and defining true Ras binding domains I: biochemical analysis. *J Mol Biol*, **348**, 741-758.

Yamada, T., Sakisaka, T., Hisata, S., Baba, T. and Takai, Y. (2005) RA-RhoGAP, Rap-activated

Rho GTPase-activating protein implicated in neurite outgrowth through Rho. *J Biol Chem*, **280**, 33026-33034.

Yamamoto, T., Harada, N., Kano, K., Taya, S., Canaani, E., Matsuura, Y., Mizoguchi, A., Ide, C. and Kaibuchi, K. (1997) The Ras target AF-6 interacts with ZO-1 and serves as a peripheral component of tight junctions in epithelial cells. *J Cell Biol*, **139**, 785-795.

Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K. and Matsuda, M. (2000) CalDAG-GEFIII activation of Ras, R-ras, and Rap1. *J Biol Chem*, **275**, 25488-25493.

Yoshikawa, Y., Satoh, T., Tamura, T., Wei, P., Bilasy, S.E., Edamatsu, H., Aiba, A., Katagiri, K., Kinashi, T., Nakao, K. and Kataoka, T. (2007) The M-Ras-RA-GEF-2-Rap1 pathway mediates tumor necrosis factor-alpha dependent regulation of integrin activation in splenocytes. *Mol Biol Cell*, **18**, 2949-2959.

Zaldua, N., Gastineau, M., Hoshino, M., Lezoualc'h, F. and Zugaza, J.L. (2007) Epac signaling pathway involves STEF, a guanine nucleotide exchange factor for Rac, to regulate APP processing. *FEBS Lett*, **581**, 5814-5818.

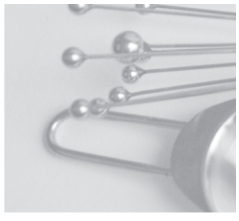
Zhang, Z., Rehmann, H., Price, L.S., Riedl, J. and Bos, J.L. (2005) AF6 negatively regulates Rap1-induced cell adhesion. *J Biol Chem*, **280**, 33200-33205.

chapter 3

cAMP-induced Epac-Rap activation inhibits epithelial cell migration by modulating focal adhesion and leading edge dynamics

Karen S. Lyle, Judith H. Raaijmakers, Wytse Bruinsma, Johannes L. Bos and Johan de Rooij

Cellular Signaling 20 (2008) 1104-1116



cAMP-induced Epac-Rap activation inhibits epithelial cell migration by modulating focal adhesion and leading edge dynamics

Karen S. Lyle[1,a], Judith H. Raaijmakers[1,a], Wytse Bruinsma[1], Johannes L. Bos[1] and Johan de Rooij[2]

[1]Department of Physiogical Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands
[2]Department of Cell Biology, the Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands
[a]These autors contributed equally to this paper

Abstract

Epithelial cell migration is a complex process crucial for embryonic development, wound healing and tumor metastasis. It depends on alterations in cell-cell adhesion and integrin-extracellular matrix interactions and on actomyosin-driven, polarized leading edge protrusion. The small GTPase Rap is a known regulator of integrins and cadherins that has also been implicated in the regulation of actin and myosin, but a direct role in cell migration has not been investigated. Here, we report that activation of endogenous Rap by cAMP results in an inhibition of HGF- and TGFβ-induced epithelial cell migration in several model systems, irrespective of the presence of E-cadherin adhesion. We show that Rap activation slows the dynamics of focal adhesions and inhibits polarized membrane protrusion. Importantly, forced integrin activation by antibodies does not mimic these effects of Rap on cell motility, even though it does mimic Rap effects in short-term cell adhesion assays. From these results, we conclude that Rap inhibits epithelial cell migration, by modulating focal adhesion dynamics and leading edge activity. This extends beyond the effect of integrin affinity modulation and argues for an additional function of Rap in controlling the migration machinery of epithelial cells.

Introduction

Epithelial cell migration is a complex process induced by specific growth factors that takes place during certain stages of embryonic development, organogenesis and wound healing. In response to oncogenic signals, epithelial cell migration also mediates tumor invasion and metastasis (Thiery, 2002). Epithelial cell migration requires the disruption of cell-cell adhesion (Thiery, 2002), the modification of the integrin-extracelllularmatrix (ECM) interactions (Danen, 2005) and engagement of the actomyosin-based migration machinery that induces polarized membrane protrusion (Horwitz and Webb, 2003). Beneath this leading edge protrusion of a migrating cell, integrin-mediated focal contacts are initiated and subsequently reinforced by tension generated in the actomyosin cytoskeleton (Sheetz et al., 1998). As a consequence, they grow larger and alter their composition to become focal adhesions (FAs) (Zamir and Geiger, 2001). Contraction of the

actomyosin cytoskeleton attached to leading edge FAs pulls the cell body forward and is used to disassemble rear-end FAs (Ridley et al., 2003; Webb et al., 2002). The efficiency of migration in two-dimensional culture also depends on the balance between ECM concentration and the extent of integrin activation (Huttenlocher et al., 1996).

Several growth factors implicated in tumor metastasis can induce the processes described above in cultured cells, resulting in the scattering of initially clustered epithelial cells. The most well-known inducers of epithelial cell scattering are transforming growth factor-β (TGFβ) and hepatocyte growth factor (HGF) (Thiery and Sleeman, 2006). TGFβ induces scattering in many different cell lines, invariantly accompanied by silencing of the E-cadherin gene through Smad signaling (Deckers et al., 2006). The most prominent induction of scattering by HGF occurs in MDCK cells (Stoker et al., 1987) and does not involve down-regulation of E-cadherin protein levels or adhesive capacity, but correlates with increased integrin-mediated adhesion and depends on actomyosin-based tension (de Rooij et al., 2005). Given the lethal consequences of tumor metastasis, we aim to understand the cellular machinery that governs epithelial cell migration.

cAMP is a pivotal second messenger that regulates a wide range of cellular processes. Signaling through cAMP and protein kinase A (PKA) has been implicated in cytoskeletal regulation and cell migration (Howe, 2004). The effects of PKA on cell migration can be both stimulatory and inhibitory, depending on the cell type and matrix used (Dormond and Ruegg, 2003; Edin et al., 2001; Howe, 2004; Kim et al., 2000). cAMP also activates the guanine nucleotide exchange factor (GEF) Epac that can subsequently activate the small GTPase Rap (de Rooij et al., 1998). Rap is an important regulator of both integrin- and cadherin-mediated adhesion (reviewed in (Bos, 2005); (Caron, 2003) and (Kooistra et al., 2007)). Although it is not yet completely understood how Rap regulates these two processes, several proteins that interact with its GTP-bound form have been identified that may serve as effector proteins (Bos, 2005). In the case of integrin-mediated adhesion, Rap regulates both integrin affinity and integrin avidity, or clustering, depending on the type of integrin and the cell type (Caron et al., 2000; de Bruyn et al., 2002; Katagiri et al., 2000; Reedquist et al., 2000). Two effectors of Rap1, Riam and RAPL, have been shown to be important in the regulation of integrin affinity (Katagiri et al., 2003; Lafuente et al., 2004), although they induce integrin activation via distinct mechanisms (Han et al., 2006; Katagiri et al., 2006). In the regulation of (V)E-cadherin-mediated adhesion, Rap effectors likely recruit junctional proteins to sites of developing cell-cell contacts to stabilize the connection between the actin cytoskeleton and the junctional complex (Glading et al., 2007; Hoshino et al., 2005; Kooistra et al., 2007). Because of its

established function as a regulator of integrin-mediated cell adhesion, a role for Rap in cell migration has been suggested. Direct evidence comes from studies in leukocytes, where chemokine-induced integrin activation by Rap1 leads to an increase in adhesion to the endothelium and subsequent endothelial transmigration (Lorenowicz et al., 2007; Wittchen et al., 2005). Clearly, in addition to PKA, also Epac/Rap signaling may be involved in regulation of cell migration via cAMP.

Previously, we reported that Rap is involved in cell surface expression of E-cadherin and the stabilization of cell-cell junctions and that cAMP-induced activation of Rap through Epac1 inhibits HGF-induced cell scattering. These observations suggested that scattering is inhibited by stabilization of adherens junctions (Price et al., 2004). However, a deficiency in C3G, another RapGEF, results in increased migration velocity (Ohba et al., 2001), indicating that Rap might also have a restraining effect on cell migration itself. Moreover, Zhang et al. recently described that TGFβ-induced transformation of cells is inhibited by cAMP, independently of PKA (Zhang et al., 2006). As TGFβ, in contrast to HGF, down-regulates the E-cadherin expression level, cAMP-Epac-Rap signaling may regulate cell migration directly rather than through a stabilizing effect on cell-cell junctions.

Here, we show that activation of Rap through Epac1 inhibits epithelial cell migration in a number of different model systems in response to both HGF and TGFβ, irrespective of the presence of cell-cell junctions. Interestingly, forced integrin activation, by the integrin-activating antibody TS2/16, does not inhibit migration, even though it induces adhesion to the ECM to the same extent as Rap activation does. Apparently, the effects of Rap on cadherin-mediated adhesion and integrin activation are not sufficient to inhibit epithelial cell migration, indicating that inhibition of the basal cell migration machinery is the critical step downstream of Rap that mediates its effects on scattering. To further understand the mechanism of Rap-induced inhibition of cell migration, we studied the migration machinery in more detail and observed that Rap activation impairs the dynamics of focal adhesions and blocks protrusive activity at the leading edge in migrating cells. These effects are also not mimicked by integrin-activating antibodies. Together, these data show that Rap regulates focal adhesion and leading edge dynamics, independently of integrin activation, to restrain epithelial cell migration.

Materials and Methods

Cell lines and culture

Stable MDCK-GFP-Epac cells were created by transfection of MDCK cells with pEGFP-C1-Epac1 followed by selection with G418. Polyclonal MDCK cells stably expressing moderate levels of GFP-Epac were isolated by fluorescence activated cell sorting (FACS) from this cell line. MDCK-Epac1 cells were described previously (Price et al., 2004). Stable Epac1-expressing A549 cells were created by infecting A549 cells with Epac1 ecotrophic virus. The Epac1 gene was linked via an IRES sequence to a zeocin resistance gene. 48 hours after infection, cells were placed under

selection with zeocin (0.2 mg/ml) to select for Epac1 expressing cells. Monoclonal A549-Epac1 cell lines expressing moderate levels of Epac1 were single-cell sorted from these polyclonal cell lines by FACS. MDCK, MDCK-GFP-Epac and MDCK-Epac cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), glutamine, and antibiotics. A549 and RCC10 cell lines were cultured in RPMI supplemented with glutamine, antibiotics, and 10% or 8% FCS, respectively. RCC10 cells were transiently transfected using FuGENE6 transfection reagent (Roche) according to the manufacturer's protocol. Cells were plated in complete medium 24 hours after transfection, and analysis of expressed proteins occurred 24 hours thereafter.

Plasmids

The GFP-CAAX construct expresses an N-terminal GFP-tagged tetra-amino acid motif (CAAX) that localizes to the plasma membrane (von der Hardt et al., 2007). The GFP-paxillin construct expresses a N-terminal EGFP-tagged fusion to human paxillin and was generously provided by Dr. Marc Ginsberg (University of California-San Diego). GFP-Epac1 contains amino acids 2-881 of Epac1 fused at its N-terminus to EGFP in the pEGFP-C1 vector.

ECM proteins

For analysis of MDCK cell motility, non-tissue culture treated 48-well plates were coated with collagen type I from calf skin (Sigma) for 2 hours at 37°C, washed 3 times with phosphate buffered saline (PBS), and blocked with 1% heat-denatured bovine serum albumin (BSA) in PBS for 1 hour at 37°C. For analysis of A549 cell motility, 48-well plates were coated with 1 µg/mL fibronectin for 2 hours at 37°C and washed 3 times in PBS. In all other cases, glass-bottomed dishes and coverslips were coated with collagen type I from rat tendon (Upstate) for 16 hours at 4°C and washed 3 times in PBS.

Rap activation assays and immunoblotting

Rap activation was assayed as described previously (Franke et al., 1997). Endogenous Rap was detected following Western blotting with polyclonal anti-Rap antibody (Santa Cruz). Polyclonal phospho-ERK (Thr202/Tyr204) antibody and phospho-Vasp (Ser157) antibody were obtained from Cell Signaling. Polyclonal phospho-Smad2 (Ser465/467), monoclonal anti-E-cadherin (HECD-1) and anti-GAPDH antibodies were from Chemicon International. Anti-β1 integrin and anti-β-catenin antibodies were from BD Transduction Laboratories and anti-tubulin antibody was from Calbiochem. Monoclonal anti-Epac1 5D3 antibody was described previously (Price et al., 2004).

Live cell microscopy

For phase-contrast imaging, MDCK, MDCK-GFP-Epac, and RCC10 cells were plated in medium containing 0.5% FCS and 10 mM Hepes, pH 7.4, 24 hours before image acquisition in non-tissue culture–treated polystyrene well plates coated with 10 µg/mL collagen (or the indicated concentrations). Prior to imaging, 100 µM 8-CPT-2OMe-cAMP, 10 µM forskolin, 300 µM N6-Bnz-cAMP or 3 µg/mL TS2/16 were added to the appropriate wells, wells were completely filled with medium and the plate was sealed using silicon grease and a glass plate. Images were acquired every 6 min using a 10x 0.5 NA Plan objective lens and a 0.5 NA ELWD condenser with a Zeiss Axiocam camera on a Zeiss Axiovert 200M microscope in climate-controlled incubator. A robotic stage (Zeiss MCU 28) was used to collect images at different stage positions. All electronic microscope functions were controlled using Axiovision software (Zeiss). The cells were imaged for 2 hours in absence of HGF, and then 5 ng/ml HGF was added to cells on the microscope stage to prevent loss of the cells of interest and imaging was continued for 16 hours. At least three timelapse series were acquired for each condition in each separate experiment.

A549 and A549-Epac cells were plated on a fibronectin-coated (1 µg/mL) 48-well plate 24 hours before imaging, which was performed as above. At the indicated timepoint 10 ng/mL TGFβ, 5 ng/ml HGF and 100 µM 007 or 3 µg/ml integrin-activating antibody TS2/16 and/or LIBS6 were added to the appropriate wells. GFP-tagged proteins were imaged in a 8-well Lab-tek chambered coverglass (Nalge Nunc International, Rochester, NY) coated with 10 µg/mL collagen on a Zeiss Axiovert 200M microscope using a Lambda DG-4 Ultra High Speed Wavelength Switcher from Sutter Instruments as a light source. Fluorescent images were acquired every

2 or 3 minutes using either a 40X/1.3 oil or a 63X/1.25 oil Neofluar objective lens.

Fixation and immunolocalization
Cells were fixed in freshly prepared 4% paraformaldehyde for 10 minutes, permeabilized in 0.2% Triton X-100 for 5 minutes, and blocked in PBS containing 0.2% BSA and 5% horse serum for 1 hour at room temperature. Cells were incubated with monoclonal paxillin antibody (BD Biosciences) for 1 hour in PBS containing 0.2% BSA, followed by incubation with the appropriate secondary antibody for 45 minutes at room temperature. Images were acquired using a Zeiss Axioskop 2 microscope fitted with a Zeiss Axiocam CCD camera and 100X Plan APO objective lens.

Image analysis and processing
To determine cell trajectories in phase-contrast timelapse image series, the centroids of the nuclei were followed. To automate this and allow for unbiased analysis of many cells in multiple timelapses, a program was written in Matlab (Mathworks) that segments images based on pixel intensity and determines the presence of nuclei based on phase-density, size and shape. Nuclei are then linked in consecutive frames using a neural network algorithm and cells tracked for less than 5 consecutive frames are automatically discarded (manuscript in preparation, JdR and Danuser G.). Detection fidelity in our experiments was over 80%, which was confirmed by eye for each individual timelapse. To distinguish single cells from clustered cells in this program, areas occupied by cells were determined by edge-detection and overlayed with the detected nuclei to determine if one (single cell) or more (clustered cells) nuclei were present in a detected cell-area. Similar results were obtained when a smaller number of randomly selected cells from a number of timelapses were analyzed using the track objects function in MetaMorph (Universal Imaging Corp.). Focal adhesion and total cell areas from images of fixed and stained cells were measured in ImageJ. First, the fluorescence intensities of images from 2 independent experiments were normalized, and then a bandpass filter was applied to remove background staining that was consistently observed around the nucleus. FAs were segmented using the analyze particles function in ImageJ, and the total segmented area was normalized to the total cell area. The effectiveness of our segmentation procedure was inspected visually. FAs were manually tracked in ImageJ. Approximately 27 FAs were randomly selected in similarly localized regions of the cell and marked in the first frame (t=0) of each timelapse series using the Cell Counter ImageJ plugin. The selected FAs were tracked until they were no longer visible and the frame at which the focal adhesion disappeared was recorded. To quantify protrusion dynamics, we determined the changes in membrane area between sequential timelapse images of RCC10 cells expressing GFP-CAAX. Using Metamorph software, an exclusive threshold was applied to each normalized image series to define the outer cell membrane. We created a journal in Metamorph that defined and measured the thresholded areas and produced a stack of binary images. The subtract function in the ImageJ Image Calculator was applied to sequential binary timelapse images to determine areas of protrusion and retraction, and differences in membrane area were normalized to the total cell area. Images showing the net protrusion and retraction were made by applying the sum slices option in the z-project function of ImageJ to sequentially subtracted images.

Adhesion assays
Cells were trypsinized, washed once in RPMI containing 10% FCS, and allowed to recover surface proteins for 1.5 hours in suspension in RPMI containing 0.5% FCS, glutamine, antibiotics, and 10 mM Hepes, pH 7.4, at 37°C with constant, gentle rolling. 8-CPT-2OMe-cAMP (200 µM) and TS2/16 antibody (2 µg/mL, unless indicated otherwise) were diluted in 100 µL of RPMI and added to the wells of a 48-well polystyrene cell culture dish coated with 3 µg/ml collagen (for RCC10 cells) or 1 µg/ml fibronection (A549-Epac cells). After rolling, 50,000 cells in 100 µL were plated per well, making the final concentrations of 8-CPT-2OMe-cAMP and TS2/16 100 µM and 1 µg/mL, respectively. Adhesion was allowed to proceed for the indicated times at 37°C, and unbound cells were discarded by washing three times with PBS preheated to 37°C. Adhered cells were lysed in the wells by adding 200 µl of assay buffer containing 0.4%

Triton X-100, 50 mM sodium citrate, and 10 mg/ml phosphatase substrate (Sigma-Aldrich). The total amount of cellular protein per well was quantified by acid phosphatase activity as previously described (Schwartz and Denninghoff, 1994). The reaction was incubated for 20 hours at 37°C and terminated by addition of 100 µl of 1N NaOH. Absorbance was measured at 405 nm. Every condition was measured in quadruplicate.

Statistical Analysis

Statistical analysis was performed in Kaleidagraph (Synergy Software) using the unpaired Student's t-test for samples of unequal variance.

Results

cAMP-induced Rap activation inhibits HGF-stimulated epithelial cell migration

To investigate the effect of Rap activation on epithelial cell migration we used MDCK cells, which do not express endogenous Epac, and MDCK cells stably expressing GFP-tagged human Epac1 (MDCK-GFP-Epac cells). Cells were plated in a 48-well plate coated with collagen (10 µg/mL), simultaneously filmed by phase-contrast microscopy for 2 hours, stimulated with HGF, and filmed for an additional 18 hours. Parental MDCK cells exhibited a typical response to HGF; the cells initially spread, disrupted cell-cell contacts and migrated away from their neighbors (Movie S1 and Figure 1A). MDCK-GFP-Epac cells showed a similar response to HGF treatment compared to parental MDCK cells, but scattering was inhibited in HGF-stimulated MDCK-GFP-Epac cells treated with the cell-permeable cAMP analogue 8-bromo-cAMP (Movie S1 and Figure 1A) or the cAMP-elevating drug forskolin (Movie S2 and Figure S1). 8-Bromo-cAMP did not inhibit scattering in parental MDCK cells, suggesting that activation of Epac mediated this response. To confirm that PKA is not needed for the inhibition of scattering by cAMP, we used the Epac-specific cAMP analog 8-CPT-2OMe-cAMP (Enserink et al., 2002) and the PKA-specific cAMP analog N6-Bnz-cAMP (Christensen et al., 2003). 8-CPT-2OMe-cAMP inhibited scattering in MDCK-GFP-Epac, but not in parental MDCK cells (Movie S1 and Figure 1A). In contrast, activation of PKA with N6-Bnz-cAMP did not inhibit scattering in MDCK-GFP-Epac cells (Movie S2 and Figure S1).

To quantify these observations, the migration velocity of parental and MDCK-GFP-Epac cells was determined by tracking cell nuclei using custom-written, automated cell-tracking software. For each condition 3 independent time-lapse image series were analyzed, resulting in the tracking of at least 300 individual cells per condition. MDCK and MDCK-GFP-Epac cells increased their migration velocity from 0.5 to 1.2 µm/minute, within 16 hours of HGF stimulation (Figure 1B, solid and dashed black lines). MDCK cells treated with 8-bromo-cAMP showed the same response to HGF (Figure 1B, solid grey line). In contrast, MDCK-GFP-Epac cells exposed to HGF and 8-bromo-cAMP or 8-CPT-2OMe-cAMP showed no significant increase in cell velocity (Figure 1B, grey dashed line and Figure 1C, black dashed line, respectively). To evaluate the statistical significance of the inhibition of migration by cAMP, the velocity values for each time point

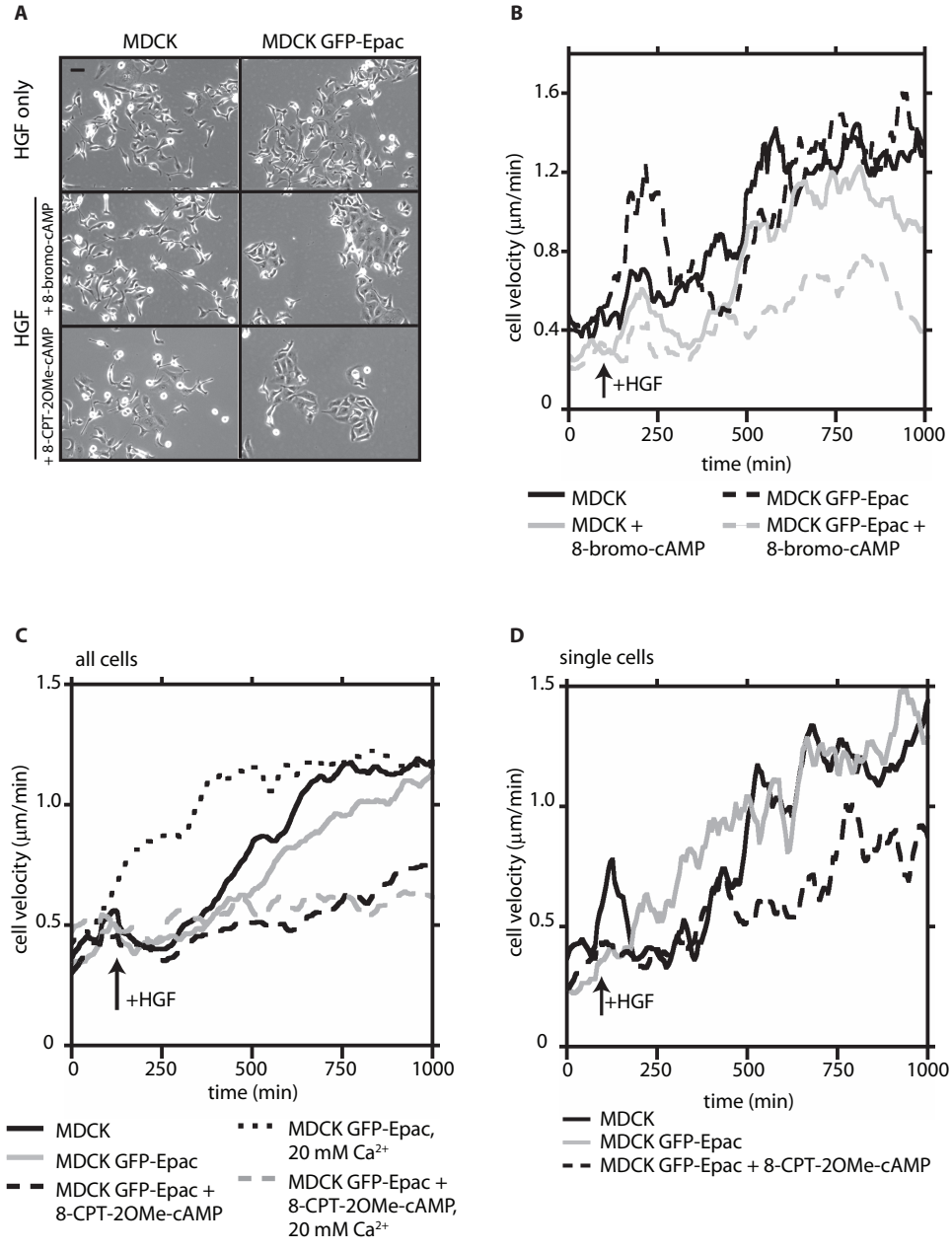


Figure 1. cAMP-induced Rap activation inhibits HGF-induced cell migration in the absence or presence of cell-cell adhesion.

(A) Representative images from phase contrast timelapse image series showing the inhibitory effect of 8-bromo-cAMP and 8-CPT-2OMe-cAMP on HGF-induced cell motility in MDCK-GFP-Epac, but not parental MDCK cell lines. Scale bar is 100 μm. (B) Velocity time-course, by automated tracking of approximately 300 cells from three independent time-lapse image series, to quantify HGF-induced migration and cAMP-induced inhibition in these cells.

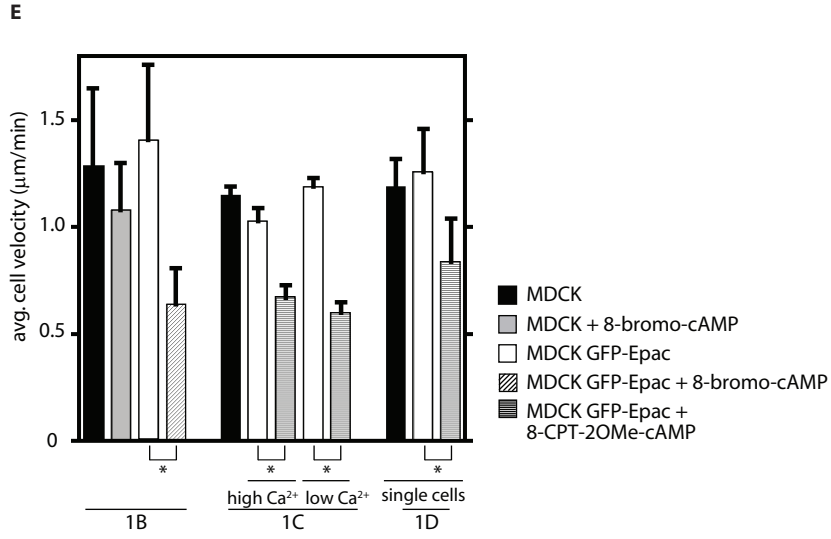


Figure 1, continued

(C) Velocity time-course by tracking of approximately 600 cells from 3 independent time-lapse image series to quantify the inhibitory effect of 8-CPT-2OMe-cAMP on HGF-induced MDCK and MDCK-GFP-Epac cell velocity in the presence (1.8 mM Ca^{2+}) and absence (20 µM Ca^{2+}) of cell-cell junctions. (D) Velocity time-course by tracking of approximately 60 cells from 3 independent time-lapse image series, showing the effect of 8-CPT-2OMe-cAMP on the migration of non-contacted MDCK-GFP-Epac cells in high Ca^{2+}. (E) Average cell velocity (averaging values from all time-points at the plateau phase of maximum velocity (750-1000 minutes)) (± SD) from each of the velocity time-courses in B, C, and D. An asterisk (*) indicates $p<0.0001$.

after the plateau of maximal velocity was reached (t=750 – 1000 min post-HGF) were averaged (Figure 1E) and subjected to a Students t-test. Thus we conclude that cAMP-induced activation of Epac (and not PKA) and subsequent activation of endogenous Rap in MDCK cells strongly inhibits HGF-induced scattering.

Rap activation inhibits HGF-induced cell migration in the absence of cell-cell junctions

As Rap has been previously shown to modulate both cell-cell and cell-ECM adhesion receptors (Caron et al., 2000; Hogan et al., 2004; Katagiri et al., 2000; Knox and Bron, 2002; Price et al., 2004; Reedquist et al., 2000), we aimed to investigate whether the cAMP-induced inhibition of cell migration is simply caused by the stabilization of cell-cell adhesion through Rap. To this end, we analyzed the velocity of MDCK-GFP-Epac cells in the absence of cell-cell junctions using two approaches. First, we examined the HGF-induced motility in low Ca^{2+} (20 µM Ca^{2+})-containing medium that does not support homotypic E-cadherin adhesion. Second, we used our tracking software to distinguish clearly single cells from cells in contact with neighboring cells in high Ca^{2+} conditions to specifically analyze the HGF-induced motility of single cells.

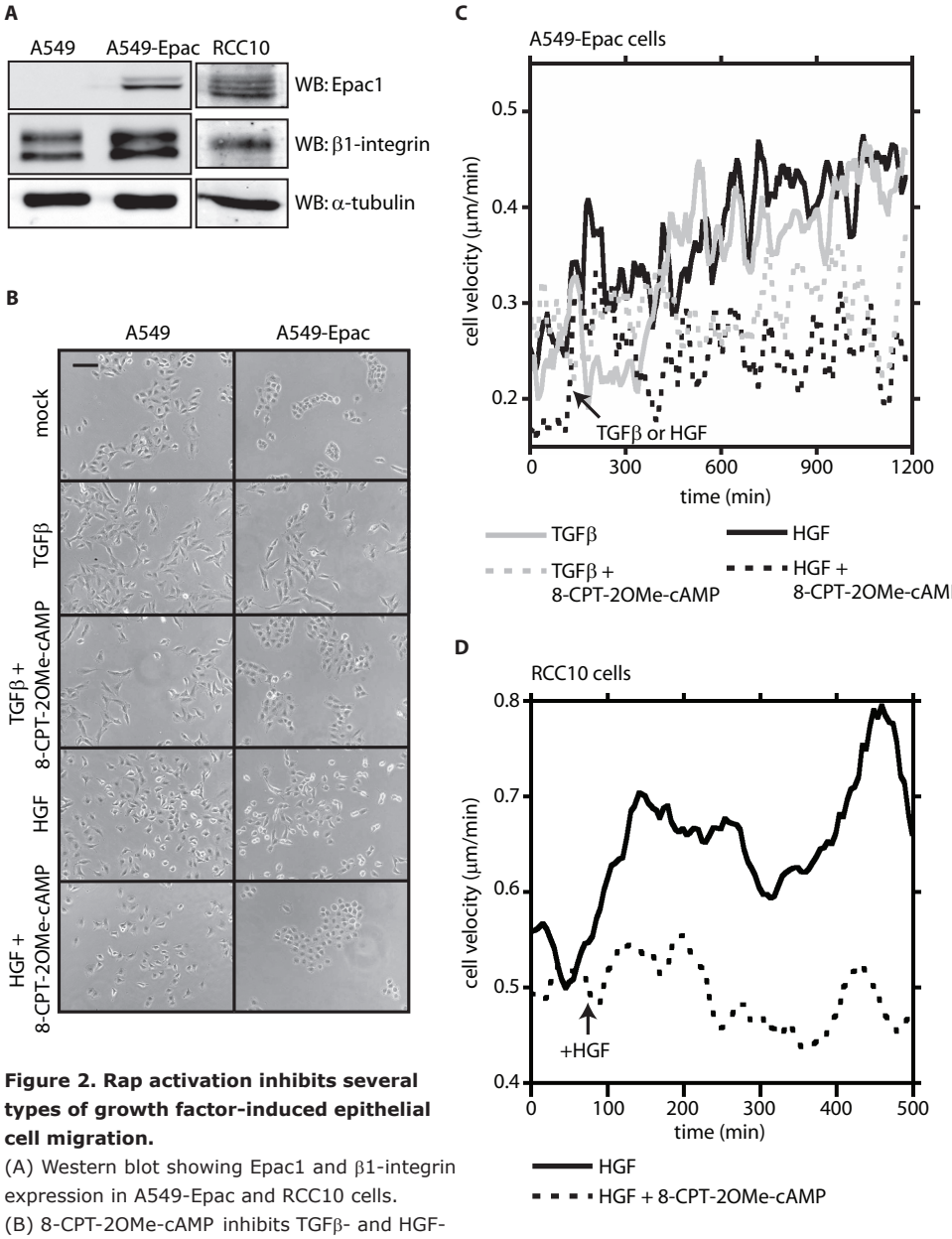


Figure 2. Rap activation inhibits several types of growth factor-induced epithelial cell migration.

(A) Western blot showing Epac1 and β1-integrin expression in A549-Epac and RCC10 cells.

(B) 8-CPT-2OMe-cAMP inhibits TGFβ- and HGF-induced scattering of A549-Epac cells. Representative phase contrast images of A549-Epac cells showing the effect of 8-CPT-2OMe-cAMP on TGFβ- and HGF-induced cell scattering. Scale bar is 100 μm. Cells were stimulated with TGFβ or HGF for 24 hours, in the presence or absence of 8-CPT-2OMe-cAMP. (C) Velocity time-course showing the effect of 8-CPT-2OMe-cAMP on TGFβ- and HGF-induced A549-Epac cell migration. (D) Velocity time-course showing the inhibitory effect of 8-CPT-2OMe-cAMP on HGF-induced RCC10 cell migration. (E) Average cell velocity (± SD) at the plateau phase (1100-1200 minutes) of A549 cells and RCC10 cells (400-500 minutes) from each of the velocity time-courses in C and D, respectively.

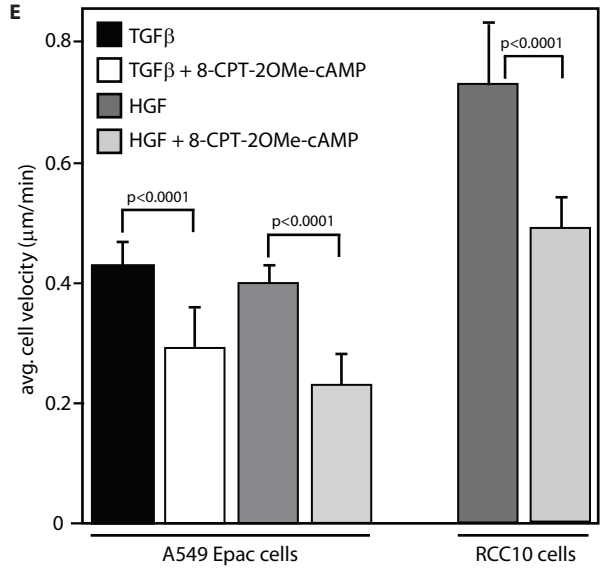


Figure 1C (dotted black line and Movie S3) shows that HGF-stimulated MDCK-GFP-Epac cells in low Ca^{2+}-containing medium achieved a similar maximum velocity of 1.2 μm/minute. Notably, these cells showed an earlier increase in cell velocity compared to MDCK-GFP-Epac cells cultured in high Ca^{2+} (Figure 1C, solid grey line), indicating that cell-cell junctions do need to be disrupted before the velocity increase is observed. In the presence of 8-CPT-2OMe-cAMP, MDCK-GFP-Epac cells in low Ca^{2+} showed no increase in velocity, similar to 8-CPT-2OMe-cAMP-treated cells in the presence of high Ca^{2+} (Figure 1C, dashed grey and dashed black lines, respectively and Movie S3). To corroborate this conclusion, the velocity increase of clearly non-contacted cells present in high Ca^{2+} conditions (usually less than 10% of all cells) was inhibited as well (Figure 1D, dashed black line). Comparison of maximum velocities and statistical analysis was performed as above and is depicted in figure 1E. These data demonstrate that Rap activation strongly inhibits epithelial cell migration even in the absence of functional adherens junctions.

Rap inhibits epithelial cell migration induced by several different growth factors

To investigate if the inhibitory effect of Rap activation on cell migration is restricted to HGF-induced motility, we used A549 cells that scatter in response to HGF or TGFβ, another notorious metastasis-promoting growth factor (Kasai et al., 2005) that disrupts cell-cell junctions by silencing the E-cadherin gene (Deckers et al., 2006). In analogy to MDCK cells, we constructed a cell line stably expressing Epac1 (A549-Epac, Figure 2A) to specifically activate endogenous Rap by 8-CPT-2OMe-cAMP. As shown in figure 2B, both HGF and TGFβ induce scattering in A549 and A549-Epac cells. In A549-Epac

cells, scattering is abolished by 8-CPT-2OMe-cAMP, whereas in parental A549 cells scattering is normal.
We used time-lapse phase-contrast microscopy to further characterize the scattering process and the effect of Rap activation on it in these cells (Movie S4) and measured cell migration to quantify this. HGF and TGFβ both induce cell migration (Figure 2C, solid black and grey lines, respectively), which is completely abolished by 8-CPT-2OMe-cAMP (Figure 2C, dotted black and grey lines, respectively). Quantification and statistical evaluation is shown in Figure 2E. Thus, Rap activation efficiently blocks scattering induced by two distinct signaling pathways, one of which results in a loss of E-cadherin expression (even in the presence of active Rap, see Figure 3F).

cAMP induction of endogenous Epac-Rap signaling inhibits epithelial cell migration

To investigate whether endogenous Epac-Rap signaling also blocks cell migration, we used renal cell carcinoma RCC10 cells. These cells are Von Hippel-Lindau-defective and express little to no E-cadherin (Esteban et al., 2006). RCC10 cells respond to HGF stimulation by activating ERK1/2 and express the β1 integrin required for migration on a collagen matrix, as well as Epac1 (Figure 2A and 3C). These cells showed a 1.5 fold increase in velocity upon HGF stimulation (Figure 2D, solid black line and Movie S5). The motogenic response of these cells was more rapid compared to MDCK cells, and cells achieved their maximal velocity after 1 hour of HGF stimulation. This probably reflects the absence of cell-cell junctions in these cells. Importantly, treating cells with 8-CPT-2OMe-cAMP to specifically activate Epac/Rap, completely inhibited the HGF-induced increase in cell motility (Figure 2D, dotted black line and Movie S5 and quantification in 2E). Together, these data show that cAMP-mediated activation of endogenous Rap via both exogenously- and endogenously-expressed Epac1 leads to the inhibition of epithelial cell scattering induced in different cell lines and by different growth factors. As this inhibition is independent of the level of E-cadherin expression and the presence of cell-cell junctions, this suggests that Rap has an inhibitory effect on the induction of cell migration itself.

