Spatiotemporal Regulation of Rap Guanine Nucleotide Exchange Factors

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Cover picture: “Enough already!” by Aaron de la Cruz, part of “In the family” project aimed at raising awereness about breast cancer.
Patients diagnosed with breast cancer filled in their “cell” within the piece.

Invitation picture by Jacob, 14 months old.

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Spatiotemporal Regulation of Rap Guanine Nucleotide Exchange Factors

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Regulatie van Rap Guanine Nucleotide

Exchange Factors in Plaats en Tijd

(met een samenvatting in het Nederlands)

Regolazione dei Fattori di Scambio dei

Nucleotidi Guaninici per Rap

(con un riassunto in Italiano)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, Prof. Dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 15 mei 2014 des middags te 2.30 uur

door

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geboren op 12 mei 1986 te Mariano Comense (CO), Italië

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What would life be if we had no
courage to attemp anything?

Vincent van Gogh

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General Introduction

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Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) orchestrate the activity of small G-proteins. In response to extracellular stimuli, GEFs and GAPs activate signaling cascades regulated by G-proteins by controlling their regulation in time and in space. Generally, GEFs function as activators of G-proteins by promoting their GTP-bound state while GAPs serve as inhibitors by increasing the rate of GTP hydrolysis (Bos, Rehmann et al. 2007). Our understanding of the mechanisms of regulation of signal transduction networks by GEFs and GAPs is still growing. Here we try to address this issue by focusing on exchange factors for the small G-protein Rap.

The small G-protein Rap

Small GTPases are low molecular weight monomeric G proteins of around 20-25 kDa. Their core GTPase domain is subject to regulation by GEFs and GAPs, which drive either their active GTP-bound or inactive GDP-bound stable state (Figure 1) (Bourne, Sanders et al. 1990).

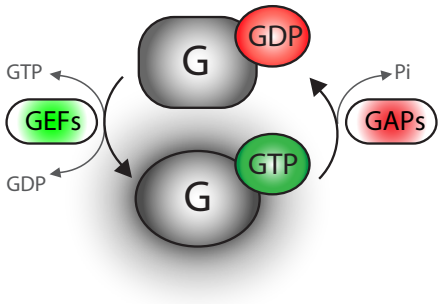

Figure 1: **Small G-protein regulatory cycle**
Small G-proteins (G) cycle between an inactive GDP-bound and an active GTP-bound state. This switch is regulated by GTPase accelerating proteins (GAPs), which are responsible for GTP hydrolysis, and by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP.

The human genome comprises more than 150 members that can be subdivided into five families according to their sequence homologies. These are the Rho, Rab, Arf, Ran and Ras family. Each family comprises a set of specific regulators and structural studies revealed their very distinct structural features (Colicelli 2004, Wennerberg, Rossman et al. 2005). The Ras family includes proteins involved in the control of gene expression, cell proliferation and survival (Bos, Rehmann et al. 2007). Rap is a member of the Ras family of G-proteins, firstly identified as a negative regulator of Ras transforming activity (Kitayama, Sugimoto et al. 1989). We now know that Rap functions independently of Ras and controls a variety of cellular processes such as cell adhesion (Enserink, Price et al. 2004, Bos 2005), endothelial barrier function (Pannekoek, van Dijk et al. 2011) and epithelial cell polarity (Gloerich, ten Klooster et al. 2012). The mammalian genome comprises two Rap1 proteins, Rap1a and Rap1b, and three Rap2 isoforms, Rap2a, Rap2b and Rap2c. Although Rap1 and Rap2 proteins share 60% sequence similarity, effectors can discriminate between Rap1 and Rap2 and thus link extracellular stimuli to distinct cellular responses (Gloerich and Bos 2011). Moreover, Rap proteins localize at membrane compartments by means of distinct lipid modifications at their C-terminus (Farrell, Yamamoto et al. 1993, Wright and Philips 2006, Canobbio, Trionfini et al. 2008, Gloerich and Bos 2011). Regulatory mechanisms of Rap by GAPs and GEFs were first unveiled in the yeast S. cervisiae (Park and Bi 2007). The positioning of the bud site, where the daughter cell will grow and separate, is under control of the Rap homolog Bud1/Rsr1. Moreover, depletion of the GEF and GAP of Bud1/Rsr1, Bud5 and Bud2 respectively, leads to randomized localization of the bud site (Park, Chant et al. 1993, Cabib, Drgonova et al. 1998). Bud5 and Bud2 localize to the nascent bud site by cortical proteins and recruit and activate Bud1/Rsr1 at the growing bud site. GTP-bound Bud1/Rsr1 then interacts with the Cdc42 GEF, Cdc24, for the establishment of cell polarity and bud formation. The assembly of such complex is under control of the Bud2 GAP, which ensures localized signaling of Bud1/Rsr1 and activation of the effector Cdc42 (Figure 2) (Park, Bi et al. 1997). Likewise, in

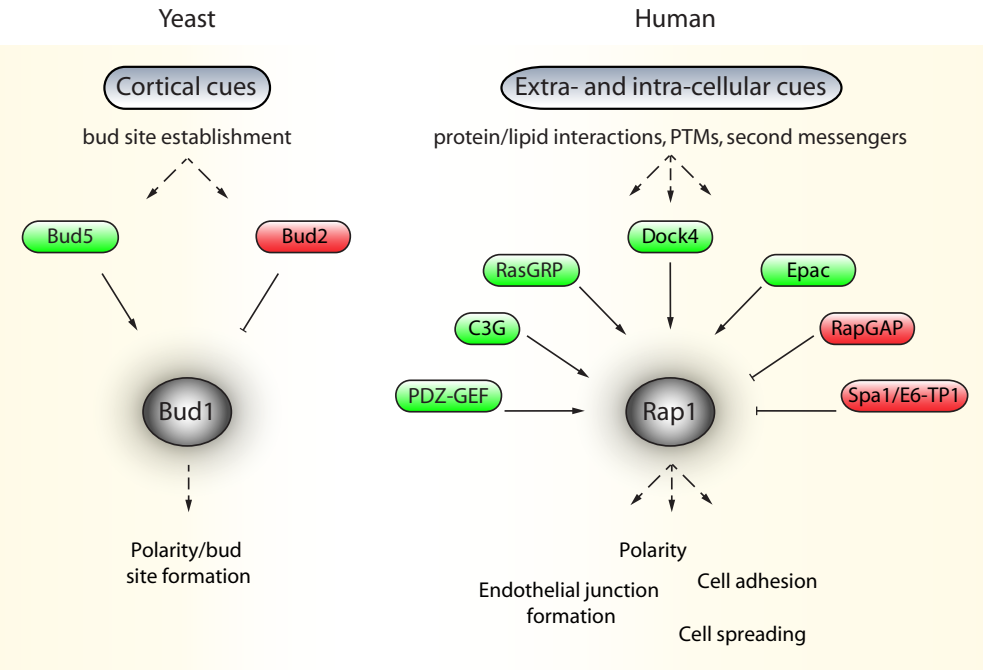

Figure 2: Overview of Rap1 signaling network in yeast and in humans
Left side: In yeast, the Rap homolog Bud1 controls polarity and bud site formation via a single pathway through the action of one guanine nucleotide exchange factor (GEF) and one GTPase accelerating protein (GAP). Right side: In humans, Rap1 is under control of numerous GEFs and GAPs to regulate a variety of cellular pathways in response to several stimuli. GEFs are indicated in green and GAPs in red.

multicellular organisms the Rap1 signaling network is under tight control of GEFs and GAPs, which translate different cues to specific cellular responses by regulating small GTPases in time and in space (Figure 2).

RapGEFs

Exchange factors in charge of controlling the GDP-GTP switch of the Rap subfamily are characterized by the presence of a catalytic CDC25 homology domain (CDC25-HD). This conserved protein module was firstly identified in the yeast S. cervisiae and is exclusive to GEFs for Ras-like GTPases (van Dam, Rehmann et al. 2009). Several classes of Rap GEFs exist, all originally identified by database searches for CDC25-HD domain-containing proteins. These comprise C3G, PDZGEF1 and PDZGEF2, Epac1 and Epac2, Repac, RasGRP1 and RapGRP3, PLCε and RasGEF1 (Figure 3). Their distinctive CDC25-HD module is usually found adjacent to a Ras exchange motif (REM) and a Ras association (RA) domain (Figure 3). Some Rap GEFs specifically activate only certain Rap members. This is the case for C3G, which can exert a higher exchange activity towards Rap1 (van den Berghe, Cool et al. 1997), and RasGEF1 for Rap2 (Yaman, Gasper et al. 2009). In contrast, others can act on a broader range of G-proteins, as shown for RasGRP3 and its ability to activate Ras, R-Ras and Rap1 (Yamashita, Mochizuki et al. 2000). By responding to specific signaling cascades, RapGEFs are themselves subject to regulation. Some of the regulatory mechanisms will be discussed below.

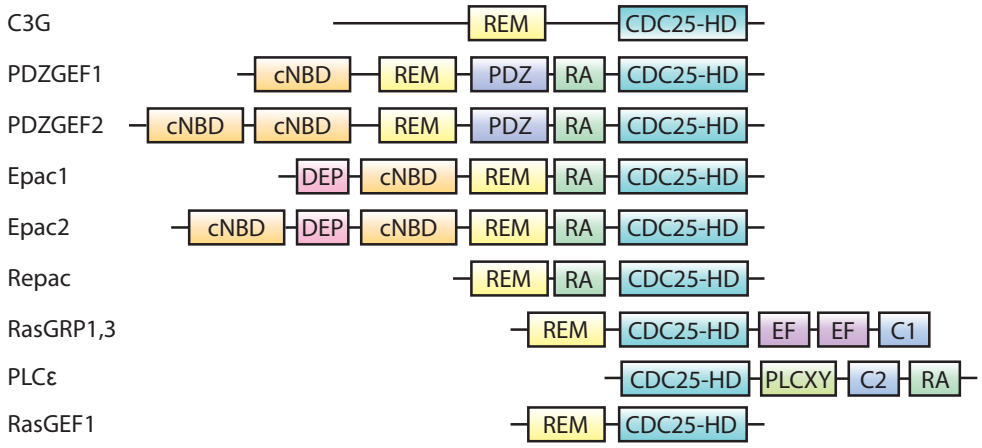

Figure 3: Domain organization of RapGEFs
REM: Ras exchange motif; CDC25-HD: CDC25 homology domain; cNBD: cyclic nucleotide binding domain; PDZ: PSD-95/discs-large/ZO-1; RA: Ras associating; DEP: Dishevelled/Egl-10/Pleckstrin; EF: EF hands; C1 and C2: protein kinase C conserved region 1 and 2; PLCXY: catalytic lipase domain.

Regulation of Epac

The two Epac variants are multidomain proteins containing an N-terminal regulatory region comprising of one (Epac1) or two (Epac2) cyclic nucleotide binding domain (cNBD) and a DEP domain. The catalytic region at their C-terminus harbors the CDC25-HD domain for exchange activity, an RA and REM domain (Figure 3). Epac1 and Epac2 are directly activated by the second messenger cAMP and are subject to diverse mechanisms of spatial and temporal regulation. By binding to diverse proteins or lipids via different domains, Epac proteins link cAMP signaling to specific cellular responses (summarized in Figure 4). cAMP not only activates Epac but also induces its translocation towards the plasma membrane (PM) where it binds to phosphatidic acid (PA) (discussed in chapter 2). This dynamic redistribution of Epac1 requires the cAMP-induced conformational change as well as its DEP domain and is needed for activation of PM-located Rap (Ponsioen, Gloerich et al. 2009). In addition to its PM targeting, Epac1 has been reported to localize at different cellular compartments. Indeed, the N-terminal 49 amino acids of Epac1 interact with proteins of the Ezrin, Radixin, Moesin (ERM) family and this binding recruits Epac1 to a more clustered localization at the PM. This interaction is triggered by activation of ERM proteins and is needed for Rap-mediated cell adhesion (Gloerich, Ponsioen et al. 2010). Epac1 can regulate cell adhesion also via a different pathway. In endothelial cells, Epac1 is recruited via its REM domain to a complex consisting of phosphodiesterase 3 (PDE3B) and PI3Kγ that results in stimulation cAMP-dependent cell adhesion and spreading (Wilson, Baillie et al. 2011). During cardiac hypertrophy, another phosphodiesterase, PDE4D3, tethers Epac1 to perinuclear membranes in order to attenuate ERK5 signaling through Rap (Dodge-Kafka, Soughayer et al. 2005). Epac1 has also been linked to microtubule stabilization processes by binding via its cNBD domain to the light chain 2 of microtubule associated protein 1 (MAP1A) (Magiera, Gupta et al. 2004). This interactions entails Epac1 localization in the cytoplasm, where it associates with the cytoskeleton via MAP1A, and enhances Epac1 exchange activity by increasing its sensitivity to activation by cAMP (Gupta and Yarwood 2005). Moreover, a fraction of Epac1 can also be found in the nucleus and at the nuclear pore complex. Nuclear Epac1 is employed in the

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cytoplasmic transport of the DNA repair component DNA-dependent protein kinase (DNA-PK) in response to cAMP (Huston, Lynch et al. 2008), whereas localization of Epac1 at the nuclear envelope has an inhibitory effect. Binding of the nucleoporin RanBP2 to the catalytic CDC25-HD domain of Epac1 results in negative regulation of Epac1 activity (Gloerich, Vliem et al. 2011). This interaction keeps a pool of inactive Epac1 at the nuclear pore until it is relieved upon phosphorylation of the zinc fingers of RanBP2 during mitosis (Gloerich, Vliem

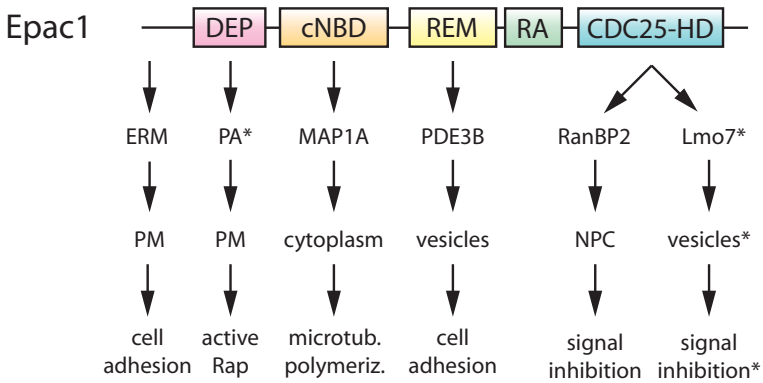

Figure 4: Epac1 interacting partners
Overview of known domain-specific binding partners of Epac1. The resulting subcellular localization and function of the interaction is indicated. Asterisks indicate novel binding partners and mechanisms of interaction found and discussed in this thesis.

et al. 2011). Epac1 is differentially distributed during the cell cycle. Indeed, during prophase, Epac moves away from the nuclear envelope and associates with the mitotic spindle during metaphase. Epac1 then localizes around the chromosomes during anaphase and accumulates on the nuclear envelope and at the cleavage furrow during telophase and cytokinesis (Qiao, Mei et al. 2002). Finally, Epac1 is tethered to cytoplasmic vesicles by the LIM domain-containing protein Lmo7. This results in negative regulation of cAMP-Epac1 signaling and is regulated by phosphorylation events (discussed in Chapter 5).

Regulation of PDZGEF

The multiple isoforms of PDZGEF comprise a characteristic PDZ (PSD-95/discs-large/ZO-1) domain, and the RapGEF-specific CDC25-HD, RA and REM modules (Figure 3). At their N-terminus they contain one or two cyclic nucleotide binding domains with auto-inhibitory functions (de Rooij, Boenink et al. 1999) similarly to Epac. Despite this, no second messenger tested so far was able to enhance the activity of the proteins (Kuiperij, Rehmann et al. 2006), thus suggesting the involvement of other regulatory mechanisms (summarized in Figure 5). The small G-protein M-Ras was found to be involved in the spatial regulation on PDZGEF2 (Gao, Satoh et al. 2001). Indeed, PDZGEF2 translocates to the membrane through the binding of its RA domain with M-Ras. In this way PDZGEF2 activates Rap downstream of TNFα receptor activation (Gao, Satoh et al. 2001). Similarly, during neurite outgrowth, TrkA receptor after neutrophins binding is internalized and localizes at endosomes. PDZGEF1 is then recruited to the surface of late endosomes for sustained activation of Rap1 (Hisata, Sakisaka et al. 2007). Recently, PDZGEF was found to play a role in establishment of in-

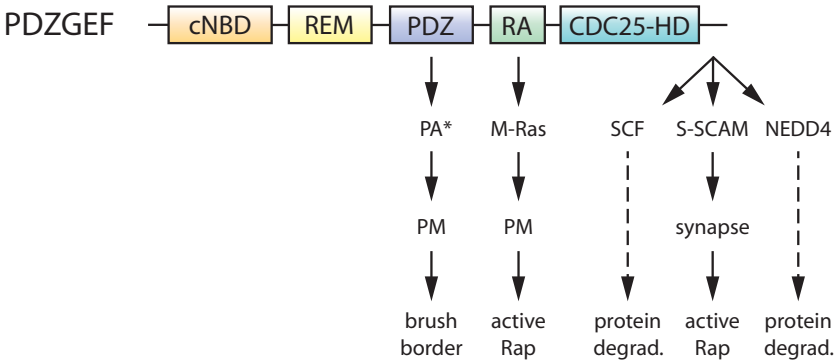

Figure 5: PDZGEF interacting partners
Overview of known domain-specific binding partners of PDZGEF. The resulting subcellular localization and function of the interaction is indicated. Asterisk indicates novel mechanism of interaction found and discussed in this thesis.

testinal cell polarity (Gloerich, ten Klooster et al. 2012). PDZGEF is recruited to the apical membrane by binding to locally-generated PA via its PDZ domain (discussed in Chapter 4), thereby activating a pool of Rap2a needed for induction of brush border formation (Gloerich, ten Klooster et al. 2012). The unstructured C-terminus of PDZGEF is subject to several mechanisms of regulation and post translational modifications. Indeed, the synaptic scaffolding molecule (S-SCAM) was shown to bind to the C-terminus of PDZGEF (Ohtsuka, Hata et al. 1999). This interaction localizes PDZGEF at synaptic junctions and is needed for Rap activation (Mino, Ohtsuka et al. 2000). The E3 ubiquitin ligase NEDD4 can also bind to the C-terminus of PDZGEF, thereby regulating its stability in cells by targeting it for proteasomal degradation (Pham and Rotin 2001). Likewise, in response to metastatic factors, PDZGEF is phosphorylated by casein kinase-1α at its C-terminus and targeted for degradation by the SCF ubiquitin ligase complex (Magliozzi, Low et al. 2013). This is needed for inactivation of Rap1 and induction of cell migration. Finally, PDZGEF1 catalytic activity towards Rap was shown to depend on phosphorylation of its C-terminus by PKCε downstream of the T-cell receptor (Letschka, Kollmann et al. 2008).

Regulation of PLCε and RasGRP

Phospholipase Cε (PLCε) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol triphosphate. PLCε is an atypical RapGEF as it comprises, in addition to an RA and the catalytic CDC25-HD domain, also a phospholipase domain (PLCXY) and a Ca2+-dependent lipid binding domain (C2) (Figure 3). Its CDC25-HD can bind to Rap1 thereby amplifying EGF-induced Rap activation at specific cellular compartments such as the Golgi apparatus (Jin, Satoh et al. 2001). A similar signal amplification also occurs in response to cAMP in cardiomyocites for regulation of cardiac contraction. Downstream of Epac1-induced cardiac contractility, PLCε hydrolytic activity enhances calcium release in the sarcoplasmic reticulum and its GEF activity is required for sustained Rap1 activation (Oestreich, Malik et al. 2009).
RasGRP is another RapGEF that can interact with calcium ions (Ca2+) and phospholipids (specifically DAG) via its EF-hands and C1 domains. In addition, it contains a CDC25-HD and

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REM domain required for interacting with Rap (Figure 3). Production of these messengers by phospholipase C in required for activation of RasGRP (Ebinu, Bottorff et al. 1998). Indeed, binding of DAG and Ca2+ to RasGRP is needed for Rap activation during platelet aggregation (Crittenden, Bergmeier et al. 2004). RasGRP1 can also bind to F-actin via its N-terminus. This results in its recruitment to membrane ruffles and in local activation of Rap (Caloca, Zugaza et al. 2004).

Regulation of C3G, RasGEF1 and Repac

C3G is a Rap1-specific GEF (van den Berghe, Cool et al. 1997) consisting only of a CDC25-HD and a REM domain (Figure 3). Its unstructured N-terminus contains proline-rich sequences able to interact with SH3 domain-containing proteins such as Crk, Hck, c-Abl and Cas (Knudsen, Feller et al. 1994, Kirsch, Georgescu et al. 1998, Shivakrupa, Radha et al. 2003, Radha, Rajanna et al. 2007). Crk is a bona fide binding partner and regulator of C3G. Crk binding releases C3G from its auto-inhibited state and directs its localization to the PM. Together with phosphorylation by Src, this results in full activation of C3G and of Rap1-mediated signaling ((Matsuda, Hashimoto et al. 1994, Feller 2001), Popovic M. and Rehmann H., unpublished data).
RasGEF1 shares the domain architecture with C3G, although lacking a long unstructured N-terminus (Figure 3). RasGEF1 acts as GEF for Rap2 and not for Rap1 or any other Ras subfamily members. By interacting with a particular side chain unique to Rap2, RasGEF1 can discriminate between Rap isoforms and activate specifically Rap2 (Yaman, Gasper et al. 2009).
Repac consists of a REM, RA and the catalytic CDCD25-HD domain (Figure 3). Repac is regulated by biding to M-Ras via its RA domain in a GTP-dependent manner (Rebhun, Castro et al. 2000). It was initially thought that this interaction would inhibit the exchange activity of Repac towards Rap (Rebhun, Castro et al. 2000), but it was later found that the binding brings Repac to the PM, where it can activate Rap1 downstream of M-Ras (Gao, Satoh et al. 2001).

Scope of this thesis

GEFs regulate a variety of cellular pathways by activating G-protein signaling. They do so not only by accelerating the rate of GDP dissociation and GTP binding of small GTPases, but also by controlling their cellular localization. In order to grasp how GEFs specifically and selectively signal to particular G-proteins, we need to understand how these regulators are themselves regulated.