8-CPT-2OMe-cAMP activates Rap, but does not interfere in growth factor signaling

One of the mechanisms via which Rap may interfere in growth factor-induced cell migration, is through direct inhibition of growth factor signaling. Rap pulldowns were used to confirm that 8-CPT-2OMe-cAMP activates Epac to induce Rap-GTP in Epac-expressing (endogenous or exogenous) cell lines. This activation of Rap was present for the complete duration of our time lapse experiments and was not affected by HGF or TGFβ stimulation (Figure 3A-C). Next, we examined if Epac1 expression or Rap activation with 8-CPT-2OMe-cAMP could suppress HGF- and TGFβ-

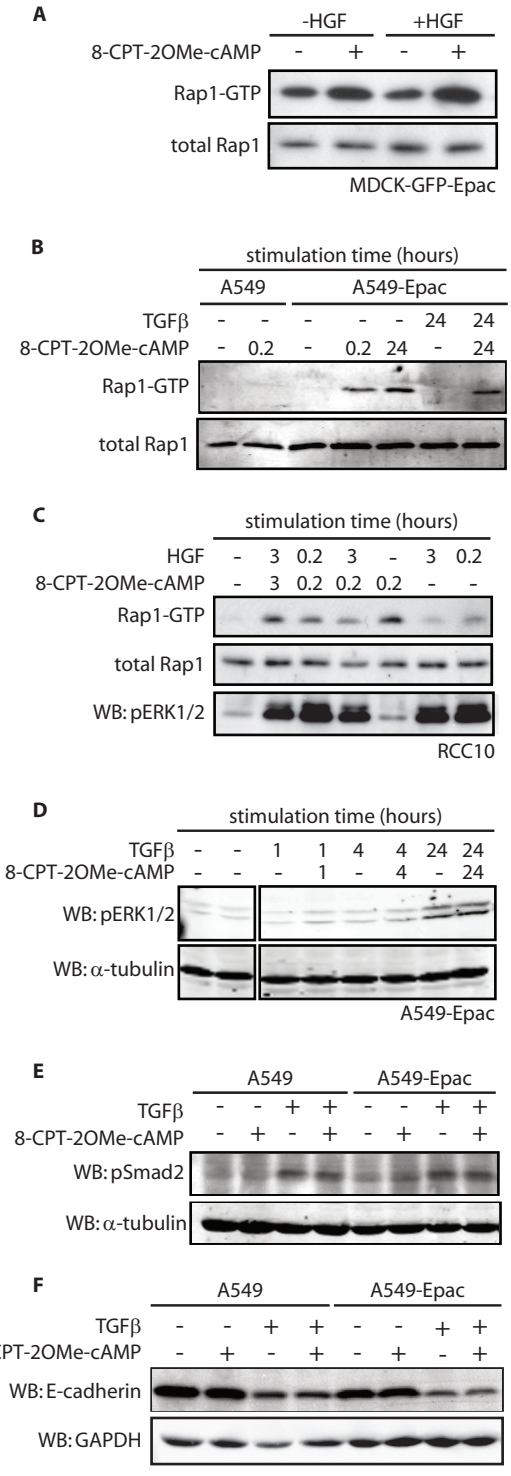


Figure 3. Rap activation does not interfere with growth factor signaling. (A) Rap is activated by 8-CPT-2OMe-cAMP in MDCK-GFP-Epac cells in the presence and absence of HGF. Cells were stimulated for 16 hours with HGF and/or 8-CPT-2OMe-cAMP. Blots shown are representative of at least 3 independent experiments. (B) Rap activation in parental and Epac-expressing A549 cells. Cells were treated for the indicated time periods with TGFβ and 8-CPT-2OMe-cAMP prior to performing a Rap-GTP pulldown assay. Blots shown are representative of at least 4 independent experiments. (C) Rap activation does not interfere with HGF-induced ERK activation and 8-CPT-2OMe-cAMP-induced Rap activity is not significantly affected by short- or long-term HGF stimulation in RCC10 cells. Cells were treated for the indicated time periods with HGF and/or 8-CPT-2OMe-cAMP prior to performing a Rap-GTP pulldown assay and probing of whole cell lysates for Erk activation. Blots shown are representative of 3 independent experiments. (D) Rap activation does not affect TGFβ-induced ERK activation in A549-Epac cells. Cells were incubated for 24 hours with the indicated stimuli. Whole cell lysates were probed with the appropriate antibodies as indicated to show activation and equal loading. Blots shown are representative of at least 3 independent experiments. (E) Epac expression and Rap activation do not affect TGFβ-induced phosphorylation of Smad2. Cells were incubated for 24 hours with the indicated stimuli. Whole cell lysates were probed with the appropriate antibodies as indicated to show activation and equal loading. Blots shown are representative of 3 independent experiments. (F) Rap activation does not restore TGFβ-induced downregulation of E-cadherin in A549-Epac cells. Cells were incubated for 24 hours with the indicated stimuli. Whole cell lysates were probed with the appropriate antibodies as indicated to show activation and equal loading. Blots shown are representative of at least 3 independent experiments.

mediated ERK activation (Kasai et al., 2005). In agreement with our previous experiments in MDCK cells (Price et al., 2004), activation of Rap in RCC10 cells does not interfere with HGF-induced ERK1/2 phosphorylation at any of the time-points investigated (Figure 3C). Rap activation also did not modulate the TGFβ-induced phosphorylation of ERK1/2 in A549-Epac cells (Figure 3D). Furthermore, neither exogenous Epac1 expression nor 8-CPT-2OMe-cAMP stimulation suppressed Smad2 phosphorylation after TGFβ addition (Figure 3E), as was reported in 293T cells (Conrotto et al., 2007). Finally, Rap activation did not affect the down-regulation of E-cadherin protein levels that is observed in the presence of TGFβ (Figure 3F). These data show that Rap activation does not interfere in the major growth factor receptor signaling events important for scattering, but rather acts to prevent the induction of migration at a more downstream level. For instance, Rap may directly inhibit the migration machinery.

Forced integrin activation does not mimic the inhibitory effects of Rap on cell migration

Besides the disruption of cell-cell adhesion (Thiery, 2002), cells also require modification of the integrin-mediated adhesion to the ECM (Danen, 2005) and engagement of the actomyosin-based migration machinery that induces polarized membrane protrusion (Horwitz and Webb, 2003) for efficient cell migration. To investigate whether the induction of integrin affinity downstream of Rap (Han et al., 2006; Katagiri et al., 2000), could explain the inhibition of cell migration, we examined the effect of integrin-activating antibodies on HGF-induced cell motility. These antibodies force the integrins into their high-affinity conformation and thus mimic inside-out integrin activation as induced by Rap. Because these antibodies recognize human, but not dog integrins, we used RCC10 and A549 cells and not MDCK cells. For RCC10 cells plated on collagen, which mainly binds to β1 integrins to mediate cell migration, we used the β1 integrin-activating antibody TS2/16 (van de Wiel-van Kemenade et al., 1992). For A549 cells plated on fibronectin (Fn), which can bind to β1 and β3 integrins to facilitate cell migration, we used a combination of TS2/16 and the β3 integrin-activating antibody LIBS6 (Frelinger et al., 1990; Huttenlocher et al., 1996). In short-term adhesion assays performed with RCC10 cells on collagen, TS2/16 and 8-CPT-2OMe-cAMP induce adhesion to a similar extent (Figure 4C), indicating that indeed β1 integrins are the main collagen receptors in these cells. In A459 cells on Fn, TS2/16 induces adhesion to a higher extent than 8-CPT-2OMe-cAMP, whereas LIBS6 results in a very small induction of adhesion (Figure 4F), indicating that β1 integrins are the main receptors for Fn in these cells. However, in migration experiments TS2/16 did not inhibit HGF-induced cell motility in RCC10 cells (Figure 4A, solid grey line), and TS2/16 + LIBS6 failed to inhibit HGF-induced migration in

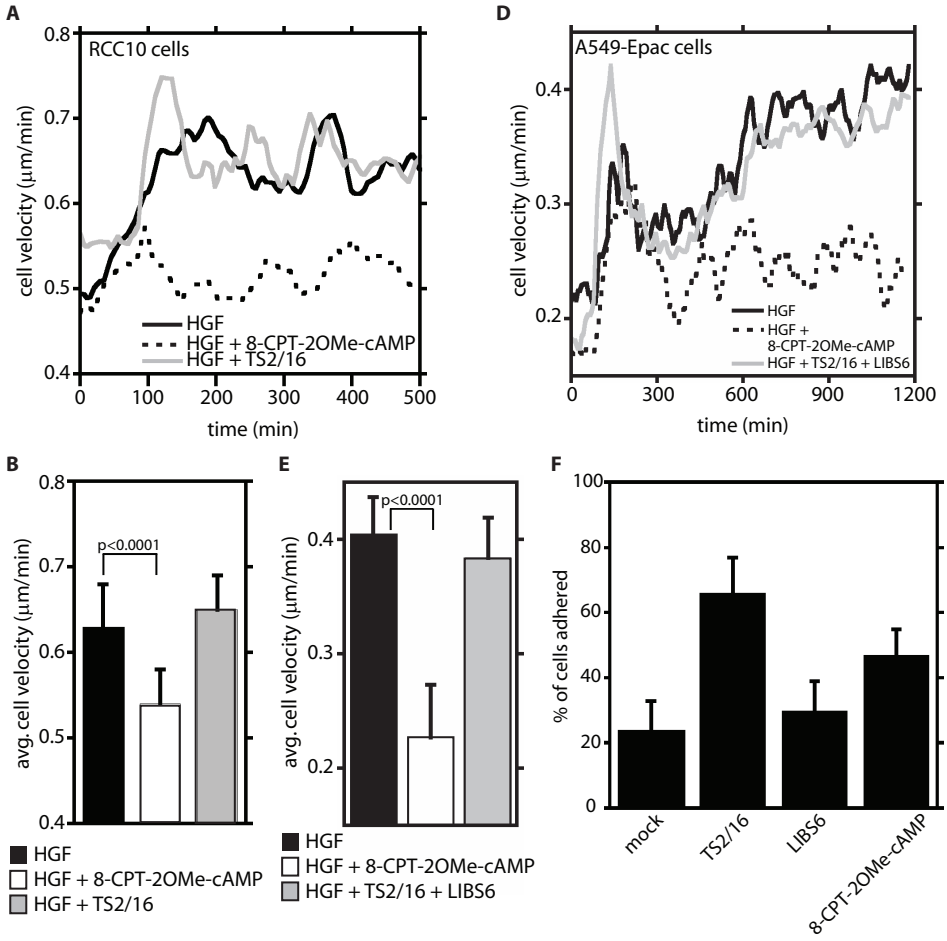


Figure 4. Forced integrin-activation by antibodies does not mimic the effect of Rap activation on cell migration.

(A) Velocity increase of RCC10 cells by HGF is inhibited by 8-CPT-2OMe-cAMP, but not TS2/16. In a live-cell phase-contrast time-lapse, 100 μM 8-CPT-2OMe-cAMP or 3 μg/ml TS2/16 were added at t=60 min. Velocity of approximately 300 automatically tracked cells from 3 independent time-lapse image series was measured. (B) Average cell velocity (± SD) of RCC10 cells at the plateau-phase (400-500 minutes) from the velocity time-course in A. (C) Adhesion time course showing comparable 8-CPT-2OMe-cAMP- and TS2/16-induced adhesion kinetics in RCC10 cells on 3 μg/ml collagen. Cells were plated in the absence or presence of 100 μM 8-CPT-2OMe-cAMP or 3 μg/ml TS2/16 and left to adhere for the indicated time points.

Figure 4, continued
Data are means ± SD; n = 4. (D) Velocity increase of A549-Epac cells by HGF is not inhibited by TS2/16 and LIBS6. HGF and antibodies (3 μg/ml TS2/16 and 3.7 μg/ml LIBS6) were added during imaging at t=120 min and velocity was determined by automated cell-tracking of approximately 300 cells from 3 independent time-lapse image series. (E) Average cell velocity (± SD) at the plateau-phase (1100-1200 minutes) of A549 cells from the velocity time-course in C. (F) TS2/16 and 8-CPT-2OMe-cAMP, but not LIBS6, induce adhesion of A549-Epac cells to fibronectin. A549-Epac cells were plated on 1 μg/ml fibronectin in the absence or presence of 100 μM 8-CPT-2OMe-cAMP or 3 μg/ml TS2/16 or 3.7 μg/ml LIBS6, allowed to adhere for 30 min, washed and quantified. Data are means ± SD; n = 4.

A459 cells (Figure 4D, solid grey line). Quantification of plateau-velocity and statistical evaluation is shown in Figure 4B (RCC10 cells, 400-500 min) and 4E (A549 cells, 1100-1200 min). This shows that simply inducing integrin activation does not inhibit HGF-induced motility and indicates that a different effect downstream of Rap is involved.

Rap activation inhibits focal adhesion turnover and front-rear polarity in contrast to forced β1-integrin activation

Because just simple integrin activation and ECM adhesion does not seem to be enough to inhibit migration, we focused on focal adhesions (FAs), to investigate if Rap might affect integrin signaling downstream of the integrin-ECM connection. FAs regulate cell motility by connecting the cytoskeleton to the ECM (Burridge and Chrzanowska-Wodnicka, 1996). To determine if Rap activation modulates these structures in epithelial cells, we examined the effect of 8-CPT-2OMe-cAMP on FA morphology in MDCK-Epac cells (MDCK cells stably expressing un-tagged Epac1 (Price et al., 2004)) plated on collagen and stained for paxillin, a major component of FAs. Three hours after plating, MDCK-Epac cells (in the absence or presence of 8-CPT-2OMe-cAMP) were adhered and spread onto the collagen-coated glass coverslip. In the presence of 8-CPT-2OMe-cAMP, FAs were larger and more elongated and paxillin staining was more intense (Figure 5A). The same effect on focal adhesion area was observed in A549-Epac and RCC10 cells (data not shown). Measuring the area of paxillin intensity that clearly surmounted background fluorescence, revealed a 1.3-fold increase in the relative focal adhesion area in 8-CPT-2OMe-cAMP-treated compared to untreated cells (Figure 5B, $p < 0.0001$). These data show that Rap activation enhances FAs in MDCK-Epac cells.

Next, we examined the dynamic behavior of FAs in migrating RCC10 cells. For these studies we used RCC10 cells because they express endogenous Epac, exhibit a rapid response to HGF and do not form tightly clustered cell colonies. RCC10 cells were transfected with GFP-paxillin, plated and imaged using widefield fluorescence microscopy. Cells transfected with GFP-paxillin appeared indistinguishable from untransfected cells in phase-contrast images demonstrating that exogenous GFP-paxillin expression does not

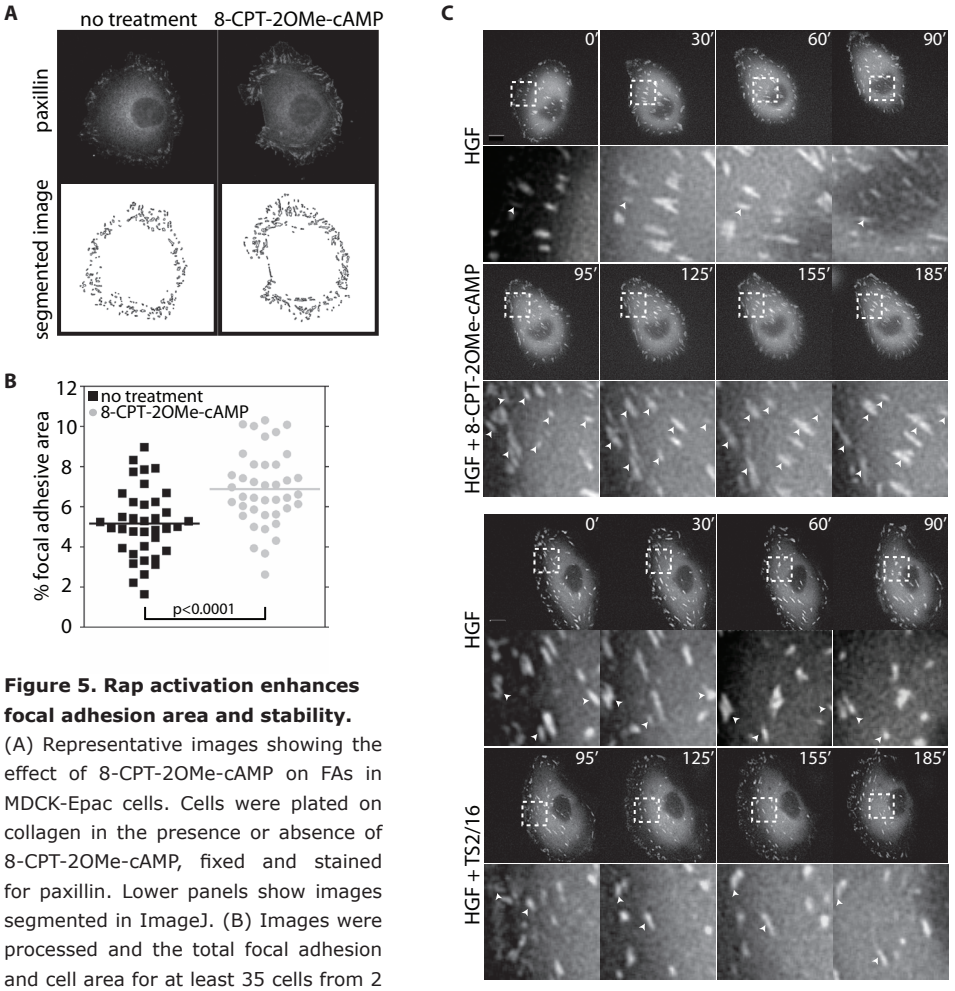


Figure 5. Rap activation enhances focal adhesion area and stability.
(A) Representative images showing the effect of 8-CPT-2OMe-cAMP on FAs in MDCK-Epac cells. Cells were plated on collagen in the presence or absence of 8-CPT-2OMe-cAMP, fixed and stained for paxillin. Lower panels show images segmented in ImageJ. (B) Images were processed and the total focal adhesion and cell area for at least 35 cells from 2 independent experiments were quantified in ImageJ as described in Materials and Methods. (C) Representative images from timelapse series showing the effect of 8-CPT-2OMe-cAMP and TS2/16 on the stability of GFP-paxillin-labeled FAs in HGF-treated RCC10 cells. A 7.6 μm x 7.6 μm area of the cell is highlighted (white dashed box), and white arrowheads show FAs that persist for more than 60 minutes within this area. Scale bar is 5 μm. (D) Focal adhesion persistence analysis was performed in ImageJ as described in the Materials and Methods for eight cells (~200 FAs tracked per condition) and the data are expressed as the mean ± SD.

significantly alter the morphology of RCC10 cells (data not shown). In the presence of HGF, we observed small FAs forming at the leading edge of protrusions, and larger FAs in the body and trailing edge of the cell (Movie S6 and Figure 5C (panels from 0 to 90 minutes)). In Figure 5C, a dashed grey box outlines a magnified region at the leading edge of the cell, and white arrowheads identify the FAs that persist for longer than 60 minutes. Upon stimulation with 8-CPT-2OMe-cAMP, cells rapidly altered their focal adhesion dynamics with fewer FAs forming and disappearing within the 90 minute time period (Figure 5C, panels from 95 minutes onwards and Movie S6), resulting in a loss of small leading edge adhesions and an increase in large central FAs. As a consequence, cells appeared to rapidly lose their distinct front/rear polarity and migration was inhibited, when stimulated with 8-CPT-2OMe-cAMP, but not with TS2/16.

The lifetime of individual FAs is highly variable in migrating cells. To quantify focal adhesion lifetime in a comprehensive manner, we randomly selected FAs in the first frame of the image sequence and determined how long these persisted. Cells treated with HGF showed a nearly equal percentage of FAs that persisted for a short time (less than 15 minutes), intermediate times (16-60 minutes) and a very long time (>61 minutes) (Figure 5D). When Rap was activated in these cells, the percentage of FAs lasting for more than 61 minutes was increased 2-fold (Figure 5D, grey and white bars, $p=0.002$), whereas we observed a concomitant 2-fold decrease in the percentage of FAs lasting 15 minutes or shorter ($p=0.04$). TS2/16 treatment did not mimic the effect of 8-CPT-2OMe-cAMP, as it did not result in any increase in the percentage of long-lasting FAs, but rather in a 1.5-fold increase of small, short-lived FAs (Figure 5C and D, grey and black bars, $p=0.06$). Furthermore, the presence of TS2/16 enhanced the polarized phenotype of these cells (as judged by the asymmetric distribution of small and large FAs).

In conclusion, Rap activation reduces FA dynamics and results in the loss of front rear polarity. This response is very different from the response to treatment with an integrin-activating antibody, which shows increased FA dynamics and no loss of cell-polarity.

Rap activation reduces lamellipodial activity

In RCC10 GFP-paxillin time-lapses, we noted a decrease in the number of leading edge protrusions after Rap activation (Figure 6A, arrowheads). To clearly determine whether Rap activation indeed affects HGF-induced protrusion and retraction, we examined the membrane dynamics of RCC10 cells in the presence and absence of 8-CPT-2OMe-cAMP. RCC10 cells were transiently transfected with the membrane marker GFP-CAAX (von der Hardt et al., 2007), plated on collagen-coated glass-bottom dishes overnight and imaged using widefield fluorescence microscopy. In the presence of HGF, RCC10 cells formed approximately equivalent and polarized areas of net protrusion

and retraction (Figure 6B, solid black areas and Movie S7). The total protrusion and retraction area was markedly reduced when Rap was activated with 8-CPT-2OMe-cAMP in HGF-treated cells (Figure 6B), but remained in cells treated with TS2/16. Furthermore, protrusion and retraction were not polarized in 8-CPT-2OMe-cAMP-treated cells as no persistence in any direction was observed. These differences were not due to toxic or otherwise disturbing effects of the expression of GFP-CAAX as expression was similar in HGF-only and HGF+8-CPT-2OMe-cAMP

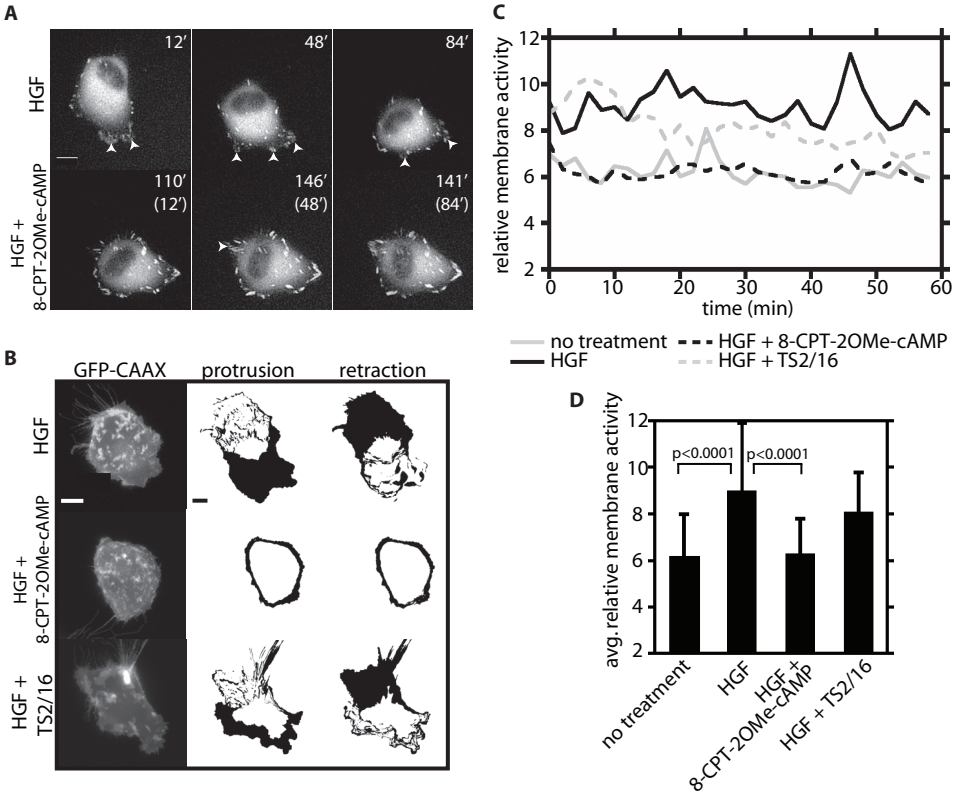


Figure 6. Rap activation inhibits HGF-induced membrane protrusions.
(A) 8-CPT-2OMe-cAMP decreases the number of protrusions in RCC10 cells treated with HGF. The arrowheads indicate newly formed protrusions in GFP-paxillin expressing RCC10 cells in each timelapse frame. (B) Rap activation reduces the HGF-induced increase in membrane protrusion and retraction area. Representative images of RCC10 cells transiently expressing GFP-CAAX and their corresponding total protrusion and retraction area (black areas) over 60 minutes after treatment with HGF alone, HGF + 8-CPT-2OMe-cAMP, and HGF + TS2/16. The difference in membrane area between sequential timelapse images was determined in ImageJ as described in Materials and Methods. Scale bar is 5 μm. (C) Timecourse showing the difference in membrane area relative to the total area of the cell, due to protrusion and retraction, in sequential frames of 60 minutes timelapses. The data are the averages from 9, 27, 18, and 19 cells for mock-, HGF-, HGF + 8-CPT-2OMe-cAMP-, and HGF + TS2/16-treated cells, respectively. (D) Average membrane activity (± SD) of RCC10 cells during the time-course in C.

or HGF+TS2/16-treated cells used for this analysis (as determined by fluorescence intensity). Furthermore, HGF-only treated cells that expressed GFP-CAAX migrated indistinguishable from untransfected RCC10 cells (data not shown).

To quantify the protrusive activity, we divided the area of leading-edge extension in consecutive frames by the total cell area during the 60 minutes of the experiment (Figure 6C). The average membrane activity of HGF-treated cells was 1.4-fold higher than basal activity (Figure 6C, black and grey lines, respectively, and 6D). The membrane activity was reduced 1.4-fold when Rap was activated with 8-CPT-2OMe-cAMP (Figure 6C, black dashed line and 6D). Addition of TS2/16 antibody only resulted in a small difference in protrusive activity (Figure 6B and C, grey dashed line, and 6D) and polarization was fully maintained. Clearly, forced integrin activation by TS2/16 did not mimic the inhibitory effect of Rap activation. These data demonstrate that Rap activation blocks the HGF-induced increase in polarized membrane protrusion and retraction.

Discussion

For cells to migrate efficiently, both cell-cell junctions and cell-ECM interactions need to be regulated and tension within the actomyosin cytoskeleton needs to be induced (Danen, 2005; Horwitz and Webb, 2003; Thiery, 2002). Polarized membrane protrusion and efficient turnover of focal adhesions are also required for efficient cell migration (Webb et al., 2002). As the small GTPase Rap is a known regulator of cell junctions and integrin-mediated adhesion (Bos, 2005) and has been suggested to be involved in cell migration (Ohba et al., 2001), we investigated how Rap interferes in epithelial cell migration. Surprisingly, neither the stabilization of cell-cell junctions nor the activation of integrins could account for this effect on migration. Major receptor signaling pathways like ERK and Smad (unlike previously reported (Conrotto et al., 2007)), are also not affected, indicating that signaling through Epac/Rap does not interfere in HGF or TGFβ signaling, but rather acts downstream to block the induction of cell migration by these transforming growth factors.

During migration on the ECM, integrin-based FAs provide linkage between the cytoskeleton and the ECM to transmit forces, sense the environment and initiate intracellular signaling cascades (Burridge and Chrzanowska-Wodnicka, 1996). Regulated, efficient formation and turnover of FAs is required for optimal cell migration. Small adhesions at the leading edge turn over rapidly to facilitate protrusion or mature into larger FAs that provide the tension for the disassembly of adhesions at the rear of the cell (Webb et al., 2002; Zaidel-Bar et al., 2003).

We examined the effect of Rap activation on FAs using paxillin as a marker (Webb et al., 2003) and found a rapid alteration of focal adhesion dynamics. After Rap was activated, fewer FAs formed at the leading

edge and focal adhesion disassembly was inhibited, resulting in an overall increase in FA persistence and an apparent loss of the polarized morphology that is characteristic of migrating cells. One explanation for the observed effects on focal adhesion complexes is an increase in integrin-mediated cell adhesion. However, the β1-integrin-activating antibody TS2/16 did not affect FA dynamics in the same way as Rap activation, although both induced a similar level of adhesion. This suggests that increased integrin affinity per se is not causing the inhibition of cell migration. In accordance with these observations, Huttenlocher et al. showed that forcing integrins into a high affinity state using antibodies does not influence FA morphology and does not strongly inhibit cell migration, but shifts the optimal migration conditions to a lower concentration of ECM substrate. In contrast, mutant integrins that have lost proper regulation of their linkage to the cytoskeleton and exhibit forced cytoskeletal linkage, show strongly enhanced FAs and a severely impaired capacity to migrate (Huttenlocher et al., 1996). We observe similar effects when Rap is activated in epithelial cells that are treated with growth factors. Our results, therefore, suggest that Rap has an effect on the integrin-cytoskeletal linkage and that this effect is more important for the inhibition of cell migration than the effect of Rap on integrin affinity. Whether this is achieved by the same molecules that mediate the affinity modulation (Riam and Talin (Han et al., 2006)), remains to be shown. Thus far we could not detect endogenous Riam or Riam-GFP in FAs in the cells that we used for our experiments. In addition to stabilizing FAs, Rap inhibits the formation of polarized membrane protrusions during migration. This process is driven by actin polymerization (the motor), but also depends on a regulation of the integrin-cytoskeletal linkage (the clutch) as has been illustrated by two recent papers (Brown et al., 2006; Hu et al., 2007). Several reports have indicated that Rap may influence actin polymerization, the driving force behind lamellipodial protrusion. For instance, Rap has strong stabilizing effects on cortical actin in endothelial cells (Cullere et al., 2005; Kooistra et al., 2005) and the Rap interacting protein Riam was shown to increase the amount of filamentous actin, presumably through its interaction with profillin (Lafuente et al., 2004). Furthermore, Rap interacts with several RacGEFs, known regulators of actin polymerization, to increase cell spreading (Arthur et al., 2004), although it has also been reported that Rap can function as an antagonist of Rac signaling (Valles et al., 2004). Because we also observe an effect on FA dynamics, the effect of endogenous Rap activity on lamellipodial protrusion could be explained by a stabilization of the connection between the integrins and the cytoskeleton. In other words, the clutch is engaged too long, leading to a loss of productive protrusion. Further studies are required to determine whether Rap regulates the connection between the actin cytoskeleton and

integrins and to identify the molecular mechanism that mediates the Rap-induced inhibition of cell migration. Finally, contractile tension generated within the actomyosin cytoskeleton is also required for efficient migration. Increased actomyosin contraction results in increased FA size (Burridge and Chrzanowska-Wodnicka, 1996) and dynamic regulation of myosin is critical to efficient protrusion (Gupton and Waterman-Storer, 2006). An alternative explanation for the effects on FA size and dynamics and for the effects on protrusion could be the recent finding that Rap1 regulates myosin in *Dictyostelium* (Jeon et al., 2007). However, we could not detect any effects of Rap activation on myosin light chain phosphorylation downstream of HGF or TGFβ, arguing against this possibility. Also, the Rap1 effector that mediates the myosin induction by Rap1 is not conserved between *Dictyostelium* and mammals.

Conclusion

We conclude that activation of endogenous Rap leads to an inhibition of growth factor-induced epithelial cell migration by targeting the basal migration machinery. This effect is independent of E-cadherin stabilization and cannot be explained solely by affinity modulation of β1-integrins. Rap inhibits epithelial cell migration through the stabilization of focal adhesions and the inhibition of membrane protrusion, possibly by stabilizing the connection between the actin cytoskeleton and integrins.

Acknowledgements

We thank members of our labs for comments and discussion, Livio Kleij for assistance with live cell imaging experiments, dr. Jeroen Bakkers for providing the GFP-CAAX construct and dr. Mark Ginsberg for pEGFP-paxillin. We thank dr. Jan Willem Akkerman for sparing precious LIBS6 antibody, which originated from dr. Mark Ginsberg, whom we thank for it as well. We thank Arnoud Sonnenberg for support, suggestions and critical reading of the manuscript. This work was supported by a Long-Term Fellowship from the Human Frontier Science Program (KSL), and grants from the Dutch Cancer Society (KWF Kankerbestrijding) to JHR (2003–2956) and JdR (2006-3714).

References

Arthur, W.T., Quilliam, L.A. and Cooper, J.A. (2004) Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. *J. Cell. Biol.*, **167**, 111-122.

Bos, J.L. (2005) Linking Rap to cell adhesion. *Curr Opin Cell Biol*, **17**, 123-128.

Brown, C.M., Hebert, B., Kolin, D.L., Zareno, J., Whitmore, L., Horwitz, A.R. and Wiseman, P.W. (2006) Probing the integrin-actin linkage using high-resolution protein velocity mapping. *J Cell Sci*, **119**, 5204-5214.

Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol*, **12**, 463-518.

Caron, E. (2003) Cellular functions of the Rap1 GTP-binding protein: a pattern emerges. *J Cell Sci*, **116**, 435-440.

Caron, E., Self, A.J. and Hall, A. (2000) The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators. *Curr Biol*, **10**, 974-978.

Christensen, A.E., Selheim, F., de Rooij, J.,

Dremier, S., Schwede, F., Dao, K.K., Martinez, A., Maenhaut, C., Bos, J.L., Genieser, H.G. and Doskeland, S.O. (2003) cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J Biol Chem*, **278**, 35394-35402.

Conrotto, P., Yakymovych, I., Yakymovych, M. and Souchelnytskyi, S. (2007) Interactome of transforming growth factor-beta type I receptor (TbetaRI): inhibition of TGFbeta signaling by Epac1. *J Proteome Res*, **6**, 287-297.

Cullere, X., Shaw, S.K., Andersson, L., Hirahashi, J., Luscinskas, F.W. and Mayadas, T.N. (2005) Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. *Blood*, **105**, 1950-1955.

Danen, E.H. (2005) Integrins: regulators of tissue function and cancer progression. *Curr Pharm Des*, **11**, 881-891.

de Bruyn, K.M., Rangarajan, S., Reedquist, K.A., Figdor, C.G. and Bos, J.L. (2002) The small GTPase Rap1 is required for Mn(2+)- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion. *J Biol Chem*, **277**, 29468-29476.

de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M.A. and Waterman-Storer, C.M. (2005) Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol*, **171**, 153-164.

de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A. and Bos, J.L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*, **396**, 474-477.

Deckers, M., van Dinther, M., Buijs, J., Que, I., Lowik, C., van der Pluijm, G. and ten Dijke, P. (2006) The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. *Cancer Res*, **66**, 2202-2209.

Dormond, O. and Ruegg, C. (2003) Regulation of endothelial cell integrin function and angiogenesis by COX-2, cAMP and Protein Kinase A. *Thromb Haemost*, **90**, 577-585.

Edin, M.L., Howe, A.K. and Juliano, R.L. (2001) Inhibition of PKA blocks fibroblast migration in response to growth factors. *Exp Cell Res*, **270**, 214-222.

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Doskeland, S.O., Blank, J.L. and Bos, J.L. (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat. Cell. Biol.*, **4**, 901-906.

Esteban, M.A., Tran, M.G., Harten, S.K., Hill, P., Castellanos, M.C., Chandra, A., Raval, R., O'Brien T, S. and Maxwell, P.H. (2006) Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer Res*, **66**, 3567-3575.

Franke, B., Akkerman, J.W. and Bos, J.L. (1997) Rapid Ca2+-mediated activation of Rap1 in human platelets. *Embo J*, **16**, 252-259.

Frelinger, A.L., 3rd, Cohen, I., Plow, E.F., Smith, M.A., Roberts, J., Lam, S.C. and Ginsberg, M.H. (1990) Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J Biol Chem*, **265**, 6346-6352.

Glading, A., Han, J., Stockton, R.A. and Ginsberg, M.H. (2007) KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. *J Cell Biol*, **179**, 247-254.

Gupton, S.L. and Waterman-Storer, C.M. (2006) Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. *Cell*, **125**, 1361-1374.

Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J. and Ginsberg, M.H. (2006) Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol*, **16**, 1796-1806.

Hogan, C., Serpente, N., Cogram, P., Hosking, C.R., Bialucha, C.U., Feller, S.M., Braga, V.M., Birchmeier, W. and Fujita, Y. (2004) Rap1 regulates the formation of E-cadherin-based cell-cell contacts. *Mol Cell Biol*, **24**, 6690-6700.

Horwitz, R. and Webb, D. (2003) Cell migration. *Curr Biol*, **13**, R756-759.

Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T. and Takai, Y. (2005) Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. *J Biol Chem*, **280**, 24095-24103.

Howe, A.K. (2004) Regulation of actin-based cell migration by cAMP/PKA. *Biochim Biophys Acta*, **1692**, 159-174.

Hu, K., Ji, L., Applegate, K.T., Danuser, G. and Waterman-Storer, C.M. (2007) Differential transmission of actin motion within focal adhesions. *Science*, **315**, 111-115.

Huttenlocher, A., Ginsberg, M.H. and Horwitz, A.F. (1996) Modulation of cell migration by integrin-mediated cytoskeletal linkages and ligand-binding affinity. *J Cell Biol*, **134**, 1551-1562.

Jeon, T.J., Lee, D.J., Merlot, S., Weeks, G. and Firtel, R.A. (2007) Rap1 controls cell adhesion and cell motility through the regulation of myosin II. *J Cell Biol*, **176**, 1021-1033.

Kasai, H., Allen, J.T., Mason, R.M., Kamimura, T. and Zhang, Z. (2005) TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res*, **6**, 56.

Katagiri, K., Hattori, M., Minato, N., Irie, S., Takatsu, K. and Kinashi, T. (2000) Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase. *Mol Cell Biol*, **20**, 1956-1969.

Katagiri, K., Imamura, M. and Kinashi, T. (2006) Spatiotemporal regulation of the kinase Mst1 by binding protein RAPL is critical for lymphocyte polarity and adhesion. *Nat Immunol*, **7**, 919-928.

Katagiri, K., Maeda, A., Shimonaka, M. and Kinashi, T. (2003) RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nat Immunol*, **4**, 741-748.

Kim, S., Harris, M. and Varner, J.A. (2000) Regulation of integrin alpha vbeta 3-mediated endothelial cell migration and angiogenesis by integrin alpha5beta1 and protein kinase A. *J Biol Chem*, **275**, 33920-33928.

Knox, A.L. and Brown, N.H. (2002) Rap1 GTPase regulation of adherens junction positioning and cell adhesion. *Science*, **295**, 1285-1288.

Kooistra, M.R., Corada, M., Dejana, E. and Bos, J.L. (2005) Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. *FEBS Lett*, **579**, 4966-4972.

Kooistra, M.R., Dube, N. and Bos, J.L. (2007) Rap1: a key regulator in cell-cell junction formation. *J Cell Sci*, **120**, 17-22.

Lafuente, E.M., van Puijenbroek, A.A., Krause, M., Carman, C.V., Freeman, G.J., Berezovskaya, A., Constantine, E., Springer, T.A., Gertler, F.B. and Boussiotis, V.A. (2004) RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. *Dev. Cell*, **7**, 585-595.

Lorenowicz, M.J., Fernandez-Borja, M. and Hordijk, P.L. (2007) cAMP signaling in leukocyte transendothelial migration. *Arterioscler Thromb Vasc Biol*, **27**, 1014-1022.

Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B.J., Maki, K., Miyazaki, J. and Matsuda, M. (2001) Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. *Embo J*, **20**, 3333-3341.

Price, L.S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F.J., Collard, J.G. and Bos, J.L. (2004) Rap1 regulates E-cadherin-mediated cell-cell adhesion. *J Biol Chem*, **279**, 35127-35132.

Reedquist, K.A., Ross, E., Koop, E.A., Wolthuis, R.M., Zwartkruis, F.J., van Kooyk, Y., Salmon, M., Buckley, C.D. and Bos, J.L. (2000) The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *J Cell Biol*, **148**, 1151-1158.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T. and Horwitz, A.R. (2003) Cell migration: integrating signals from front to back. *Science*, **302**, 1704-1709.

Schwartz, M.A. and Denninghoff, K. (1994) Alpha v integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they play a minor role in adhesion. *J Biol Chem*, **269**, 11133-11137.

Sheetz, M.P., Felsenfeld, D.P. and Galbraith,

C.G. (1998) Cell migration: regulation of force on extracellular-matrix-integrin complexes. *Trends Cell Biol*, **8**, 51-54.

Stoker, M., Gherardi, E., Perryman, M. and Gray, J. (1987) Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature*, **327**, 239-242.

Thiery, J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*, **2**, 442-454.

Thiery, J.P. and Sleeman, J.P. (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol*, **7**, 131-142.

Valles, A.M., Beuvin, M. and Boyer, B. (2004) Activation of Rac1 by paxillin-Crk-DOCK180 signaling complex is antagonized by Rap1 in migrating NBT-II cells. *J Biol Chem*, **279**, 44490-44496.

van de Wiel-van Kemenade, E., van Kooyk, Y., de Boer, A.J., Huijbens, R.J., Weder, P., van de Kasteele, W., Melief, C.J. and Figdor, C.G. (1992) Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the beta subunit of VLA. *J Cell Biol*, **117**, 461-470.

von der Hardt, S., Bakkers, J., Inbal, A., Carvalho, L., Solnica-Krezel, L., Heisenberg, C.P. and Hammerschmidt, M. (2007) The Bmp gradient of the zebrafish gastrula guides migrating lateral cells by regulating cell-cell adhesion. *Curr Biol*, **17**, 475-487.

Webb, D.J., Brown, C.M. and Horwitz, A.F. (2003) Illuminating adhesion complexes in migrating cells: moving toward a bright future. *Curr Opin Cell Biol*, **15**, 614-620.

Webb, D.J., Parsons, J.T. and Horwitz, A.F. (2002) Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat Cell Biol*, **4**, E97-100.

Wittchen, E.S., van Buul, J.D., Burridge, K. and Worthylake, R.A. (2005) Trading spaces: Rap, Rac, and Rho as architects of transendothelial migration. *Curr Opin Hematol*, **12**, 14-21.

Zaidel-Bar, R., Ballestrem, C., Kam, Z. and Geiger, B. (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci*, **116**, 4605-4613.

Zamir, E. and Geiger, B. (2001) Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci*, **114**, 3583-3590.

Zhang, A., Dong, Z. and Yang, T. (2006) Prostaglandin D2 inhibits TGF-beta1-induced epithelial-to-mesenchymal transition in MDCK cells. *Am J Physiol Renal Physiol*, **291**, F1332-1342.

Supplementary information

Supplemental movies can be found online at doi:10.1016/j.cellsig.2008.01.018

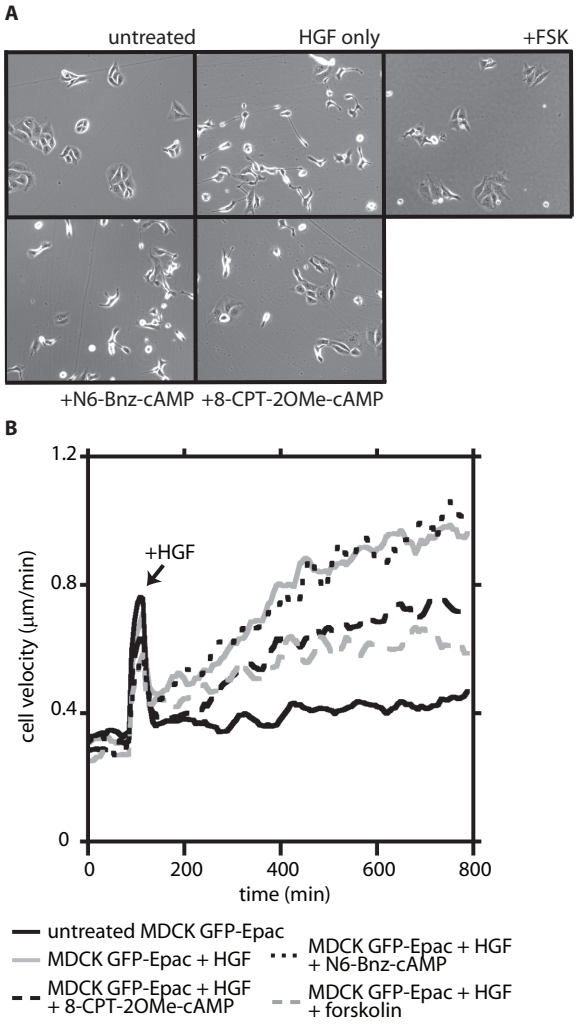
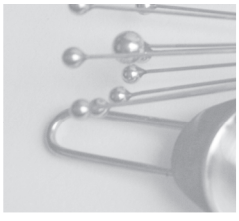


Figure S1. Selective PKA activation does not inhibit HGF-induced MDCK cell scattering. (A) Representative images from phase contrast timelapse image series showing that 10 μM forskolin and 100 μM 8-CPT-2OMe-cAMP inhibit cell scattering, but 300 μM of the PKA selective analog N6-Bnz-cAMP does not. (B) Velocity time-course to quantify the effect of forskolin and cAMP analogues on HGF-induced migration in MDCK-GFP-Epac cells. (C) Forskolin and N6-Bnz-cAMP activate PKA in the presence of HGF as assessed by VASP phosphorylation. MDCK-GFP-Epac cells were pretreated for 90 minutes with the indicated analogs or forskolin before incubation with HGF for 2 hrs. Whole cell lysates were probed with the appropriate antibodies as indicated to show activation of PKA and equal loading (n=3).

Addendum

Gene expression profiling of A549-Epac cells treated with 007, TGFβ or both

Judith H. Raaijmakers, Marian J.A. Groot Koerkamp, Diane Bouwmeester, Dik van Leenen, Frank C. Holstege and Johannes L. Bos



Gene expression profiling of A549-Epac cells treated with 007, TGFβ or both

Judith H. Raaijmakers, Marian J.A. Groot Koerkamp, Diane Bouwmeester, Dik van Leenen, Frank C. Holstege and Johannes L. Bos

Department of Physiogical Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands

Abstract

In cell culture, A549-Epac-B14 cells grow in foci and do not migrate. When treated with TGFβ, these cells start to scatter and migration velocity is increased. We have previously shown that the activation of Epac results in a complete inhibition of TGFβ-induced scattering and migration. Here, we investigated the effect of activation of Epac on gene expression by microarray analysis. We compared mock stimulated cells to cells stimulated with TGFβ, the Epac activator 007, or both stimuli together and measured expression profiles after 24 hours. We note that although differences are observed, only minor changes in gene expression were apparent in all three conditions after 24 hours. This indicates that the modulation of gene expression is not a major consequence of the activation of Epac. The fact that no obvious TGFβ or EMT signature was apparent from these expression changes is surprising. This implies that, in these cells, the effect of TGFβ on gene expression is less than expected and that the induction of cell scattering and migration may not require dramatic changes in gene expression, like the down-regulation of E-cadherin. Together, we conclude for A549-B14 cells that the induction of cell scattering and migration by TGFβ and subsequent inhibition by 007 only have a limited effect on the gene expression profile.

Results and Discussion

Previously, we found that the activation of endogenous Rap1 through Epac by the cAMP analogue 8-CPT-2'OMe-cAMP inhibits growth factor-induced migration of several epithelial cell lines. We observed that this inhibition was instantaneous, thus the effect of 007 to inhibit migration is a direct one. However, since both integrins as well as Ras-like small GTPases can also have downstream transcriptional effects (Coleman et al., 2004; Hynes, 2002), we wondered whether the 007-induced migration inhibition has effects on gene expression as well, and how this gene expression profile is affected by treatment with TGFβ. Therefore, we isolated RNA from A549-Epac-B14 cells stimulated either mock for 24 hours or with 100 μM 007, 10 ng/ml TGFβ, or both. Treatment of A549-B14 cells with TGFβ results in a scattering response. Cells change their morphology, become more elongated and fibroblast-like, separate themselves from neighboring cells and exhibit increased migration velocities (Kasai et al., 2005; Lyle et al., 2008). When cells are treated with 007, they flatten due to increased cell adhesion and cell spreading. When

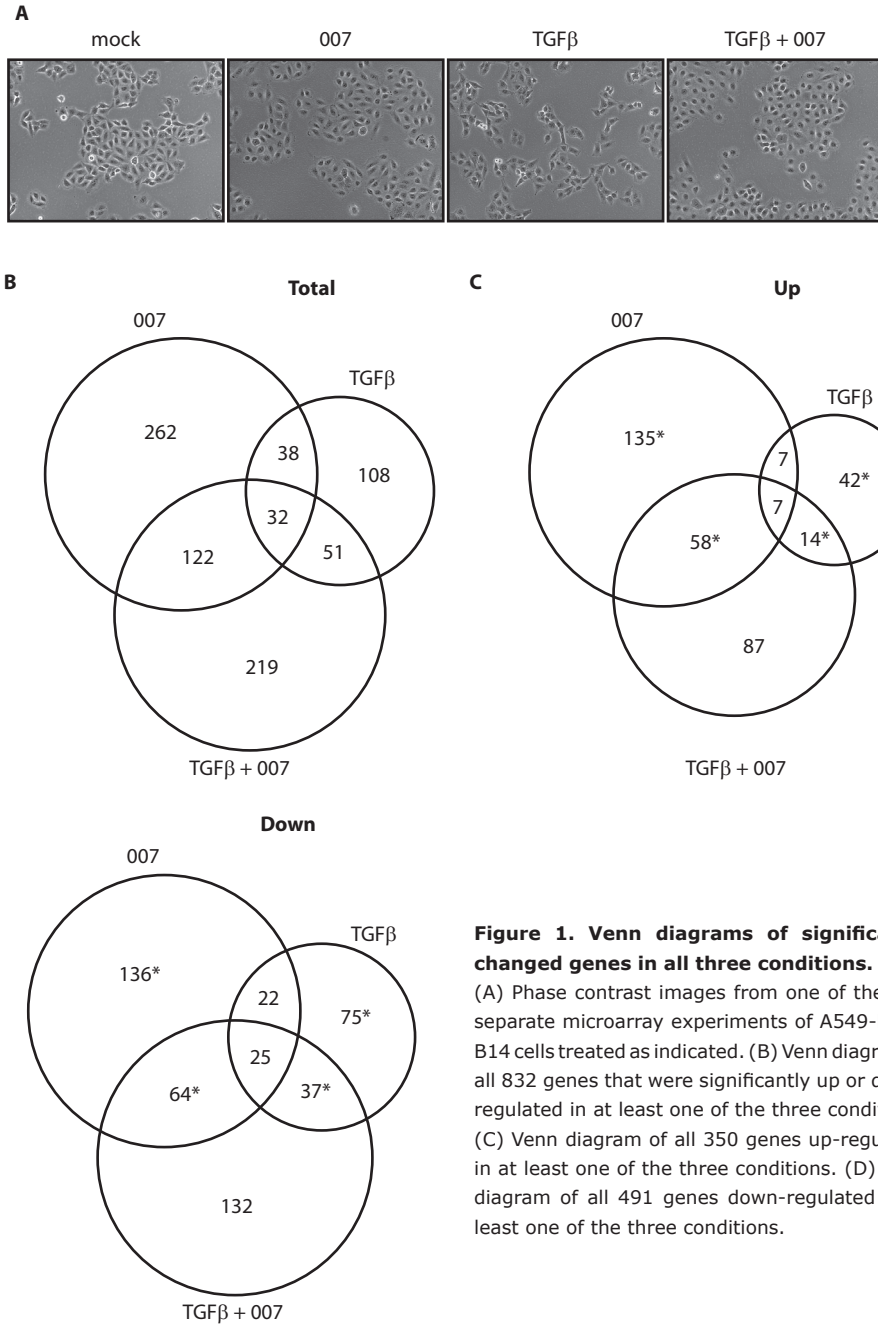


Figure 1. Venn diagrams of significantly changed genes in all three conditions.
(A) Phase contrast images from one of the four separate microarray experiments of A549-Epac-B14 cells treated as indicated. (B) Venn diagram of all 832 genes that were significantly up or down-regulated in at least one of the three conditions. (C) Venn diagram of all 350 genes up-regulated in at least one of the three conditions. (D) Venn diagram of all 491 genes down-regulated in at least one of the three conditions.

cells are treated with TGFβ + 007, the cells fail to migrate and scatter, but do spread and flatten out (Figure 1A and Chapter 3 of this thesis (Lyle et al., 2008)).

For the microarray experiment, RNA amplifications and labeling were performed as described in Roepman et al. (2005). Labeled cRNA of each of the three conditions was mixed with alternatively labeled mock cRNA and hybridized on microarrays (Human Array-Ready Oligo set (version 2.0; Qiagen) printed on Codelink activated slides (GE Healthcare)). At the same time, dye swap experiments were performed. Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7). Genes with $p < 0.05$ after family wise error correction were considered significantly changed. In total, 832 genes are significantly up- or down-regulated in this experiment, in one or more of the three conditions (Figure 1B-D).

007-induced gene expression

First, we inspected which genes are up- or down-regulated by 007 (Figures 1C and D and Table 1). Strikingly, with 007, more genes are significantly changed than with TGFβ, or TGFβ + 007. However, the induced fold changes are rather low (Table 1). This indicates that 007 has a limited effect on gene expression. Table 1 shows the ten genes most up- or down-regulated after 007 treatment. Interesting examples in Table 1 are the down-regulation of Rap1GAP1, a negative regulator of Rap1 signaling, and the up-regulation of Ezrin, an anchor for Epac (Gloerich et al., manuscript in preparation). This suggests that there may be positive feedback mechanisms in place to sustain 007 signaling.

To see whether different biological processes are affected in cells treated with 007, we performed GO searches (http://integromics.holstegelab.nl/, $p = 0.05$, mt = simulation) on all gene lists depicted as numbers with an asterisk in the Venn diagrams (Figures 1C-D). We used only genes that were at least 1.2 fold up- or down-regulated. Surprisingly, the GO terms cytoskeletal anchoring at the plasma membrane as well as cell-cell adhesion, were significantly enriched within the gene list of 45 genes up-regulated by only 007. The genes contributing to this enrichment are shown in Table 2. Interestingly, the aforementioned gene encoding for Ezrin appears in both lists. The most down-regulated genes by 007 also returned two GO terms, although these are more general ones: negative regulation of cellular and biological processes. The genes contributing to this enrichment are shown in Table 3.

TGFβ-induced gene expression

EMT is characterized by a loss of cell-cell adhesion and polarity, and the acquisition of a more mesenchymal, migratory phenotype. This is accomplished by the down-regulation of epithelial genes, such as genes encoding cadherins, claudins and other tight junction proteins and cytokeratins (Moreno-Bueno et al., 2008; Peinado et al., 2007; Zavadil and Bottinger, 2005). Markers that describe the induction of the mesenchymal phenotype are less specific, but the induction of EMT generally requires a shift in expression of integrin types, basal lamina components and the induction of matrix metalloproteinases, such as

Table 1. Top 10 genes most up- and most down-regulated in the only 007 condition.

Genes most up-regulated by 007 alone			
Accession number	*Gene symbol*	*Description*	*Fold change*
MI0003627	hsa-mir-614	hsa-mir-614	1.57
Q9P2E8	March4	E3 ubiquitin-protein ligase MARCH4	1.53
NP_653253	LETM2	leucine zipper-EF-hand containing transmembrane protein 2	1.49
P29279	CTGF	Connective tissue growth factor	1.47
Q9Y2J4	AMOTL2	Angiomotin-like protein 2	1.42
O75293	GADD45B	Growth arrest and DNA-damage-inducible protein GADD45 beta	1.41
P30530	AXL	Tyrosine-protein kinase receptor UFO	1.41
Q01804	OTUD4	OTU domain-containing protein 4	1.40
P12429	ANXA3	Annexin A3	1.39
Q99685	MGLL	Monoglyceride lipase	1.36
Genes most down-regulated by 007 alone			
Accession number	*Gene symbol*	*Description*	*Fold change*
P15428	HPGD	15-hydroxyprostaglandin dehydrogenase	-1.74
Q07973	CYP24A1	Cytochrome P450 24A1	-1.56
O14503	BHLHB2	Class B basic helix-loop-helix protein 2	-1.46
O75911	DHRS3	Short-chain dehydrogenase/ reductase 3	-1.46
Q9UKB3	DNAJC12	DnaJ homolog subfamily C member 12	-1.44
Q96HC4	PDLIM5	PDZ and LIM domain protein 5	-1.42
P55011	SLC12A2	Solute carrier family 12 member 2	-1.40
Q02388	COL7A1	Collagen alpha-1(VII) chain	-1.39
Q16654	PDK4	Pyruvate dehydrogenase kinase isoform 4	-1.39
P47736	RAP1GAP	Rap1 GTPase-activating protein 1	-1.34

MMP2 and -9 (Peinado et al., 2007; Willis and Borok, 2007; Zavadil and Bottinger, 2005). In A549 cells, TGFβ-induced EMT is regulated by Smad2-dependent transcriptional regulation of genes encoding E-cadherin, fibronectin, collagen I and III (Kasai et al., 2005). Although the induction of scattering by TGFβ in A549-Epac-B14 cells is phenotypically

Table 2. Genes contributing to GO terms returned by gene list of up-regulated genes by 007 only.

Genes contributing to GO term: cytoskeletal anchoring at plasma membrane			
Accession number	*Gene symbol*	*Description*	*Fold change*
O75369	FLNB	Filamin-B	1.26
P15311	EZR	Ezrin	1.23
Genes contributing to GO term: cell-cell adhesion			
Accession number	*Gene symbol*	*Description*	*Fold change*
P29279	CTGF	Connective tissue growth factor	1.47
O95832	CLDN1	Claudin-1	1.28
Q99959	PKP2	Plakophilin-2	1.27
P15311	EZR	Ezrin	1.23
P15151	PVR	Poliovirus receptor (Nectin-like protein 5)	1.21

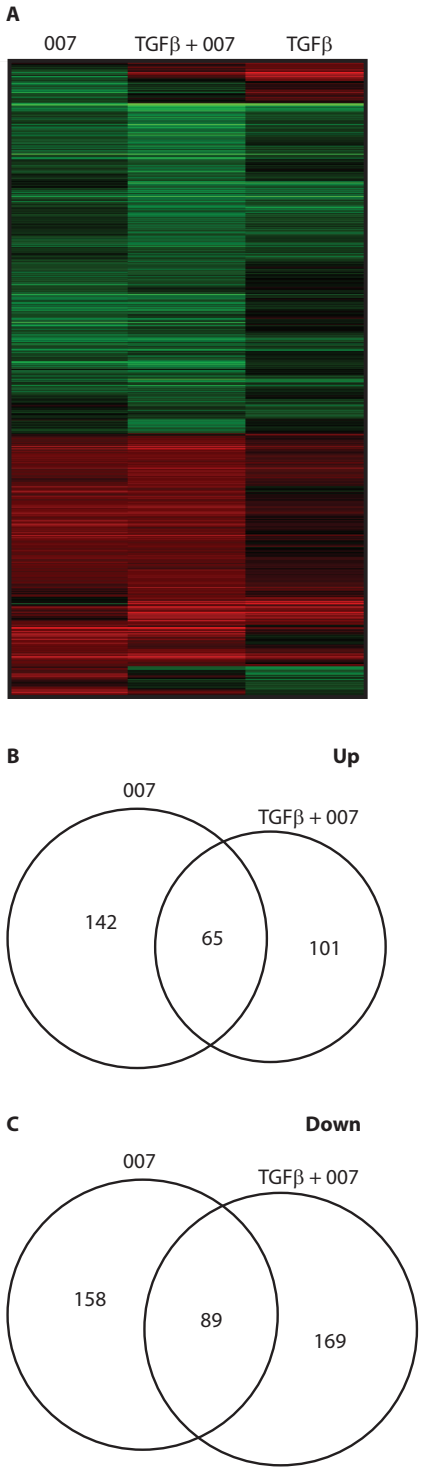
Table 3. Genes contributing to GO terms returned by gene list of down-regulated genes by 007 only.

Genes contributing to GO term: negative regulation of cellular/biological process			
Accession number	*Gene symbol*	*Description*	*Fold change*
P15428	HPGD	15-hydroxyprostaglandin dehydrogenase	-1.74
O14503	BHLHB2	Class B basic helix-loop-helix protein 2	-1.46
Q92743	HTRA1	Serine protease HTRA1	-1.32
Q86SJ2	AMIGO2	Amphoterin-induced protein 2	-1.30
P06396	GSN	Gelsolin	-1.27
O14544	SOCS6	Suppressor of cytokine signaling 6	-1.23
P09601	HMOX1	Heme oxygenase 1	-1.23
Q9Y5V3	MAGED1	Melanoma-associated antigen D1	-1.23
P04083	ANXA1	Annexin A1	-1.21

Table 4. EMT-related genes significantly affected in at least one of the three conditions.

Genes regulated by TGFβ only			
Accession number	*Gene symbol*	*Description*	*Fold change*
Q99456	KRT12	Cytokeratin-12	-1.26
Q14512	FGFBP1	Fibroblast growth factor-binding protein 1	-1.35
Genes regulated by TGFβ and TGFβ + 007			
Accession number	*Gene symbol*	*Description*	*Fold change*
P41134	ID1	DNA-binding protein inhibitor ID-1	-1.33/-1.25
P19022	CDH2	N-cadherin	1.20/1.15
Q8NC51	SERBP1	Plasminogen activator inhibitor 1 RNA-binding protein	1.17/1.19
P05783	KRT18	Cytokeratin-18	-1.34/-1.24
Genes regulated by 007 only			
Accession number	*Gene symbol*	*Description*	*Fold change*
P10451	SPP1	Osteopontin	-1.15
P29279	CTGF	Connective tissue growth factor	1.47
Q99959	PKP2	Plakophilin-2	1.27
Genes regulated by TGFβ, TGFβ+ 007 and 007			
Accession number	*Gene symbol*	*Description*	*Fold change*
O95857	TSPAN13	Tetraspanin-13	-1.14/ -1.29/-1.25
O15551	CLDN3	Claudin-3	-1.21/ -1.36/-1.18

indistinguishable from A549 cells, in our experiment the number of genes affected by TGFβ and the fold difference are low compared to published results (Kasai et al., 2005; Keating et al., 2006; Miyazaki et al., 2002; Ranganathan et al., 2007). Also, the genes encoding E-cadherin, fibronectin, collagen I and III are not significantly affected, and GO searches with the genes regulated solely by TGFβ did not return any significant GO terms. However, as mentioned above, a clear genetic signature for EMT has not been defined yet. Using literature, we therefore compiled a list of 89 genes claimed to be involved in EMT, either in general, or downstream of TGFβ stimulation (Moreno-Bueno et al., 2006; Moreno-Bueno et al., 2008; Peinado et al., 2007; Willis and Borok, 2007; Zavadil and Bottinger, 2005). In our experiment, only 11 of these genes were significantly affected in any of



the three conditions, of which 6 in the TGFβ only or TGFβ + 007 condition. These 11 genes are shown in Table 4. Taken together, although the cellular response to TGFβ is normal in this experiment, we observe only a weak transcriptional response to TGFβ. This weak response could be due to the fact that A549 cells, although responsive to TGFβ, are already tumor cells and thus may respond differently (perhaps less strong) than non-transformed cells.

Comparing the 007- to the 007+TGFβ-induced gene expression profile

We next wondered whether the expression profile induced by 007 would change in the presence of TGFβ. This could tell us if effects induced by 007 are different when cells are migrating (or actually, are being induced to migrate by TGFβ). Figure 2A shows a cluster diagram of the 832 significantly changed genes in the three conditions. It may be noted that the profiles induced by 007 alone or in the presence of TGFβ are most similar to each other. This is also demonstrated by how the three different conditions cluster when making a condition tree (not shown), with 007 and TGFβ + 007 always clustering together, separate from TGFβ. However, of the 454 genes regulated by 007, only 154 genes are also significantly affected by TGFβ +

Figure 2.
(A) Cluster diagram of the 832 significant genes. Venn diagrams showing overlap in genes (B) up and (C) down-regulated in 007 and TGFβ + 007 treated cells. Color figure on page 154.

007 (Figures 2B and C), indicating that the other 300 genes are no longer significantly changed by 007 in the presence of TGFβ. Illustrating this difference, the GO terms enriched in the 007-induced gene lists (Tables 2 and 3) were not returned by the list of TGFβ + 007-induced genes, indicating that the presence of TGFβ indeed affects these 007-induced gene expression changes.

We also performed indirect Maanova analyses to compare the three gene lists to each other instead of to the mock condition, to identify differentially regulated genes. Although genes that were up-regulated by 007 when compared to mock such as Ezrin, Filamin and Claudin-1, are no longer up-regulated in the TGFβ + 007 condition, these genes are not significantly down-regulated in the TGFβ + 007 condition when comparing it to the 007 condition. However, we do observe the significant up-regulation of several putative TGFβ-induced genes such as transforming growth factor-beta-induced protein, latent-transforming growth factor beta-binding protein 2 and the Myc proto-oncogene protein, when comparing the TGFβ + 007 profile to the 007-induced profile directly.

Comparing the TGFβ- to the 007+TGFβ-induced gene expression profile

Vice versa, some of the TGFβ-induced genes are not affected in the TGFβ + 007 condition. This may indicate that the presence of 007 directly affects these genes. It is also possible that these gene expression changes are only induced when cells start migrating, which is inhibited by 007. Thus, 007 could also indirectly affect these gene expression changes. As the TGFβ-induced gene list by definition contains genes that are only significant in the TGFβ-only condition, some more genes that contribute to TGFβ-induced EMT can be found when comparing the TGFβ and TGFβ + 007 gene lists. Such genes include transforming growth factor-beta-induced protein, N-cadherin, keratin-18, SERBP1, β5 integrin and DNA-binding protein inhibitor ID-1. These are EMT-related, TGFβ-induced genes that are not affected by the presence of, or the cellular response to, 007. Interestingly, there are also genes that are up- or down-regulated only in the presence of both 007 and TGFβ, indicating a possible synergy between these signaling networks, *i.e.* genes that require input from both pathways to regulate their expression. Examples of these genes include up-regulated genes such as c-Myc and kindlin-1, and down-regulated genes such as TGF-beta receptor type-2 and smooth muscle myosin light chain kinase. As indicated by the minimal changes in gene expression, the limited returns of GO terms by the different gene lists and by the similarities in the cluster diagram, in this experiment there seem to be limited differences in gene expression changes induced by either TGFβ or 007. Although the RNA for this experiment was gathered in four biologically separate experiments, we only used one time-point for all stimulations. We know from previous

experiments that the effects induced by both stimuli do occur after twenty-four hours (see Chapter 3 (Lyle et al., 2008) and data not shown), but having only one data point does limit the conclusions that can be drawn from such experiments.

In conclusion, although different expression profiles are found in all three conditions, the fold change induced by each stimulus is low and clear profiles belonging to TGFβ signaling were not found. Of course, the return of the cell-cell adhesion GO term belonging to the 007-induced gene list is very interesting, but whether this is a direct effect of 007 on gene transcription, or an indirect one due to the known effects of Rap1 activation on cell adhesion, is still unclear.

References:

Coleman, M.L., Marshall, C.J. and Olson, M.F. (2004) RAS and RHO GTPases in G1-phase cell-cycle regulation. Nat Rev Mol Cell Biol, 5, 355-366.

Hynes, R.O. (2002) Integrins: bidirectional, allosteric signaling machines. Cell, 110, 673-687.

Kasai, H., Allen, J.T., Mason, R.M., Kamimura, T. and Zhang, Z. (2005) TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respir Res, 6, 56.

Keating, D.T., Sadlier, D.M., Patricelli, A., Smith, S.M., Walls, D., Egan, J.J. and Doran, P.P. (2006) Microarray identifies ADAM family members as key responders to TGF-beta1 in alveolar epithelial cells. Respir Res, 7, 114.

Lyle, K.S., Raaijmakers, J.H., Bruinsma, W., Bos, J.L. and de Rooij, J. (2008) cAMP-induced Epac-Rap activation inhibits epithelial cell migration by modulating focal adhesion and leading edge dynamics. Cell Signal, 20, 1104-1116.

Miyazaki, Y.J., Hamada, J., Tada, M., Furuuchi, K., Takahashi, Y., Kondo, S., Katoh, H. and Moriuchi, T. (2002) HOXD3 enhances motility and invasiveness through the TGF-beta-dependent and -independent pathways in A549 cells. Oncogene, 21, 798-808.

Moreno-Bueno, G., Cubillo, E., Sarrio, D., Peinado, H., Rodriguez-Pinilla, S.M., Villa, S., Bolos, V., Jorda, M., Fabra, A., Portillo, F., Palacios, J. and Cano, A. (2006) Genetic profiling of epithelial cells expressing E-cadherin repressors reveals a distinct role for Snail, Slug, and E47 factors in epithelial-mesenchymal transition. Cancer Res, 66, 9543-9556.

Moreno-Bueno, G., Portillo, F. and Cano, A. (2008) Transcriptional regulation of cell polarity in EMT and cancer. Oncogene, 27, 6958-6969.

Peinado, H., Olmeda, D. and Cano, A. (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer, 7, 415-428.

Ranganathan, P., Agrawal, A., Bhushan, R., Chavalmane, A.K., Kalathur, R.K., Takahashi, T. and Kondaiah, P. (2007) Expression profiling of genes regulated by TGF-beta: differential regulation in normal and tumour cells. BMC Genomics, 8, 98.

Roepman, P., Wessels, L.F., Kettelarij, N., Kemmeren, P., Miles, A.J., Lijnzaad, P., Tilanus, M.G., Koole, R., Hordijk, G.J., van der Vliet, P.C., Reinders, M.J., Slootweg, P.J. and Holstege, F.C. (2005) An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. Nat Genet, 37, 182-186.

Willis, B.C. and Borok, Z. (2007) TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol, 293, L525-534.

Zavadil, J. and Bottinger, E.P. (2005) TGF-beta and epithelial-to-mesenchymal transitions. Oncogene, 24, 5764-5774.

chapter 4

Effect of Rap1 activation on different signaling pathways involved in focal adhesion regulation

Judith H. Raaijmakers, Ingrid Verlaan and Johannes L. Bos

Effect of Rap1 activation on different signaling pathways involved in focal adhesion regulation

Judith H. Raaijmakers, Ingrid Verlaan and Johannes L. Bos

Department of Physiogical Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands

Abstract

In epithelial cells, activation of Rap inhibits growth factor-induced migration. This effect is independent of E-cadherin-mediated cell-cell junction regulation, but correlates with a decrease in focal adhesion dynamics and membrane protrusive activity (Lyle et al., 2008). The coordinated formation and turnover of focal adhesions is crucial for efficient cell migration and is a tightly regulated process. Here, we investigated a number of major signaling pathways involved in the regulation of focal adhesion dynamics to determine which one is perturbed by activation of Rap1. We found that in the presence of 8-pCPT-2'OMe-cAMP (hereafter referred to as 007), there is no apparent difference in the composition of these focal adhesions or in the level of phosphorylation of central components. Also, Rap1 activation does not increase the level of contractility in these cells and the focal adhesions are still sensitive to breakdown by microtubules and by inhibition of Rock. Thus, from these experiments we conclude that it is unlikely that Rap1 modulates FA dynamics by regulating the cytoskeleton directly. We propose that Rap1 may regulate the connection between FAs and the actin cytoskeleton.

Introduction

The process of migration occurs in a multi-step cycle, in which the formation and release of sites of attachment to the extracellular matrix (ECM) is coordinated. First, the cell needs to polarize in the direction of migration. Then, at the front of the cell, adhesions sites are formed to attach the protruding lamellipodia to the ECM. Subsequent contraction, mediated by the actin cytoskeleton that is linked to these adhesions, translocates the cell body forward. At the same time, adhesions at the rear of the cell are released to allow the cell to move forward (Ridley et al., 2003; Webb et al., 2002). One of the most important aspects of regulated cell migration is the coordinated formation and turnover of focal adhesions (FAs) (Webb et al., 2002). Previously, we have shown that activation of Rap1 inhibits growth factor-induced epithelial cell migration. The effect of Rap1 on migration could not be mimicked by forced integrin activation, nor did it depend on strengthening of E-cadherin mediated cell-cell junctions, so we hypothesized that Rap1 might affect the migration machinery directly. Indeed, we have shown that the activation of Rap1 results in an increase in number, size and stability of FAs (Lyle et al., 2008). There are many different pathways controlling FA formation and breakdown, all of

which are potential targets for Rap1 in the inhibition of migration.

Rho-induced contractile tension regulates FA maturation

After Rac-dependent focal complex formation, activation of RhoA is required for the maturation of these complexes into FAs (Chrzanowska-Wodnicka and Burridge, 1996). RhoA drives FA assembly via the phosphorylation and activation of myosin light chain (MLC). The Rho effector, Rho kinase (Rock), can phosphorylate MLC both directly and indirectly, through inhibition of the MLC phosphatase (Riento and Ridley, 2003). MLC phosphorylation then leads to increased actomyosin contractility, clustering of integrins and maturation of focal complexes into focal adhesions (Ridley, 2001). If contractility is inhibited, for instance by the Rock inhibitor Y27632, integrins disperse from FAs and stress fibers and FAs disassemble (Chrzanowska-Wodnicka and Burridge, 1996). At the cell rear, regulation of tension is required to ensure proper tail retraction (Ridley et al., 2003). The speed of cell migration depends on a proper level of adhesive strength. When adhesion strength is low, the cell cannot form strong adhesions at the cell front to generate enough traction force, whereas under conditions of high adhesion, the release of cell-ECM adhesions at the rear is inhibited. Thus, intermediate amounts of adhesive strength provide the optimal amount of tension for fast cell migration (Palecek et al., 1997).

FAK and paxillin are central regulators of FA maturation

Focal adhesions and focal complexes do not only differ with respect to their dependence on tension for their formation. There are also differences in their composition. Focal complexes and adhesions both contain high levels of integrins, and the integrin-binding proteins talin and paxillin, but differ with respect to other FA components, such as vinculin, FAK, VASP and α-actinin (Zaidel-Bar et al., 2003; Zaidel-Bar et al., 2004). The level of phosphorylation and/or activity of proteins in an FA can also differ from that in FCs. For instance, the phosphorylation of FAK is regulated during FA formation. FAK is one of the first proteins to be recruited to a nascent FC. After FAK auto-phosphorylation on tyrosine 397, Src family kinases are recruited, leading to phosphorylation of additional sites in FAK (Schlaepfer et al., 2004). The disassembly of adhesions depends on phosphorylation of tyrosine 397 and requires Src activity (Webb et al., 2004). One of the mechanisms via which FAK can regulate FA disassembly is by decreasing tension through inhibition of RhoA and Rock (Ren et al., 2000; Schober et al., 2007). The importance of FAK in regulating FA dynamics is illustrated by the fact that FAK-/- fibroblasts have altered FA dynamics, which leads to large, refractory FAs, cell spreading defects and inhibited cell migration (Ilic et al., 1995).

The regulation of paxillin phosphorylation is also involved in FA dynamics. In FCs and FAs,

upon integrin activation, paxillin is phosphorylated at tyrosines 31 and 118 (Schaller, 2001; Turner, 2000). The binding affinity of paxillin for FAK is much lower when it is in an unphosphorylated state. Tyrosine phosphorylation of paxillin thus enhances the recruitment of FAK, which in turn stimulates adhesion turnover (Zaidel-Bar et al., 2007).

The role of microtubules in FA disassembly

It is well established that actin and actomyosin-based contractility are required for FA formation and cell migration. Additionally, the microtubule network is involved in regulating FA dynamics, by inducing FA disassembly. When FAs behind the leading edge or at the cell rear are targeted by microtubules, they are induced to disassemble (Kaverina et al., 1999). Several mechanisms have been proposed to explain this. Firstly, microtubules (MTs) could facilitate disassembly by locally reducing the amount of tension, perhaps by inhibiting RhoA, or by inducing calpain-driven proteolysis of certain FA proteins (Broussard et al., 2008). Also, targeting of the GTPase dynamin to the FAs via the MTs results in their destabilization, in a FAK-dependent manner (Ezratty et al., 2005).

As these are the major mechanisms controlling FA dynamics, any, or all, of the mechanisms described above might be involved in the stabilizing effect of 007 on FAs. Here, we have investigated the effect of 007 stimulation on these regulators of FA dynamics. We observe that 007 does not affect contractility or the requirement of tension for FA formation. Furthermore, it does not affect the apparent composition of FAs or the phosphorylation of known key components, nor does it affect the sensitivity of FAs to MT-induced breakdown. Thus, from these experiments, we conclude that it is unlikely that Rap1 modulates the regulation of FAs by the cytoskeleton directly. We propose that Rap1 may regulate the connection between FAs and the actin cytoskeleton.

Materials and Methods

Cell Lines and Culture

Stable Epac1-expressing A549 cells (A549-Epac-B14) and MDCK-Epac1 cells were created as described previously (Lyle et al., 2008; Price et al., 2004). MDCK-Epac cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics. A549-Epac cells were cultured in RPMI supplemented with glutamine, antibiotics, and 10% FCS. A549-Epac cells were transfected with Fugene6 (Roche), according to manufacturer's instructions.

Plasmids

The GFP-FP4-mito construct was a kind gift from Dr. F. Gertler (Bear et al., 2000).

Chemicals and antibodies

TGFβ (10 ng/mL), HGF (25 ng/mL), Y-27632 (10 μM) and nocodazole (200 ng/mL) were from Sigma and 8-pCPT-2'OMe-cAMP (007; 100 μM) was from Biolog. Fibronectin was purified from plasma as described previously (Poulouin et al., 1999).

Anti-phospho-MLC2-T18/S19, anti-phospho-MLC2-S19 and anti-MLC2 were from Cell Signaling, anti-vinculin was from Sigma, anti-phospho-paxillin-Y118 was from BD Transduction Laboratories, anti-FAK-Y397 from Biosource and anti-α-tubulin was from Calbiochem.