In this thesis we aim to further investigate the temporal and spatial regulation of GEFs for the Rap subfamily of G-proteins. We will focus on the cAMP-activated Epac1 and on PDZGEF. We will firstly investigate the molecular mechanism behind the DEP domain-dependent membrane localization of Epac1, required for Rap signaling (Chapter 2). In Chapter 3 we will discuss in details how the DEP domain, also present in other exchange factors, can be regulated and its contribution to signal specificity (reviewed in Chapter 3). In Chapter 4 we reveal how PDZGEF is comparably recruited to specific cellular compartments by interacting with membrane anchors. Finally, in chapter 5 we describe an additional mechanism of regulation of Epac1 by an interacting protein which results in negative control of cAMP-Epac1 signaling.

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cAMP regulates DEP domain-mediated binding of Epac1 to phosphatidic acid at the plasma membrane

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Epac1 is a cAMP-regulated guanine nucleotide exchange factor (GEF) for the small G protein Rap. Upon cAMP binding, Epac1 undergoes a conformational change that results in its release from auto-inhibition. In addition, cAMP induces the translocation of Epac1 from the cytosol to the plasma membrane. This relocalization of Epac1 is required for efficient activation of plasma membrane located Rap and for cAMP-induced cell adhesion. This translocation requires the DEP domain, but the molecular entity that serves as the plasma membrane anchor and the possible mechanism of regulated binding remains elusive. Here we show that Epac1 binds directly to phosphatidic acid. Similar to the cAMP-induced Epac1 translocation, this binding is regulated by cAMP and requires the DEP domain. Furthermore, depletion of phosphatidic acid by inhibition of phospholipase D1 prevents cAMP-induced translocation of Epac1 as well as the subsequent activation of Rap at the plasma membrane. Finally, mutation of a single basic residue within a polybasic stretch of the DEP domain, which abolishes translocation, also prevents binding to phosphatidic acid. From these results we conclude that cAMP induces a conformational change in Epac1 that enables DEP domain-mediated binding to phosphatidic acid resulting in the tethering of Epac1 at the plasma membrane and subsequent localized activation of Rap.

Introduction

Epac1 and Epac2 are cAMP responsive guanine nucleotide exchange factors (GEFs) for the small G protein Rap and are involved in the spatio-temporal regulation of a variety of processes, among others endothelial cell-cell junction modulation, myocardial contraction and insulin secretion (Gloerich and Bos 2010). Epac1 and Epac2 are multi-domain proteins, comprising an N-terminal regulatory region and a C-terminal catalytic region. The regulatory region contains one (Epac1) or two (Epac2) cAMP binding domains and a Dishevelled, Egl-10, Pleckstrin (DEP) domain, while the catalytic region harbors a CDC25-homology domain for GEF activity, a REM (Ras exchange motif) and a putative RA (Ras association) domain. In the absence of cAMP, Epac adopts an auto-inhibitory conformation in which the regulatory region occludes the binding site for Rap, which is relieved upon cAMP binding (Rehmann, Das et al. 2006, Rehmann, Arias-Palomo et al. 2008). In addition to activating Epac1, cAMP also induces its translocation to the plasma membrane (PM). This diffusion-driven translocation requires the DEP domain of Epac1 and functions only in the context of the full length protein, suggesting that a binding site for a plasma membrane anchor is provided in its open conformation (Ponsioen, Gloerich et al. 2009). Both activation and translocation of Epac1 are required for efficient cAMP-induced activation of Rap1 at the PM and for subsequent downstream responses, such as induction of cell adhesion (Ponsioen, Gloerich et al. 2009). Epac1 can also be recruited to a more clustered localization at the PM, independent of its DEP domain and cAMP binding, through an interaction with activated Ezrin, Radixin, Moesin (ERM) proteins. This translocation is regulated by ERM-activating stimuli such as thrombin, and is mediated by the N-terminal 49 amino acid residues of Epac1 (Gloerich, Ponsioen et al. 2010). Other membrane anchors for Epac1 within different cellular compartments have been identified (Gloerich and Bos 2010), however the PM anchor for the cAMP-induced, DEP domain-mediated localization is currently unclear.

The DEP domain is a 90 amino acid residue globular domain, firstly identified in Drosophila Dishevelled, C. elegans EGL-10 and mammalian Pleckstrin, and present in a number of mammalian protein families (Ponting and Bork 1996, Pan, Pang et al. 2004, Civera, Simon et al. 2005, Ballon, Flanary et al. 2006). Of these, the most extensively studied is the DEP domain of Dishevelled (Dvl), an adaptor protein in Wnt-induced signaling. This DEP domain contains a cluster of exposed basic residues that enables membrane recruitment through interactions with negatively charged phospholipids, such as phosphatidic acid, required for both canonical and non-canonical Wnt signalling (Axelrod, Miller et al. 1998, Boutros, Paricio et al. 1998, Wong, Mao et al. 2000, Jung, Kim et al. 2009, Simons, Gault et al. 2009). Additional protein interactions by the DEP domain of Dvl further modulate Wnt signaling, which includes its binding to the μ2 subunit of the AP2 clathrin complex to mediate internalization of the Frizzled receptor (Yu, Rual et al. 2007, Yu, Xing et al. 2010). Another DEP domain-containing family of proteins is the R7 family of regulators of G-protein signaling (RGS). RGS proteins are GTPase-accelerating proteins that facilitate GTP hydrolysis of Gα subunits of heterotrimeric G proteins (reviewed in (De Vries, Zheng et al. 2000)). RGS proteins form stable trimeric complexes in a DEP domain-dependent manner with the Gβ5 subunit and specific membrane anchor proteins such as members of the syntaxin family of SNARE proteins, R7BP and R9AP (Hu, Zhang et al. 2003, Martemyanov, Lishko et al. 2003, Kovoor, Seyffarth et al. 2005, Drenan, Doupnik et al. 2006, Anderson, Posokhova et al. 2009). Moreover, the DEP domain of R7-RGS proteins also enables direct interactions with G-protein coupled receptors (Sandiford and Slepak 2009, Sandiford, Wang et al. 2010). These studies point out a

DEP domain-mediated selectivity for PM anchors and their involvement in multiple distinct molecular interactions controlling membrane recruitment.
The aim of this study was to identify the anchor at the PM for Epac1, and to elucidate the regulatory mechanism for its DEP domain-mediated translocation. We found that in the presence of cAMP, Epac1, but not Epac1 lacking its DEP domain (ΔDEP-Epac1), directly binds to phosphatidic acid (PA). Importantly, this interaction is regulated by cAMP. Furthermore, cellular depletion of PA prevents cAMP-induced Epac1 translocation and subsequent Rap activation at the PM. Finally, we identified a positively charged residue within a polybasic region in the DEP domain of Epac1 that mediates binding to PA. Combined with a recent observation that cAMP increases solvent exposure of this region of the DEP domain (Li, Tsalkova et al. 2011), we conclude that a cAMP-induced conformational change enables DEP domain-mediated binding of Epac1 to PA at the PM.

Materials and Methods

Reagents and DNA constructs
8-pCPT-2'-O-Me-cAMP (007) and 8-pCPT-2'-O-Me-cAMP-AM (007-AM) were from Biolog Life Sciences (Bremen, Germany); the PLD inhibitor CAY10593 was from Cayman chemical (Ann Arbor, USA) and the thrombin receptor-activating peptide (TRP, SFLLRNPDKYEPF sequence) was a kind gift from Kees Jalink. The PLD1 antibody was from Sigma (Saint Louis, USA), the anti-HA antibody was from Covance (Princeton, USA) and the 5D3 anti-Epac1 antibody has been previously described (Price, Hajdo-Milasinovic et al. 2004). ON-TARGET plus SMARTpool siRNAs directed against PLD1 (J-009413) and scrambled control siRNAs were from Thermo Scientific Dharmacon (Lafayette, USA). The wildtype Epac1, ΔDEP-Epac1 (amino acids 50 to 148 deleted), and R82A Epac1 constructs have been described previously (Ponsioen, Gloerich et al. 2009). Mutations were introduced by site-directed mutagenesis. Purification of recombinant Epac1 and ΔDEP-Epac1 was previously described (de Rooij, Rehmann et al. 2000), YFP-RalGDS RBD was kindly provided by Mark Philips and GFP-Spo20p PABD was a kind gift from Nicolas Vitale.

Cell culture and transfection
HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum and antibiotics. Cells were transfected with expression plasmids using X-tremeGENE 9 transfection reagent (Roche Inc.) and with SMARTpool siRNA using HiPerfect (Qiagen), according to manufacturer´s protocol.

Cell imaging
For confocal live-imaging, one day after transfection cells were seeded overnight in WillCo wells (WillCo Wells B.V.) in the presence of CAY10593 (1 µM) where indicated, and examined in L-15 Leibovitz medium (Invitrogen) at 37°C on an inverted Zeiss LSM510 confocal microscope equipped with 63x magnification objective lens (N.A. 1.4, Leica, Mannheim, Germany). For TIRF imaging, cells expressing CFP-Epac1 and YFP-RalGDS RBD, pre-incubated overnight with CAY10593 (1 µM) where indicated, were imaged on a TIRF microscope (Ti, Nikon) with a 60x 1.49 NA Apo TIRF objective lens and an electron microscopy charge-coupled device camera (Luka, Andor) at 37°C. A series of images were taken at 30 seconds intervals. For quantification of TIRF microscopy, images were corrected for background, measured in

a region of the image that did not contain cells, and the average pixel intensity was measured over time in a region of interest enclosing a cell expressing CFP-Epac1 and YFP-RalGDS RBD and normalized to the start value. For quantification of the translocation of Epac1 constructs, time series images (Figure 2) or images of cells taken after stimulation with 007-AM (1 µM) (Figure 4) were imported into a custom-made visual studio program (Professional edition 2008; Microsoft, Redmond, WA), in which the PM/cytoplasmic fluorescence ratio was measured. Regions of interest at the PM, cytoplasm, and background were determined automatically for every image and the fluorescence pixel intensity was measured (Leyman, Sidani et al. 2009).

Protein-lipid overlay assays

Nitrocellulose membranes spotted with a variety of lipids or with increasing concentrations of lipids (PIP and Membrane strips and Membrane lipid array, Echelon Biosciences) were blocked for 1 hour in 3% fatty acid-free BSA in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1 % Tween 20) and then incubated with 1 µg/ml of recombinant full length His-Epac1 or GST-ΔDEP-Epac1 in presence or absence of 8-pCPT-2'O-Me-cAMP (007, 100 µM) for the indicated time periods. For experiments using Epac1 isolated from HEK293T cells, cells were transfected with HA-Epac1 or HA-R82A Epac1 and then lysed in buffer containing 50 mM Tris-HCl p.H. 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM MgCl2 and protease and phosphatase inhibitors. Lysates were then incubated overnight with protein G sepharose beads and anti-HA antibody. After washing of the beads, the bound proteins were eluted with 3 x HA pepetide (250 µg/ml) in BC300 buffer (20 mM Tris-HCl pH 7.9, 20% glycerol, 300 mM KCl) and protein recovery was determined by western blotting. Equal amounts of Epac1 and R82A Epac1 were incubated with lipid strips in the presence of 007 (100 µM). Bound protein was detected using the 5D3 anti-Epac1 antibody and visualized by Odyssey Infrared Imaging (Li-Cor).