Western blotting

Cells were plated in 6-well dishes (Corning),

grown to 50% confluency for 24 hours, treated with indicated growth factors for 24 hours in the presence or absence of Y27632 or 007, as indicated. Cells were washed twice with cold PBS and lysed in Laemmli Sample Buffer. Protein samples were separated by SDS-PAGE and transferred to PVDF (Immobilon). Western blot analysis was performed under standard conditions using the indicated antibodies. Membranes were probed with fluorophore-conjugated secondary antibodies (Westburg) and analyzed using the Odyssey Infra-red imaging system and software according to the manufacturer (LI-COR).

Immunofluorescence

Cells were plated and treated as indicated. Cells were washed twice in PBS, fixed in freshly-prepared 3.8% formaldehyde for 15 minutes and permeabilized in 0.2% Triton X-100 for 5 minutes where the normal fixation method was used. In case of treatment with the cytoskeletal buffer, cells were washed twice with PBS, twice with cytoskeletal buffer (0.5% TX-100, 10 mM Pipes pH 7.0, 50 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 300 mM sucrose and inhibitors (NaF, aprotinin, leupeptin and sodium orthovanadate)) once with PBS and then fixed in 3.8% formaldehyde for 15 minutes. Coverslips were blocked in PBS containing 2% BSA overnight at 4 ºC. Cells were incubated with the indicated antibodies for 2-3 hours in PBS containing 0.2% BSA, followed by incubation with the appropriate secondary antibodies (Invitrogen) for 1 hour at room temperature. Coverslips were mounted using Immu-mount (Thermo Scientific). Images were acquired using a Zeiss Axioskop 2 microscope fitted with a Zeiss Axiocam CCD camera and 40X and 100X Plan APO objective lenses.

Replating of A549-Epac cells for immunofluorescence

Cells were trypsinized, washed once in RPMI containing 10% FCS, and allowed to recover surface proteins for 1.5 hours in suspension in RPMI containing 0.5% FCS, glutamine, antibiotics, and 20 mM Hepes, pH 7.4, at 37°C with constant, gentle rolling. Cells were replated onto coverslips coated with 10 µg/mL fibronectin overnight at 4ºC, for 3 hours in the presence or absence of 8-CPT-2'OMe-cAMP or Y27632 before fixation.

Wash-out experiments

Cells grown for 24 hours on (uncoated) coverslips were treated for 3 hours with either Y27632 or nocodazole (and in the presence or absence of 007). After removal of the medium, fresh medium containing serum and antibiotics, with or without 007, was added and wash-out was left to proceed for the indicated time periods, before fixation of the cells.

Results

Rap1 activation does not affect the level of MLC phosphorylation

One of the most obvious effects of 007 stimulation in A549-Epac cells is the induction of more and larger FAs. One possibility is that activation of Rap1 leads to inappropriate or excessive activation of Rock, causing increased MLC phosphorylation and thus leading to too much tension and too many FAs. We therefore examined the effect of Rap activation on the phosphorylation of MLC. Figure 1 shows the levels of phosphorylated MLC in A549-Epac cells treated for 24 hours with either TGFβ or HGF, in the absence or presence of the Rock inhibitor Y27632, and after 007 stimulation for various time-points (24 hours, 3 hours or 10 minutes for A-C, respectively). In these cells, TGFβ induces MLC phosphorylation, whereas HGF has no effect. The Rock inhibitor Y27632 reduces both basal and TGFβ-induced MLC phosphorylation. Presence of 007 does not affect the induction of phosphorylation by TGFβ, or prevent the inhibition by Y27632. In MDCK cells, HGF does induce MLC phosphorylation, which is maximal after 3 hours of stimulation (de Rooij et al., 2005). Similarly, in MDCK-Epac cells, we observe an increase of

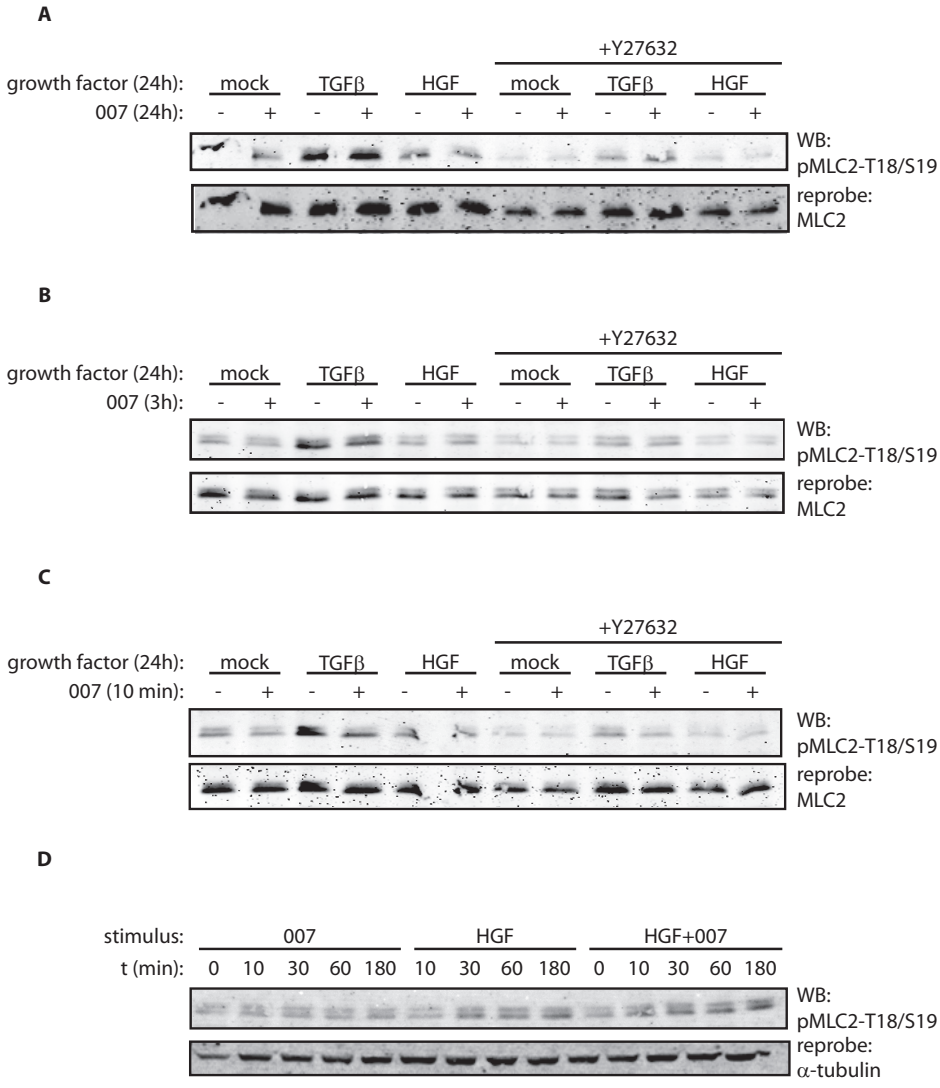


Figure 1. 007 treatment does not affect the level of phosphorylated MLC in A549-Epac or MDCK-Epac cells.

(A) Western blot showing phosphorylated MLC levels in A549-Epac cells after 24-hour stimulation with either TGFβ or HGF, in the presence or absence of Y27632 and/or 007. Blot was reprobed to show equal levels of total MLC. (B-C) Western blots showing phosphorylated MLC levels in A549-Epac cells after 24-hour stimulation with either TGFβ or HGF, in the presence or absence of Y27632, followed by 3 hour or 10 minutes treatment with 007. Blots were reprobed to show equal levels of total MLC. (D). Blot showing phosphorylated MLC levels in MDCK-Epac cells after stimulation with 007, HGF, or HGF+007 for the indicated amounts of time. Blot was reprobed with anti-α-tubulin to show equal protein levels.

phosphorylated MLC in the presence of HGF, but this is unaffected by stimulation with 007 (Figure 1D). Stimulation with 007 alone does not affect MLC phosphorylation either. When staining fixed cells for phosphorylated MLC, we observe the same effect. In control or 007-treated cells, some phospho-MLC-positive fibers can be observed along the cell cortex; these fibers disappear upon Y27632 treatment (Figure 2A-D). In cells stimulated with TGFβ, several small phospho-MLC fibers are present throughout the cells. These fibers are not affected by 007 treatment, and are still sensitive to Y27632, also in the presence of 007 (2E-H). Although all of the TGFβ-induced intracellular phospho-MLC fibers disappear upon Y27632, some staining along the cell cortex remains in these cells (3F and H). From these results we conclude that 007 treatment does not affect the regulation of MLC phosphorylation. This implies that the 007-induced increase in FA size and stability is unlikely to be caused by increased tension.

FAs induced by Rap1 activation are sensitive to inhibition of contractility

As Rap activation does not lead to increased levels of tension in these cells, we wanted to determine whether these 007-induced FAs still require tension. We therefore treated A549-Epac cells for 3 hours with or without 007, in the presence or absence of Y27632. As shown in Figure 3A, Rock inhibition by Y27632 results in an almost complete loss of FAs, both in the presence and

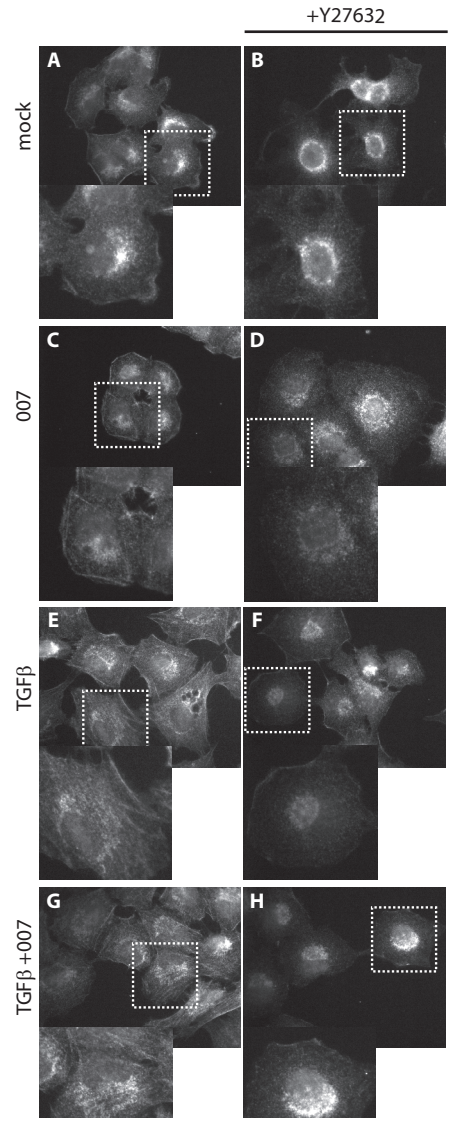


Figure 2. Effect of 007 and TGFβ stimulation on pMLC fibers in the presence and absence of the Rock inhibitor Y27632.

(A-H) A549-Epac cells were treated for 24 hours as indicated and stained with anti-phospho-MLC-S19 antibody to detect phosphorylated myosin fibers indicative of contractility. Panels E and G show increased fibers (insets), that are gone after treatment with Y27632 (F and H).

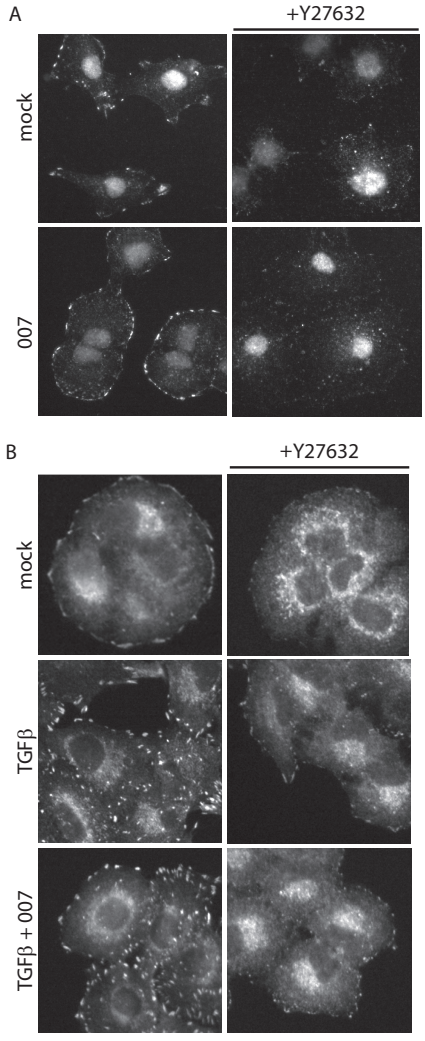


Figure 3. Inhibition of Rock leads to breakdown of 007- and TGFβ-induced focal adhesions in A549-Epac cells.

(A) Cells grown on coverslips were treated for 3 hours as indicated before treatment with cytoskeletal wash buffer followed by fixation. Slides were stained with anti-vinculin antibody to show focal adhesions. Upon treatment with Y27632, focal adhesions disperse (right panels). (B) Cells were treated for 24 hours as indicated, before fixation and staining for vinculin. Note that cells in A) were treated with CSK buffer, thus permeabilizing the cells prior to fixation, whereas in B) permeabilization occurred after fixing the cells.

absence of 007. The FAs induced by TGFβ are normally affected by Rock inhibition as well (Figure 3B, lowest 4 panels). Thus, the presence of 007 does not rescue the breakdown of FAs by Y27632. We performed the same experiment with blebbistatin, another inhibitor of actomyosin contractility that inhibits myosin's ATPase activity. Treatment with blebbistatin results in the disappearance of FAs, which is not inhibited by 007 stimulation (data not shown). We next examined whether the presence of 007 affects the formation of new FAs. We therefore treated cells for 3 hours with Y27632 to remove all FAs, followed by a wash-out of the inhibitor with normal medium, or with medium containing 007. The cells were fixed at different time-points after the wash-out. During the recovery, the first FAs start appearing after 5-10 minutes. A full recovery is seen after about 60 minutes (Figure 4). The presence of 007 during the wash-out period does not increase the speed of FA formation. Both the normal fixation method (not shown), as well as the use of cytoskeletal wash buffer, show the same kinetics of FA recovery, in the absence and presence of 007. Similarly, the recovery of FAs after breakdown by blebbistatin is not affected by activation of Rap1 (data not shown). We thus conclude that Rap1 does not affect FA dynamics by acting at the level of tension induction. In addition, we conclude that Rap1 does not interfere with the requirement of tension for FA formation.

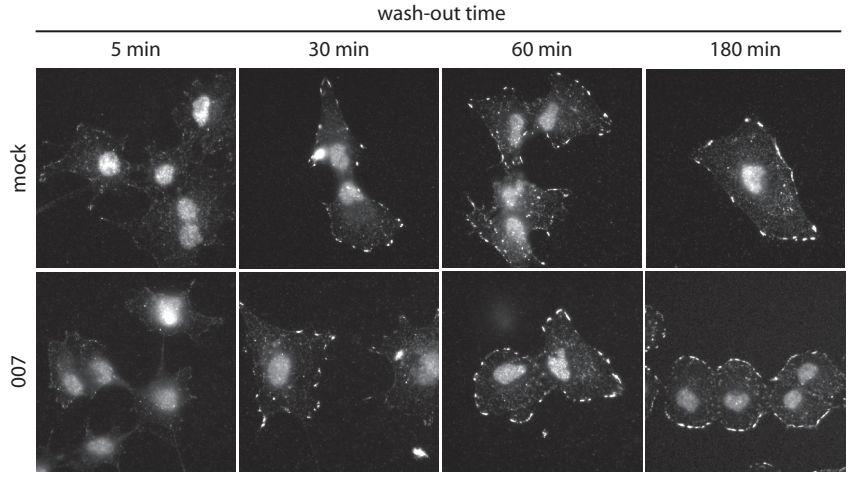


Figure 4. Formation of focal adhesions in the presence or absence of 007 after breakdown by Y27632.

Cells grown on coverslips were treated for 3 hours with Y27632, followed by removal of Y27632 and addition of fresh medium with or without 007. Cells were fixed after the indicated time periods using the cytoskeletal buffer protocol and stained for vinculin.

Phosphorylation of FA regulators is not affected by Rap1 activation

FAs form and mature in a coordinated fashion. Small, nascent FAs (the ones required for optimal cell migration) contain different proteins as compared to more mature, less dynamic FAs (Zaidel-Bar et al., 2004). To test whether 007-induced FAs might be more mature than 'normal' FAs, we investigated the presence of different FA proteins within these FAs. We trypsinized cells and, after allowing the cells to recover in suspension, replated them on high fibronectin for 90 minutes followed by fixation and staining for vinculin (a core FA protein) and for specific phosphorylated forms of FAK and paxillin. We observe no differences in the presence or absence of these proteins upon stimulation with 007. The only difference is that 007-induced FAs are consistently larger and stained more strongly (Figure 5A). We also examined the stainings for talin, zyxin and α-actinin, and examined cells replated for different periods of time, ranging from 30 to 90 minutes, but we did not find any differences between these FAs with respect to the presence or absence of these marker proteins (not shown). We also examined the level and timing of FAK and paxillin phosphorylation on these sites with Western blotting (5B). As could be expected, paxillin phosphorylation increases during adhesion and spreading, but the presence of 007 does not affect the level or timing of phosphorylation. We observed the same for FAK phosphorylation (data not shown). As we inspected the major markers of FA maturation used throughout literature, we tentatively conclude that 007 treatment does not induce

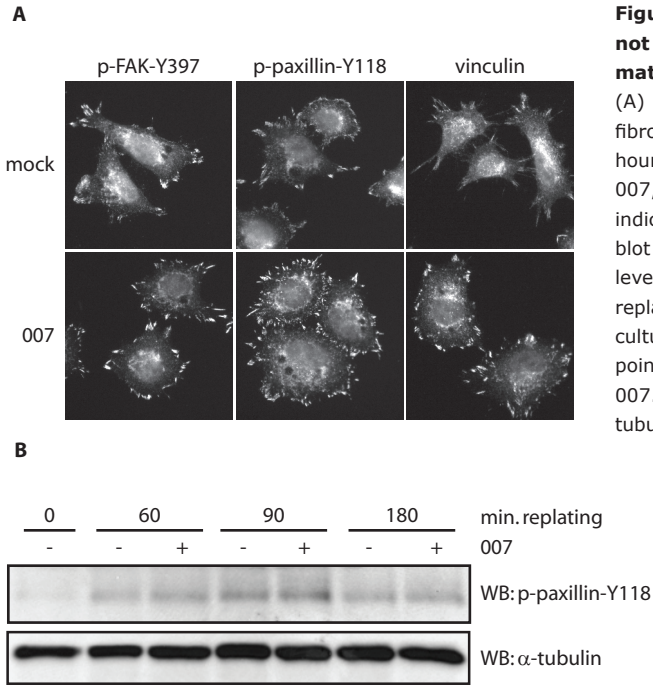


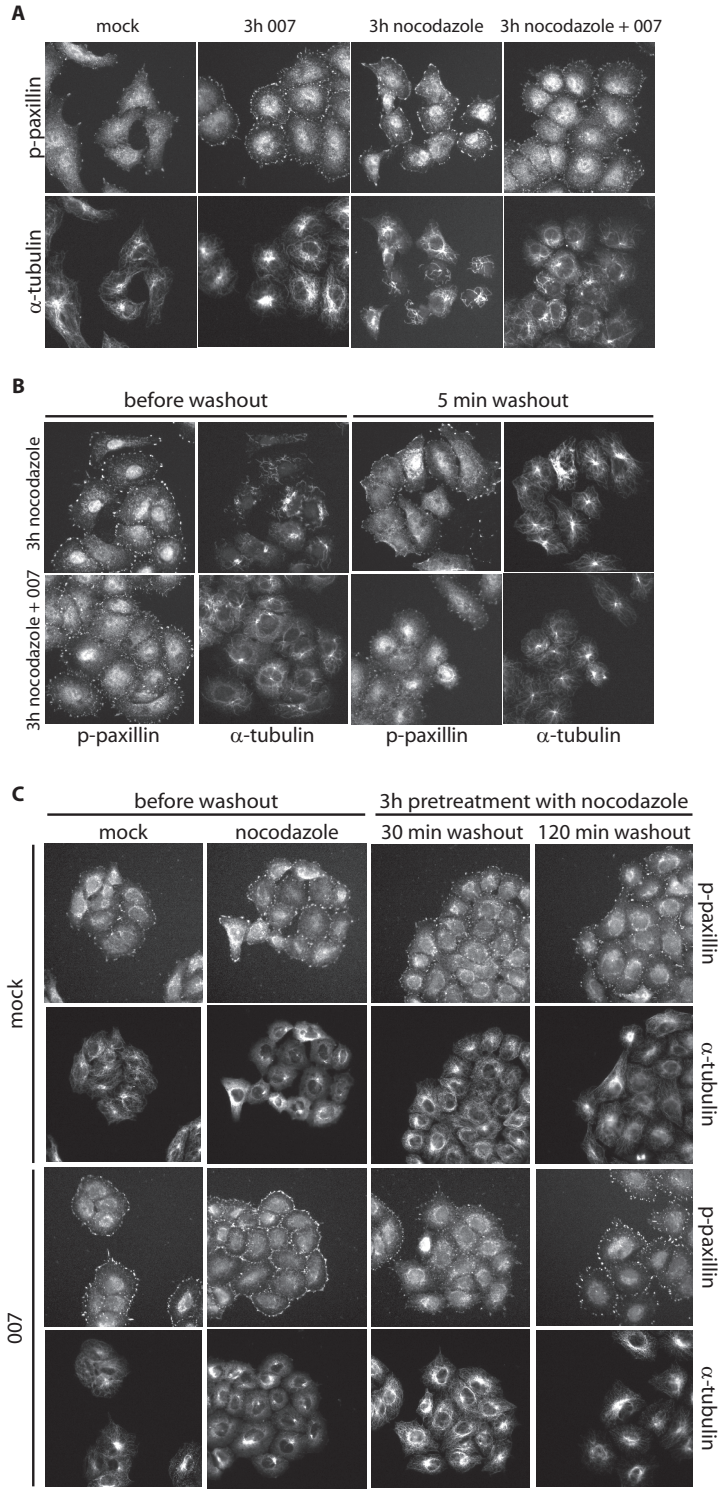
Figure 5. 007 treatment does not affect the composition or maturation of FAs.
(A) Cells were replated onto fibronectin-coated coverslips for 3 hours in the absence or presence of 007, followed by staining with the indicated antibodies. (B) Western blot showing phosphorylated paxillin levels in A549-Epac cells. Cells were replated onto fibronectin-coated culture dishes for the indicated time-points, in the presence or absence of 007. Blot was also probed with α-tubulin to show equal protein levels.

an obvious change in the composition or maturation of these FAs.

007-induced FAs are sensitive to microtubule-induced turnover

Besides the actomyosin cytoskeleton, the microtubules also play a role in regulating focal adhesions. Targeting of a microtubule (MT) to an FA will result in its turnover and breakdown (Kaverina et al., 1999). To test whether disrupted MT targeting or a difference in MT dynamics is the cause of increased FA stability in 007-treated cells, we treated cells with nocodazole to disrupt the MTs and induce stable FAs, followed by a nocodazole washout by normal medium, to induce MT regrowth and FA turnover. A three-hour stimulation with nocodazole was sufficient to induce a complete loss of MTs and to stabilize the focal adhesions, specifically at the cell edge (Figure 6A). Although the FAs in cells treated for 3 hours with nocodazole and 007 look slightly different than

Figure 6. 007-induced focal adhesions are still turned over by growing microtubules.
(A) Cells grown on coverslips were treated as indicated and stained for phospho-paxillin-Y118 or α-tubulin. (B) Cells grown on coverslips were treated for 3 hours as indicated on the left (all panels), before removal of nocodazole followed by wash-out with medium with (lower panels) or without 007 (upper panels) for 5 minutes (right panels only). Cells were fixed and stained for phospho-paxillin-Y118 or α-tubulin. (C) Cells grown on coverslips were treated for 3 hours as indicated (left panels). Right panels were treated for 3 hours with nocodazole, before removal of nocodazole followed by wash-out with medium with (lower panels) or without 007 (upper panels) for various time lengths. Cells were fixed and stained for phospho-paxillin-Y118 or α-tubulin.


A
mock
3h 007
3h nocodazole
3h nocodazole + 007
p-paxillin
α-tubulin
B
before washout
5 min washout
3h nocodazole
3h nocodazole + 007
p-paxillin
α-tubulin
p-paxillin
α-tubulin
C
before washout
3h pretreatment with nocodazole
mock
nocodazole
30 min washout
120 min washout
mock
007
p-paxillin
α-tubulin
p-paxillin
α-tubulin

those of cells treated with either one of the stimuli, there is no additive effect with respect to an increase in FA size. When MTs were allowed to grow back in normal medium, we observed that both the nocodazole- as well as the nocodazole + 007-induced FAs disappeared as a consequence of MT targeting (Figure 6B). In these cells, MT recovery is quite fast. MTs could already be observed again after 2-3 minutes, and after 5 minutes a normal MT network was visible (Figure 6B). Concomitantly, the FAs started disappearing after 3 minutes, and after 5 minutes of nocodazole washout, less FAs were present and those that remained, were reduced to a normal size (Figure 6B). This indicates that 007-induced FAs are also sensitive to breakdown induced by regrowing MTs. In addition, we examined whether the presence of 007 during the washout would affect the turnover kinetics of nocodazole-induced FAs. At short time-points, the presence of 007 has no effect, indicating that 007 does not prevent FA targeting by MTs. As expected, at longer time-points after wash-out in the presence of 007, the more and larger "007" FAs re-appear (Figure 6C). We conclude from these results that in the presence of 007 the FAs are still sensitive to MT-induced breakdown.

Mislocalization of Ena/VASP proteins does not inhibit Rap1-induced spreading

Besides increased spreading and FAs, cells treated with 007 also exhibit limited membrane protrusive activity (Lyle et al., 2008). The Ena/VASP proteins (Mena, VASP and EVL) are key modulators of the actin cytoskeleton and control lamellipodia formation and cell motility (Krause et al., 2003). They can also be localized in FAs, to which they are recruited by zyxin and vinculin after binding of their EVH1

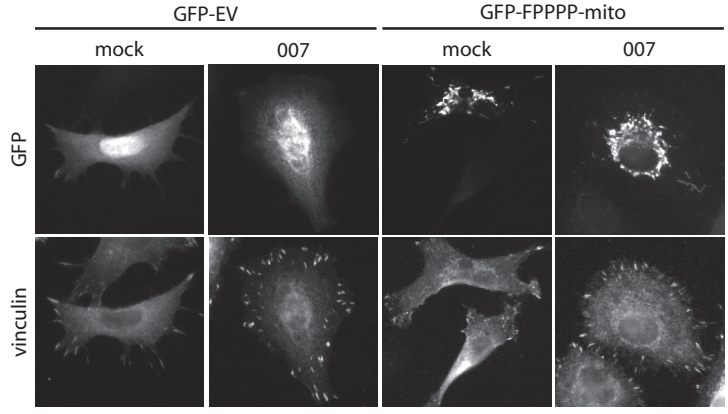


Figure 7. Mislocalization of Ena/VASP proteins does not inhibit the induction of spreading and FAs by 007.

48 hours after transfection with either GFP-EV (left) or GFP-FPPPP-mito (right), cells were trypsinized and replated on coated coverslips for 3 hours, in the presence or absence of 007. Cells were fixed and stained for vinculin. Upper panels show GFP-expressing cells, lower panels show vinculin staining.

domain to the proline-rich FPPPP motif in these proteins (Drees et al., 2000; Gertler et al., 1996). To test for the involvement of Ena/VASP proteins in Rap1-induced spreading and FA formation, a GFP-tagged FPPPP-mito construct was used to sequester Ena/VASP proteins at the mitochondria, to mislocalize and inactivate them (Bear et al., 2000). If these proteins are essential to the 007 effect, cells expressing the sequestering construct should not be able to respond to 007 anymore. However, cells with the sequestering construct still respond to 007 by spreading and by increased FA formation (Figure 7, right panels). In addition, the expression of a membrane-targeting construct to induce Ena/VASP activation does not increase spreading or FA formation in the untreated condition (not shown). Although we did not examine the actin cytoskeleton in detail to confirm the effect of Ena/VASP sequestration, we did confirm this result using VASP siRNA, which had no effect on 007-induced FA formation and spreading of replated cells (data not shown, see Chapter 5). Thus, over-activation of Ena/VASP proteins does not appear to be the determinant of the Rap1 activation phenotype on focal adhesions in these cells.

Discussion

Rap1 activation does not affect Rho-induced contractile tension

Rap1 activation by 007 in migrating cells results in an inhibition of cell migration and a decrease in FA and membrane dynamics (Lyle et al., 2008). In normally adhered cells, 007 treatment causes an increase in cell spreading and in the number and size of FAs. To determine how Rap1 might mediate these effects on FA dynamics, we investigated the major pathways known from earlier studies to regulate FAs. Firstly, FA formation requires tension and cells need contractility for movement. We hypothesized that the activation of Rap1 may regulate the activity of Rho or Rho kinase, thereby increasing tension, resulting in too many, too big, or too stable FAs and an inability of cells to move. However, we did not find increased levels of tension in these cells, as determined by the level of phosphorylated MLC. Also, the regulation of these FAs by tension appeared normal. Furthermore, we did not observe major changes in RhoGTP levels in A549-Epac cells after 007 stimulation, but we were unable to reproducibly repeat these assays (data not shown). Together, these results indicate that Rap1 does not affect FA dynamics by affecting contractile tension.

We also examined whether the effect of Rap1 activation on FAs might be more direct, for instance through changing their composition. We stained FAs for different FA marker proteins, including both adaptors and proteins with enzymatic activities. Although we did not analyze these stainings in a quantitative manner, we observed no difference in the presence of any of the proteins examined when comparing control and 007-stimulated cells. Since FAs are large protein complexes, with typically over a 100 distinct proteins present, these experiments are far from exhaustive.

Thus, to examine these types of differences in a quantitative manner and in more detail, quantitative mass spectrometry is required.

The role of microtubules in disassembly of Rap1-induced FAs

Another regulator of FA dynamics is the microtubule network (Kaverina et al., 1999). In stable adherent cells, 007 treatment results in more and larger FAs, even though also in these cells, continuous targeting of FAs by MTs occurs. This may indicate that 007-induced FAs are resistant to MT-induced breakdown. Also the observation that there is no additive effect of nocodazole treatment on 007-induced FAs may indicate that the 007 effect indeed includes the MT network. On the other hand, it might also mean that both stimuli on their own are able to induce the maximal FA size and/or number possible, and that it is just not possible for FAs to grow further when the second stimulus is added. In our experiments, we observe that 007-induced FAs are still induced to turn over when targeted by regrowing MTs during a wash-out. We observed no clear differences in the kinetics of FA disappearance between cells pretreated with 007, 007 during nocodazole, or 007 during wash-out, indicating that 007 does not block the ability of MTs to dissociate FAs. However, these experiments do not exclude that under stationary conditions, *i.e.* in stably adherent cells or at longer time-points after wash-out, 007 affects MT targeting to FAs. Interestingly, Sehrawat et al. reported that 007 affects MT turnover in endothelial cells, resulting in increased MT length, but whether increased length represents a reduced targeting of FAs is unclear (Sehrawat et al., 2008).

In conclusion, we have analyzed several pathways essential for the regulation of FA dynamics. We found that 007-induced FAs are still sensitive to tension and to MT-induced breakdown, indicating that 007 does not interfere in the mechanism of FA dissociation by these two pathways. Since we do not observe any effect of 007 on actomyosin-induced contractility, it is unlikely that 007 regulates FA stabilization by increasing tension. However, currently we cannot exclude that 007 stabilizes FAs by a reduction of MT targeting. Recently, the connection between integrins and the actin cytoskeleton has gained a lot of attention in the migration field. The proteins mediating this link ensure that movement induced by actin polymerization is properly transmitted to the integrin. In order to regulate this linkage, integrins must be able to sense changes in the rigidity of their surroundings. These mechano-sensing mechanisms are thought to induce biochemical events in response to force. For instance, it was recently shown that tension-induced stretching of talin results in the exposure of additional vinculin binding sites, thus strengthening the adhesion upon sensing force (del Rio et al., 2009). Interestingly, Rap1 has been shown to be activated by tension-sensing cytoskeletal extractions. Upon physical stretching of p130Cas, Crk and C3G are

activated, leading to Rap1 activation (Sawada et al., 2006; Tamada et al., 2004). The role of this Rap1 activation upon mechanical force is currently unknown. However, something similar is observed in adherens junctions. There, Rap1 becomes activated upon breakdown of junctions, possibly to restore junctional integrity (Balzac et al., 2005). The same could apply to integrin-mediated adhesions. One intriguing possibility is that Rap1, without affecting the level of tension, can strengthen the integrin-actin linkage. A stronger linkage would lead to a more efficient use of lower amounts of tension, causing more force to be exerted by the same amount of contractility. Over-activation of Rap1 would then make the integrin-actin link less efficient in transmitting information about tension and/or speed, leading to defects in FA dynamics and possibly migration.

References

Balzac, F., Avolio, M., Degani, S., Kaverina, I., Torti, M., Silengo, L., Small, J.V. and Retta, S.F. (2005) E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. *J Cell Sci*, **118**, 4765-4783.

Bear, J.E., Loureiro, J.J., Libova, I., Fassler, R., Wehland, J. and Gertler, F.B. (2000) Negative regulation of fibroblast motility by Ena/VASP proteins. *Cell*, **101**, 717-728.

Broussard, J.A., Webb, D.J. and Kaverina, I. (2008) Asymmetric focal adhesion disassembly in motile cells. *Curr Opin Cell Biol*, **20**, 85-90.

Chrzanowska-Wodnicka, M. and Burridge, K. (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol*, **133**, 1403-1415.

de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M.A. and Waterman-Storer, C.M. (2005) Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol*, **171**, 153-164.

del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J.M. and Sheetz, M.P. (2009) Stretching single talin rod molecules activates vinculin binding. *Science*, **323**, 638-641.

Drees, B., Friederich, E., Fradelizi, J., Louvard, D., Beckerle, M.C. and Golsteyn, R.M. (2000) Characterization of the interaction between zyxin and members of the Ena/vasodilator-stimulated phosphoprotein family of proteins. *J Biol Chem*, **275**, 22503-22511.

Ezratty, E.J., Partridge, M.A. and Gundersen, G.G. (2005) Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol*, **7**, 581-590.

Gertler, F.B., Niebuhr, K., Reinhard, M., Wehland, J. and Soriano, P. (1996) Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics. *Cell*, **87**, 227-239.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, **377**, 539-544.

Kaverina, I., Krylyshkina, O. and Small, J.V. (1999) Microtubule targeting of substrate contacts promotes their relaxation and dissociation. *J Cell Biol*, **146**, 1033-1044.

Krause, M., Dent, E.W., Bear, J.E., Loureiro, J.J. and Gertler, F.B. (2003) Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. *Annu Rev Cell Dev Biol*, **19**, 541-564.

Lyle, K.S., Raaijmakers, J.H., Bruinsma, W., Bos, J.L. and de Rooij, J. (2008) cAMP-induced Epac-Rap activation inhibits epithelial cell migration by modulating focal adhesion and leading edge dynamics. *Cell Signal*, **20**, 1104-1116.

Palecek, S.P., Loftus, J.C., Ginsberg, M.H., Lauffenburger, D.A. and Horwitz, A.F. (1997) Integrin-ligand binding properties govern cell migration speed through cell-

substratum adhesiveness. *Nature*, **385**, 537-540.

Poulouin, L., Gallet, O., Rouahi, M. and Imhoff, J.M. (1999) Plasma fibronectin: three steps to purification and stability. *Protein Expr Purif*, **17**, 146-152.

Price, L.S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F.J., Collard, J.G. and Bos, J.L. (2004) Rap1 regulates E-cadherin-mediated cell-cell adhesion. *J. Biol. Chem.*, **279**, 35127-35132.

Ren, X.D., Kiosses, W.B., Sieg, D.J., Otey, C.A., Schlaepfer, D.D. and Schwartz, M.A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci*, **113 (Pt 20)**, 3673-3678.

Ridley, A.J. (2001) Rho GTPases and cell migration. *J Cell Sci*, **114**, 2713-2722.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T. and Horwitz, A.R. (2003) Cell migration: integrating signals from front to back. *Science*, **302**, 1704-1709.

Riento, K. and Ridley, A.J. (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol*, **4**, 446-456.

Sawada, Y., Tamada, M., Dubin-Thaler, B.J., Cherniavskaya, O., Sakai, R., Tanaka, S. and Sheetz, M.P. (2006) Force sensing by mechanical extension of the Src family kinase substrate p130Cas. *Cell*, **127**, 1015-1026.

Schaller, M.D. (2001) Paxillin: a focal adhesion-associated adaptor protein. *Oncogene*, **20**, 6459-6472.

Schlaepfer, D.D., Mitra, S.K. and Ilic, D. (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim Biophys Acta*, **1692**, 77-102.

Schober, M., Raghavan, S., Nikolova, M., Polak, L., Pasolli, H.A., Beggs, H.E., Reichardt, L.F. and Fuchs, E. (2007) Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J Cell Biol*, **176**, 667-680.

Sehrawat, S., Cullere, X., Patel, S., Italiano, J., Jr. and Mayadas, T.N. (2008) Role of Epac1, an exchange factor for Rap GTPases, in endothelial microtubule dynamics and barrier function. *Mol Biol Cell*, **19**, 1261-1270.

Tamada, M., Sheetz, M.P. and Sawada, Y. (2004) Activation of a signaling cascade by cytoskeleton stretch. *Dev Cell*, **7**, 709-718.

Turner, C.E. (2000) Paxillin and focal adhesion signalling. *Nat Cell Biol*, **2**, E231-236.

Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T. and Horwitz, A.F. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol*, **6**, 154-161.

Webb, D.J., Parsons, J.T. and Horwitz, A.F. (2002) Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat Cell Biol*, **4**, E97-100.

Zaidel-Bar, R., Ballestrem, C., Kam, Z. and Geiger, B. (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci*, **116**, 4605-4613.

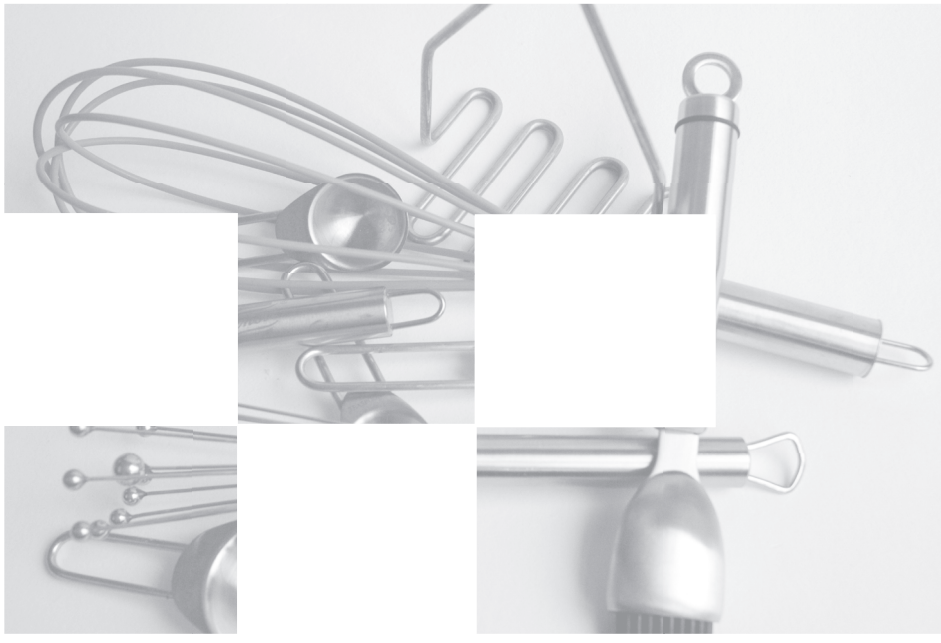
Zaidel-Bar, R., Cohen, M., Addadi, L. and Geiger, B. (2004) Hierarchical assembly of cell-matrix adhesion complexes. *Biochem Soc Trans*, **32**, 416-420.