Liposome assay

The liposome binding assay was performed as described previously (Van Galen, Van Balkom et al. 2010). In brief, liposomes containing phophatidyl choline (PC) as the only phospholipid or 80% (mol) PC and in addition either 20% phophatidic acid (PA), phosphatidylethanolamine (PE) or phosphatidylinositol (PI) were made by mixing stock solutions of lipids with cholesterol in a molecular ration of 2.28:1. The chloroform/methanol solvent was evaporated using a flow of N2 with subsequent drying at room temperature in a speedvac (Savant SVC100H, Farmingdale, USA) for at least 90 min. Lipids were resuspended in 50-NT buffer (50 mM NaCl, 25 mM Tris-HCl pH 7.4) buffer to a final concentration of 6.8 µmol/ml and liposomes were generated by sonicating on ice using an ultrasonic probe (MSE Soniprep 150, London, UK). 50 µl of the liposome suspension was incubated with 1 µg of recombinant His-Epac1 in 50-NT buffer for 90 min at 37°C in the presence of 100 µM 8-Br-cAMP. Sucrose (60% (w/v)) was mixed with the sample to a final concentration of 36.5% and samples were overlayed with 500 µl 25% (w/v) sucrose in 50-NT buffer and subsequently with 100 µl 50-NT buffer. Subsequently samples were centrifuged in a TLA-55 rotor (Beckman, Fullerton, USA) for 90 min at 136,000 g at 4°C. After centrifugation, liposome-bound protein was collected in 300 µl from the top of the gradient and unbound protein was collected in 300 µl from the pellet fraction. Collected proteins were analysed by SDS-PAGE and western blotting using the Epac1 5D3 antibody.

Results

cAMP regulates the direct binding of Epac1 to phosphatidic acid

We have previously shown that cAMP induces the translocation of Epac1 to the PM, a process which requires the DEP domain of Epac1. This domain is present in a number of proteins and shown to bind to phospholipids (Hurley, Anderson et al. 2002, Simons, Gault et al. 2009). To investigate whether Epac1, in the presence of cAMP, binds to phospholipids, we carried out protein-lipid overlay assays. Nitrocellulose membranes onto which a variety of lipids were spotted were incubated with bacterially-produced recombinant Epac1 in the presence of 8-pCPT-2'OMe-cAMP (also known as 007), a cAMP analog that selectively activates Epac. We observed binding of Epac1 to phosphatidic acid (PA) that was at least four times higher than the binding to other phospholipids (Figure 1A and B). Importantly, an Epac1 mutant lacking the DEP domain (ΔDEP-Epac1) did not bind to PA (Figure 1A and B). A similar preference of Epac1 for binding to PA was observed using a liposome binding assay (Figure 1C). To further study the relative binding affinity of Epac1 to PA, we made use of membranes spotted with increasing concentrations of PA. Epac1 showed clear binding to less than 10 nM PA, whereas ΔDEP-Epac1 did not show any binding even at the highest concentration of PA (Figure 1D). From these results we conclude that Epac1 binds selectively and directly to PA and that this interaction requires the DEP domain.

We next investigated whether cAMP is required for efficient binding of Epac1 to PA, as cAMP is also required for its PM translocation. To that end, recombinant Epac1 was incubated in the presence or absence of 007 with PA containing membranes. In the absence of 007, no binding of Epac1 to PA could be observed after 5 minutes of incubation, and only limited binding after prolonged incubation for 30 minutes. However, in the presence of 007 a clear binding was already observed within 5 minutes, which was further increased after 30 minutes of incubation (Figure 1E). These results indicate that cAMP binding increases the affinity of Epac1 for PA, and implies that its translocation to the PM results from acquired affinity for PA in its open conformation.

PA is required for the cAMP-induced translocation of Epac1 to the PM

We next investigated whether PA is indeed required for cAMP-induced tethering of Epac1 to the PM. We therefore inhibited one of the PA-generating enzymes, phospholipase D (PLD), using the PLD inhibitor CAY10593. To monitor PA levels we used the PA-binding domain of the Spo20p protein (Spo20p PABD) linked to GFP. This PA sensor resides in the nucleus due to a nuclear localization signal, unless it is trapped at the membrane by binding to PA (Figure 2A, left panel, and (Zeniou-Meyer, Zabari et al. 2007)). In the presence of CAY10593, PM-bound GFP-Spo20p PABD was no longer observed, indicating that PA in the PM was successfully depleted (Figure 2A, left panel). Next, we examined the translocation of YFP-Epac1 in the presence of this PLD inhibitor. CAY10593 completely abolished the translocation of YFP-Epac1 upon stimulation with 007-AM (a more cell permeable AM ester of 007 (Vliem, Ponsioen et al. 2008)) (Figure 2A, right panel). To validate the role of PLD in PA formation in HEK293T cells and in Epac1 translocation, we depleted PLD1, the most abundant PLD in these cells (Chae, Kim et al. 2008), using siRNAs. This resulted in loss of Spo20p PABD-GFP from the PM and in inhibition of the cAMP-induced translocation of Epac1 to the PM (Figure 2B).

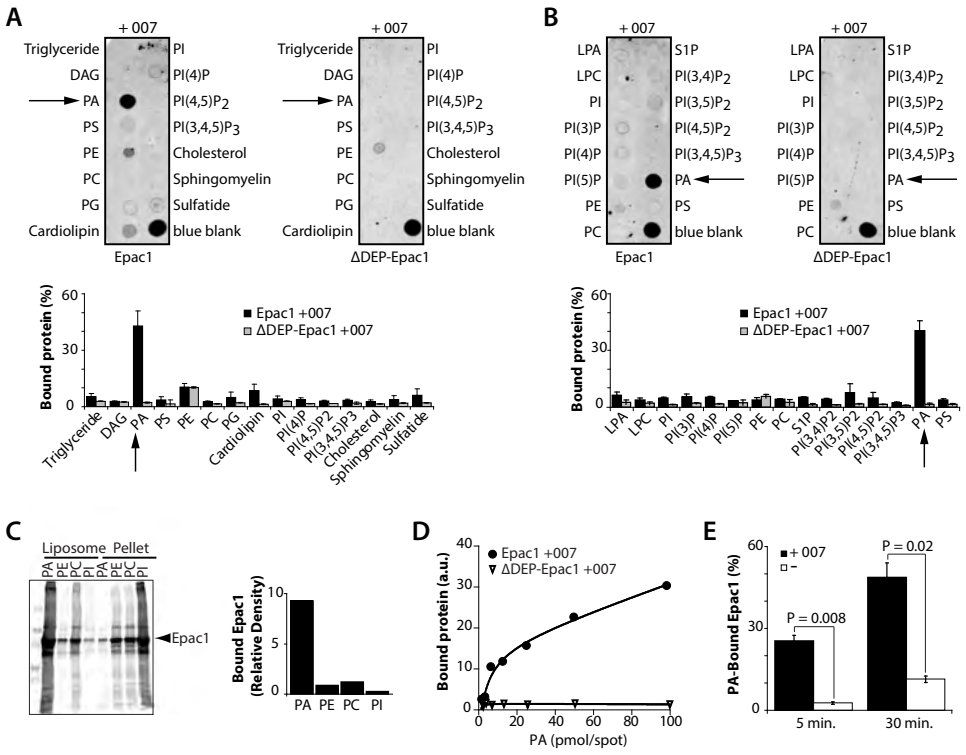

Figure 1: Epac1 binds to PA in a DEP domain- and cAMP-dependent manner
A) Top panel: Protein-lipid overlay assay of recombinant Epac1 in the presence of 007 (100 µM) using membrane strips containing 100 pmol/spot of the following lipids: Triglyceride, Diacylglycerol (DAG), Phosphatidic acid (PA), Phosphatidylserine (PS), Phosphatidylethanolamine (PE), Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Cardiolipin, Phosphatidylinositol (PI), Phosphatidylinositol 4-phosphate (PI(4)P), Phosphatidylinositol 4,5-bisphosphate, (PI(4,5)P2), Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), Cholesterol, Sphingomyelin, or 3-sulfogalactosylceramide (Sulfatide). Binding of Epac1 was detected using the Epac1 antibody 5D3. Bottom panel: Quantification of the binding of Epac1 to the different lipids, showing the mean binding with SD from three independent experiments expressed as percentage normalized to control (blue blank). B) Top panel: Protein-lipid overlay assay as in (A) but using PIP strips containing 100 pmol/spot of the following lipids: Lysophosphatidic acid (LPA), Lysophosphocholine (LPC), Phosphatidylinositol (PI), Phosphatidylinositol 3-phosphate (PI(3)P), Phosphatidylinositol 4-phosphate (PI(4)P), Phosphatidylinositol 5-phosphate (PI(5)P), Phosphatidylethanolamine (PE), Phosphatidylcholine (PC) Sphingosine 1-Phosphate (S1P), Phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), Phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2), Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), Phosphatidic acid (PA), or Phosphatidylserine (PS). Bottom panel: Quantification of the binding of Epac1 to the different lipids, showing the mean binding with SD from three independent experiments expressed as percentage normalized to control (blue blank). C) Epac1 in the presence of 100 µM 8-Br-cAMP was added to liposomes containing phosphatidylcholine (PC) as the only phospholipid or in addition phosphatidic acid (PA), phosphatidylethanolamine (PE) or phosphatidylinositol (PI). Liposomes and bound Epac1 protein were isolated by a sucrose gradient and Epac1 present in the liposome fractions was analyzed by western blotting. A representative experiment with the quantification of the relative amount of Epac1 in the different liposome fractions (liposome/pellet ratio) is shown. D) Graph showing the representative binding of recombinant Epac1 or ΔDEP-Epac1, in presence of 007 (100 µM), to PA spotted at increasing concentrations ranging from 1.56 to 100 nM onto nitrocellulose membrane. Non-linear binding curves were fitted using GraphPad Prism software. E) Quantification of the binding of Epac1 to the different lipids, showing the mean binding with SD from three independent experiments expressed as percentage normalized to control (blue blank). Lipid-containing membrane strips were incubated for the indicated time periods with recombinant Epac1 as in Figure 1A, in the presence or absence of 007 (100 µM), and bound Epac1 was visualized using the Epac1 antibody 5D3. Statistical analysis was performed using a one-tailed Student's t-Test.

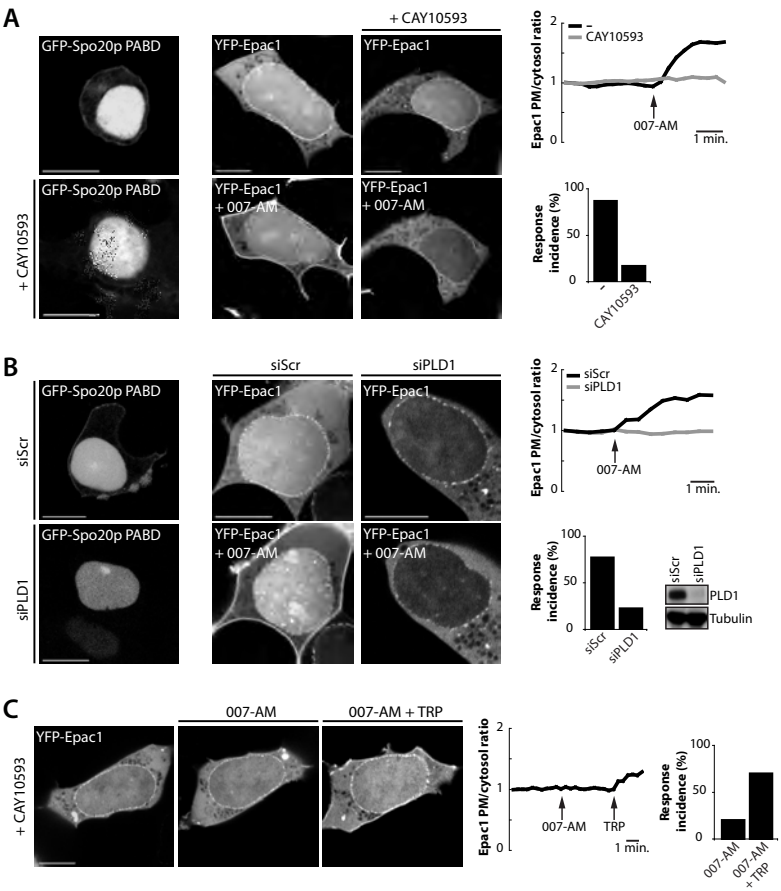

Figure 2: PA is required for the cAMP-induced translocation of Epac1 to the PM
A) Left panel: Confocal live-imaging of the PA-binding domain of Spo20p (GFP-Spo20p PABD) in HEK293T cells, showing its localization at the PM where PA is present. The unbound probe is nuclear due to the presence of a nuclear localization signal. Treatment of cells with the inhibitor of the PA producing enzyme PLD, CAY10593 (1 μM), results in PA depletion as it abolishes PM recruitment of GFP-Spo20p PABD. Right panel: Confocal live-imaging of the translocation of YFP-Epac1 to the PM in HEK293T cells by stimulation with 007-AM (1 μM). This translocation of Epac1 is inhibited when cellular PA is depleted by treatment with CAY10593 (1 μM). The graph shows the average fluorescence pixel intensity of YFP-Epac1 at the PM versus the cytosol during stimulation with 007-AM (1 μM) relative to pre-stimulus levels, in the absence or presence of CAY10593 (1 μM), calculated as described in Materials and Methods. The bar graph shows the percentage of cells showing translocation of Epac1 to the PM upon 007-AM stimulation (response incidence; 13/15 cells expressing YFP-Epac1, and 2/9 cells expressing YFP-Epac1 in presence of CAY10593 (1 μM)). B) Left panel: Confocal live-imaging of GFP-Spo20p PABD in HEK293T cells showing its accumulation at the PM in scr siRNA transfected (siScr) cells, but not in cells depleted of PLD1 (siPLD1). Right panel: Confocal live-imaging of YFP-Epac1 In HEK293T cells, showing that the 007-AM-induced translocation of YFP-Epac1 observed in scr siRNA (siScr) transfected cells is lost upon knockdown of PLD1 (siPLD1). The western blot shows the efficiency of PLD1 depletion. The graph shows the average fluorescence pixel intensity of YFP-Epac1 at the PM versus the cytosol during stimulation with 007-AM (1 μM) relative to pre-stimulus levels in scr and PLD1 siRNA-transfected cells, calculated as described in Materials and Methods. The bar graph shows the percentage of cells showing translocation of Epac1 to the PM upon 007-AM stimulation (response incidence; 10/13 cells expressing YFP-Epac1, and 2/12 cells expressing YFP-Epac1 upon siRNA depletion of PLD1). C) Confocal live-imaging of HEK293T cells depleted of PA by treatment with CAY10593 (1 μM). In the absence of PA, after 007-AM stimulation (1 μM), YFP-Epac1 remains cytosolic, but can still be recruited to the PM via activation of ERM proteins by stimulation with thrombin receptor activating peptide (TRP, 50 μM). The graph shows the average fluorescence pixel intensity of YFP-Epac1 at the PM versus the cytosol during stimulation with 007-AM (1 μM) and TRP (50 μM) relative to pre-stimulus levels, in the absence or presence of CAY10593 (1 μM) , calculated as described in Materials and Methods. The bar graph shows the percentage of cells showing translocation of Epac1 to the PM upon 007-AM stimulation (response incidence; 2/10 cells expressing YFP-Epac1 after 007-AM treatment, and 7/10 cells following TRP stimulation). All scale bars: 10μm.

To exclude that PA depletion from the PM had a more general overall effect on Epac1 translocation, we made use of our previous observation that thrombin receptor stimulation results in the translocation of Epac1 to the PM by a DEP domain-independent mechanism (Gloerich, Ponsioen et al. 2010). Binding of Epac1 to activated ERM (Ezrin, Radixin, Moesin) proteins requires the first N-terminal 49 amino acid residues of Epac1. In cells depleted of PA, thrombin receptor activating peptide (TRP) still induced Epac1 translocation to the PM (Figure 2C). Taken together, we conclude that PA is required for the cAMP-induced membrane translocation of Epac1.

PA binding is needed for Epac1-mediated activation of Rap at the PM

We next investigated whether PA is also required for Epac1-mediated activation of Rap at the PM. To that end, we measured the PM recruitment of YFP-RalGDS RBD, which recog-

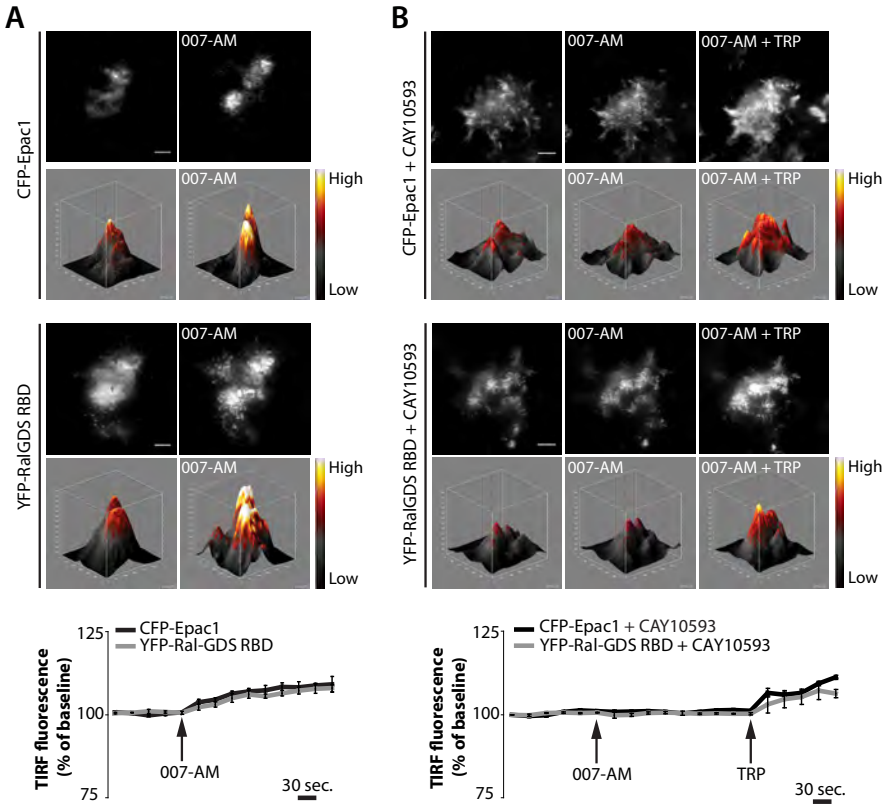

Figure 3: PA binding is needed for Epac1-mediated activation of Rap at the PM

A) TIRF live-imaging of CFP-Epac1 together with YFP-RalGDS-RBD, which binds Rap only in the active conformation, in HEK293T cells. Both Epac1 and RalGDS-RBD accumulate at the PM after 007-AM stimulation (1 μM). Representative TIRF images and 3D surface plots (fluorescence intensity of TIRF signal plotted against the surface area of the shown image using the smart LUT scale) are shown. The graph shows the average fluorescence intensity with SEM of five independent experiments, relative to pre-stimulus levels. B) TIRF live-imaging of CFP-Epac1 and YFP-RalGDS RBD similar to A, upon inhibition of cellular PA production by addition of CAY10593 (1 μM). CAY10593 prevents the recruitment of CFP-Epac1 and YFP-RalGDS RBD to the PM after 007-AM (1 μM) stimulation. In contrast, activation of the ERM (Ezrin, Radixin, Moesin) proteins and DEP-domain independent membrane targeting of Epac1 upon thrombin receptor activation (TRP, 50 μM), results in accumulation of both Epac1 and RalGDS RBD at the PM. The graph shows the average fluorescence intensity with SEM of five independent experiments, relative to pre-stimulus levels. All scale bars: 10μm.

nizes Rap only in its active state (Bivona and Philips 2005), upon depletion of PA. We used TIRF microscopy, which specifically excites fluorophores that are within ~100 nm of the coverslip, to measure the concentration of fluorescent proteins at the basal cell surface. In control HEK293T cells, 007-AM stimulation resulted in accumulation of both CFP-Epac1 and YFP-RalGDS RBD at the PM (Figure 3A). This was completely abolished when cells were depleted of PA by pre-incubation with CAY10593. Treatment of cells with TRP following 007-AM stimulation restored PM recruitment of both CFP-Epac1 and YFP-RalGDS RBD (Figure 3B). From these results we conclude that PA is required for Epac1-mediated activation of Rap at the PM.

Arginine 82 within a polybasic stretch in the DEP domain is required for binding to PA

The DEP domain of Epac1 contains a stretch of 17 residues that includes six basic side-chains (Figure 4A). Since PA binds to positively charged residues at membranes (Kooijman, Tieleman et al. 2007), the polybasic cluster in the DEP domain may provide the binding site for PA. One of the residues in this cluster, arginine 82 (R82), was previously found to be essential for cAMP-induced translocation, and is in close proximity of a region which becomes more solvent exposed after cAMP binding (Figure 4A and B) (Ponsioen, Gloerich et al. 2009, Li, Tsalkova et al. 2011). To determine whether the other positively charged side chains within the polybasic stretch of the DEP domain contribute to membrane targeting of Epac1, a series of mutants was analyzed. Albeit some of the mutants displayed reduced response (R85A or R91A), only the R82A showed a clear inhibition of translocation, as illustrated by both the lack of increased fluorescence at the PM and of response rate (Figure 4B). This indicates that the R82 is the most critical residue for cAMP-induced translocation of Epac1. We therefore tested whether the R82 residue is involved in PA binding, using the protein-lipid overlay assay. Indeed, mutation of arginine 82 to alanine (R82A) completely abolished binding of Epac1 to PA (Figure 4C), indicating that R82 is part of the binding site for PA.