Zaidel-Bar, R., Milo, R., Kam, Z. and Geiger, B. (2007) A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *J Cell Sci*, **120**, 137-148.

chapter 5

A small-scale siRNA screen to identify regulators of Rap1-induced focal adhesion formation

Judith H. Raaijmakers, Sarah Ross, Ingrid Verlaan
and Johannes L. Bos



A small-scale siRNA screen to identify regulators of Rap1-induced focal adhesion formation

Judith H. Raaijmakers, Sarah Ross, Ingrid Verlaan and Johannes L. Bos

Department of Physiogical Chemistry, Centre for Biomedical Genetics and Cancer Genomics Centre, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands

Abstract

In several epithelial cell lines, activation of Rap1 by 007 affects focal adhesion dynamics, correlating with a decrease in cell migration velocity. Upon replating of A549-Epac1 cells, 007 stimulation induces an increase in focal adhesions as well, which is accompanied by increased cell spreading and changes in the actin cytoskeleton. To identify proteins involved in 007-induced focal adhesion formation, we designed a replating assay and performed a small siRNA screen using a library enriched for Rap1 effectors and adhesion proteins. We show that these effects are mediated by Rap1A, and not Rap1B. We also identified important roles for talin1, RockII and Ezrin in the regulation of these effects. Furthermore, we identified a number of proteins that partially modulate the Rap1 effect. In conclusion, these results suggest that Rap1 uses redundant effector pathways to regulate focal adhesion formation and spreading.

Introduction

The small GTPase Rap1 is a known regulator of cell-cell adhesion and integrin-mediated cell adhesion to the extracellular matrix (ECM) (reviewed in (Bos et al., 2003; Kooistra et al., 2007; Menasche et al., 2007)). Although a detailed understanding of how Rap1 affects cell-ECM adhesion is lacking, we know that Rap1 regulates all integrins that are connected to the actin cytoskeleton but not those connected to intermediate filaments (Enserink et al., 2004). Furthermore, depending on the integrin and the cell type, Rap1 can regulate integrins by both affecting the binding of the integrin to its ligand (affinity) or by regulating the clustering of integrins on the cell surface (Bos, 2005; Kinbara et al., 2003). Although the mechanisms of how Rap1 induces adhesion in different cell lines remain elusive, several effectors have been identified that mediate Rap1 signaling. Most of these proteins are involved in one of the many aspects of cell adhesion and regulation of the actin cytoskeleton (reviewed in (Raaijmakers and Bos, 2008)).

Upon integrin activation during cell adhesion and spreading, various proteins are targeted to the site of adhesion in order to form adhesion complexes. Focal adhesions (FAs) start as small adhesions at the protruding edge, where integrins bind to the ECM. Subsequent binding and activation events of proteins in the nascent adhesion complex determine whether the complex becomes stabilized and matures into a stable FA, or whether it is turned over directly (Burridge and Chrzanowska-Wodnicka, 1996; Webb et al., 2002). FAs are large protein complexes that form the connection

between the integrin adhesions to the ECM and the actin cytoskeleton. By linking these two, FAs maintain cell shape, transmit signals, respond to the environment and contribute to the regulation of cell migration (Geiger et al., 2001).

To study the molecular mechanism of Rap-mediated regulation of focal adhesions, we have developed an assay using Epac1-expressing A549 cells. When these cells are replated on fibronectin, we observe a dramatic increase in the number and size of FAs after Epac1 activation using the Epac-selective agonist 8-pCPT-2'OMe-cAMP (007). We have used this assay to perform a small-scale siRNA screen. Indeed, siRNAs directed against proteins directly involved in the process, like Epac1 and Rap1A, inhibited the 007-induced increase in FA formation. Also proteins essential for adhesion, like talin1, were found, validating this assay for siRNA screening. Surprisingly however, none of the siRNAs targeting assigned Rap1 effectors, including Riam, were capable of inhibiting the 007-induced increase in FAs. This suggests that Rap1 uses redundant effector pathways to regulate this process or that not all components of this pathway have been identified and we have to search for further mediators of Rap1-induced focal adhesion regulation.

Materials and Methods

Cell Lines and Culture

The monoclonal Epac1-expressing A549-Epac-B14 (from now on: A549-Epac) cell line was created as previously described (Lyle et al., 2008). A549-B14 cells were cultured in RPMI supplemented with glutamine, antibiotics, and 10% FCS.

Replating assay

Cells were plated sparsely for 24 hours before transfection with 50 nm ON-target*plus* SMARTpool siRNA oligos (Dharmacon) using oligofectamine (Invitrogen) according to manufacturer's protocol. 48 hours after transfection, cells were trypsinized, washed once in 10% RPMI and allowed to recover surface proteins for 1.5 hours in suspension in 0.5 % RPMI containing glutamine, antibiotics and 20 mM Hepes. Next, $2*10^4$ cells/ml were replated on glass coverslips coated with high fibronectin (10 µg/ml in PBS, o/n at 4°C), in the presence or absence of 100 µM 007. Three hours after replating, cells were fixed in freshly prepared 3.8% paraformaldehyde for 15 minutes, permeabilized in 0.1% Triton X-100 for 5 minutes and blocked in PBS containing 2% BSA o/n at 4°C. Cells were stained for FAs using a monoclonal anti-vinculin antibody and the Alexa 568-coupled secondary antibody and for actin using Alexa 488-coupled phalloidin (Invitrogen). Images were acquired using a Zeiss Axioskop 2 microscope fitted with a Zeiss Axiocam CCD camera and 100X Plan APO objective lens.

Western blotting

Cells treated with siRNA as described above were replated directly after trypisinization into 6-well dishes (Corning) for an additional 24 hours, after which cells were washed twice with cold PBS and lysed in Laemmli Sample Buffer. Protein samples were separated by SDS-PAGE and transferred to PVDF (Immobilon). Western blot analysis was performed under standard conditions using the indicated antibodies. Membranes were probed with fluorophore-conjugated secondary antibodies (Westburg) and analyzed using the Odyssey Infra-red imaging system and software according to the manufacturer (LI-COR). Antibodies were from Santa Cruz (Rap1), BD Biosciences (RockII, Ezrin), Chemicon (α-tubulin), Sigma (vinculin, talin).

Results

007 stimulation induces specific effects during cell adhesion and spreading

To determine the molecular mechanism of Rap1-mediated regulation of focal adhesions, we have designed a replating assay. In short, cells were treated with either control or targeted SMARTpool siRNA oligos and replated for 3 hours on fibronectin-coated coverslips in the presence or absence of 007. Cells were fixed and stained for vinculin to assess the size and number of FAs and with phalloidin to examine the morphology of the actin cytoskeleton. Although differences can already be observed at shorter time-points when replating cells in the presence of 007, the three hour time-point was chosen because all cells are adhered and spread at that point. At short time-points, 007 also affects the number of cells that adhere. At intermediate time-points, the differences in cell size and shape start to become clear (data not shown). However, at these time-points, the cells are too small and round to judge the FA phenotype clearly. After three hours, both mock and 007-treated cells are adhered and spread and the difference in FAs is clearest. Untreated control cells show several focal adhesions per cell, preferentially at the corners of the cell. The actin cytoskeleton is not very organized, except for the strong cortical fibers running along the cell periphery. When cells treated with control siRNA are plated in the presence of 007, they spread more and concomitantly become round. Rap activation also induces an increase in the number of FAs that are now formed all around the cell periphery, instead of just at the corners (Figure 1A). A difference in the actin cytoskeleton can also be observed; the actin cytoskeleton now forms a broad band around the cell periphery, seemingly consisting of multiple small fibers. Because adhesion and spreading are increased at all time-points up to three hours in 007-treated cells, we wondered whether the phenotype we observe at three hours might be due to a speeding up of the normal adhesion process. To test this, we let cells treated with control siRNA adhere for up to seven hours (Figure 1B). However, even after seven hours of spreading, these cells do not obtain the same round shape as 007-treated cells (see Figure 1A), nor is the number of FAs increased compared to cells that adhered for three hours. The actin around the cell periphery also still shows the strongly stained cortical actin fibers and not the broad band of small fibers around the cell periphery (compare lower panels in Figure 1A to 1B). Thus, activation of Rap1 induces specific effects in these cells during cell adhesion and spreading.

Knockdown of Rap1A, but not Rap1B, abolishes 007-induced FA formation and spreading

To validate the assay, we first tested siRNA against Epac1 and Rap1A and Rap1B. As expected, knockdown of Epac1 completely abolishes the 007-induced phenotype (Figure 2B). Knockdown of Rap1A, but not Rap1B, has the same effect (Figure 2C and

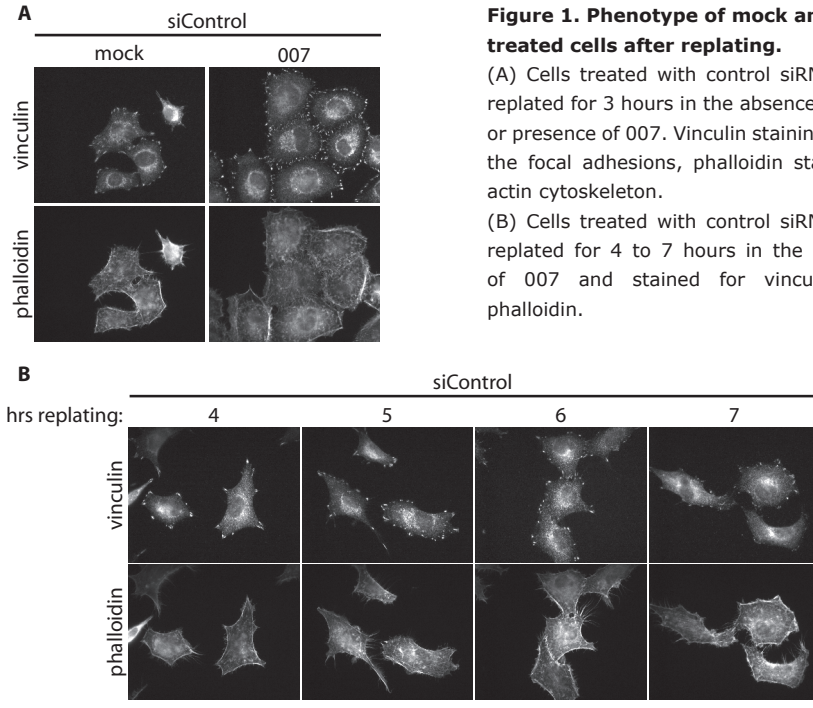


Figure 1. Phenotype of mock and 007-treated cells after replating.
(A) Cells treated with control siRNA were replated for 3 hours in the absence (mock) or presence of 007. Vinculin staining shows the focal adhesions, phalloidin stains the actin cytoskeleton.
(B) Cells treated with control siRNA were replated for 4 to 7 hours in the absence of 007 and stained for vinculin and phalloidin.

D). This indicates that the set-up of this assay is robust enough to identify key proteins required for the 007-induced effects. Additionally, it implies that in these cells, activation of Rap1A downstream of Epac1 mediates this effect, and that Rap1B cannot compensate for the loss of Rap1A. Furthermore, knockdown of Rap1A affects basal cell spreading and adhesion as well, indicating that, also in these cells, Rap1A is important for integrin-mediated adhesion.

Knockdown of talin1 inhibits Rap1-induced spreading and FA formation
To identify proteins involved in this phenotype downstream of Rap1, we made use of a custom library containing siRNAs against (putative) Rap1 effectors, Rap1GEFs and -GAPs and several adhesion- and actin-regulatory proteins. In total, 69 siRNAs were tested. The resulting phenotypes are listed in Table 1. As indicated above, knockdown of both Epac1 and Rap1A completely abolishes the 007-induced phenotype (Figure 2A). Knockdown of the integrin-activating protein talin1 also inhibits 007-induced spreading and FA formation, indicating that talin1 is a crucial player downstream of Rap1 in this pathway (Figure 3B). However, untreated cells are also affected by talin1 knockdown. After replating, fewer cells adhere to the coverslip as compared to control siRNA-transfected cells (not shown) and many cells that do adhere, stay rounded-up and do not spread (Figure 3B). Since talin1 is crucial for integrin activation (Zhang et al., 2008), this effect on 007- and untreated cells is not entirely unexpected. Talin1 was

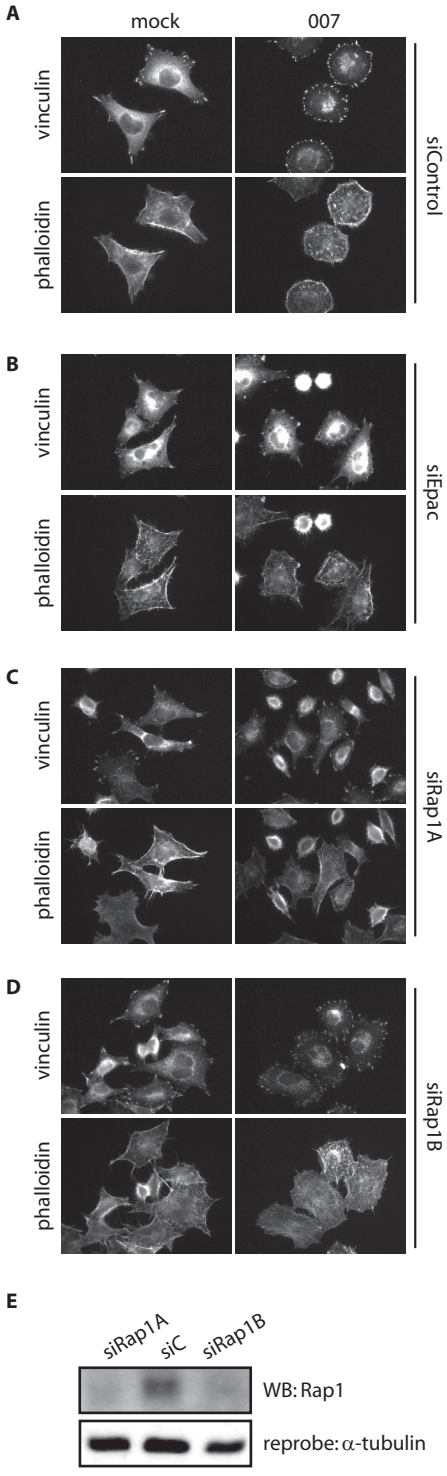


Figure 2. Example of screen showing results of Epac1, Rap1A and Rap1B siRNA. (A-D) Cells were treated with the indicated siRNAs and replated for three hours in the absence or presence of 007. Vinculin staining shows the focal adhesions, phalloidin stains the actin cytoskeleton. (E) Western blot showing knockdown of Rap1A and Rap1B.

also shown to be required downstream of Rap1 and its effector Riam, in the regulation of β3 integrins in αIIbβ3-expressing CHO cells (Han et al., 2006).

RockII and Ezrin

siRNA to RockII results in a defect in basal spreading and focal adhesion formation, and completely blocks the 007 effect. However, this block is only observed in about 50% of the cells, suggesting variations in the level of knockdown (Figure 3C). RockII is an effector of RhoA that is responsible for creating the actomyosin contractility required for FA growth (Riento and Ridley, 2003). Previously, we have shown that, in stably adherent cells, Rap1 does not affect the induction or regulation of contractility. In addition, Rap1-induced focal adhesions in adhered cells still required tension for their formation and were sensitive to treatment with the Rock inhibitor Y27632 (Chapter 4, this thesis). The identification of RockII in this screen as a component of the pathway downstream of Rap1 activation, indicates that RockII and the induction of tension are indeed required for the effect of Rap1 on focal adhesions. Similar to RockII siRNA, Ezrin siRNA inhibits about 50% of the cells from obtaining the 007 morphology (Figure

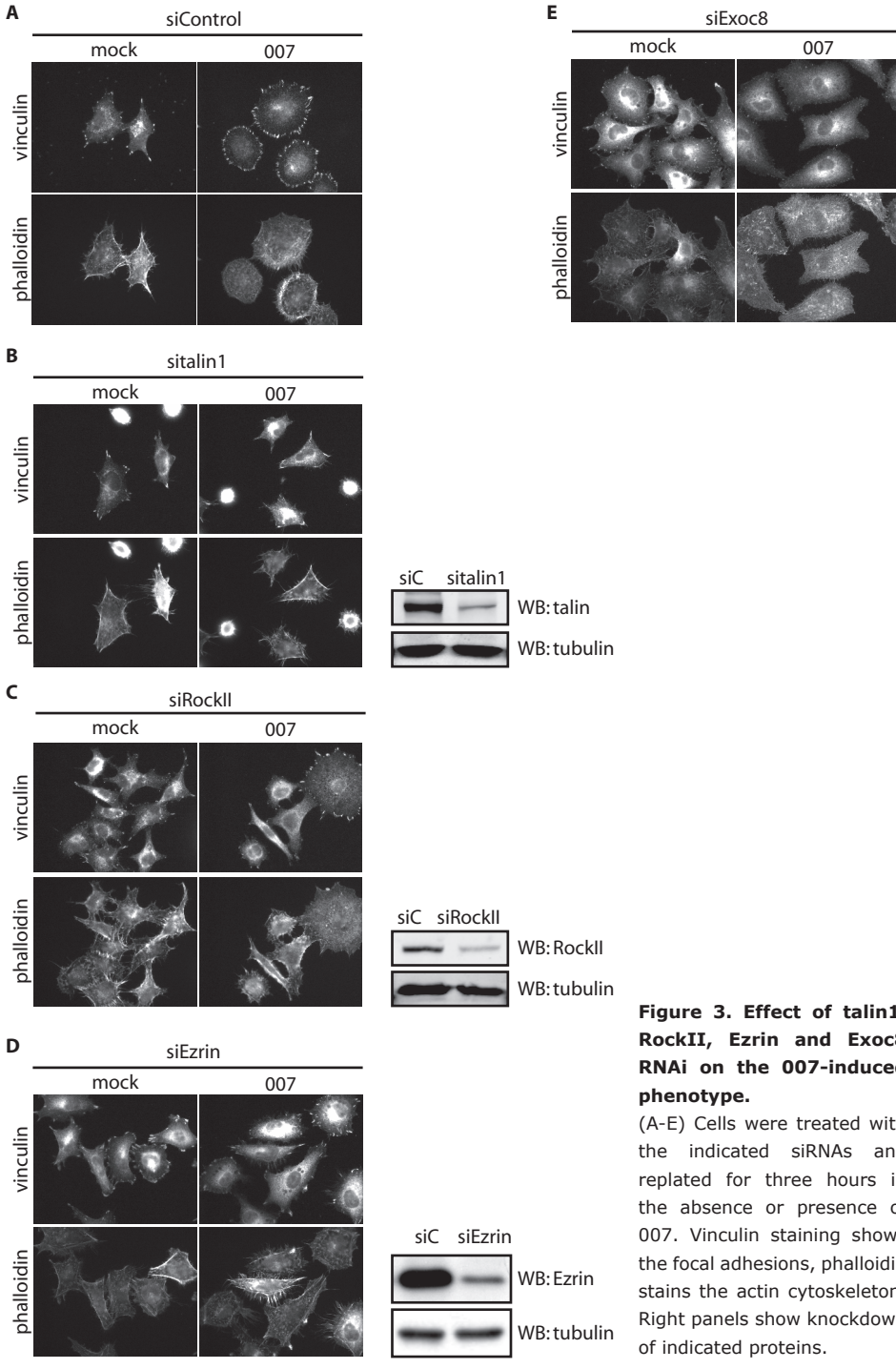


Figure 3. Effect of talin1, RockII, Ezrin and Exoc8 RNAi on the 007-induced phenotype.

(A-E) Cells were treated with the indicated siRNAs and replated for three hours in the absence or presence of 007. Vinculin staining shows the focal adhesions, phalloidin stains the actin cytoskeleton. Right panels show knockdown of indicated proteins.

3D). However, Ezrin siRNA does not affect the basal level of spreading. Ezrin is an actin-binding protein of the ERM family, which provides linkage between the plasma membrane and the actin cytoskeleton. Ezrin is an anchor protein for Epac1 at the plasma membrane (Gloerich et al., manuscript in preparation), suggesting that Ezrin is required for the proper positioning of Epac1 in these cells, to activate Rap1 in its correct location.

Exoc8

One siRNA with a very distinct effect on both mock- and 007-treated cells is siRNA to Exoc8. Exoc8 is a member of the exocyst complex, which is involved in polarized protein transport. The exocyst complex is an effector complex of the small GTPase Ral (Moskalenko et al., 2002). Knockdown of Exoc8 results in very flat, spread cells, with small, dot-like focal adhesions (Figure 3E). In addition, defects in the strong cortical actin fibers along the cell periphery can be observed. Upon stimulation with 007, somewhat more spreading is induced, but 007 fails to increase the number and size of the FAs. This implies a role for the exocyst in the regulation of the actin cytoskeleton and FAs. Furthermore, it suggests that the 007 effect on FAs is dependent on effects on the actin cytoskeleton, but independent of induction of spreading.

Multiple proteins partially affect the 007-induced phenotype

Surprisingly, none of the other siRNAs investigated, affected the 007 phenotype as completely as knockdown of the aforementioned siRNAs did. We did identify a number of proteins that partially block the 007 effect. These hits affect one or more of the different aspects of the 007 phenotype (*i.e.* cell spreading, cell rounding, FA increase or the morphology of the actin cytoskeleton). These proteins and their effects are indicated in Table 1. Several of these are proposed Rap1 effectors. For example, knockdown of the Rap1 effector AF-6, which was shown to inhibit Rap1-induced adhesion (Zhang et al., 2005), results in an increase in 007-induced FAs, supporting this earlier notion. Knockdown of the effectors RAPL and Riam as well as of the RA-domain containing protein Lamellipodin affects the actin cytoskeleton upon 007 stimulation, but does not affect the increase in spreading and focal adhesion formation. In these cells, the F-actin staining shows thin actin fibers all throughout the cells, instead of the broad band of small fibers around the cell periphery. No effects were observed with the siRNA against the Rap effector Krit1 (CCM1). However, knockdown of CCM2, a binding partner of Krit1, results in a strong increase in stress fibers upon 007 treatment. Knockdown of a different Krit1-binding protein, CCM3, has the same effect, although these cells already display more stress fibers in the untreated condition. Interestingly, knockdown of the Rap1GEF PDZGEF2 results in a similar phenotype as CCM2 knockdown. The phenotype resulting from knockdown of the related GEF, PDZGEF1, is also rather striking, as

some of these cells display an 007-like phenotype in the untreated condition. These subtle effects of known Rap1 effectors suggest that Rap1 uses redundant effector pathways to regulate this phenotype. In addition, it may be that not all components of this pathway have been identified yet.

Protein	*Spreading and focal adhesion induction*	*Actin phenotype*
α-catenin	Normal	Normal
AF-6	Normal. Some cells show increased FAs underneath cell surface.	Normal
AF-6-like	Partial phenotype, slight spreading and FA induction defect.	Mixed
αPKC	Normal	Normal
Arap1	Normal	Normal
Arap3	Small basal adhesion/spreading defect, normal response to 007.	Normal
ARHGAP20	Small basal adhesion/spreading defect, normal response to 007.	Normal
β-catenin	Mock cells are elongated and display increased protrusions. Normal induction of spreading and FAs, but cells remain elongated.	Increase in thin actin fibers.
C3G	Basal spreading defect, which is rescued and further induced by 007.	Normal
CCM1	Normal	Normal
CCM2	Extra induction of FAs underneath cell surface.	Increase in stress fibers.
CCM3	Mock cells already more spread, with many protrusions. Spreading and FAs still induced upon 007.	Increase in stress fibers.
CDC42	Normal	Normal
DGKQ	Normal	Normal
DLG5	Normal	Normal
EBP50	Normal	Normal
E-cadherin	Normal	Normal
Epac1	No spreading, no FA induction.	Indistinguishable from mock cells.
Epac2	Normal	Normal
Exoc2	Normal	Normal
Exoc8	Mock cells already spread, with small FAs. 007 still induces spreading but FAs remain small.	Basal increase in actin protrusions/filopodia. 007 induces small stress fibers throughout cells.

Table 1. Overview of focal adhesion screen results.

Phenotypes listed below were determined by visual inspection of replated cells.

Ezrin	Partial spreading and FA induction defect.	Mixed
FRMPD1	Normal	Normal
ICAP	Normal	Normal
IQGAP	Normal	Normal
Lamellipodin	Normal	Thin unorganized actin fibers throughout cells.
LIMK	Normal	Normal
Occludin	Normal	Normal
p120-catenin	Normal	Normal
Par3	Normal	Normal
Par6	Normal	Normal
PDZGEF1	Mixed; some mock cells show 007-phenotype. Normal response to 007.	Mixed; some mock cells show 007-phenotype.
PDZGEF2	Normal. Some cells show increased FAs underneath cell surface.	Increase in small stress fibers throughout cells.
PDZK10	Normal	Normal
Rac1	Normal	Normal
RalA	Normal	Normal
RalB	Normal	Normal
RalGDS	Normal	Normal
Rap1A	Basal spreading defect. No spreading, no FA induction upon 007.	Indistinguishable from mock cells.
Rap1B	Normal	Normal
Rap1GAP1	Normal. Some cells show increased FAs underneath cell surface.	Increase in small stress fibers throughout cells.
Rap1GAP2	Normal	Normal
Rap2A	Normal	Normal
Rap2B	Normal	Normal
Rap2C	Normal	Normal
RAPL	Normal	Increase in small stress fibers throughout cells.
RasGRP1	Normal. Some cells show increased FAs underneath cell surface.	Normal
RasGRP2	Normal	Normal
RasGRP3	Normal	Normal
Rgl1	Normal. Some cells show increased FAs underneath cell surface.	Normal
RhoA	Normal	Normal
Riam	Normal	Increase in small stress fibers throughout cells.

Rin1	Normal. Some cells show increased FAs underneath cell surface.	Normal
RockI	Normal	Normal
RockII	Partial spreading and FA induction defect.	Mixed
R-Ras	Normal	Normal
Sec15L1	Normal	Normal
Sec15L2	Normal	Normal
Sec8	Normal	Normal
SHIP2	Normal	Normal
Spa1	Normal	Normal
Talin1	Defect in basal adhesion and spreading. Not rescued by 007.	Indistinguishable from mock cells.
Talin2	Normal	Normal
Tiam1	Normal	Normal
VASP	Normal	Normal
Vav1	Normal	Normal
Vav2	Normal. Cells do not become very round.	Normal
Vav3	Normal	Normal
ZAK	Normal	Increase in small actin fibers.

Discussion

Here, we describe a screening approach to identify proteins downstream of activation of Epac1 by 007 that are essential for 007-induced cell spreading and FA formation. This screen was set up in such a way that we can expect to identify two classes of proteins. Firstly, proteins that are essential to the adhesion and FA formation process and second, proteins that mediate 007-induced FA formation only. The screen was validated by showing that knockdown of Epac1 completely blocked the 007-induced effects. In addition, we show that Rap1A, and not Rap1B, mediates this effect, although both proteins are expressed. A differential effect of Rap1A and Rap1B knockdown has been reported previously in junction formation (Dube et al., 2008).

Important roles for talin1, RockII and Ezrin

We tested 69 different siRNAs selected based on their reported role as effector for Rap1 or their potential role in adhesion. Three siRNAs completely inhibited the 007-effect, indicating a crucial role in 007-induced adhesion and/or spreading.

Talin is required for integrin affinity changes and links integrin to the actin cytoskeleton (Ginsberg et al., 2005). Two isoforms of talin exist, of which talin1 is ubiquitously expressed. Knockdown of talin1 completely inhibited all 007-induced effects, indicating that talin1 is essential for

these Rap1-induced effects. Talin had already been implicated downstream of Rap1 in the regulation of β3 integrins in αIIbβ3-expressing CHO cells (Han et al., 2006; Watanabe et al., 2008). In these cells, the Rap effector Riam is involved in stimulus-induced, Rap1-mediated recruitment of talin. Talin then binds to and activates the β-chain of integrin αIIbβ3 (Han et al., 2006). Recently, complete knockdown of both talin isoforms showed that talin is required for sustained spreading and adhesion, but not for initial adhesion. Focal adhesion formation was also severely affected (Zhang et al., 2008). Clearly, 007 cannot induce focal adhesion maturation in cells depleted for talin1, stressing the notion that talin1 is required for Rap1-induced focal adhesion formation. However, this siRNA also caused strong defects in untreated cells. Interestingly, we observed similar results with a single siRNA oligo against vinculin, a binding partner of talin and important component of FAs. However, we could not confirm these data with the vinculin SMARTpool siRNAs and note that side-effects of this vinculin oligo have been observed in other systems (Q. leDuc, personal communication).

siRNA to RockII had a somewhat similar affect as talin1 siRNA, as it also completely inhibited the 007 effect, but slightly inhibited basal spreading. The presence of contractile forces is one of the main requirements for FA formation; this is illustrated by the tight regulation of Rho activity during adhesion (Ren et al., 2000). Again, this effect on untreated cells makes it difficult to determine whether Rock is involved in 007-induced FA formation directly or in a parallel pathway. Previous data do confirm that Rap1-induced FAs require tension normally. In stably adherent cells, 007-induced focal adhesions were sensitive to treatment with the Rock inhibitor Y27632 (Chapter 4, this thesis). This indicates that RockII and the induction of tension are indeed required for the effect of Rap1 on focal adhesions.

Another siRNA that completely inhibited the 007 effect in 50% of the cells, but did not affect basal spreading or FA formation, was siRNA to the ERM protein Ezrin. Ezrin RNAi, thus, specifically affected 007-induced spreading and FA formation. ERM proteins are membrane-cytoskeleton linkers that control actin-based functions including adhesion and motility (Bretscher et al., 2000). They are characterized by the conserved N-terminal FERM domain, which is also present in the integrin-binding head domain of talin. Although it is not exactly understood how they function, ERM proteins are thought to act as scaffolds by recruiting the appropriate downstream effectors to specific locations (Poullet et al., 2001). Normally, they exist in an auto-inhibited conformation, which is relieved upon phosphorylation. Interestingly, one of the kinases implicated in the regulation of ERM protein activation is Rock (Matsui et al., 1998). Furthermore, one of the proteins recruited to the plasma membrane by Ezrin is Epac1 (Gloerich et al., manuscript in preparation). Although siRNAs against the two other family members, Moesin and Radixin,

were not included in this screen, it seems Ezrin has a non-redundant function in this assay, which may be to correctly position Epac1 in these cells, to activate Rap1 in its correct location.

A surprising result was found with siRNA to Exoc8. Exco8 is a member of the exocyst complex, an effector complex of Ral, that is involved in polarized protein and membrane transport (Moskalenko et al., 2002). One of the proteins targeted to the basolateral membrane by the exocyst complex is E-cadherin (Shipitsin and Feig, 2004). Interestingly, recently a role for the exocyst in plasma membrane delivery of integrins was also shown (Spiczka and Yeaman, 2008). Knockdown of Exoc8 affected FA size under basal and 007-stumlated conditions and resulted in an increase in basal spreading and defects in the actin cytoskeleton. Stimulation with 007 was unable to further increase the size of these FAs, suggesting a role for Exoc8 in the maturation of adhesion complexes. This result has been repeated with this SMARTpool siRNA several times. Unfortunately, the Exoc8 antibody does not work well enough to show expression or knockdown of the endogenous protein. We are currently investigating the effect of other exocyst components as well. The effect of Exoc8 on the actin cytoskeleton and the inability of 007 to increase FA size in these cells, again suggests that a connection with the cytoskeleton is required for the 007 effect on FAs.

Several Rap1 effector proteins partially affect the 007 phenotype

Surprisingly, the strong phenotypes as described above were not observed with any of the known or putative Rap1 effectors. This may be due to the fact that our knockdown was insufficient to identify the critical effector, or that the true effector was not present in our screen. However, alternatively, there may be redundancy in the signaling from Rap1 towards focal adhesions. Indeed, many effectors have been described to mediate Rap1-induced adhesion, *i.e.* the RacGEFs Tiam and Vav, the RhoGAPs Arap3 and RA-RhoGAP and the adaptor proteins RAPL, Riam and protein kinase D (reviewed in (Raaijmakers and Bos, 2008)).

We did identify a number of hits that have a phenotype affecting one or more of the different aspects of the 007 phenotype (*i.e.* cell spreading, reorganization of the actin cytoskeleton or FA formation). Interestingly, knockdown of the two effectors RAPL and Riam as well as of the RA-domain containing protein Lamellipodin affected the actin cytoskeleton in a similar manner. Also CCM2 and CCM3, both binding partners of the Rap effectors Krit1, gave similar phenotypes. The roles of such proteins may be addressed by investigating whether the knockdown of more than one protein at the same time will have a bigger effect on the 007 phenotype. For instance, preliminary results indicate that when both Riam and its close homologue Lamellipodin are knocked down at the same time, more cells display

defects in 007-induced changes in the morphology of the actin cytoskeleton. Within a single experiment, some cells transfected with both siRNAs look similar to cells treated with Riam siRNA, while other cells show more severe defects. These range from the presence of more stress fibers to the loss of cell spreading. The observed variability in these phenotypes may be the result of differential knockdown of both proteins within individual cells. This suggests that both proteins may be involved in some aspects of 007-induced effects, as was suggested before for the regulation of the αIIbβ3 integrin (Lee et al., 2008).

The lack of identification of proteins with smaller, or redundant, roles downstream of Rap1 may also be due to the set-up of this screen. For instance, the three-hour adhesion time-point allows the cells to overcome the possibly subtle effects of a siRNA on adhesion or spreading. Furthermore, although necessary in this set-up, the replating itself can affect the outcome. When proteins crucial for adhesion, such as talin1 or Rap1A are knocked down, only cells with the least amount of knockdown will be able to adhere and spread normally. By replating, we thus lose some of the cells with the best transfection efficiency or knockdown.

In conclusion, we showed here that the 007-induced effects on spreading and FA formation are mediated by Rap1A and not Rap1B and identified important roles for talin1, RockII and Ezrin. In addition, we identified a number of proteins that partially modulate the Rap1 effect. These results further suggest that Rap1 uses redundant effector pathways to regulate these 007-induced effects.

References

Bos, J.L. (2005) Linking Rap to cell adhesion. *Curr Opin Cell Biol*, **17**, 123-128.

Bos, J.L., de Bruyn, K., Enserink, J., Kuiperij, B., Rangarajan, S., Rehmann, H., Riedl, J., de Rooij, J., van Mansfeld, F. and Zwartkruis, F. (2003) The role of Rap1 in integrin-mediated cell adhesion. *Biochem Soc Trans*, **31**, 83-86.

Bretscher, A., Chambers, D., Nguyen, R. and Reczek, D. (2000) ERM-Merlin and EBP50 protein families in plasma membrane organization and function. *Annu Rev Cell Dev Biol*, **16**, 113-143.

Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol*, **12**, 463-518.

Dube, N., Kooistra, M.R., Pannekoek, W.J., Vliem, M.J., Oorschot, V., Klumperman, J., Rehmann, H. and Bos, J.L. (2008) The RapGEF PDZ-GEF2 is required for maturation of cell-cell junctions. *Cell Signal*, **20**, 1608-1615.

Enserink, J.M., Price, L.S., Methi, T., Mahic, M., Sonnenberg, A., Bos, J.L. and Tasken, K. (2004) The cAMP-Epac-Rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the alpha3beta1 integrin but not the alpha6beta4 integrin. *J Biol Chem*, **279**, 44889-44896.

Geiger, B., Bershadsky, A., Pankov, R. and Yamada, K.M. (2001) Transmembrane extracellular matrix-cytoskeleton crosstalk. *Nat Rev Mol Cell Biol*, **2**, 793-805.

Ginsberg, M.H., Partridge, A. and Shattil, S.J. (2005) Integrin regulation. *Curr Opin Cell Biol*, **17**, 509-516.

Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J. and Ginsberg, M.H. (2006) Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol*, **16**, 1796-1806.

Kinbara, K., Goldfinger, L.E., Hansen, M.,

Chou, F.L. and Ginsberg, M.H. (2003) Ras GTPases: integrins' friends or foes? *Nat Rev Mol Cell Biol*, **4**, 767-776.

Kooistra, M.R., Dube, N. and Bos, J.L. (2007) Rap1: a key regulator in cell-cell junction formation. *J Cell Sci*, **120**, 17-22.

Lee, H.S., Lim, C.J., Puzon-McLaughlin, W., Shattil, S.J. and Ginsberg, M.H. (2008) RIAM activates integrins by linking talin to Ras GTPase membrane-targeting sequences. *J Biol Chem*.

Lyle, K.S., Raaijmakers, J.H., Bruinsma, W., Bos, J.L. and de Rooij, J. (2008) cAMP-induced Epac-Rap activation inhibits epithelial cell migration by modulating focal adhesion and leading edge dynamics. *Cell Signal*, **20**, 1104-1116.

Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S. and Tsukita, S. (1998) Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. *J Cell Biol*, **140**, 647-657.

Menasche, G., Kliche, S., Bezman, N. and Schraven, B. (2007) Regulation of T-cell antigen receptor-mediated inside-out signaling by cytosolic adapter proteins and Rap1 effector molecules. *Immunol Rev*, **218**, 82-91.

Moskalenko, S., Henry, D.O., Rosse, C., Mirey, G., Camonis, J.H. and White, M.A. (2002) The exocyst is a Ral effector complex. *Nat Cell Biol*, **4**, 66-72.

Poullet, P., Gautreau, A., Kadare, G., Girault, J.A., Louvard, D. and Arpin, M. (2001) Ezrin interacts with focal adhesion kinase and induces its activation independently of cell-matrix adhesion. *J Biol Chem*, **276**, 37686-37691.

Raaijmakers, J.H. and Bos, J.L. (2008) Specificity in Ras and Rap signaling. *J Biol Chem*.

Ren, X.D., Kiosses, W.B., Sieg, D.J., Otey, C.A., Schlaepfer, D.D. and Schwartz, M.A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci*, **113 (Pt 20)**, 3673-3678.

Riento, K. and Ridley, A.J. (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol*, **4**, 446-456.

Shipitsin, M. and Feig, L.A. (2004) RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. *Mol Cell Biol*, **24**, 5746-5756.