Discussion

We have previously shown that cAMP binding activates Epac1 by releasing it from auto-inhibition, but also induces its translocation to the PM (Rehmann, Prakash et al. 2003, Rehmann, Rueppel et al. 2003, Rehmann, Das et al. 2006, Rehmann, Arias-Palomo et al. 2008, Ponsioen, Gloerich et al. 2009). This translocation of Epac1 is required for efficient Rap1 activation and subsequent cell adhesion (Ponsioen, Gloerich et al. 2009). We now show that Epac1 can bind to PA in vitro in a DEP domain-dependent manner and that this interaction is regulated by cAMP. Depletion of PA by inhibition or depletion of PLD1 abolishes DEP domain-mediated, but not ERM-mediated, recruitment of Epac1. Furthermore, we illustrate that depletion of PA inhibits cAMP-induced activation of Rap at the PM, which can be restored by thrombin-induced tethering of Epac1 to active ERM proteins. Finally, we show that R82 within the DEP domain, which is required for cAMP-induced translocation, is also required for PA binding. Collectively, our results show that PA is the cAMP-regulated DEP domain anchor for Epac1 at the PM.

The observation that cAMP facilitates the binding of PA to the DEP domain suggest that the PA binding site in the DEP domain may become exposed upon the conformational opening of Epac1, however the crystal structure of Epac2 in its closed conformation indicates the

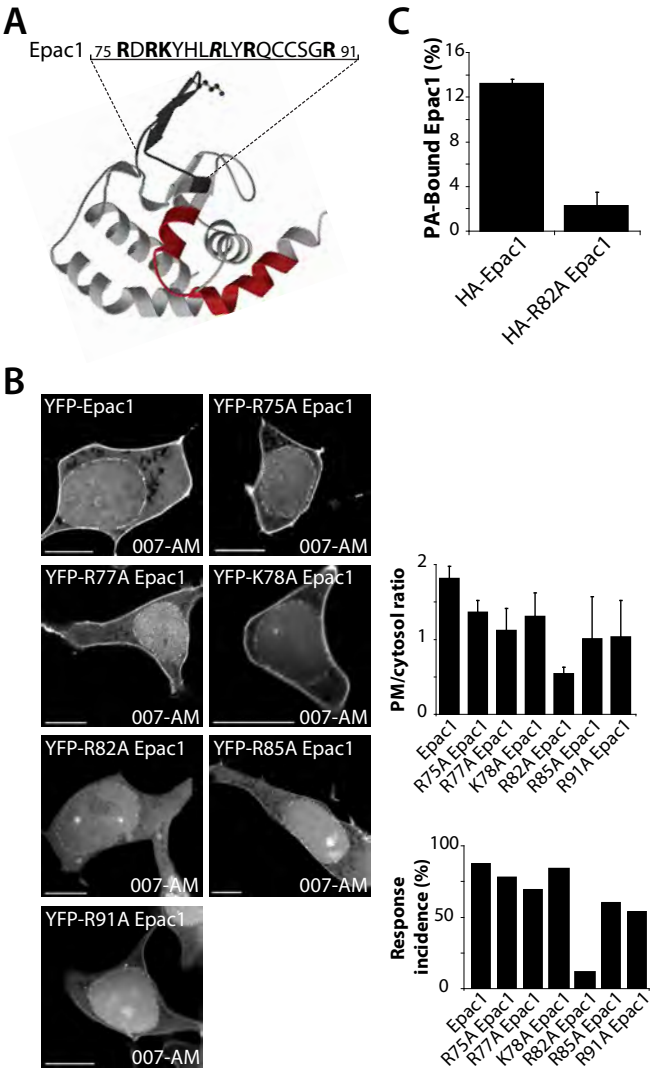

Figure 4: Arginine 82 within a polybasic stretch mediates binding of Epac1 to PA
A) Ribbon diagram of the DEP domain of Epac2 with indicated positively charged residues. Residue K212 in Epac2, corresponding to R82 in Epac1, is presented as ball-and-stick. The region that becomes more solvent exposed after cAMP (Li et al. 2011) is highlighted in red and the amino acid sequence of the cluster of basic residues present in the DEP domain of Epac1 (amino acids 75 to 91) are shown in dark grey. Positively charged residues are represented in bold, R82 residue in bold italic. B) Confocal live-imaging of HEK293T cells stimulated with 007-AM (1 µM) showing deficient translocation of YFP-R82A Epac1, but not of YFP-R75A Epac1, YFP-R77A Epac1 and YFP-K78A Epac1, and reduced membrane localization of YFP-R85A Epac1 and YFP-R91A Epac1 as compared to wildtype YFP-Epac1. The graph shows the ratio of average fluorescence pixel intensity of Epac1 at the PM versus the cytosol after stimulation with 007-AM (1 µM), calculated as described in Materials and Methods. The error bars show mean with SD (N=4). The bar graph shows the percentage of cells showing translocation of Epac1 to the PM upon 007-AM stimulation (response incidence; 7/8 cells expressing YFP-Epac1, 7/9 cells expressing YFP-R75A Epac1, 9/13 cells expressing YFP-R77A Epac1, 10/12 cells expressing YFP-K78A Epac1, 1/9 cells expressing YFP-R82A Epac1, 9/15 cells expressing YFP-R85A Epac1, and 7/13 cells expressing YFP-R91A Epac1). C) Quantification of the interaction of wildtype Epac1 and R82A Epac1 with PA. Equal amounts of HA-Epac1 and HA-R82A Epac1 isolated from HEK293T cells were incubated with lipid strips in the presence of 007 (100 µM). Anti-Epac1 5D3 antibody was used to detect binding and the graph shows percentage of PA-bound Epac1 as compared to control (blue blank), showing mean with SD from three independent experiments.

DEP domain is not sterically hindered by the catalytic region. Furthermore, DEP-domain mediated PM-anchoring is only observed in the context of the full protein, and not with the individual regulatory region (containing the DEP domain and the cAMP binding domain) or the isolated DEP domain (Ponsioen, Gloerich et al. 2009). Possibly, cAMP binding induces additional conformational changes to allow interactions of the DEP domain with PA. Importantly, deuterium exchange mass spectrometry revealed a change in solvent exposed areas in the DEP domain of Epac after cAMP binding, suggesting a cAMP-induced conformational change in the DEP domain (Li, Tsalkova et al. 2011). Interestingly, the polybasic stretch containing R82 is partially overlapping with the region that became more solvent exposed after cAMP binding (Figure 4A). We therefore propose that cAMP induces a conformational change that induces inter- and intradomain conformational changes in Epac1 that results in activation of its guanine nucleotide exchange activity, but also reorients the loop containing R82, thereby regulating the binding to PA at the PM. However, the presence of a cAMP-induced PA binding site in the catalytic domain that cooperates with the binding site in the DEP domain is not excluded.

Although PA is distributed uniformally at the PM of most cells, it also contributes to compartmentalization of distinct cellular signaling events (van Meer, Voelker et al. 2008). For instance, PA enables the correct localization of the RacGEF DOCK2 at the leading edge of neutrophils during chemotaxis (Nishikimi, Fukuhara et al. 2009), and recruits the RasGEF Sos to cell-free membranes but not to cell-cell contact areas (Zhao, Du et al. 2007, Zhang and Du 2009, Nishioka, Frohman et al. 2010). Although not observed in the cell lines we have studied, it may imply that PA localizes Epac1 to restricted areas of the PM. In this respect, it is interesting to note that several additional membrane anchors for Epac1 have been described (Gloerich and Bos 2010), indicating a complex spatial regulation of Epac1.

In summary, we show that the DEP domain of Epac1 binds to PA and that this binding is regulated by cAMP. Furthermore we show that PA is required for cAMP-induced translocation and Rap1 activation. This provides further insight in the mechanism of spatio-temporal control of Epac1 and, more general, in the molecular mechanism of how DEP domains tether proteins to membranes.

Acknowledgements

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A

Addendum:
Critical differences between Epac1 and Epac2 account for their differential spatiotemporal regulation

A

In chapter 2 we have provided insights into the spatial regulation of Epac1. We have shown that upon cAMP binding, Epac1 undergoes a conformational change that allows its translocation to the plasma membrane (PM) and subsequent activation of Rap. This dynamic redistribution of Epac1 requires the cAMP-induced conformational change as well as the critical arginine 82 (R82) residue within the DEP domain that mediates binding to phosphatidic acid (PA) at the PM. Despite sharing sequence and structural similarities, Epac proteins are subject to very diverse mechanisms of spatial and temporal regulation. Indeed, Epac2 does not translocate via its DEP domain to the plasma membrane in response to cAMP (Figure 1a) but is targeted to the PM by interacting with Ras-GTP via its RA domain (Li, Asuri et al. 2006, Liu, Takahashi et al. 2008). Although the DEP domain of Epac1 is required for its cAMP-induced translocation, this domain is not sufficient for membrane targeting as the separate DEP domain or the separate regulatory region do not localize at the PM before or after cAMP (Ponsioen, Gloerich et al. 2009). Moreover, when the separate regulatory and catalytic region of Epac1 are co-expressed in cells, they are able to bind each other and translocate to the PM (Ponsioen, Gloerich et al. 2009). This indicates that the DEP domain and the regulatory region function only in conjunction with the catalytic region. This is probably due to the fact that the DEP domain is in a proper conformation for binding to PA at the PM only after undergoing conformational changes induced by cAMP that result in exposure of the critical R82 residue and that involve rearrangements in the full length protein (Li, Tsalkova et al. 2011). Interestingly, the regulatory region of Epac1 (Epac1 Reg.) is able to co-immunoprecipitate with the catalytic region of Epac2 (Epac2 Cat.) both in absence or presence of the cAMP analogue 8-pCPT-2'-O-Me-cAMP-AM (007-AM) (Figure 1b). Accordingly, in HEK293T cells, CFP-Epac2 Cat. and YFP-Epac1 Reg. show combined translocation to the PM of both regions upon stimulation with 007-AM (Figure 1c). This indicates that the regulatory region of Epac1 is a critical determinant for membrane localization and might account for the differential regulation of the two isoforms.

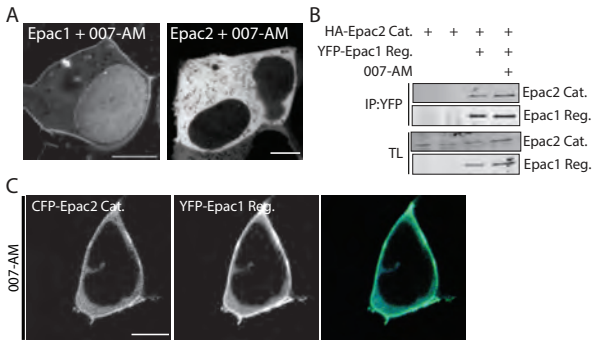

Figure 1: Differential regulation of Epac1 and Epac2
A) Live-imaging of YFP-Epac1 and YFP-Epac2 in HEK293T cells showing PM translocation of Epac1 but not of Epac2 after 10 minutes stimulation with 10 µM 007-AM. Scale bars: 10 µm. B) Co-immunoprecipitation from HEK293T cells of Epac2 catalytic region (HA-Epac2 Cat.) and Epac1 regulatory region (YFP-Epac1 Reg.) before and after stimulation with 10 µM 007-AM. C) Live-imaging of HEK293T cells showing combined translocation to the PM of CFP-Epac2 Cat. and YFP-Epac1 Reg. after 10 minutes stimulation with 10 µM 007-AM. Scale bars: 10 µm.