Spiczka, K.S. and Yeaman, C. (2008) Ral-regulated interaction between Sec5 and paxillin targets Exocyst to focal complexes during cell migration. *J Cell Sci*, **121**, 2880-2891.

Watanabe, N., Bodin, L., Pandey, M., Krause, M., Coughlin, S., Boussiotis, V.A., Ginsberg, M.H. and Shattil, S.J. (2008) Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin alphaIIbbeta3. *J Cell Biol*, **181**, 1211-1222.

Webb, D.J., Parsons, J.T. and Horwitz, A.F. (2002) Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat Cell Biol*, **4**, E97-100.

Zhang, X., Jiang, G., Cai, Y., Monkley, S.J., Critchley, D.R. and Sheetz, M.P. (2008) Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nat Cell Biol*, **10**, 1062-1068.

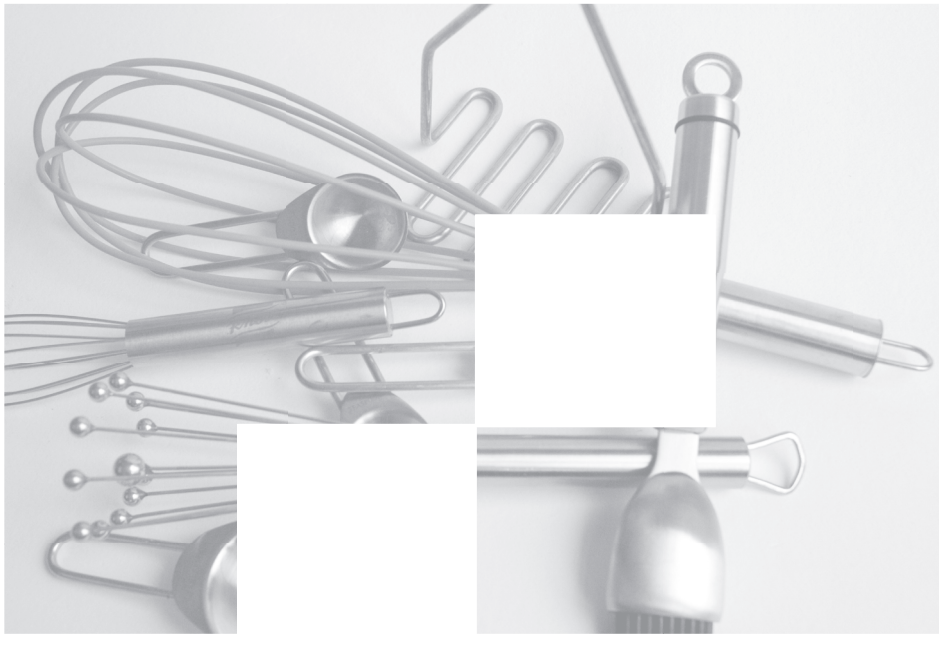
Zhang, Z., Rehmann, H., Price, L.S., Riedl, J. and Bos, J.L. (2005) AF6 negatively regulates Rap1-induced cell adhesion. *J Biol Chem*, **280**, 33200-33205.

chapter 6

The PI3K effector Arap3 interacts with the PI(3,4,5)P_3 phosphatase SHIP2 in a SAM domain-dependent manner

Judith H. Raaijmakers, Laurence Deneubourg, Holger Rehmann, John de Koning, Zhongchun Zhang, Sonja Krugmann, Christophe Erneux and Johannes L. Bos

Cellular Signaling 19 (2007) 1249-1257



The PI3K effector Arap3 interacts with the PI(3,4,5)P_3 phosphatase SHIP2 in a SAM domain-dependent manner

Judith H. Raaijmakers[1], Laurence Deneubourg[2], Holger Rehmann[1], John de Koning[3], Zhongchun Zhang[1], Sonja Krugmann[4], Christophe Erneux[2] and Johannes L. Bos[1]

[1]Department of Physiogical Chemistry and Centre for Biomedical Genetics, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands
[2]Interdisciplinary Research Institute (IRIBHM), Université Libre de Bruxelles, Campus Erasme, 808 Route de Lennik, Brussels, Belgium
[3]Hybrigenics SA, 3-5 Impasse Reille, 75014 Paris, France
[4]Inositide Laboratory, The Brabraham Institute, Cambridge CB2 4AT, United Kingdom

Abstract

Arap3 is a phosphoinositide (PI) 3 kinase effector that serves as a GTPase activating protein (GAP) for both Arf and Rho G-proteins. The protein has multiple pleckstrin homology (PH) domains that bind preferentially phosphatidyl-inositol-3,4,5–trisphosphate (PI(3,4,5,)P_3) to induce translocation of Arap3 to the plasma membrane upon PI3K activation. Arap3 also contains a Ras association (RA) domain that interacts with the small G-protein Rap1 and a sterile alpha motif (SAM) domain of unknown function. In a yeast two-hybrid screen for new interaction partners of Arap3, we identified the PI 5'-phosphatase SHIP2 as an interaction partner of Arap3. The interaction between Arap3 and SHIP2 was observed with endogenous proteins and shown to be mediated by the SAM domain of Arap3 and SHIP2. *In vitro*, these two domains show specificity for a heterodimeric interaction. Since it was shown previously that Arap3 has a higher affinity for PI(3,4,5,)P_3 than for PI(3,4)P_2, we propose that the SAM domain of Arap3 can function to recruit a negative regulator of PI3K signaling into the effector complex.

Introduction

The phosphoinositide (PI) 3 kinase (PI3K) pathway plays an important role in various signaling pathways, such as insulin signaling, membrane trafficking and the regulation of cell dynamics, via production of the second messenger PI(3,4,5,)P_3 (Leevers et al., 1999; Vanhaesebroeck et al., 2001). Arap3 (Arf GAP, Rho GAP, Ankyrin repeat and PH domains) is a PI3K effector protein that was first identified through its ability to bind PI(3,4,5,)P_3 lipids (Krugmann et al., 2002). Upon binding of PI(3,4,5,)P_3 lipids to one of its pleckstrin homology (PH) domains, Arap3 translocates to the plasma membrane and is activated to serve as a dual GTPase activating protein (GAP) for Arf and Rho G-proteins (Krugmann et al., 2004). Arap3 is implicated in the regulation of the actin cytoskeleton, lamellipodia formation and cell spreading (Krugmann et al., 2006; Stacey et al., 2004). In addition, Arap3 contains an RA domain that binds specifically to the small G-protein Rap1 and a SAM domain of unknown function (Krugmann et al., 2004) (Figure 1A).

The SAM domain is a 60-70 amino acid motif that mediates protein-protein, protein-RNA and protein-lipid interactions (Barrera et al., 2003; Kim and Bowie, 2003). SAM domains are found in over 1000 proteins with diverse cellular functions and in organisms from yeast to man (Qiao and Bowie, 2005). They mediate protein-protein interactions by either homo- or heterodimerization or through oligomerization (Kwan et al., 2006; Stapleton et al., 1999; Thanos et al., 1999). Thus far, the function and the binding partner of the Arap3 SAM domain are unknown. Besides PI(3,4,5,)P_3 lipids and $Rap1^{GTP}$, the only known interaction partner for Arap3 is the adaptor protein CIN85 that is involved in the internalization of monoubiquitinated membrane proteins (Dikic, 2002; Kowanetz et al., 2004). This interaction is mediated by a proline-arginine motif in Arap3 that is specific for the CIN85 SH3 domain (Figure 1A).

The SH2 domain-containing inositol 5'-phosphatase SHIP2 hydrolyzes PI(3,4,5)P_3 to PI(3,4)P_2 (Pesesse et al., 1997; Pesesse et al., 1998). It is ubiquitously expressed and, together with SHIP1 and PTEN, inhibits PI3K-activated signaling pathways (Backers et al., 2003). Studies in SHIP2 knock-out mice suggest that SHIP2 plays a role in controlling insulin sensitivity and obesity, probably by decreasing the level of active protein kinase B (PKB) (Clement et al., 2001; Sleeman et al., 2005). Like Arap3, SHIP2 has several protein-protein interaction domains. Besides its SH2 domain that mediates the recruitment of SHIP2 to activated receptor tyrosine kinases (Koch et al., 2005; Pesesse et al., 2001) and its catalytic phosphatase domain, SHIP2 has a proline-rich region followed by a C-terminal SAM domain. Several interaction partners are known for SHIP2, including the HGF receptor c-Met (Koch et al., 2005), the E3 ubiquitin ligase Cbl and Cbl-associated protein (CAP) (Vandenbroere et al., 2003). In addition to its function in down-regulating the insulin pathway, SHIP2 is also linked to the regulation of the actin cytoskeleton and cell adhesion, mainly by its ability to bind proteins such as filamin (Wang et al., 2004), vinexin (Paternotte et al., 2005), p130Cas (Prasad et al., 2001) and Shc (Habib et al., 1998; Wisniewski et al., 1999). A role in endocytosis and the down-regulation of the EGF and EphA2 receptors has also been proposed for SHIP2 (Prasad et al., 2002; Zhuang et al., 2006). Furthermore, after growth factor stimulation or adhesion, SHIP2 becomes phosphorylated and can relocalize to membrane ruffles (Habib et al., 1998; Prasad et al., 2002). Thus, SHIP2 can regulate changes in PI(3,4,5,)P_3 levels and is involved in the organization of the actin cytoskeleton. Until recent, binding partners were known only for the SHIP2 SH2 domain (Koch et al., 2005; Prasad et al., 2001; Wisniewski et al., 1999) and proline-rich region (Paternotte et al., 2005; Vandenbroere et al., 2003; Wang et al., 2004), not for its SAM domain, but it has now been shown that the EphA2 receptor binds to SHIP2 through dimerization of both SAM domains (Zhuang et al., 2006).

To get more insight into the role of Arap3 as a downstream PI3K effector protein, we looked for new interaction partners of Arap3. Here, we describe the identification of the lipid phosphatase SHIP2 as a binding partner of Arap3 and show that the interaction is mediated by heterodimerization of their SAM domains. We show that the SAM domains are both necessary and sufficient for this interaction and that the two domains have a high affinity for one another. Since Arap3 is a protein regulated by PI(3,4,5)P_3, the substrate for SHIP2, we propose that the SAM domain of Arap3 can function to recruit a negative regulator of PI3K signaling into the effector complex.

Materials and methods

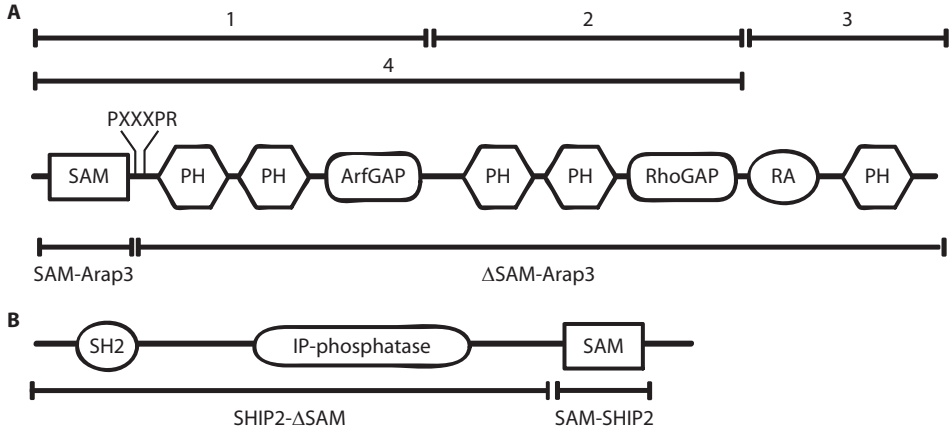
Antibodies and reagents

Monoclonal antibodies recognizing the FLAG-M2 epitope and the penta-His epitope were obtained from Sigma and Qiagen, respectively. Rabbit polyclonal anti-SHIP2 antibody and sheep anti-Arap3 antibody were described before (Pesesse et al., 1998; Krugmann et al., 2002). Where indicated, cells were stimulated with 20 ng/ml EGF (ICN Biomedicals Inc), 1 µg/ml insulin (Sigma), 10 µM LY294002 (Sigma).

Plasmids and constructs

GFP-ΔSAMArap3 (residues 71-1544) was made using mutagenesis PCR with GFP-Arap3 (Krugmann et al., 2002) as a template. FlagHis-tagged Arap3 was created using Gateway Technology (Invitrogen). FlagHis-ΔSAMArap3 (residues 71-1544) was also made using mutagenesis with FlagHis-Arap3 as a template. His-tagged SHIP2 and His-t-SHIP2 have been described before (Pesesse et al., 2001). His-SHIP2-ΔSAM (residues 1-1192) was made using mutagenesis PCR. The GST-tagged SAM domains of Arap3 (residues 1-75) and SHIP2 (residues

Figure 1. Domain composition of Arap3 and SHIP2.
Schematic representation of the domain structure of (A) Arap3 and (B) SHIP2. Numbers above indicated parts of Arap3 show the truncation mutants used in the Y2H screen. Both Arap3-1 and Arap3-4 interacted with SHIP2. The CIN85-binding proline-arginine motif in Arap3 is indicated as well.



1192-1258) were made by inserting *Sal*I/*Not*I-digested PCR products into *Xho*I/*Not*I digested pGEX-4T3 vector (Pharmacia). HA-RapV12 and HA-RapGAP were described previously (Reedquist et al., 2000; Zwartkruis et al., 1998).

Yeast two-hybrid screen
Four different Arap3 truncation constructs (residues 1-607, 608-1089, 1089-1544 and 1-1089) were PCR-amplified, cloned into a plasmid derived from pBTM116 using Gateway Technology and sequence verified. Yeast two-hybrid screening was carried out by Hybrigenics S.A. (Paris, France) as previously described (Colland et al., 2004).

Cell culture and transfections
HEK293T, HeLa and MEF cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and glutamine. Cells were transfected using FuGENE6 transfection reagent according to manufacturer's instructions (Roche). Typically, for a 10 cm culture dish, 2 µg DNA was used per construct. Where indicated, cells were serum starved overnight in DMEM with supplements but without FBS.

Co-immunoprecipitations
Cells were washed twice in ice-cold PBS, lysed in lysis buffer (1% Triton X-100, 50 mM Tris HCl pH 7.5, 150 mM NaCl and protease inhibitors) and lysates were centrifuged at 14000 rpm at 4°C for 8 minutes. After centrifugation, samples were taken to analyze total cell lysate, the rest was incubated with protein agarose beads and either non-immune serum or the appropriate antibody for 2 hours at 4°C. After incubation, precipitates were washed 3 times with lysis buffer before dissolving bound proteins in Laemmli sample buffer. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF, Immobilon). Western blot analysis was performed under standard conditions using the indicated antibodies. Membranes were probed with fluorophore-conjugated secondary antibodies and analysed using the Odyssey Infra-red imaging system and software according to the manufacturer (LI-COR) or with horseradish peroxidase-coupled secondary antibodies and standard enhanced chemiluminescence (Amersham).

GST pull-down assays
Cells were lysed and centrifuged as described. Glutathion-agarose beads were washed twice in lysis buffer and incubated with equal amounts of GST, GST-SAM-Arap3 or GST-SAM-SHIP2 for 30 minutes at 4°C. Beads were washed three times with lysis buffer and incubated with lysate for 1 hour at 4°C. After incubation, beads were washed three times with lysis buffer before dissolving bound protein in Laemmli sample buffer. Bound proteins were analyzed as described above.

Protein purification, gel filtration and ITC
The SAM domains of Arap3 and SHIP2 were expressed from pGEX-4T3 (Pharmacia) as GST-fusion proteins in BL21 cells. Bacteria were grown at 37ºC and 170 rpm in Standard I medium (Merck). Protein expression was induced after an OD_{600} of 0.8 was reached and the bacteria were cultured overnight at room temperature, collected by centrifugation, resuspended in 50 mM Tris HCl pH 7.5, 50 mM NaCl, 5% glycerol, 5 mM DTE and 5mM EDTA and lysed by sonication. Insoluble material was removed by centrifugation at 30,000x*g* and the soluble fraction was loaded onto a 20 ml Glutathione-column (Pharmacia). The column was washed with at least 5 volumes of 50 mM Tris HCl pH 7.5, 400 mM NaCl, 5% glycerol and 5 mM DTE and 2 volumes of 50 mM Tris HCl pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, 5% glycerol and 5 mM DTE (buffer T). The column was loaded with 200 Units Thrombin (Serva) in buffer T, incubated overnight at 4ºC and eluted with buffer T. Protein-containing fractions were concentrated using a Millipore concentrator unit (cut off 5 kDa) to a concentration of approximately 200 g/l.
Gel filtration experiments were carried out on a Sephacryl 100 (26/60) column (Pharmacia) equilibrated with 50 mM Tris HCl pH 7.5, 50 mM NaCl, 2,5% glycerol and 5 mM DTE.
For ITC experiments, the buffer was exchanged to 100 mM K-phosphate, 50 mM NaCl and 5 mM DTE by gel filtration. Prior to loading onto the column, the protein solution was diluted in an equal volume of phosphate buffer and calcium phosphate was removed by centrifugation. ITC experiments were carried out at 25ºC using a VP-ITC instrument (MicroCal, USA).

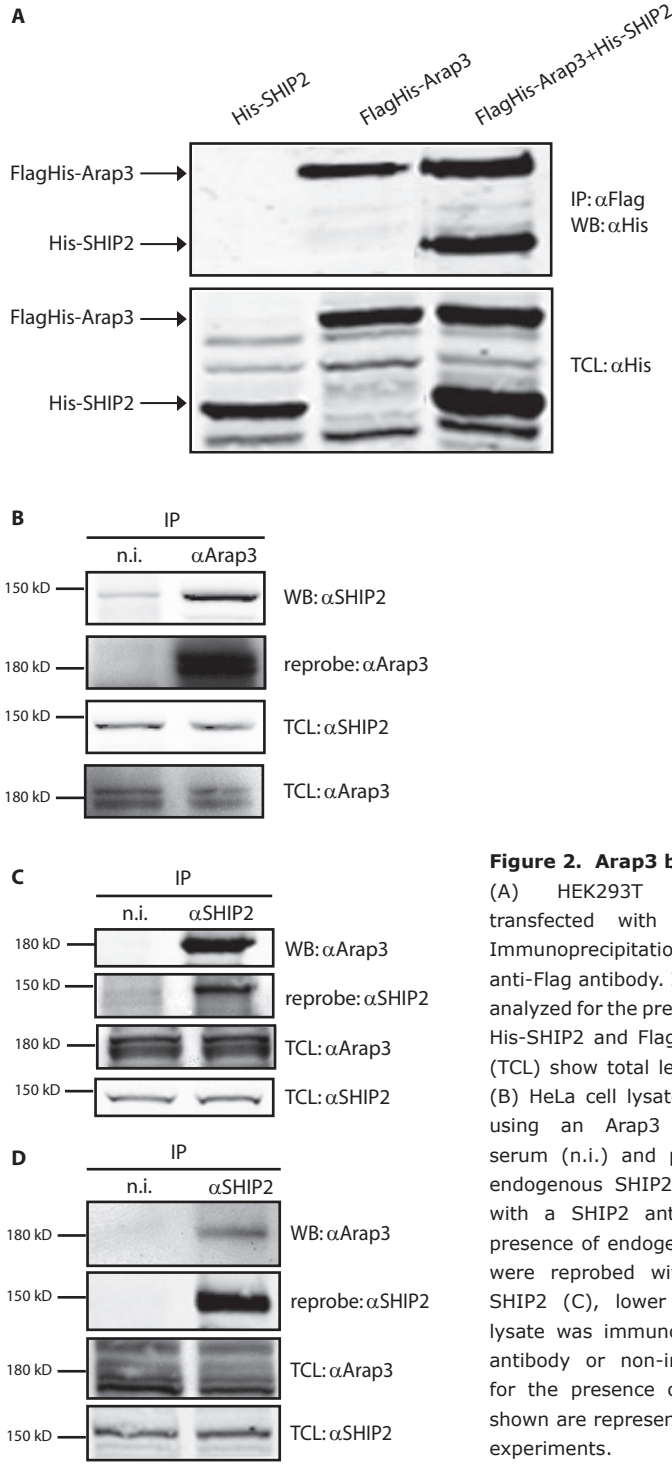


Figure 2. Arap3 binds SHIP2 in vivo.
(A) HEK293T cells were transiently transfected with the indicated constructs. Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates IP) were analyzed for the presence of both over-expressed His-SHIP2 and FlagHis-Arap3. Total cell lysates (TCL) show total levels of transfected proteins. (B) HeLa cell lysates were immunoprecipitated using an Arap3 antibody or non-immune serum (n.i.) and probed for the presence of endogenous SHIP2 (B) or immunoprecipitated with a SHIP2 antibody and probed for the presence of endogenous Arap3 (C). Membranes were reprobed with anti-Arap3 (B) or anti-SHIP2 (C), lower panels. (D) HEK293T cell lysate was immunoprecipitated using a SHIP2 antibody or non-immune serum and probed for the presence of endogenous Arap3. Blots shown are representatives of at least 3 identical experiments.

Results

Identification of a SAM domain-mediated interaction between Arap3 and SHIP2

To identify putative regulators of Arap3, we performed a yeast two-hybrid screen of a human placenta cDNA library using four different truncation mutants of Arap3 as baits (Figure 1A). Table 1 shows a list of ten proteins that were identified with the various Arap3 constructs. Interestingly, several of these proteins possess a SAM domain as is present in Arap3. For two of these proteins, ANKS1 and SHIP2, the fragments recovered from the yeast two-hybrid screen included the SAM domain, suggesting that these interactions were mediated by the dimerization of the SAM domains. As the inositol 5'-phosphatase SHIP2 is a known regulator of the PI3K pathway, we focused on the characterization of the interaction between Arap3 and SHIP2 (Figure 1B). To validate the result from the yeast two-hybrid screen, we performed a co-immunoprecipitation experiment with over-expressed Arap3 and SHIP2 in HEK293T cells (Figure 2A). Indeed, Arap3 is able to pull down full-length SHIP2 *in vivo*.

To verify that the endogenous proteins are in the same complex, we performed co-immunoprecipitations in HeLa cells with either anti-Arap3 or anti-SHIP2 antibody (Figures 2B and 2C, respectively) and in 293T cells with anti-SHIP2 antibody (Figure 2D). Although there is some unspecific binding of SHIP2 in the control samples where non-immune serum was used, the amount of co-precipitated protein is far higher in the lanes where anti-Arap3 antibody was used to precipitate the complex (Figure 2B). Interestingly, while the Arap3 antibody recognizes a double band in both whole cell lysate and after immunoprecipitation with the same antibody (Figure 2B), only the slower migrating protein is recovered with SHIP2 (Figures 2C-D). Although a doublet has been observed in other cell lines expressing Arap3 (SK, unpublished observation), the nature of these different bands is still unclear.

The SAM domains are both necessary and sufficient to mediate heterodimerization of Arap3 and SHIP2

As the yeast two-hybrid screen identified interacting fragments of Arap3 and SHIP2 both containing a SAM domain, we made deletion mutants of both proteins in this region (Table 1 and Figure 1). When comparing these mutants in co-immunoprecipitation experiments, we observed that only the full-length proteins were capable of binding, confirming that the presence of both SAM domains is needed for the interaction (Figure 3A).

We next investigated whether the SAM domains are sufficient to mediate the interaction. We made GST-fusion proteins of both SAM domains and performed in vitro GST pull-down assays. As shown in Figure 3B, the SAM domain of Arap3 indeed pulls down full-length SHIP2, but not a mutant of SHIP2 that lacks the proline-rich and SAM domain-

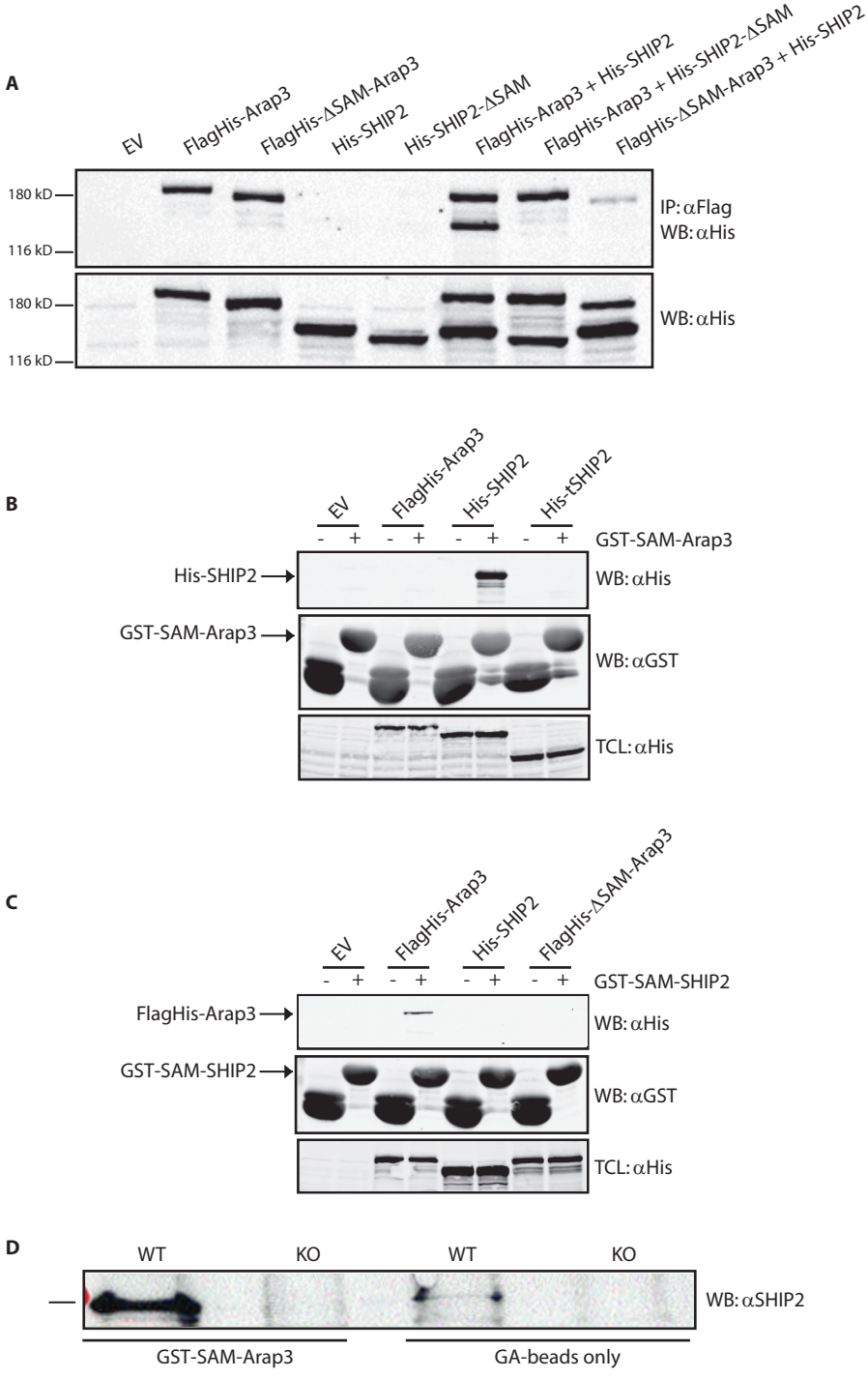

A
EV
FlagHis-Arap3
FlagHis-ΔSAM-Arap3
His-SHIP2
His-SHIP2-ΔSAM
FlagHis-Arap3 + His-SHIP2
FlagHis-Arap3 + His-SHIP2-ΔSAM
FlagHis-ΔSAM-Arap3 + His-SHIP2
180 kD
116 kD
IP: αFlag
WB: αHis
180 kD
116 kD
WB: αHis
B
EV
FlagHis-Arap3
His-SHIP2
His-tSHIP2
- + - + - + - +
GST-SAM-Arap3
His-SHIP2
WB: αHis
GST-SAM-Arap3
WB: αGST
TCL: αHis
C
EV
FlagHis-Arap3
His-SHIP2
FlagHis-ΔSAM-Arap3
- + - + - + - +
GST-SAM-SHIP2
FlagHis-Arap3
WB: αHis
GST-SAM-SHIP2
WB: αGST
TCL: αHis
D
WT
KO
WT
KO
WB: αSHIP2
GST-SAM-Arap3
GA-beads only

Figure 3. The SAM domains are both necessary and sufficient to mediate the interaction between Arap3 and SHIP2.

(A) HEK293T cells were transiently transfected with the indicated constructs or empty vector (EV). Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates were analyzed for the presence of both over-expressed His-SHIP2 and FlagHis-Arap3. HEK293T cells were transiently transfected with the indicated constructs. GST pull downs were performed with either (B) GST or GST-SAM-Arap3 or (C) GST or GST-SAM-SHIP2. Membranes were probed for presence of His-SHIP2 or FlagHis-Arap3 and with anti-GST antibody to show equal loading of GST proteins. (D) Lysates of either WT MEF cells or SHIP2-/- (KO) MEF cells were used in a GST pull down assay using GST-SAM-Arap3 (first two lanes) or GA (glutathione agarose) beads alone (last two lanes). Binding of SHIP2 was detected using anti-SHIP2 antibody. Blots shown are representatives of at least 3 identical experiments.

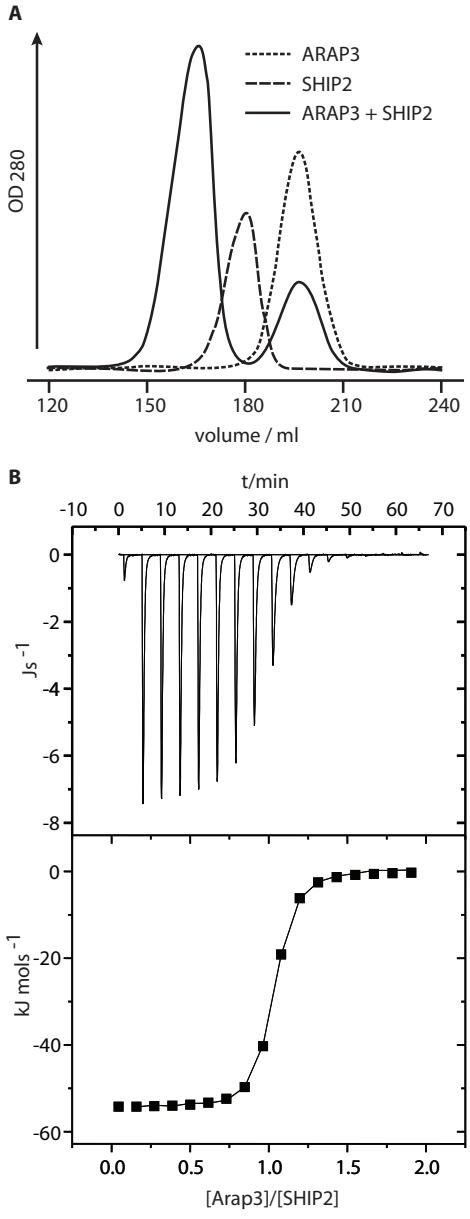
containing C-terminus (t-SHIP2 (Pesesse et al., 2001)). Similarly, the SAM domain of SHIP2 only interacts with full-length Arap3, and not with the mutant lacking the SAM domain (Figure 3C). As it is known that SAM domains can mediate the formation of both homo- and heterodimers, we wanted to determine the specificity of the SAM domains of both proteins for each other. As shown in Figures 3B and C, neither isolated SAM domain interacted with its full-length protein, showing specificicity of the SAM domains for heterodimerization.

SAM domains show specificity for a heterodimeric interaction

To further test the specificity of the interaction, we performed a GST pull-down assay with the SAM domain of Arap3 in both wild type (WT) and SHIP2 knock-out mouse embryonic fibroblasts (MEFs) (Paternotte et al., 2005). As shown in Figure 3D, the SAM domain of Arap3 is sufficient to pull down endogenous SHIP2 from WT MEFs. From these experiments, we conclude that the SAM domains are both required and sufficient to mediate the formation of a heterodimer between Arap3 and SHIP2.

To further analyze the properties of the heterodimeric interaction, we performed gel filtration experiments with the purified SAM domains alone or both (Figure 4A). On a Sephacryl 100 column, we observed that the Arap3 SAM domain had a slightly longer retention time than the SAM domain of SHIP2, which could be due to differences in protein charge since the theoretical pI of the SAM domains is 7.1 and 4.3 for SHIP2 and Arap3, respectively. However, when both domains were combined on the column, the retention time was decreased further, indicating an increase in size due to the formation of a dimer.

To determine the affinity of the interaction, isothermal titration calorimetry (ITC) was used. Upon titration of the Arap3 SAM domain into a solution of the SHIP2 SAM domain, the two SAM domains dimerized with an enthalpy change (ΔH) of 54 kJ/mol and an affinity (K_d) of 100 nM. Also, the ITC measurements indicate that the interaction indeed occurs at a 1:1 stoichiometry (Figure 4B). ITC carried out with titration of SHIP2 into a solution of Arap3 SAM domain



gave the same enthalpy change and affinity data (not shown). We thus conclude that Arap3 and SHIP2 interact as a dimer with an affinity that is physiologically relevant.

Presence of the Arap3 SAM domain does not affect SHIP2 phosphatase activity

As both proteins are involved in the PI3K pathway, we next investigated whether dimerization of its SAM domain would modulate the catalytic activity of SHIP2. To this end, His-SHIP2 was purified from COS-7 cells and PI(3,4,5)P_3 5'-phosphatase activity was measured in an *in vitro* phosphatase assay in the presence or absence of an excess (5 μM) of purified Arap3 or SHIP2 SAM domain (Paternotte et al., 2005). The SHIP2 PI(3,4,5)P_3 5'-phosphatase activity was comparable in all conditions (data not shown). We therefore conclude that binding of Arap3 to SHIP2 does not affect SHIP2 activity *in vitro*.

The interaction between Arap3 and SHIP2 is not regulated by Rap1 or PI3K

Since Arap3 localization is regulated by both Rap1 and PI3K (Krugmann et al., 2004), we investigated whether the interaction with SHIP2 is modulated by either of these. We

Figure 4. The SAM domains show specificity for a heterodimeric interaction.
(A) 20 mg of the SAM domain of SHIP2 (dashed line), 35 mg of the SAM domain of Arap3 (dotted line) or a mixture of 20 mg of the SAM domain of SHIP2 and 35 mg of that of Arap3 (continuous line) were subjected to gelfiltration. (B) Arap3 SAM domain (744 μM) was placed in the syringe of the ITC apparatus and titrated into a solution of the SAM domain of SHIP2 (23 μM) at a temperature of 25ºC. The release of heat was measured as changes in heating power over time (upper panel.) The lower panel shows the released heat per injection normalized to the amount of added protein plotted versus the ratio of concentration of Arap3 SAM and SHIP2 SAM domain.

performed a co-immunoprecipitation with over-expressed proteins in either the presence or absence of RapV12, a constitutively active mutant of Rap1 (Zwartkruis et al., 1998), or of Rap1GAP, the GTPase activating protein specific for Rap, to reduce the amount of GTP-bound Rap1 (Reedquist et al., 2000). We found that modulation of Rap1 activity did not affect the interaction between Arap3 and SHIP2 (Figure 5A). We next investigated whether active PI3K is required for the interaction between endogenous proteins in HeLa cells and 293T cells. However, neither activation of PI3K by growth factor stimulation nor inhibition of PI3K by LY294002 affected the interaction (Figures 5B and C). We therefore conclude that the interaction appears to be constitutive and is not modulated by the activation of Arap3 by PI3K or Rap1.

Arap3 is part of a multimeric protein complex

Previously, it was shown that Arap3 is present in a multimeric protein complex with the SH3 domain-containing protein CIN85 that binds Arap3 via a specific proline-arginine motif (Kowanetz et al., 2004). Our screen also identified the CIN85-related protein, CMS. To investigate whether Arap3, SHIP2 and CIN85 or CMS can form a multimeric protein complex, we performed a co-immunoprecipitation experiment between CIN85 or CMS and SHIP2, either in the presence or absence of Arap3. As shown in Figure 5D, Arap3 is indeed co-immunoprecipitated with both CIN85 and CMS. In addition, SHIP2 is also co-immunoprecipitated with Arap3 and both CIN85 and CMS. In the absence of co-transfected Arap3, SHIP2 is still co-immunoprecipitated with CIN85 and CMS, albeit to a much reduced level. This residual co-immunoprecipitation is presumably due to the presence of endogenous Arap3. From these results we conclude that SHIP2, Arap3 and CIN85/CMS form a multimeric protein complex.

Discussion

In this paper, we show a direct interaction between the PI3K effector Arap3 and the inositol 5'-phosphatase SHIP2. We identified SHIP2 as an Arap3 binding partner in a yeast two-hybrid screen and confirmed the interaction by co-immunoprecipitation of the endogenous proteins. Furthermore, by mutational analysis and ITC experiments, we demonstrate that the interaction is mediated by heterodimerization of the SAM domains present in both proteins. The interaction appears to be constitutive as it is not affected by regulators of Arap3, *i.e.* Rap1 and PI3K. Furthermore, we show that Arap3 and SHIP2 together can form multimeric protein complexes with the SH3 domain-containing adaptor proteins CIN85 and CMS. The relevance of this finding comes from our previous observation that Arap3 is regulated by PI3K signaling, whereas SHIP2 is a negative regulator of PI3K signaling. PI3K phosphorylates $PI(4,5)P_2$ to create $PI(3,4,5,)P_3$ (Vanhaesebroeck et al., 1997) and SHIP2 is a negative regulator of the PI3K pathway (Backers et al., 2003) that

dephosphorylates $PI(3,4,5)P_3$ lipids to $PI(3,4)P_2$ (Pesesse et al., 1998). Importantly, as shown previously by us, Arap3 binds $PI(3,4,5,)P_3$ stronger than it binds $PI(3,4)P_2$ (Krugmann et al., 2002). Since binding of Arap3 to $PI(3,4,5)P_3$ is required for efficient membrane localization of Arap3, dephosphorylation of $PI(3,4,5,)P_3$ by SHIP2 implies a reduced affinity of Arap3 for the plasma membrane. We therefore conclude that Arap3 forms a complex with a negative regulator of its signaling pathway.

Previously, we have shown that one of the biological effects of Arap3 is to inhibit PDGF-induced lamellipodia formation (Krugmann et al., 2004). We have investigated whether deletion of the SAM domain has any affect on this process. However, both wild-type Arap3 and a mutant of Arap3 lacking the SAM domain have a similar inhibitory effect on PDGF-induced lamellipodia formation (data not shown). Furthermore, both wild-type SHIP2 and the mutant of SHIP2 lacking the SAM domain have a similar inhibitory effect on lamellipodia formation, presumably due to a general inhibition of PI3K signaling (data not shown). We therefore concluded that currently no biological systems are present to test our model that SHIP2 negatively regulates Arap3. Alternatively, since PI3K signaling has a strong spatial element, it may well be that the presence of SHIP2 in the Arap3 complex is important to restrict the distribution of $PI(3,4,5)P_3$ to local environments.

SAM domains are conserved modular domains that are widespread and common in nature. With a wide capacity to mediate interactions in signaling pathways (Qiao and Bowie, 2005), they can mediate protein-protein interactions and also regulate protein-lipid and protein-RNA binding. SAM domains mediate many forms of protein-protein interactions by homo-, hetero-, or oligomerization with target proteins (Kim and Bowie, 2003). Interestingly, some protein families have differential conservation of the SAM domain, as is the case for SHIP1 and SHIP2. As SHIP1, that does not contain a SAM domain, is mainly expressed in hematopoietic cells and SHIP2 is more ubiquitously expressed (Schurmans et al., 1999), this may indicate that SHIP2 has acquired additional functions in these cells and the presence of the SAM domain is required to mediate these functions by recruiting new interaction partners.

For instance, both Arap3 and SHIP2 have binding partners involved in endocytosis. It was shown before that SHIP2 binds the E3 ligase Cbl and Cbl-associated protein (CAP) and SHIP2 is therefore suggested to have a role in endocytosis (Prasad and Decker, 2005; Zhuang et al., 2006). Furthermore, it was reported that Arap3 binds the adaptor protein CIN85 (Kowanetz et al., 2004) and our screen identified the CIN85-related protein CMS as an Arap3 binding partner as well (Table 1). These two adaptor proteins both function in Cbl-mediated endocytosis (Dikic, 2002). We have found that SHIP2, Arap3 and either CIN85 or CMS are present in a complex, demonstrating that different

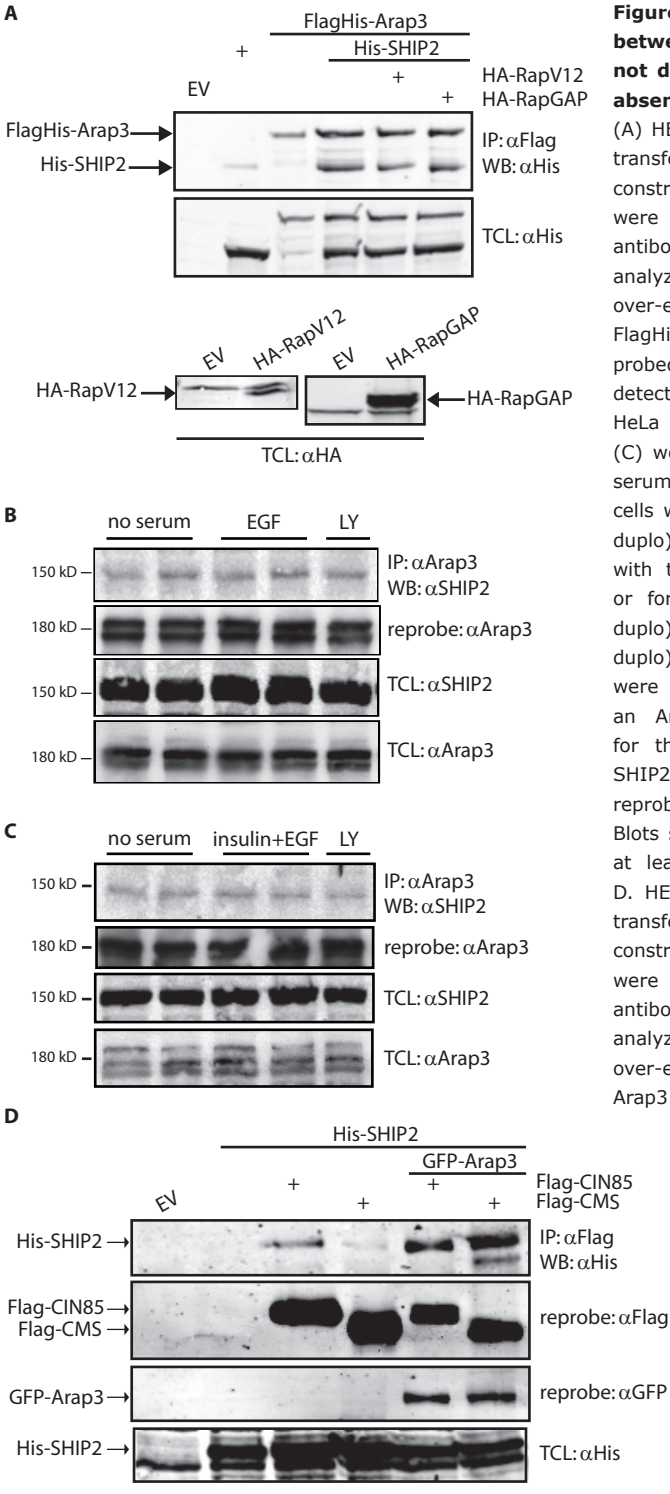


Figure 5. The interaction between Arap3 and SHIP2 does not depend on the presence or absence of active Rap1 or PI3K. (A) HEK293T cells were transiently transfected with the indicated constructs. Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates were analyzed for the presence of both over-expressed His-SHIP2 and FlagHis-Arap3. Total cell lysates were probed with an anti-HA antibody to detect HA-RapV12 and HA-RapGAP. HeLa cells (B) and HEK293T cells (C) were grown to confluency and serum starved overnight. Starved cells were either left untreated (in duplo) or stimulated for 30 minutes with the PI3K inhibitor LY294002 or for 10 minutes with EGF (in duplo) (B) or insulin and EGF (in duplo) (C) as indicated. The lysates were immunoprecipitated using an Arap3 antibody and probed for the presence of endogenous SHIP2. Membranes were also reprobed with Arap3 antibody. Blots shown are representatives of at least 3 identical experiments. D. HEK293T cells were transiently transfected with the indicated constructs. Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates were analyzed for the presence of both over-expressed His-SHIP2 and GFP-Arap3.

Protein	*Interacts with*	*Identified with*	*Function*
SHIP2*	SAM domain	Arap3_1 and Arap3_4	Inositol 5' phosphatase - Down-regulation of insulin signaling, regulation of actin cytoskeleton
CIN85	SH3 domain (SH3B)	Arap3_1 and Arap3_4	Adaptor protein - Endocytosis
CMS	SH3 domain (SH3B)	Arap3_1 and Arap3_4	Adaptor protein - Endocytosis
TNKS2*	Ankyrin repeats	Arap3_1 and Arap3_4	ADP-ribose polymerase - Regulation of telomere length
ANKS1*	SAM domain	Arap3_1 and Arap3_4	Unknown
αPix/ARHGEF6	SH3 and part of RhoGEF domain (DH)	Arap3_1	Guanine nucleotide exchange factor for Rac and CDC42 Regulation of actin cytoskeleton
GGA3	Alpha-adaptin C2 domain	Arap3_3	Adaptor protein - Trafficking between the trans-Golgi network and the lysosome
AP3 μ subunit	C-terminus	Arap3_3	Member of the clathrin-associated adaptor complex, involved in vesicle budding and protein sorting
Par-6 beta	PDZ domain and C-terminus	Arap3_1	Asymmetrical cell division and cell polarization
SAMHD1*	SAM and HD domain	Arap3_1	Phosphodiesterase

Table 1. Proteins identified as Arap3 binding partners in a yeast-two-hybrid screen.

Several binding partners of Arap3 identified in a yeast two-hybrid screen are listed. The interacting regions are indicated in the second column and previously reported functions of the identified proteins are mentioned. Proteins indicated with an * contain a SAM domain. Full names of all abbreviated proteins: SH2 domain containing inositol phosphatase 2 (SHIP2); Cbl interacting protein of 85 kDa (CIN85) (Kowanetz et al., 2004); Cas ligand with multiple SH3 domains (CMS); Tankyrase 2 (TNKS2); ankyrin repeat and SAM domain containing 1(ANKS1); Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 (ARHGEF6); Partitioning defective 6 homolog beta (Par6 beta); Golgi associated, gamma adaptin ear containing, ARF binding protein 3 (GGA3); SAM domain and HD domain 1 (SAMHD1).

binding surfaces on Arap3 are used for these interactions. This indicates that both Arap3 and SHIP2 (through its SAM and SH2 domain), can function as scaffold proteins, perhaps binding proteins that depend on their enzymatic activities. For instance, one of the other proteins identified in the yeast two-hybrid screen, ARHGEF6, or α-pix, is regulated by PI3K as well and is a GEF for Rac and Cdc42 (Baird et al., 2005). As it is often seen that the GTP levels of Rac and Cdc42 are inversely regulated with Rho, it is quite interesting that Arap3 complexes with a Rac GEF.

It was also proposed before that SHIP2 is involved in the regulation of the actin cytoskeleton and cell adhesion, like Arap3, and that it interacts with multiple proteins in the cytoskeleton network (Krugmann et al., 2006; Paternotte et al., 2005; Prasad et al., 2001; Prasad et al., 2002; Stacey et al., 2004). It will therefore be interesting to see which of these proteins are found in the same complex together, and what exactly is the role of all these different interactions in the complex signaling pathways that eventually lead to cell adhesion.

Acknowledgements

We thank members of our laboratories for continuous discussions. Access to an Isothermal Titration Calorimeter was kindly provided by Alfred Wittinghofer, MPI-Dortmund. We thank Joost Das for help with protein purifications. J.H.R. was supported by a grant from the Dutch Cancer Society (KWF Kankerbestrijding 2003-2956). H.R. was supported by the Chemical Sciences of the Netherlands Organization for Scientific Research (NWO-CW). Z.Z. was supported by a grant from the Netherlands Genomics Initiative through the Cancer Genomics Centre. S.K. holds a BBSRC David Phillips Fellowship.

References

Backers, K., Blero, D., Paternotte, N., Zhang, J. and Erneux, C. (2003) The termination of PI3K signalling by SHIP1 and SHIP2 inositol 5-phosphatases. *Adv Enzyme Regul*, **43**, 15-28.

Baird, D., Feng, Q. and Cerione, R.A. (2005) The Cool-2/alpha-Pix protein mediates a Cdc42-Rac signaling cascade. *Curr Biol*, **15**, 1-10.

Barrera, F.N., Poveda, J.A., Gonzalez-Ros, J.M. and Neira, J.L. (2003) Binding of the C-terminal sterile alpha motif (SAM) domain of human p73 to lipid membranes. *J Biol Chem*, **278**, 46878-46885.

Clement, S., Krause, U., Desmedt, F., Tanti, J.F., Behrends, J., Pesesse, X., Sasaki, T., Penninger, J., Doherty, M., Malaisse, W., Dumont, J.E., Le Marchand-Brustel, Y., Erneux, C., Hue, L. and Schurmans, S. (2001) The lipid phosphatase SHIP2 controls insulin sensitivity. *Nature*, **409**, 92-97.

Colland, F., Jacq, X., Trouplin, V., Mougin, C., Groizeleau, C., Hamburger, A., Meil, A., Wojcik, J., Legrain, P. and Gauthier, J.M. (2004) Functional proteomics mapping of a human signaling pathway. *Genome Res*, **14**, 1324-1332.

Dikic, I. (2002) CIN85/CMS family of adaptor molecules. *FEBS Lett*, **529**, 110-115.

Habib, T., Hejna, J.A., Moses, R.E. and Decker, S.J. (1998) Growth factors and insulin stimulate tyrosine phosphorylation of the 51C/SHIP2 protein. *J Biol Chem*, **273**, 18605-18609.

Kim, C.A. and Bowie, J.U. (2003) SAM domains: uniform structure, diversity of function. *Trends Biochem Sci*, **28**, 625-628.

Koch, A., Mancini, A., El Bounkari, O. and Tamura, T. (2005) The SH2-domian-containing

inositol 5-phosphatase (SHIP)-2 binds to c-Met directly via tyrosine residue 1356 and involves hepatocyte growth factor (HGF)-induced lamellipodium formation, cell scattering and cell spreading. *Oncogene*, **24**, 3436-3447.

Kowanetz, K., Husnjak, K., Holler, D., Kowanetz, M., Soubeyran, P., Hirsch, D., Schmidt, M.H., Pavelic, K., De Camilli, P., Randazzo, P.A. and Dikic, I. (2004) CIN85 associates with multiple effectors controlling intracellular trafficking of epidermal growth factor receptors. *Mol Biol Cell*, **15**, 3155-3166.

Krugmann, S., Anderson, K.E., Ridley, S.H., Risso, N., McGregor, A., Coadwell, J., Davidson, K., Eguinoa, A., Ellson, C.D., Lipp, P., Manifava, M., Ktistakis, N., Painter, G., Thuring, J.W., Cooper, M.A., Lim, Z.Y., Holmes, A.B., Dove, S.K., Michell, R.H., Grewal, A., Nazarian, A., Erdjument-Bromage, H., Tempst, P., Stephens, L.R. and Hawkins, P.T. (2002) Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices. *Mol Cell*, **9**, 95-108.

Krugmann, S., Andrews, S., Stephens, L. and Hawkins, P.T. (2006) ARAP3 is essential for formation of lamellipodia after growth factor stimulation. *J Cell Sci*, **119**, 425-432.

Krugmann, S., Williams, R., Stephens, L. and Hawkins, P.T. (2004) ARAP3 is a PI3K- and rap-regulated GAP for RhoA. *Curr Biol*, **14**, 1380-1384.

Kwan, J.J., Warner, N., Maini, J., Chan Tung, K.W., Zakaria, H., Pawson, T. and Donaldson, L.W. (2006) Saccharomyces cerevisiae Ste50 binds the MAPKKK Ste11 through a head-to-tail SAM domain interaction. *J Mol Biol*, **356**, 142-154.

Leevers, S.J., Vanhaesebroeck, B. and Waterfield, M.D. (1999) Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Curr Opin Cell Biol*, **11**, 219-225.

Paternotte, N., Zhang, J., Vandenbroere, I., Backers, K., Blero, D., Kioka, N., Vanderwinden, J.M., Pirson, I. and Erneux, C. (2005) SHIP2 interaction with the cytoskeletal protein Vinexin. *Febs J*, **272**, 6052-6066.

Pesesse, X., Deleu, S., De Smedt, F., Drayer, L. and Erneux, C. (1997) Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. *Biochem Biophys Res Commun*, **239**, 697-700.

Pesesse, X., Dewaste, V., De Smedt, F., Laffargue, M., Giuriato, S., Moreau, C., Payrastre, B. and Erneux, C. (2001) The Src homology 2 domain containing inositol 5-phosphatase SHIP2 is recruited to the epidermal growth factor (EGF) receptor and dephosphorylates phosphatidylinositol 3,4,5-trisphosphate in EGF-stimulated COS-7 cells. *J Biol Chem*, **276**, 28348-28355.

Pesesse, X., Moreau, C., Drayer, A.L., Woscholski, R., Parker, P. and Erneux, C. (1998) The SH2 domain containing inositol 5-phosphatase SHIP2 displays phosphatidylinositol 3,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate 5-phosphatase activity. *FEBS Lett*, **437**, 301-303.

Prasad, N., Topping, R.S. and Decker, S.J. (2001) SH2-containing inositol 5'-phosphatase SHIP2 associates with the p130(Cas) adapter protein and regulates cellular adhesion and spreading. *Mol Cell Biol*, **21**, 1416-1428.

Prasad, N., Topping, R.S. and Decker, S.J. (2002) Src family tyrosine kinases regulate adhesion-dependent tyrosine phosphorylation of 5'-inositol phosphatase SHIP2 during cell attachment and spreading on collagen I. *J Cell Sci*, **115**, 3807-3815.

Prasad, N.K. and Decker, S.J. (2005) SH2-containing 5'-inositol phosphatase, SHIP2, regulates cytoskeleton organization and ligand-dependent down-regulation of the epidermal growth factor receptor. *J Biol Chem*, **280**, 13129-13136.

Qiao, F. and Bowie, J.U. (2005) The many faces of SAM. *Sci STKE*, **2005**, re7.

Reedquist, K.A., Ross, E., Koop, E.A., Wolthuis, R.M., Zwartkruis, F.J., van Kooyk, Y., Salmon, M., Buckley, C.D. and Bos, J.L. (2000) The small GTPase, Rap1, mediates CD31-induced integrin adhesion. *J Cell Biol*, **148**, 1151-1158.

Schurmans, S., Carrio, R., Behrends, J., Pouillon, V., Merino, J. and Clement, S.

(1999) The mouse SHIP2 (Inppl1) gene: complementary DNA, genomic structure, promoter analysis, and gene expression in the embryo and adult mouse. *Genomics*, **62**, 260-271.

Sleeman, M.W., Wortley, K.E., Lai, K.M., Gowen, L.C., Kintner, J., Kline, W.O., Garcia, K., Stitt, T.N., Yancopoulos, G.D., Wiegand, S.J. and Glass, D.J. (2005) Absence of the lipid phosphatase SHIP2 confers resistance to dietary obesity. *Nat Med*, **11**, 199-205.

Stacey, T.T.I., Nie, Z., Stewart, A., Najdovska, M., Hall, N.E., He, H., Randazzo, P.A. and Lock, P. (2004) ARAP3 is transiently tyrosine phosphorylated in cells attaching to fibronectin and inhibits cell spreading in a RhoGAP-dependent manner. *J Cell Sci*, **117**, 6071-6084.

Stapleton, D., Balan, I., Pawson, T. and Sicheri, F. (1999) The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization. *Nat Struct Biol*, **6**, 44-49.

Thanos, C.D., Goodwill, K.E. and Bowie, J.U. (1999) Oligomeric structure of the human EphB2 receptor SAM domain. *Science*, **283**, 833-836.

Vandenbroere, I., Paternotte, N., Dumont, J.E., Erneux, C. and Pirson, I. (2003) The c-Cbl-associated protein and c-Cbl are two new partners of the SH2-containing inositol polyphosphate 5-phosphatase SHIP2. *Biochem Biophys Res Commun*, **300**, 494-500.

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J. and Waterfield, M.D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem*, **70**, 535-602.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G. and Waterfield, M.D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci*, **22**, 267-272.

Wang, Y., Keogh, R.J., Hunter, M.G., Mitchell, C.A., Frey, R.S., Javaid, K., Malik, A.B., Schurmans, S., Tridandapani, S. and Marsh, C.B. (2004) SHIP2 is recruited to the cell membrane upon macrophage colony-stimulating factor (M-CSF) stimulation and regulates M-CSF-induced signaling. *J Immunol*, **173**, 6820-6830.

Wisniewski, D., Strife, A., Swendeman, S., Erdjument-Bromage, H., Geromanos, S., Kavanaugh, W.M., Tempst, P. and Clarkson, B. (1999) A novel SH2-containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase (SHIP2) is constitutively tyrosine phosphorylated and associated with src homologous and collagen gene (SHC) in chronic myelogenous leukemia progenitor cells. *Blood*, **93**, 2707-2720.

Zhuang, G., Hunter, S., Hwang, Y. and Chen, J. (2006) Regulation of EphA2 receptor endocytosis by SHIP2 lipid phosphatase via PI3 kinase-dependent Rac1 activation. *J Biol Chem*.

Zwartkruis, F.J., Wolthuis, R.M., Nabben, N.M., Franke, B. and Bos, J.L. (1998) Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. *Embo J*, **17**, 5905-5912.

chapter 7

Summary and Discussion

Summary and discussion

The small GTPase Rap1 is a key regulator of tissue architecture, with important functions in cell-cell and cell-ECM adhesion (Bos, 2005; Bos et al., 2003). Although our understanding of the Rap1 signaling network has increased significantly over the recent years, the continuing identification of Rap1 effectors also complicates our view of its role in the regulation of adhesion. One of the processes where coordinated regulation of cell-cell adhesion and adhesion to the ECM is required, is epithelial cell migration. In this thesis, I have described our efforts in elucidating the role of Rap1 in this process.

Rap1 inhibits growth-factor induced cell migration

A role for Rap1 in cell migration is most established in lymphocytes and vascular endothelial cells (Fujita et al., 2005; Lorenowicz et al., 2006; Shimonaka et al., 2003; Tohyama et al., 2003). Impaired adhesion and spreading and an increase in cell migration velocity in C3G-/- MEFs pointed to a role for the GEF C3G and Rap1 in regulating adhesion and migration in other cell types (Ohba et al., 2001). More recently, Rap1 was shown to counteract HGF-induced scattering of MDCK-Epac cells through the stabilization of cell-cell junctions. This indicated that, in epithelial cells, modulation of cell-cell adhesion may be more important in regulating cell scattering (Price et al., 2004). In contrast, Rap1 was shown to inhibit cell migration velocity of the NBT-II carcinoma cell line through the inhibition of the GTPase Rac1 downstream of paxillin phosphorylation, indicating that Rap might have a restraining effect on the process of cell migration itself (Valles et al., 2004). Currently, it is unclear which effects downstream of Rap1 activation are most important in regulating cell migration.

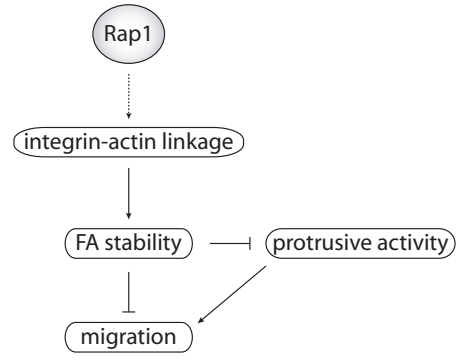
There are various different ways to measure cell migration, which are all being used to determine the role of Rap1. A wound healing assay measures the ability of cells in a confluent monolayer to close a gap. Transwell migration assays measure the percentage of cells able to migrate through a membrane in response to a chemotactic cue. Neither of these assays measures the actual migration velocity like the tracking of single cells in time-lapse recordings of two-dimensional cell culture. We employed this assay to be able to assess the role of endogenous Rap1 in cell migration in a more direct manner.

Rap1 is activated by several extracellular signals and second messengers, one of them being cAMP. cAMP activates Rap1 via the Rap1GEF Epac. The Epac-specific cAMP analogue 8-CPT-2'OMe-cAMP (also termed 007) is a useful tool to activate endogenous Rap1 and identify Rap1-specific cellular effects. To investigate the molecular mechanism via which Rap1 affects cell migration, we used cell lines (over-) expressing Epac1, to be able to activate endogenous Rap1 by 007. We filmed cell in two-

dimensional culture conditions and used custom-written software to track all cells in these time-lapse series to determine the average cell velocity in an unbiased manner. In Chapter 3, we showed that in several epithelial cell lines, activation of Rap1 inhibits growth factor-induced migration velocity. Interestingly, we found that when cells were cultured in low Ca^{2+}-containing medium (which does not support E-cadherin-mediated adhesion), activation of Rap1 still inhibited the migration velocity of these cells, indicating that, in this set-up, the stabilization of cell junctions is not required. Furthermore, this inhibition of cell migration was accompanied by a decrease in focal adhesion (FA) dynamics and a reduction in membrane protrusive activity. This decrease in FA dynamics resulted in an increased number of larger FAs. Such stable FAs are thought to be less compatible with fast cell migration than small FAs, since FAs need to be able to quickly turn over. This provides components for newly formed FAs, ensuring continuous membrane protrusion, stabilization and forward movement. Additionally, the proper amount of adhesive strength is important for cell migration. The speed of cell migration depends on a proper level of adhesion. When adhesion strength is low, the cell cannot generate enough traction force for translocation and rear retraction, whereas under conditions of too much adhesion, the release of cell-ECM adhesions at the rear is inhibited. Thus, intermediate amounts of adhesive strength provide the optimal amount of tension for efficient cell migration (Palecek et al., 1997).

The question then remains whether these three aspects of the 007 phenotype (inhibition of cell migration, FA stabilization and inhibition of membrane protrusion) are all part of the same Rap1 effect, or whether these are separate, independent Rap1-induced effects. Figure 1 shows a model to explain the effects of Rap1 on cell migration. In the normal situation, Rap1 may function as a modulator of the actin cytoskeleton or the integrin-actin linkage. Over-activation of Rap1 would then result in a linkage that is too strong and result in more stable FAs. A loss of FA dynamics then results in migration inhibition and reduced protrusive activity. In addition, the loss of protrusive activity may also contribute to the inhibition of migration. It would be interesting to compare the strength of the integrin-actin linkage in normal and 007-stimulated cells, to determine whether Rap1 activation affects this linkage.

Figure 1.



Important signaling pathways regulating FAs are not affected by Rap1

Since the 007-induced effects involve the migration machinery, we hypothesized that 007-induced FAs may be deficient in some aspect of the dynamic regulation of FA turnover. We therefore examined the responsiveness of 'normal' vs. Rap1-induced FAs to changes in contractile tension or microtubule (MT) targeting (Chapter 4). Surprisingly, we found that both types of FAs are regulated by tension comparably and that MT-induced disassembly functions normally. We also showed that Rap1 activation does not increase the level of phospho-MLC, the most direct biochemical readout of actomyosin contractility. These results imply that the 007-induced increase in FA size and stability is unlikely to be caused by increased contractility. However, as explained above, Rap1 may induce increased linkage between the integrins and the cytoskeleton, resulting in more tension on the Rap1-activated FAs by the same amount of contractility. This could be tested by dose-response curves for the Rock inhibitors Y27632 and blebbistatin. In our experiments so far, we have only used one concentration of both inhibitors, which may have been too high to discern possibly subtle effects. If a more efficient linkage is causing the Rap1 effect, 007-induced FAs should then be more resistant than normal ones to increasing concentrations of these inhibitors. On the other hand, the FA formation experiments after Y27632 washout show that 007-induced FAs do not form quicker or more efficiently than normal ones, suggesting they do not have the ability to use low amounts of tension more efficiently.

Does this then imply that there is no effect on the integrin-actin linkage? It could also be that the resolution of such assays is too low to clearly determine these subtle differences. Indeed, in these experiments, we fix the cells every few minutes, whereas it may be a very fast, dynamic process. Real-time imaging of FA formation, stabilization and disassembly is thus better suited to answer these questions.

The same holds for the effect of MT regrowth on FA breakdown. Live imaging of MT targeting to these FAs, both in the normal situation, as well as after nocodazole-induced breakdown, may provide more information on the ability of MTs to target and disassemble these FAs. Interestingly, it was recently shown that Epac activation resulted in a net increase in the length of microtubules, although this was independent of Rap activation. In contrast, our results do depend on activated Rap1. Furthermore, in this assay, MT growth does not correlate with stabilization, as MT growth should target FAs for turnover instead of stabilizing them. Thus, although we cannot exclude it yet, a protective effect of 007 against MT-induced disassembly seems unlikely.

We also investigated 007-induced FAs in more detail to determine in what way these differ from normal FAs. We compared the composition

of these FAs but found no differences in the presence or activity of several key regulators between 007-induced and 'normal' FAs. However, we only investigated these FAs on one specific ECM substrate and at one time-point during adhesion and spreading. Thus, we cannot exclude that we missed the crucial moment where such differences may occur. Again, real-time imaging of FA formation may answer the question whether the timing of arrival of different proteins into these FAs is affected. Also, no quantitative information was gained by these experiments. As we expect the differences between these FAs to be subtle, we plan to perform quantitative mass spectrometry comparing both types of FAs. It will be interesting to determine whether different (amounts of) protein complexes are present, or perhaps differences in the phosphorylation profiles of important regulatory proteins. In conclusion, the experiments described in Chapter 4 suggest that 007-induced FAs do not differ much from normal FAs. However, these experiments were not exhaustive. Other pathways controlling FA dynamics may be involved. For instance, protein cleavage by calpains is also involved in FA turnover (Franco and Huttenlocher, 2005). Interestingly, cells deficient in calpain show enhanced stabilization of FAs and decreased cell migration. Indeed, it is intriguing to note that for several important FA regulators the phenotypes of knockout cells resemble the active Rap1 phenotype. Several of these and the corresponding phenotypes are shown in Table 1. These proteins might be the targets whose functions are modulated by Rap1.

Rap1 uses redundant pathways to regulate adhesion, spreading and FA formation

As the experiments described in Chapter 4 suggested that both 'normal' and 007-induced FAs are normally responsive to the major FA regulatory pathways, we chose another approach to identify the role of Rap1 in Chapter 5. We used a small siRNA library with selected targets to try and identify the pathway responsible more directly downstream of Rap1, by determining which Rap1 effector protein is responsible. For Rap1, the list of effectors is rapidly expanding and contains proteins both with and without catalytic activity, which are mostly involved in different aspects of cell adhesion and modulation of the actin cytoskeleton. We set up a replating assay to screen siRNAs targeting Rap1 effectors and regulatory proteins as well as adhesion machinery proteins. Surprisingly, although the screen was very specific in demonstrating the requirement for Epac1 and Rap1A, none of the reported Rap effectors tested gave such a phenotype. We did observe differential effects of some siRNAs on actin, spreading or the focal adhesions. This is an indication that this phenotype is not mediated by a linear pathway downstream of Rap1, but by multiple pathways and more than one effector protein. For instance, Riam siRNA did not affect 007-induced focal adhesion maturation, but did have effects on

Table 1.

Knockout	*Phenotype*	*References*
FAK	• decreased migration rate, spreading and protrusiveness • increased number and size of peripherally localized adhesions • impaired adhesion disassembly	Ilic et al., 1995 Ren et al., 2000 Webb et al., 2004
SYF	• reduced motility and spreading • impaired adhesion disassembly	Klinghoffer et al., 1999 Webb et al., 2004
Src	• increased size of peripherally localized adhesions • defects in SYF-/- cells are rescued by re-expression of Src	Fincham and Frame, 1998 Webb et al., 2004 Cary et al., 2002
p130Cas	• decreased migration rate and spreading • impaired actin filament assembly • impaired adhesion disassembly	Honda et al., 1998 Honda et al., 1999 Webb et al., 2004
Calpain	• decreased cell migration • increased large peripheral adhesion complexes • stabilization of adhesion complexes • abnormal actin cytoskeleton organization • abnormal lamellipodia and filopodia formation	Dourdin et al., 2001 Franco and Huttenlocher, 2005 and refs therein
Paxillin	• impaired migration and spreading • defects in the cortical cytoskeleton • impaired adhesion disassembly	Hagel et al., 2002 Webb et al., 2004
PTP-Pest	• impaired migration • increased number and size of adhesions • increased spreading rate	Angers-Loustau et al., 1999
PTPa	• impaired migration • altered cell shape	Zeng et al., 2003
SHP-2	• impaired migration • increased number of adhesions	Yu et al., 1998 Von Wichtert et a.l., 2003

the actin cytoskeleton. In the case of the αIIbβ3 integrin, Riam is the Rap1 effector directly linking Rap1 to talin-mediated integrin activation (Han et al., 2006). In this screen, talin1 knockdown did inhibit all 007-induced effects. Although we could not confirm the efficiency of Riam knockdown in all experiments, this indicates that, in other cell types, or for other integrins, there may be more effectors capable of taking on this role. For instance, preliminary results indicate that when both Riam and its close homologue Lamellipodin are knocked down at the same time, more cells display defects in 007-induced changes in the morphology of the actin cytoskeleton. This suggests that both these proteins are involved in some aspect of the 007-induced effects in these cells. Indeed, it was recently shown

that both Riam and Lamellipodin can mediate integrin activation through a common scaffolding mechanism and the membrane recruitment of talin (Lee et al., 2008). Thus, it will be interesting to determine whether combined knockdown of several Rap1 effectors has additional effects. Moreover, it could be that the true Rap1 effector(s) mediating these effects has yet to be identified. We are currently setting up quantitative MS to identify (differences in) Rap1 complexes upon 007 stimulation.

One of the known binding partners of Epac1, Ezrin, was also identified in the screen. Upon activation, Ezrin recruits Epac1 to the plasma membrane (Gloerich et al., manuscript in preparation). Ezrin knockdown specifically blocked the 007-induced effects in these cells. This indicates that Epac1 needs to be properly localized to mediate these 007-induced effects.

Another interesting result from the screen was Exoc8. Exoc8 is a member of the exocyst complex, an effector of the GTPase Ral involved in exocytosis (Moskalenko et al., 2002). This complex is important for the targeted delivery of membrane and secretory proteins to specific sites on the plasma membrane (Hsu et al., 2004). When cells move forward over stable adhesions, adhesion components accumulate towards the cell rear. After disassembly of the FA, these proteins need to be moved towards the cell front, to be available for new adhesions. This may involve endocytic mechanisms (Webb et al., 2002). Indeed, a role for the exocyst in plasma membrane delivery of integrins was recently shown (Spiczka and Yeaman, 2008). Integrin activity itself is also regulated by recycling mechanisms (Caswell and Norman, 2006). We have examined HGF-induced integrin recycling but we did not observe an effect of 007 stimulation (data not shown). The exocyst has also been shown to coordinate vesicle trafficking with cytoskeletal remodeling (Sugihara et al., 2002). We observed an effect of Exoc8 knockdown on both the actin cytoskeleton and on FAs. The actin cytoskeleton in these cells is almost completely devoid of strong actin cables. This implies that Exoc8 may affect actin bundling. As actin bundling is required to create tension on the integrin-actin linkage, this is in accordance with the fact that these cells have very small FAs. In the presence of 007, we did observe increased cell spreading, but no effect on FA size. Firstly, this suggests that spreading and FA formation downstream of Rap1 activation are independent processes. Second, this implies that the presence of tension, or, of proper integrin-actin linkage, is required for the 007 effect. Although we did not verify the level of knockdown, siRNAs against RalA and RalB did not have an effect on the 007 phenotype in the screen. It will be interesting to determine the effects of the other components of the exocyst complex.

The effect of RockII knockdown in the screen also indicated that the presence of tension is required for the effect of Rap1 on FAs. As we have shown in Chapter 4 that the induction of tension is unaffected by 007, a

role for Rap1 in the modulation of the integrin-actin linkage seems probable. Currently, a lot of attention is focused on this linkage in the migration field, but thus far, the mechanisms involved remain elusive.

As microscopic techniques continue to improve, more details about structural and regulatory relationships between integrins and the actin cytoskeleton are becoming visible. It will be interesting to determine how, and via which effector(s), Rap1 is involved here.

Interaction of the RhoGAP Arap3 with SHIP2

Rho family proteins are critical regulators of the actin cytoskeleton. These proteins have important roles in several processes controlled by Rap1, like junction formation and cell adhesion. Rap1 has been shown to interact with the RacGEFs Vav2 and Tiam1 and with the RhoGAPs Arap3 and RA-RhoGAP, placing Rap1 upstream of Rho GTPase signaling. In Chapter 6, we have performed a yeast two-hybrid screen with Arap3 to investigate its possible role in actin cytoskeleton modulation downstream of Rap1. We identified the lipid phosphatase SHIP2 as a direct binding partner of Arap3. Interestingly, Arap3 activity is regulated by PI3K signaling, whereas SHIP2 negatively regulates PI3K signaling (Backers et al., 2003; Krugmann et al., 2004). Thus, Arap3 specifically binds a negative regulator of its signaling pathway. Both proteins were shown to regulate the actin cytoskeleton and adhesion (Krugmann et al., 2006; Prasad and Decker, 2005). In the screen, SHIP2 knockdown did not affect the 007-induced phenotype, whereas Arap3 reduced basal spreading, which was rescued by 007. One of the other proteins identified as a binding partner for Arap3 in the yeast two-hybrid screen, ARHGEF6, or alpha-pix, is also regulated by PI3K and is a GEF for Rac and Cdc42 (Baird et al., 2005). It will be interesting to determine whether these proteins can be found in the same complex and how they are involved the regulation of cytoskeletal dynamics downstream of Rap1.

The regulation of cell adhesion by Rap1 likely comprises multiple different effector proteins and pathways, controlling separate aspects of this process, such as integrin activation, spreading, protrusion and the control of actin cytoskeleton dynamics. Our growing understanding of cell adhesion processes and the emergence of refined imaging techniques should contribute to the elucidation of the molecular mechanism of Rap1 function in cell adhesion.

References

Angers-Loustau, A., Cote, J.F., Charest, A., Dowbenko, D., Spencer, S., Lasky, L.A. and Tremblay, M.L. (1999) Protein tyrosine phosphatase-PEST regulates focal adhesion disassembly, migration, and cytokinesis in fibroblasts. *J Cell Biol*, **144**, 1019-1031.

Backers, K., Blero, D., Paternotte, N., Zhang, J. and Erneux, C. (2003) The termination of PI3K signalling by SHIP1 and SHIP2 inositol 5-phosphatases. *Adv Enzyme Regul*, **43**, 15-28.

Baird, D., Feng, Q. and Cerione, R.A. (2005) The Cool-2/alpha-Pix protein mediates a Cdc42-

Rac signaling cascade. *Curr Biol*, **15**, 1-10.

Bos, J.L. (2005) Linking Rap to cell adhesion. *Curr Opin Cell Biol*, **17**, 123-128.

Bos, J.L., de Bruyn, K., Enserink, J., Kuiperij, B., Rangarajan, S., Rehmann, H., Riedl, J., de Rooij, J., van Mansfeld, F. and Zwartkruis, F. (2003) The role of Rap1 in integrin-mediated cell adhesion. *Biochem Soc Trans*, **31**, 83-86.

Cary, L.A., Klinghoffer, R.A., Sachsenmaier, C. and Cooper, J.A. (2002) SRC catalytic but not scaffolding function is needed for integrin-regulated tyrosine phosphorylation, cell migration, and cell spreading. *Mol Cell Biol*, **22**, 2427-2440.

Caswell, P.T. and Norman, J.C. (2006) Integrin trafficking and the control of cell migration. *Traffic*, **7**, 14-21.

Dourdin, N., Bhatt, A.K., Dutt, P., Greer, P.A., Arthur, J.S., Elce, J.S. and Huttenlocher, A. (2001) Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J Biol Chem*, **276**, 48382-48388.

Fincham, V.J. and Frame, M.C. (1998) The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. *Embo J*, **17**, 81-92.

Franco, S.J. and Huttenlocher, A. (2005) Regulating cell migration: calpains make the cut. *J Cell Sci*, **118**, 3829-3838.

Fujita, H., Fukuhara, S., Sakurai, A., Yamagishi, A., Kamioka, Y., Nakaoka, Y., Masuda, M. and Mochizuki, N. (2005) Local activation of Rap1 contributes to directional vascular endothelial cell migration accompanied by extension of microtubules on which RAPL, a Rap1-associating molecule, localizes. *J Biol Chem*, **280**, 5022-5031.

Hagel, M., George, E.L., Kim, A., Tamimi, R., Opitz, S.L., Turner, C.E., Imamoto, A. and Thomas, S.M. (2002) The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol*, **22**, 901-915.

Han, J., Lim, C.J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D.A., Puzon-McLaughlin, W., Lafuente, E.M., Boussiotis, V.A., Shattil, S.J. and Ginsberg, M.H. (2006) Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol*, **16**, 1796-1806.

Honda, H., Nakamoto, T., Sakai, R. and Hirai, H. (1999) p130(Cas), an assembling molecule of actin filaments, promotes cell movement, cell migration, and cell spreading in fibroblasts. *Biochem Biophys Res Commun*, **262**, 25-30.

Honda, H., Oda, H., Nakamoto, T., Honda, Z., Sakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., Katsuki, M., Yazaki, Y. and Hirai, H. (1998) Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. *Nat Genet*, **19**, 361-365.

Hsu, S.C., TerBush, D., Abraham, M. and Guo, W. (2004) The exocyst complex in polarized exocytosis. *Int Rev Cytol*, **233**, 243-265.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, **377**, 539-544.