The DEP domain within the regulatory region of Epac1 is unique in allowing membrane targeting by binding to phospholipids. Epac1 specifically interacts with PA, as reported in chapter 2, but no binding for any phospholipid was observed in the case of Epac2 (Figure 2a). BLAST alignment shows 55% sequence similarity between Epac1 and Epac2 and 48% for the DEP domain of Epac1 and the DEP domain of Epac2 alone. Moreover, the polybasic stretch in the DEP domain of Epac1, which allows binding to PA and that becomes more solvent exposed after cAMP, is not very well conserved. Indeed, residues R82 and R91 in Epac1 are replaced by a threonine and a lysine in Epac2 (indicated by arrows in Figure 2b). In

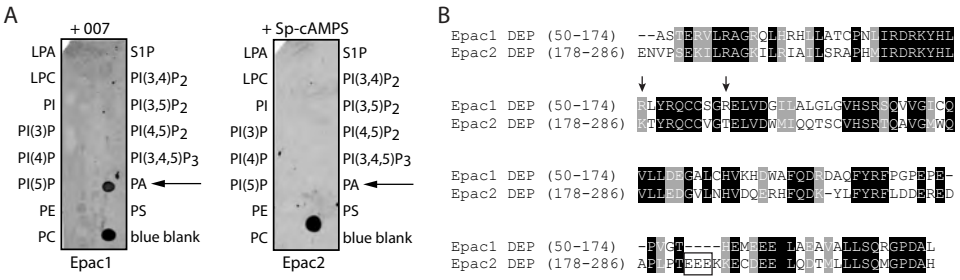

Figure 2: The DEP domain of Epac1 is unique in allowing membrane targeting
A) Protein-lipid overlay assay of recombinant Epac1 and Epac2 in presence of the specific cAMP analogues 007 or Sp-cAMPS, as indicated, using PIP strips containing 100pmol/spot of the following lipids: lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylinositol (PI), phosphatidylinositol 3-phosphate [PI(3)P], PI(4)P, phosphatidylinositol 5-phosphate [PI(5)P], phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2], phosphatidylinositol 1,2-bisphosphate [PI(4,5)P2], phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], phosphatidic acid (PA), or phosphatidylserine (PS). B) Sequence alignment of the DEP domain of Epac1 and the DEP domain of Epac2. Arrows indicate positive residues important for mediating Epac1 translocation and binding to PA at the PM, and boxed residues indicate a stretch of negatively charged amino acids present in Epac2 but absent in Epac1 DEP domain. Alignment was made using BoxShade software.

addition, Epac2 entails a stretch of negatively charged residues absent in Epac1, which may affect possible interactions with phospholipids (boxed in Figure 2b).
Thus, the DEP domain of Epac1 is unique in allowing PM targeting and dissimilarities between the DEP domain of Epac1 and Epac2 likely account for their differential mechanisms of membrane targeting.

Materials and Methods

Antibodies, reagents, DNA constructs, cell culture and transfections
The anti-HA (12CA5) antibody was produced in-house. 8-pCPT-2'-O-Me-cAMP (007) and 8-pCPT-2'-O-Me-cAMP-AM (007-AM) were from Biolog Life Sciences. Sp-cAMPS were available in house. The separate regulatory (amino acids 1 to 328 of Epac1) and catalytic (amino acids 461 to 993 of Epac2) regions were cloned into pcDNA3 with an N-terminal tag. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 1.4 mM L-glutamine (all from Lonza). Cells were transfected using Xtreme gene 9 (Roche).

Fluorescence microscopy
For confocal live-imaging, 1 day after transfection cells were seeded overnight in glass-bottomwells (WillCo Wells) and examined in L-15 Leibovitz medium (Invitrogen) at 37 °C. Images were acquired on an inverted Zeiss LSM510 confocal microscope.

Protein–Lipid Overlay Assays
Recombinant Epac1 and Epac2 (1 µg/mL) in presence of 8-pCPT-2'O-Me-cAMP (007) or Sp-cAMPS were incubated with nitrocellulose membranes spotted with a variety of lipids (Echelon Biosciences). Bound protein was detected using the 5D3 anti-Epac1 or 2B12 anti-Epac2 antibody.

3

DEP domains: structurally similar but functionally different

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(Adapted version)

The Dishevelled, EGL-10, and Pleckstrin (DEP) domain is a globular protein domain present in about 10 human protein families with well-defined structural features. A picture is emerging that DEP domains mainly function in the spatial and temporal control of diverse signal transduction events by recruiting proteins to the plasma membrane. DEP domains can interact with a variety of partners at the membrane, including phospholipids and membrane receptors, and their binding is subject to regulation.

3

Introduction

Cell surface receptors transduce extracellular signals by assembling protein complexes at the inner side of the plasma membrane. Targeting of components that are part of signaling complexes to the membrane not only increases the possibility of complex formation but also increases the strength of the signal being transduced (Kholodenko, Hoek et al. 2000). Moreover, due to the modular nature of signaling molecules, different types of complexes can easily form in time and in space (Scott and Pawson 2009). Each protein domain recognizes specific protein sequences or lipids and frequently the binding is regulated by a distinctive signaling event. One example is the DEP domain, firstly identified in Drosophila melanogaster Dishevelled, Caenorhabditis elegans EGL-10, and mammalian Pleckstrin (Ponting and Bork 1996, Kharrat, Millevoi et al. 1998, Civera, Simon et al. 2005). In mammals, the DEP domain is present in a limited number of protein families (Figure 1) that serve diverse functions in signal transduction. DEP domains have also been identified in bacteria (SMART database), indicating that the DEP domain did arise early during evolution. Some DEP domain-containing proteins, such as the regulators of G protein signaling (RGS) family of proteins, are present in yeast, whereas others, like Dishevelled (Dvl), appeared later in evolution and are only found in higher eukaryotes (Sierra, Gilbert et al. 2002, Dillman, Minor et al. 2013)RGS proteins contain both a DEP domain and an RGS domain,

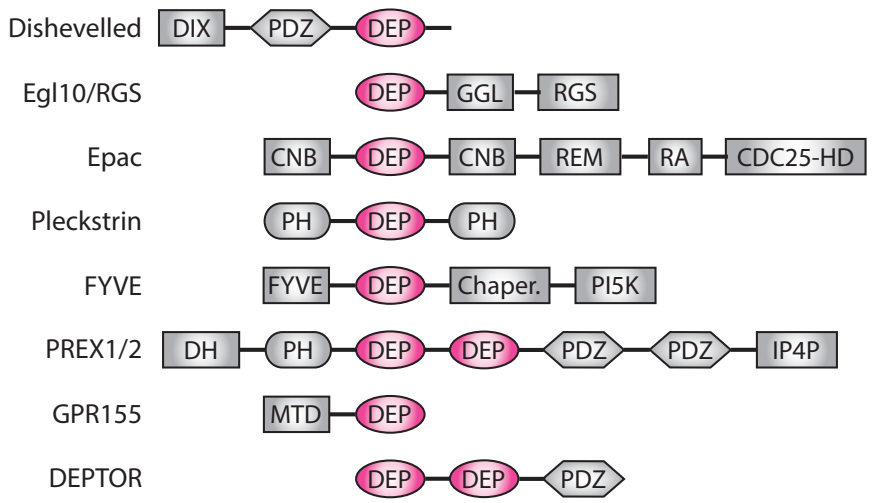

Figure 1: Domain architecture of DEP domain-containing proteins
Mammalian DEP domain-containing proteins. The DEP domain is shown in pink and other domains that are present in more than one protein are represented in an identical manner. Protein modules adjacent to the DEP domain often aid the DEP domain in its membrane binding capabilities. This is the case for Dishevelled DEP domain that requires also the PDZ domain to bind to Fz receptor and for RGS DEP domain that employs the GGL domain to bind to Gβ5. In the case of Epac, Pleckstrin and P-Rex, conformational changes that relive the protein from an auto-inhibited state or intra-moelcular interactions are needed for facilitating membrane localization of the protein (see main text for details). For other proteins the function of the DEP domain is still elusive. DEP: Dishevelled, Egl-10, Pleckstrin; DIX: Dishevelled and Axin; PDZ: Post Synaptic Density-95, Discs large and Zonula-occludens 1; GGL: G protein gamma-like; CNB: cyclic nucleotide binding; REM: Ras exchange motif; RA: Rap/Ras association; CDC25-HD: CDC25 homology domain; PH: Pleckstrin homology; FYVE: Fab 1, YOTB, Vac 1 and EEA1; Chaper: chaperonin; PI5K: Phosphatidylinositol-5-kinase; DH: Dbl homology; IP4P: inositol polyphosphate 4-phosphatase; MTD: membrane transport domain.

Table 1: Properties of DEP domains.

DEP Protein	DEP binding partner	DEP length (amino acids)	Additional requirements	Function	References
Dishevelled	PA, Fz, µ2 adaptor	~87	DEP-C region, YHEL motif	Membrane targeting	Yu et al. 2007; Tauriello et al. 2012; Simons et al. 2009
Egl10/RGS	Gβ5, R9AP and R7BP	~107	DHEX fold	Membrane targeting	Martemyanov et al. 2003; Cheever et al. 2008; Anderson et al. 2009; Ballon et al. 2006
Epac	PA	120-121	cAMP-induced conformational change	Membrane targeting	Ponsioen et al. 2009; Consonni et al. 2012
Pleckstrin	Intra-molecular interaction with PH domain	~116	PKC-induced phosphorylation	Auto-inhibition	Civera et al. 2005; Ma et al. 1997
FYVE	unknown	81	unknown	unknown	Stenmark et al. 1999
PREX1/2	Intra-molecular interaction with PDZ and IP4P domains, mTOR	~80	PI(3,4,5)P3-induced conformational change	Protein function	Urano et al. 2008; Pandiella et al. 2003
GPR155	unknown	~82	unknown	unknown	Vassilatis et al. 2003
DEPTOR	unknown	81	unknown	unknown	Peterson et al. 2009

PA: phosphatidic acid; Fz: Frizzled; DEP-C: C-terminal region; Gβ5: type 5 G protein β subunit; DHEX: DEP helical extension; PH: Pleckstrin Homology; PKC: Protein Kinase C; PDZ: Post synaptic density-95, Discs large and Zonula-occludens 1; IP4P: Inositol Polyphosphate 4-Phosphatase; mTOR: Mammalian Target of Rapamycin.

the latter of which confers GTPase-activator protein (GAP) activity (Ross and Wilkie 2000). Interestingly, the RGS domain-DEP domain module present in yeast and mammalian RGS proteins appears to be conserved in the mammalian Dvl signalosome ,with the scaffold protein Axin ,having the RGS domain (Julius, Schelbert et al. 2000, Schwarz-Romond, Metcalfe et al. 2007).

A predominant function of DEP domains is plasma membrane anchoring, but DEP domains carry out several additional functions by employing different binding interfaces (summarized in Table 1). In this respect, DEP domains seem to differ from many other protein domains with a more restricted mechanism of action. However, the use of multiple binding surfaces may be more common than originally anticipated. For instance, SH2 domains were long considered to have a single binding pocket for phosphotyrosine moieties, but more recently it was shown that SH2 domains can interact with kinase domains utilizing a different binding interface (Pawson, Gish et al. 2001, Grebien, Hantschel et al. 2011). DEP domains can be considered a paradigm for the growing group of protein domains that utilize different interfaces for different functions.