Klinghoffer, R.A., Sachsenmaier, C., Cooper, J.A. and Soriano, P. (1999) Src family kinases are required for integrin but not PDGFR signal transduction. *Embo J*, **18**, 2459-2471.

Krugmann, S., Andrews, S., Stephens, L. and Hawkins, P.T. (2006) ARAP3 is essential for formation of lamellipodia after growth factor stimulation. *J Cell Sci*, **119**, 425-432.

Krugmann, S., Williams, R., Stephens, L. and Hawkins, P.T. (2004) ARAP3 is a PI3K- and rap-regulated GAP for RhoA. *Curr Biol*, **14**, 1380-1384.

Lee, H.S., Lim, C.J., Puzon-McLaughlin, W., Shattil, S.J. and Ginsberg, M.H. (2008) RIAM activates integrins by linking talin to Ras GTPase membrane-targeting sequences. *J Biol Chem*.

Lorenowicz, M.J., van Gils, J., de Boer, M., Hordijk, P.L. and Fernandez-Borja, M. (2006) Epac1-Rap1 signaling regulates monocyte adhesion and chemotaxis. *J Leukoc Biol*, **80**, 1542-1552.

Moskalenko, S., Henry, D.O., Rosse, C., Mirey,

G., Camonis, J.H. and White, M.A. (2002) The exocyst is a Ral effector complex. *Nat Cell Biol*, **4**, 66-72.

Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B.J., Maki, K., Miyazaki, J. and Matsuda, M. (2001) Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. *Embo J*, **20**, 3333-3341.

Palecek, S.P., Loftus, J.C., Ginsberg, M.H., Lauffenburger, D.A. and Horwitz, A.F. (1997) Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature*, **385**, 537-540.

Prasad, N.K. and Decker, S.J. (2005) SH2-containing 5'-inositol phosphatase, SHIP2, regulates cytoskeleton organization and ligand-dependent down-regulation of the epidermal growth factor receptor. *J Biol Chem*, **280**, 13129-13136.

Price, L.S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F.J., Collard, J.G. and Bos, J.L. (2004) Rap1 regulates E-cadherin-mediated cell-cell adhesion. *J. Biol. Chem.*, **279**, 35127-35132.

Ren, X.D., Kiosses, W.B., Sieg, D.J., Otey, C.A., Schlaepfer, D.D. and Schwartz, M.A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci*, **113 (Pt 20)**, 3673-3678.

Shimonaka, M., Katagiri, K., Nakayama, T., Fujita, N., Tsuruo, T., Yoshie, O. and Kinashi, T. (2003) Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow. *J Cell Biol*, **161**, 417-427.

Spiczka, K.S. and Yeaman, C. (2008) Ral-regulated interaction between Sec5 and paxillin targets Exocyst to focal complexes during cell migration. *J Cell Sci*, **121**, 2880-2891.

Sugihara, K., Asano, S., Tanaka, K., Iwamatsu, A., Okawa, K. and Ohta, Y. (2002) The exocyst complex binds the small GTPase RalA to mediate filopodia formation. *Nat Cell Biol*, **4**, 73-78.

Tohyama, Y., Katagiri, K., Pardi, R., Lu, C., Springer, T.A. and Kinashi, T. (2003) The critical cytoplasmic regions of the alphaL/beta2 integrin in Rap1-induced adhesion and migration. *Mol Biol Cell*, **14**, 2570-2582.

Valles, A.M., Beuvin, M. and Boyer, B. (2004) Activation of Rac1 by paxillin-Crk-DOCK180 signaling complex is antagonized by Rap1 in migrating NBT-II cells. *J Biol Chem*, **279**, 44490-44496.

von Wichert, G., Haimovich, B., Feng, G.S. and Sheetz, M.P. (2003) Force-dependent integrin-cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2. *Embo J*, **22**, 5023-5035.

Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T. and Horwitz, A.F. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol*, **6**, 154-161.

Webb, D.J., Parsons, J.T. and Horwitz, A.F. (2002) Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat Cell Biol*, **4**, E97-100.

Yu, D.H., Qu, C.K., Henegariu, O., Lu, X. and Feng, G.S. (1998) Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. *J Biol Chem*, **273**, 21125-21131.

Zeng, L., Si, X., Yu, W.P., Le, H.T., Ng, K.P., Teng, R.M., Ryan, K., Wang, D.Z., Ponniah, S. and Pallen, C.J. (2003) PTP alpha regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. *J Cell Biol*, **160**, 137-146.

Samenvatting

(ook voor niet-ingewijden)

Ieder mens, elke plant en elk dier bestaat uit cellen. Hoewel de functies van de verschillende cellen in het menselijk lichaam erg uiteen kunnen lopen (denk bijvoorbeeld aan de verschillende functies van huidcellen, zenuwcellen, spiercellen en bloedcellen), moeten al onze cellen ook veel vergelijkbare taken kunnen volbrengen, zoals groeien, delen, bewegen en energie vrijmaken. Al deze verschillende processen worden in de cel geregeld door moleculen die we eiwitten noemen. Je kunt eiwitten vergelijken met machines die het werk in de cellen doen en die bepalen welke eigenschappen de cellen, weefsels en uiteindelijk het organisme hebben. Om alle verschillende functies in de cel uit te kunnen voeren, moeten eiwitten dus in vele soorten en maten voorkomen: groot en klein, stabiel en instabiel, met en zonder (enzymatische) activiteit. Eiwitten met activiteit zijn bijvoorbeeld in staat andere eiwitten actief te maken, te verplaatsen, of af te breken.

In een weefsel of organisme zijn cellen geen alleenstaande eenheden. Het is belangrijk dat cellen in een weefsel met elkaar kunnen communiceren en signalen vanuit hun milieu kunnen ontvangen. Zo zijn er bijvoorbeeld eiwitten die door de celmembraan heen steken om signalen van buitenaf op te pikken. Dit soort eiwitten (receptoren) kan vervolgens actief worden en dit activeringssignaal doorgeven aan andere eiwitten in de cel. Eiwitten die dit soort signalen verder doorgeven noemen we ook wel regeleiwitten. Dit proefschrift staat voornamelijk in het teken van één zo'n regeleiwit, dat Rap1 heet.

Rap1 is een belangrijk eiwit omdat het betrokken is bij het hechten van cellen aan elkaar. Dat is bijvoorbeeld belangrijk voor de laag cellen die de binnenkant van bloedvaten of de darmwand bekleedt, maar ook voor onze huid. Verder is Rap1 betrokken bij het vasthechten van cellen aan hun ondergrond. Dit noemen we cel-adhesie. Cel-adhesie wordt mogelijk gemaakt door integrines. Integrines zijn receptoreiwitten die door de celmembraan heen steken. Aan de buitenkant van de cel binden ze aan de ondergrond van cellen en aan binnenkant binden ze aan andere eiwitten in de cel. Het reguleren van cel-adhesie is onder andere belangrijk om het bewegen van cellen (cel-migratie) mogelijk te maken. Cel-migratie is essentieel voor belangrijke processen als het helen van wonden en de werking van het immuunsysteem. Maar ook sommige pathologische processen, zoals het uitzaaien van tumorcellen, zijn afhankelijk van cel-migratie.

Belangrijke eigenschappen van een regeleiwit zijn dat het andere eiwitten reguleert (bijvoorbeeld actief of inactief maakt) en zelf ook een aan/uit-schakelaar heeft. Rap1 kan inderdaad zowel actief als inactief zijn. Eén van de signaleringsroutes die bepaalt of Rap1 wel of niet actief is, wordt aangezet wanneer een hormoon aankomt bij een receptoreiwit. Als zo'n hormoon aan de buitenkant van de cel bindt aan de receptor, wordt

dit signaal doorgegeven naar eiwitten in de cel, die vervolgens het kleine signaalmolecuul cAMP maken. Het actief maken van Rap1 gebeurt door eiwitten met GEF-activiteit. Een van de belangrijkste daarvan is Epac, dat actief wordt wanneer de cel cAMP maakt. Vervolgens wordt Rap1 door Epac geactiveerd. Wanneer Rap1 actief is, kunnen bepaalde andere eiwitten aan Rap1 binden en vervolgens zelf actief worden. Dit soort eiwitten noemen we effectoren. Naast Epac kan het signaalmolecuul cAMP nog veel andere eiwitten activeren. In de cellen die wij voor onze experimenten gebruiken, willen we alleen de functie van Epac en Rap1 bestuderen. Om de effecten van andere eiwitten die door cAMP geactiveerd worden te omzeilen, hebben we een specifieke analoog van cAMP ontwikkeld (in dit proefschrift soms 007 genoemd), die alleen Epac activeert. Zo kunnen we dus specifiek Epac en vervolgens Rap1 activeren, wanneer we dat willen. Het onderzoek dat in dit proefschrift beschreven wordt, betreft de rol van Rap1 in cel-adhesie en cel-migratie.

In **hoofdstuk 1** geef ik in het kort weer wat er bekend is over de regulatie van cel-migratie in het algemeen en beschrijf ik wat we weten over de rol van Rap1 in cel-adhesie en -migratie.

In **hoofdstuk 2** vergelijken we het Rap-netwerk van eiwitten die Rap1 actief maken (activatoren) en effector-eiwitten met dat van Ras, een eiwit dat heel erg op Rap1 lijkt, maar juist een heel andere functie heeft.

In **hoofdstuk 3** hebben we cellen gefilmd om te onderzoeken wat het effect is van het activeren van Rap1 op cel-migratie. Met behulp van software kunnen we uit deze filmpjes de migratiesnelheid van cellen uitrekenen. Sommige groeifactoren die je aan cellen kunt toevoegen, zorgen ervoor dat cellen gaan migreren en losraken van elkaar. In hoofdstuk 3 laten we zien dat, als Rap1 in cellen actief is, dit niet meer gebeurt. Activatie van Rap1 remt dus de cel-migratie die veroorzaakt wordt door die groeifactoren. In deze cellen treden nog meer veranderingen op als we Rap1 actief maken via 007 en Epac. Zo worden onder andere de 'focal adhesions' minder dynamisch. Focal adhesions (letterlijk: adhesies op één plek) zijn grote eiwitcomplexen die ontstaan wanneer integrines hechten aan de ondergrond. Naast het feit dat focal adhesions bepalen hoe sterk een cel hecht aan de ondergrond, vormen ze ook de verbinding tussen de integrine en het actine cytoskelet in de cel. Dit cytoskelet bestaat uit eiwitbundels die zich door de hele cel uitstrekken en de cel zijn vorm geven. Ook zorgen ze voor de spanning en trekkracht die nodig zijn voor de cel om zich vooruit te kunnen bewegen. In cellen met actief Rap1, die niet kunnen migreren, zijn de focal adhesions veel stabieler, en worden ze ook groter. De normale regulatie van de grootte en/of sterkte van focal adhesions lijkt dus veranderd te zijn als Rap1 actief is. Maar wat is er dan precies veranderd en hoe doet Rap1 dat?

Om dit te onderzoeken hebben we een aantal van de belangrijkste regulatiemechanismen voor focal adhesions onderzocht om te kijken of

die veranderen als Rap1 actief wordt. In **hoofdstuk 4** beschrijf ik dat, voor de mechanismen die we hebben onderzocht, er geen verschil lijkt te zijn tussen normale focal adhesions and focal adhesions in cellen waarin we Rap1 hebben geactiveerd. Behalve de activiteit, wordt ook de aanmaak van eiwitten nauw gereguleerd. Alle informatie die nodig is om een eiwit te maken, zit in onze genen. Hoe veel of weinig er van een bepaald eiwit gemaakt wordt, kunnen we aflezen aan de gen-expressie. Gen-expressie wordt gemeten met behulp van micro-arrays. Om te kijken of het activeren van Rap1 leidt tot verschillen in gen-expressie die verantwoordelijk zouden kunnen zijn voor de remming van migratie, hebben we een micro-array experiment uitgevoerd. Dit is beschreven in het **addendum** bij hoofdstuk 3. Hieruit bleek dat Rap1 geen grote effecten heeft op gen-expressie, en dat het effect van Rap1 op cel-migratie waarschijnlijk niet veroorzaakt wordt doordat bepaalde eiwitten meer, of minder, gemaakt worden.

Hierna hebben we in hoofdstuk 5 een andere manier gezocht om erachter te komen via welke route Rap1 een effect uitoefent op focal adhesions. Waar we in hoofdstuk 4 eerder naar het eindpunt (de grootte van de focal adhesion) keken, zoeken we het in **hoofdstuk 5** wat dichter bij de bron: welk effector-eiwit van Rap1 is verantwoordelijk voor het effect op de focal adhesion? Hiervoor heb ik een kleine screen uitgevoerd, waarbij ik gebruik maakte van een techniek om steeds specifiek één eiwit te verwijderen uit de cel. In deze screen deed ik dat voornamelijk met al bekende effector-eiwitten van Rap1 en met eiwitten waarvan we weten dat ze belangrijk zijn voor cel-adhesie en focal adhesions. Door deze screen hebben we een aantal eiwitten kunnen identificeren die belangrijk zijn voor het effect van Rap1, maar niet het effector-eiwit gevonden dat ervoor verantwoordelijk is. Het is waarschijnlijk dat er niet één directe route is van Rap1 naar de focal adhesions, maar dat er meerdere routes en verschillende effectoren samen verantwoordelijk zijn voor de effecten van Rap1. Tot slot beschrijf ik in **hoofdstuk 6** de interactie van een van deze effectoreiwitten, Arap3, met het eiwit SHIP2. Deze eiwitten bezitten allebei een zogenaamd SAM-domein in hun structuur. Van SAM-domeinen weten we dat ze aan zichzelf of aan andere SAM-domeinen kunnen binden. In hoofdstuk 6 laat ik zien dat deze twee eiwitten inderdaad via hun SAM-domein aan elkaar binden.

De resultaten in dit proefschrift ondersteunen het idee dat Rap1 een belangrijke rol heeft in het reguleren van cel-adhesie. Verder laten we zien dat Rap1 het migreren van cellen kan remmen, en de dynamiek van focal adhesions beïnvloedt. Deze resultaten en de mogelijke implicaties hiervan worden in **hoofdstuk 7** bediscussieerd.

Curriculum Vitae

Judith Raaijmakers werd geboren in Nijmegen op 5 november 1981. Na het behalen van haar gymnasiumdiploma aan het Stedelijk Gymnasium Johan van Oldenbarnevelt te Amersfoort in 1999, begon ze in september van datzelfde jaar met de studie Medische Biologie (later: Biomedische Wetenschappen) in Utrecht. Tijdens de master Developmental Biology and Biomedical Genetics aan dezelfde universiteit, deed ze onderzoekservaring op bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht onder begeleiding van dr. Bea Kuiperij en prof. dr. Hans Bos en op het Samuel Lunenfeld Research Institute te Toronto, Canada, in het lab van prof. dr. Tony Pawson, onder begeleiding van dr. Rob Ingham. Na het afronden van deze studie in oktober 2004 (cum laude), begon ze in november 2004 met het in dit proefschrift beschreven onderzoek in de groep van prof. dr. Hans Bos, bij de afdeling Fysiologische Chemie van het UMCU.

List of publications

Raaijmakers, J.H. and Bos, J.L.
Specificity in Ras and Rap signaling
Journal of Biological Chemistry, in press

Lyle, K.S., Raaijmakers, J.H., Bruinsma, W., Bos, J.L. and de Rooij, J.
cAMP-induced Epac-Rap activation inhibits epithelial cell migration by modulating focal adhesion and leading edge dynamics
Cellular Signalling (2008); 20(6): 1104-16
(K.S.L. and J.H.R. contributed equally to this work)

Raaijmakers, J.H., Deneubourg, L., Rehmann, H., de Koning, J., Zhang, Z., Krugmann, S., Erneux, C. and Bos, J.L.
The PI3K effector Arap3 interacts with the PI(3,4,5)P3 phosphatase SHIP2 in a SAM domain-dependent manner.
Cellular Signalling (2007); 19(6): 1249-57

Ingham, R.J., Raaijmakers, J., Lim, C.S., Mbamalu, G., Gish, G., Chen, F., Matskova, L., Ernberg, I., Winberg, G. and Pawson, T.
The Epstein-Barr virus protein, latent membrane protein 2A, co-opts tyrosine kinases used by the T cell receptor
Journal of Biological Chemistry (2005); 280(40): 34133-42

Ingham, R.J., Colwill, K., Howard, C., Dettwiler, S., Lim, C.S., Yu, J., Hersi, K., Raaijmakers, J., Gish, G., Mbamalu, G., Taylor, L., Yeung, B., Vassilovski, G., Amin, M., Chen, F., Matskova, L., Winberg, G., Ernberg, I., Linding, R., O'Donnell, P., Starostine, A., Keller, W., Metalnikov, P., Stark, C. and Pawson, T.
WW domains provide a platform for the assembly of multiprotein networks
Molecular and Cellular Biology (2005); 25(16): 7092-106

Kuiperij, H.B., van der Horst, A., Raaijmakers, J., Weijzen, S., Medema, R.H., Bos, J.L., Burgering, B.M. and Zwartkruis, F.J.
Activation of FoxO transcription factors contributes to the antiproliferative effect of cAMP
Oncogene, 24 (2005); 24(12): 2087-95

Dankwoord

De afgelopen tijd heb ik vaak gedacht: als ik straks klaar ben, dan mag ik mijn dankwoord schrijven, en dat is leuk! Maar nu het boekje bijna af is en het echt mag, is het stiekem best eng. Toch maak ik graag van deze unieke kans gebruik om (uitgebreid) iedereen te bedanken die heeft bijgedragen aan de totstandkoming van dit boekje, zowel op het werk, als daarbuiten!

Als belangrijkste natuurlijk Hans, mijn promotor. Ik heb ontzettend veel van je geleerd de afgelopen jaren en bewonder je niet-aflatende enthousiasme voor ons kleine eiwitje enorm. Jouw visie op wat een goede AIO maakt, heeft mij door menig dipje heengeholpen. Dankjewel voor de steun als ik weer eens door alle integrines het bos niet meer zag en voor je deur die altijd openstond, of het nu ging om goeie proeven of om snoepreisjes naar Spanje!
Pieken zijn hoger en dipjes minder dippig als je lotgenoten om je heen hebt bij wie je je ei kwijt kunt. Gelukkig is er op zo'n groot lab altijd wel een luisterend oor te vinden!
Lieve Ester, wat is het fijn om iemand te hebben die net een beetje voor loopt, om de fijne kneepjes van af te kijken. 4 jaar een kamer delen schept een band en ik ben heel blij dat dat lijntje tussen onze hoofden het nog doet! Dankjewel voor alles, maar vooral voor je gebrom, wijsheid, vibes, vitaminepillen, onze woensdagochtend- en msn-sessies, dansen met een rokje aan en je vriendschap. Lieve Carin, naast elkaar studeren, boven elkaar pipetteren en nu met maar 2 deuren ertussen op dezelfde gang. Wat een geluk om iemand die me zo goed kent zo dichtbij te hebben, tijdens en na werktijd! Dankjewel voor je steun en vertrouwen in mij en die vieze zoete bubbels op precies het goede moment! Ik vind jou ook een bikkel en vind dat je ontzettend goed bent in alles wat je doet! Meiden, het kan niet anders dan dat het met jullie naast me een geweldig (en perfect georganiseerd) feestje wordt de 26e!
Lieve Matthijs, als onderdeel van het dynamic trio had je het soms zwaar met ons, al dan niet in dezelfde stoel. Met jou is het fijn biertjes drinken, ouwehoeren en zwemmen! Thanks voor al je support en sorry nog, voor al die SMSjes. Veel geluk samen! Diaantje, enorme bikkel, eigenlijk vind ik toch de P.....-cake je meest geweldige experiment van de afgelopen jaren :). Bedankt voor alle kletsmomentjes en zet 'm op, het tegeltje is nu voor jou! Peter, het blijft heerlijk om met jou te discussiëren over wetenschap en het lab wel-en-wee. Succes met de laatste loodjes en andere grote stappen die je binnenkort gaat zetten! Wendy, je ziet ze allemaal komen en gaan, wat fijn om jou er als stabiele factor bij te hebben. Ik ben blij dat het zo goed gaat met jou en al je mannen. My students, Marrit, Wytse and Nafsika, thanks for teaching me at least as much as I hope I taught you. Wytse, koning van het klikken, dankjewel nog daarvoor. Marrit, sorrysorrysorry voor Tiam, echt! Wat fijn dat jullie allebei zijn blijven hangen, succes de komende jaren en make me proud! Marrit, ik vind het nu al gezellig met jou op de kamer. Thanks voor je fotografie-kennis, zonder jou was de cover niet zo mooi geworden! Dear Anna, mice suck! Your perseverance is admirable and I wish you all the best for this coming year. And then...maybe another experiment? Martijn, WJ en Lars, ook al vormen jullie inmiddels zelf een nieuw soort elite, voor mij blijven jullie altijd 'de jongetjes'. Martijn, sorry dat je alleen spaties mocht checken, ga je nu een hele moeilijke vraag bedenken? WJ, wat nou junctions? Tnx voor al je integrine-kennis en het er samen over brainstormen. Lars, embrace the focal adhesions (en ga ze nou eens SILACen!) Ze zijn echt gaaf! Dear Sarah en Ingrid, thanks for joining the

focal adhesion project! Ingrid, wat fijn om hulp te krijgen tijdens de laatste loodjes! Ik hoop dat je nog lang met plezier blijft pipetteren. Sarah, it was great having you around for discussions and other chats. Good luck on the project, and make sure to see some of this little country while you're here! Jantine, fijn om sommige niet-lab sores met jou te kunnen delen. Ook al weten we nog steeds niet precies wat we meten, met jouw talent voor mooie proeven doen, komen we vast een stuk verder. OY, thanks dat ik je af en toe van je werk mocht houden als ik weer even van de bank wilde genieten. Zullen we de verfilming van Eclipse maar overslaan?! Anouk, thanks voor je interesse in het schrijven en onze gezellige chats. Meiden: veel succes de komende jaren, zet 'm op! Fried, een wandelende Rap-PubMedsite, wat heerlijk! Dank voor je interesse, het lezen van mijn stukken en alle kritische vragen. Holger, always willing and able to help out, thanks for that! Send me an email the next time there's champagne, OK? Milica, Marije en Marjolein, the female part of team Epac, good luck making beautiful crystals and westerns! Maaike, van mentorkindje tot collega, het kan soms raar lopen! Zouden we het straks op een vergadering nog weleens over Krit hebben, denk je? Succes met al je projecten in het vrouwenlab :)! Bom, aan de trainer zal het volgend seizoen in ieder geval niet liggen, bedankt voor de kritische noten tijdens werkbesprekingen. Harmjan, lieve brompot, ja, het is af! Maak er wat moois van in je kleine koude hokkie! Hesther en Iris, gaan we straks weer lekker los in de Derrick? Tobias, thanks voor je ImageJ en andere hulp en succes met het uitbouwen van de groep Dansen. Paulien, van stoere brandweerbikkel tot lieve mama, veel plezier met je kerels! Wanneer mag ik een keer oppassen? Miranda, jou zien staat voor mij gelijk aan 'bijna weekend', heerlijk! Lydia, echt, zonder jou wordt het helemaal niks op het lab. Veel geluk in de toekomst, op het lab en thuis, en dankjulliewel voor de gezelligheid! The newbies, David, Evi, Astrid en Marlous, good luck to you all! David, gedraag je hè, op Spetses! Astrid, lieve brekebeen, nog 1 maandje, dan mag het raam weer dicht! John, Anne en Emma, onze gedeelde AIOs, gezellig om jullie er ook bij te hebben en succes met jullie projecten. Marc en Annelies, met de communicatie-tak van het CGC op onze gang erbij, leer je nog eens wat (anders). Ik vond het leuk om te helpen bij de diverse activiteiten, dankjulliewel! Marc, bedankt voor de gezellige praatjes, we zien elkaar vast nog vaak. Ik ben heel benieuwd hoe een communicatie-proefschrift eruit ziet, succes! Fons, lesgeven is inderdaad leuk, geniet van het klaar zijn! Lieve ladies van het secretariaat, bedankt voor het altijd een minuutje hebben om te babbelen, om te delen in mijn blijheid over het een of ander, om elkaars schoenen te bewonderen en om even in Hans zijn agenda te spieken! Wim, jij waakt niet alleen over de serverspace, maar houdt ook nog in de gaten of het met ons zelf wel goed gaat. Dank aan je hele team voor al jullie efforts om het voor ons wat makkelijker te maken. Ook Marjoleine, Richard, Kees, Marcel en Marian bedankt voor de nodige ondersteuning.
Er zijn ook alweer veel mensen weg, met wie het gezellig was toen ik nog een jong AIOtje was...Marta, ciccia bella, heel veel geluk met Bart in Bern! Het bijwonen van jullie prachtige bruiloft was echt geweldig! Dear Karen, I am glad we got to finish the paper together. Thanks for all the help, keeping in touch and good luck with the new job! Shannon, you crazy American, thanks for disturbing the peace every now and then. Bea, van jou heb ik heel veel geleerd, dankjewel daarvoor. Echt leuk dat we elkaar zo nu en dan nog tegenkomen! Arjan, altijd optimistisch en o zo enthousiast over andermans project, altijd fijn om even met jou te brainstormen, bedankt! Armando: HOEST??? Jurgen: And STAY down! En Leo: this neonate has finally grown up! Many thanks to all former BB-members for all the good times!

Johan, van jou heb ik ook ontzettend veel geleerd. Bedankt voor je interesse, hulp, enthousiasme en het ouwehoeren over van alles en nog wat! Je groep groeit nu al uit z'n voegen, veel succes met z'n allen. Ik vond het gezellig bij jullie!
Ook de andere groepen op de 2e en 3e, bedankt voor de gezelligheid. Timmertjes, fijn dat jullie er toch bij kunnen zijn. Sjoerd, niemand kan zo heerlijk chagrijnig koffie tappen als jij! Thanks voor alle praatjes en veel succes met de laatste loodjes en daarna. Nga-Chi, jij ook succes met schrijven en solliciteren! The 'new' guys, Gianpiero, Radhika, Rick en Andrée, I like your new habit of taking breaks in the coffee corner, keep it up and good luck with all of your projects! Marijke, stressen en sporten gaan bij mij niet zo goed samen, vanaf nu kom ik weer steppen, I promise. Petra, bedankt voor de momentjes van afleiding tijdens het printen. Frank, Dik en Patrick, bedankt voor de hulp met de microarray. Marian, extra bedankt voor je ontzettende geduld...Geert, doe mij ook eens zo'n T-shirt! Thanks voor het gebruiken van je mooie microscoop, ook al ging hij nogal eens stuk (o nee, dat was niet mijn schuld...)! Livio, zonder jou wordt het helemaal niks met al die microscopen. Laat je de volgende keer wel alle stekkers erin? Ook de rest van de Kopsjes, thanks voor de gezelligheid! Saskia, Nannette, Tale en Wilco, succes!
Ook de Medemaatjes en aanverwante artikelen zijn gezellig om biertjes mee te doen, dankjulliewel! Patrick, ik ben benieuwd of ik nog een muizenproef meemaak, lijkt me gaaf!
Tony, thanks for having me over and have fun at Spetses. Rob and Jerry, I enjoyed our morning Tim's a lot, good luck to both of you! Amy, you are crazy, and Spetses with you was great! To all the members of the Pawsonlab: Greetings from the creamy pineapple! I had a great time in the lab and in TO, thanks to all of you for making it that way.

Ook na werktijd zijn er lieve mensen die belangstelling tonen en die ik wil bedanken voor hun steun en vriendschap.
Kris, al meer dan 20 jaar vriendinnen, dat is bijzonder. Ik ben blij dat we elkaar zo vaak zien en zoveel kunnen delen. Bedankt voor alle afleiding tijdens het schrijven en voor de tig keer dat je liet weten dat je trots op me was! Dat doet een mens goed! Nieuwe banen, nieuwe fases, maar wij blijven vriendinnen, dat staat vast! Lieve Ing, lieve Jet, op naar meer jubilea, dineetjes en hopelijk nog heel vaak D, D en DDD (alhoewel, dat DDD laten we maar aan Kris over...)!
Olivia, ook voor jou komt het einde in zicht. Ik ben trots op jouw doorzettingsvermogen en vind het fijn dat we nog steeds onze sores kunnen delen onder het genot van een soepie! Zet 'm op, want je kunt het!
Lieve roomies, ik hou van datumprikker en etentjes met jullie! Zullen we dat nog lekker lang volhouden? Thanks voor jullie interesse, steun en vertrouwen in mij en m'n sollicitatie- en schrijfkwaliteiten.
Kris, ons 1e telefoongesprek was inderdaad meteen leuk! Ik ben blij dat we daarna vriendinnen zijn geworden. Dankjewel voor je altijd bruisende aanwezigheid en je lieve mailtjes op het goede moment!
Marjolein, mijn promotie wordt bij lange na niet zo'n genot om naar te luisteren als jouw afstuderen, maar hopelijk wel net zo leuk!
Af en toe afreageren met veel snijwerk lucht op, daarom is Smullen ook zo leuk! En omdat er zoveel leuke vrijwilligers werken en gasten komen, natuurlijk. Dankjulliewel voor de fijne afleiding en het nodige perspectief. Lieve dr. Manon, zie je wel dat je het kan?! Samen achter de pannen vormen we een geweldig team! Veel plezier in Azië, goeie reis! Joost, ik mis jou echt op het lab! Succes met de proeven voor Linda

en het verder ontdekken van Zuid-Amerika(anse schones)....

Lieve dames: et voilà! Ik ben er supertrots op dat ik als eerste een boekje mag afleveren. En nu wil ik de term SAAIO nóóit meer horen! Van 12 naar 30, en het gekakel neemt exponentieel toe! We gaan de Derrick echt heel erg onveilig maken met z'n allen straks!!! Sas, you're next! Maar met jouw speedy schrijven en layouten-voor-de-fun wordt dat een eitje, geloof ik. Succes ermee en go kick ass in NYC! Jinte, jij daarna? Al lopen we elkaar nogal eens mis, gmailchat staat voor niks, da's ook fijn! Succes met de laatste loodjes! Maike, Simone, Linda, en Lisette, ook voor jullie: veel succes! Inkie en Juud, met jullie erbij kunnen we bijna een FF-dependance beginnen in het Stratenum, gezellig hoor!

Lieve Mariet en Wouter, het is fijn om je in Maarssen ook gewoon thuis te voelen. Wat mij betreft hoeven jullie geen haast te maken met de Frieslandplannen hoor! Bedankt voor jullie interesse en support. Judith (S.), wie had ooit kunnen denken dat we nu schoonzusjes zouden zijn!?! Buzz, Party&Co of Grote Doos, met jou spelletjes doen is leuk, zeker tegen de mannen! Lieve Jolanda, Juda en Edwin, het is altijd gezellig als de Solingertjes bij elkaar zijn, in Maarssen of wherever. Bedankt!

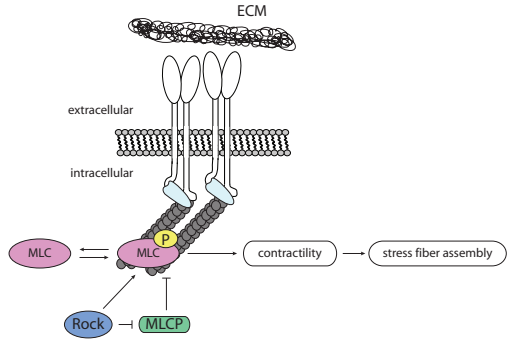
Lieve oma, wat is het jammer dat opa dit niet meer kan lezen of erbij kan zijn... Maar wat ben ik blij dat jij er wel bij bent! Ik ben trots op jou en je drukke leventje en vind het fijn dat we elkaar zoveel spreken!
Steven, my dear mon frère, ik ben trots op je! Nog even doorzetten en dan ben je ook een echte bioloog! Ik vind het heerlijk dat jij en Jis zo dichtbij wonen en dat we elkaar zo vaak zien. Met DJ Steve E erbij wordt het echt een goed dansfeestje straks! Jiska, het is fijn om jou erbij te hebben als 'zusje'!

Lieve papa en mama, weten jullie wel hoe lief ik jullie vind?! Het is zo fijn om een thuisfront te hebben dat onvoorwaardelijk achter je staat! Bedankt voor alle steun en jullie geloof in mij, wat ik ook doe.

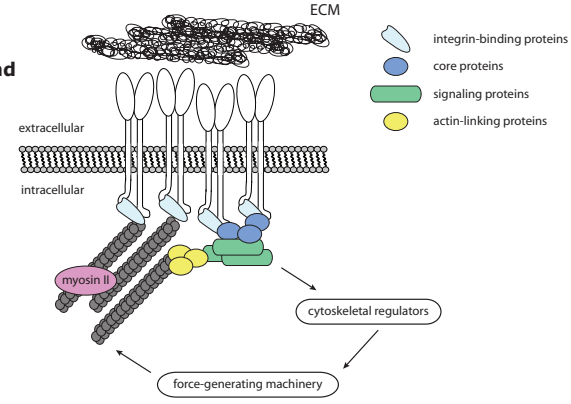
Lief schatje van me, jij bent de rust die ik niet in me heb, bij jou kan ik pas echt ontspannen. Dankjewel voor je ontzettende geduld met me de afgelopen maanden, ik weet dat ik soms niet heel gezellig was. Toch word ik altijd blij(er) van jou! Dankjewel voor je knuffels, verliefde momentjes, luisterend oor, pannenkoeken en dat je er altijd voor me bent. Ik heb zin in vanaf nu: samen reizen, samen de toekomst in, samen met jou is alles leuker! Weet ik, moet ook, thanks...!!

Color Figures

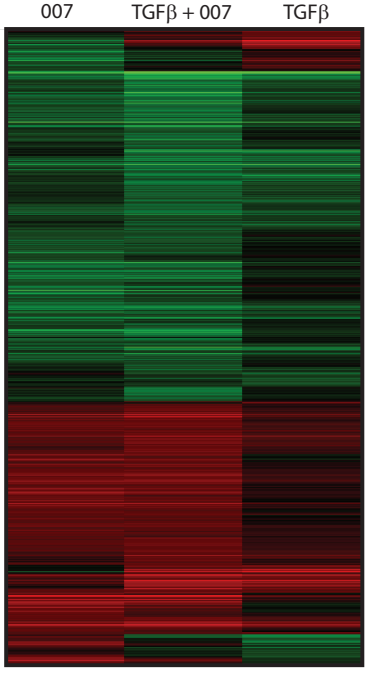
Chapter 1, Figure 2.
Regulation of MLC phosphorylation by Rho kinase

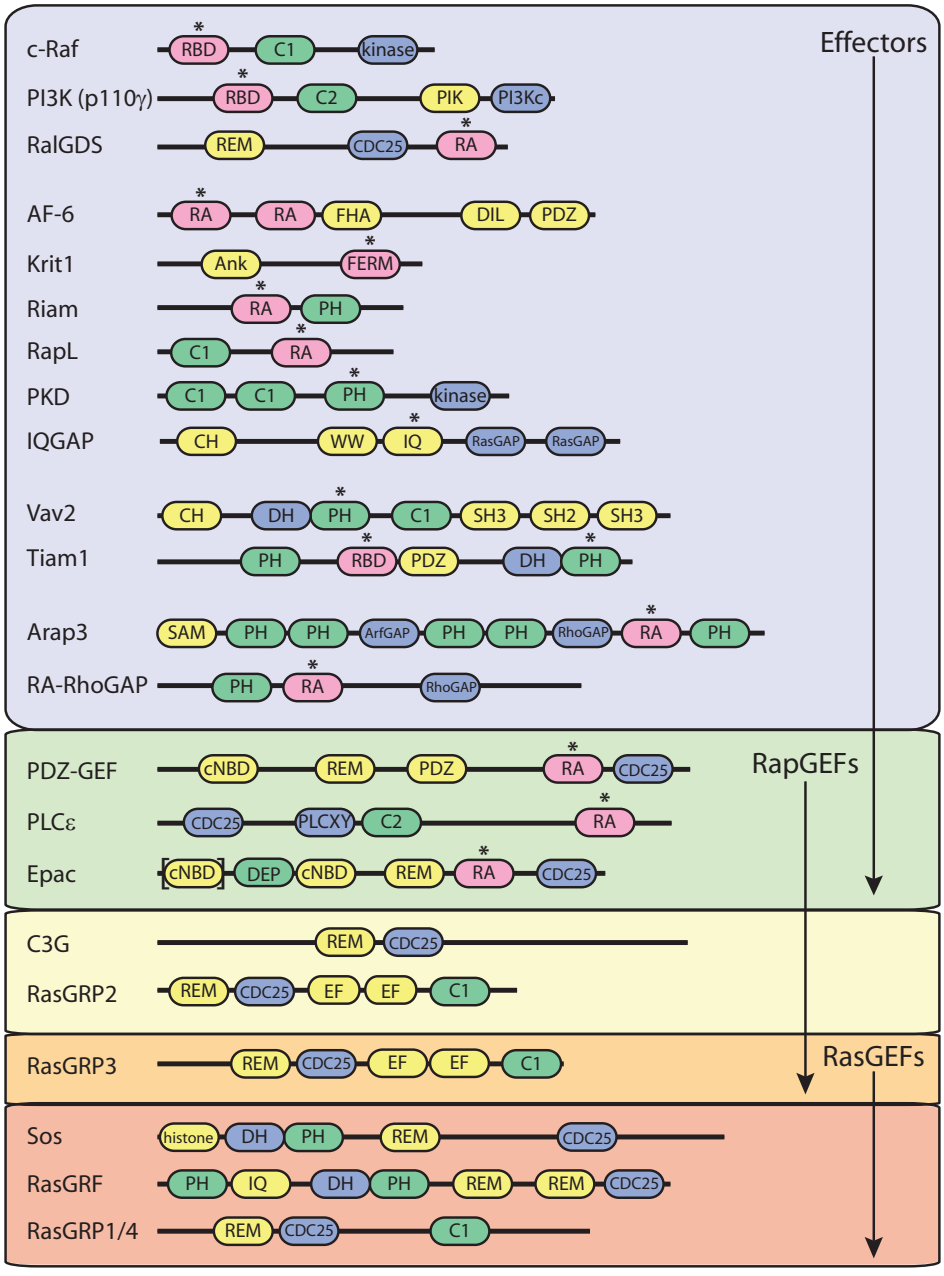


Chapter 1, Figure 4.
Focal adhesions: core proteins and linkage to the actin cytoskeleton.



Addendum, Figure 2A
Cluster diagram of the 832 significant genes.





Chapter 2, Figure 1. Ras and Rap effector proteins and GEFs

A schematic representation is shown of the domain structures of Ras- and RapGEFs and effector proteins discussed here. RA domains/RBDs are depicted in pink, catalytic domains in blue, lipid binding domains in green and other domains in yellow. Asterisks indicate domains required for Ras/Rap binding.

Say what you say,
Do what you do
Feel what you feel,
As long as it’s real.
I said take what you take
And give what you give
Just be what you want,
Just as long as it’s real.

Lily Allen - Take what you take