In the past years, analyses on the structure and function of DEP domains resulted in an intriguing picture of DEP domains and had made this protein module one of the prime examples of a regulated domain in signal transduction. In this perspective article we focus on the DEP domain-containing proteins Dvl, RGS, Epac, Pleckstrin and P-Rex. We examine the various mechanisms of DEP domain-mediated interaction with proteins and lipids and discuss how DEP domains function in the spatiotemporal control of diverse signaling events.

Structure of DEP domains

DEP domains consist of approximately 100 amino acids and they all share a high degree of sequence and structural similarities. They have a characteristic α/β fold and they are comprised of a conserved helical core and a protruding β-hairpin arm in between the core helices α1 and α2 (Figure 2) (Wong, Mao et al. 2000, Civera, Simon et al. 2005). The β-hairpin module, often bearing residues important for mediating molecular interactions, is less conserved than the helical core (Axelrod, Miller et al. 1998).

In some cases, such as for Epac and Dvl, polybasic residues in the β-hairpin are required for the recruitment to the plasma membrane (Figure 2) (Axelrod, Miller et al. 1998, Simons, Gault et al. 2009, Consonni, Gloerich et al. 2012). Furthermore, studies revealed that DEP domains encompass distinct positive and negative patches that influence their plasma membrane binding abilities. The DEP domain of RGS9 is oriented in such a way so that a cluster of positively charged residues faces the membrane (Cheever, Snyder et al. 2008). Conversely, the DEP domain of Pleckstrin has mainly a negative charge cluster on its exposed surface, whereas the DEP domain of Epac and Dvl combine both positive and negative patches. In some instances, the DEP domain requires an additional fold or motif in order to interact with membrane anchors. This is the case for the DEP domain of RGS9, which needs an adjacent DEP helical extension (DHEX) fold for membrane binding (Cheever, Snyder et al. 2008, Masuho, Wakasugi-Masuho et al. 2011). Likewise, the DEP domain of Dvl2 requires a neighboring tyrosine-based YHEL motif for membrane anchoring during non-canonical signaling and the DEP domain of Dvl1 entails a flanking region C-terminal to the DEP domain (DEP-C) in order to bind to a discontinuous motif on the sevenspan receptor Frizzled (Fz)

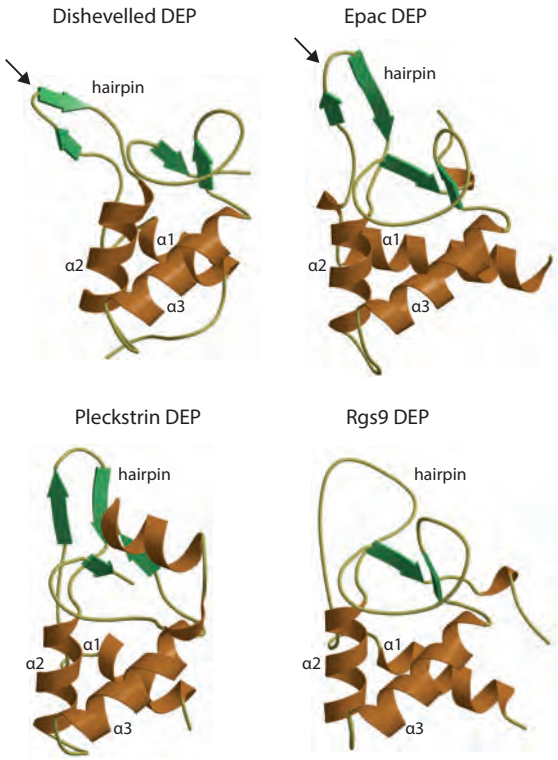

Figure 2: DEP domains structures
Cartoon structures of DEP domains showing their characteristic α/β fold. DEP domains comprise a conserved helical core, which is made of three helices and a protruding β-hairpin arm between the helices α1 and α2Such helical core is highly conserved in all DEP domains. Only in the case of Pleckstrin there is an additional helix located at the end of the DEP domain, thought to be important for protein mobility. Arrows show the location of the residues required for membrane binding. Images based on Protein Data Bank entries 1fsh (Dishevelled, (Wong, Mao et al. 2000)), 2byv (Epac, (Rehmann, Das et al. 2006)), 1w4m (Pleckstrin, (Civera, Simon et al. 2005)) and 2pbi (RGS9, (Cheever, Snyder et al. 2008)). Courtesy of Holger Rehmann.

during canonical signaling (Yu, Rual et al. 2007, Tauriello, Jordens et al. 2012).
Thus, despite the structural similarity in their core regions, small differences between DEP domains account for their differential mode of regulation and for their ability to coordinate diverse signaling events in space and in time.

Dishevelled DEP domain in signal regulation

Dvl is a cytoplasmic phosphoprotein involved in the regulation of Wnt signaling, first identified in D. melanogaster as a regulator of early embryo polarity (Perrimon and Mahowald 1987). It is composed of an N-terminal DIX auto-interaction domain responsible for the formation of multimeric complexes, a central PDZ domain required for binding to Fz receptor, and a C-terminal DEP domain important for specific membrane localization (Figure 1) (Axelrod, Miller et al. 1998, Wong, Bourdelas et al. 2003, Pan, Pang et al. 2004, Schwarz-Romond,

Fiedler et al. 2007).

Dishevelled DEP domain in membrane targeting

Activation of Fz receptors by Wnt ligands activates several down-stream signaling pathways, most notably, the canonical Wnt pathway that stabilizes β-catenin signaling to control cell fate and the non-canonical planar cell polarity (PCP) signaling pathway for cytoskeletal remodeling (Clevers and Nusse 2012, Gao 2012). In the canonical pathway, Wnt binding to Fz and the lipoprotein receptor related (LRP) 5 and 6 (Lrp5/6) receptor results in the formation of a Dvl-dependent signalosome, that includes the receptors (Fz and Lrp5/6Dvl, the RGS domain-containing adaptor protein Axin and additional proteins (Figure 3A). This interaction is mediated in part by a weak interaction of Fz with both the PDZ and the DEP domain of Dvl. The PDZ domain binds to a short linear sequence in the cytosolic tail of Fz, whereas the DEP domain together with a flanking C-terminal region (DEP-C) interacts with three discontinuous regions located in the third intracellular loop and the C-terminal tail of Fz (Tauriello, Jordens et al. 2012). Each of these interactions is required for membrane recruitment and proper functioning of Dvl in this pathway. In the context of the non-canonical PCP signaling, the DEP domain of Dvl interacts with negatively charged membrane phospholipids and, aided by the sodium/proton exchanger Nhe2 which keeps an alkaline pH suited for charge-dependent interactions, it interacts with Fz (Simons, Gault et al. 2009). Thus, the DEP-phospholipid interaction may contribute to formation of a robust Fz-Dvl complex at the plasma membrane (Figure 3B). Moreover, the DEP domain of the other isoform of Dvl, Dvl2, aided by a YHEL motif at its C-terminus, can interact with μ2 adaptor complexes at the membrane to facilitate clathrin-mediated endocytosis of Fz4 (Yu, Rual et al. 2007). This is required to regulate surface expression of Fz and thus desensitization of signaling (Schulte and Bryja 2007).

Dishevelled DEP domain in signaling complex formation

A number of important Wnt-induced post-translational modifications of Wnt pathway proteins depend on Dvl DEP domain activity. A recent study showed that the kinase RIPK4 binds to Dvl and mediates phosphorylation of both the PDZ and DEP domains (Huang, McGann et al. 2013). The combined phosphorylation events promote the assembly of Dvl2 into large signaling complexes, leading to maximal β-catenin stabilization and transcription of Wnt-responsive target genes. How DEP domain-mediated inter- and intramolecular interactions are regulated via phosphorylation remains unresolved. In addition, the Dvl DEP domain itself controls post-translational modification of other Dvl domains and protein partners. Enhanced K63-linked polyubiquitination of the Dvl DIX domain drives Wnt/β-catenin signaling in cells lacking the deubiquitinating enzyme CYLD, and this phenomenon depends on an intact DEP domain (Mlodzik 2002, Yanfeng, Tan et al. 2006). In addition, Xenopus laevis Fz3 becomes phosphorylated after association with Dvl. This phosphorylation requires the DEP domain of Dvl and is needed for Dvl-mediated Fz3 desensitization and thus for regulation of PCP signaling (Mlodzik 2002, Yanfeng, Tan et al. 2006). Thus, the interactions of the Dvl DEP domain with both protein and phospholipids mediate both the formation and modification of essential protein complexes in the regulation of Wnt signaling (Figure 3).

RGS DEP domain in signal termination

Members of the RGS family are GTPase-activating proteins (GAPs) of heterometric G pro-

teins. They use their RGS domain to promote GTP hydrolysis of the Gα subunit thereby switching off G protein-coupled receptor (GPCR) signaling (Figure 3C). Currently the best characterized of the RGS family of proteins is the R7 subfamily, whose members include RGS6, RGS7, RGS9 and RGS11. RGS proteins of the R7 family are made up of three domains, namely a DEP domain for membrane targeting at its N-terminus, a G protein gamma subunit-like (GGL) for binding G protein beta subunits and an RGS domain required for its function at its C-terminus (Figure 1) (Chasse and Dohlman 2003, Narayanan, Sandiford et al. 2007).
Structural analysis revealed that the DEP domain of RGS9 has a C-terminal helical extension (DHEX) not found in other DEP domains. The DHEX domain, together with the DEP and GGL domains, interacts with Gβ5 (Cheever, Snyder et al. 2008). In addition to binding to Gβ5, RGS proteins of the R7 family interact with RGS9 anchor protein (R9AP) and R7 binding protein (R7BP) membrane targeting proteins. The DEP domain, despite being needed for this interaction, is not sufficient for the binding (Martemyanov, Lishko et al. 2003). Recent protein-protein interaction mapping revealed that the DHEX domain, together with the DEP domain, is also involved in the interaction with R9AP and R7BP (Anderson, Posokhova et al. 2009, Masuho, Wakasugi-Masuho et al. 2011). Site-directed mutagenesis studies suggest that R7BP interacts with the DEP domain at the surface that also interacts with Gβ5, thus contributing to the stabilization of the complex and subsequent regulation of G protein-mediated signaling cascade (Masuho, Wakasugi-Masuho et al. 2011). This highlights the multifaceted nature of DEP domain-mediated complex assembly at the membrane.
The DEP domains found in Sst2, the yeast counterpart of the RGS family, mediate binding to the GPCR for pheromone mating factor (Ste2 in MAT-a and Ste3 in MAT-α cells). Interestingly, Sst2 DEP domains can only bind to GPCR in its non-phosphorylated state, suggesting that this interaction is regulated (Ballon, Flanary et al. 2006).
The DEP domain of RGS7 is required and necessary for binding to and inhibiting the muscarinic receptor type 3 M3R but not other GPCRs (Sandiford and Slepak 2009). This clearly shows that DEP domains are not just simply involved in membrane anchoring, but also in conferring specificity by selectively interacting with cognate receptors.

Epac DEP domain in phospholipid binding

Epac proteins are guanine nucleotide exchange factors (GEFs) for the small G protein Rap involved, among others, in the control of cell adhesion and actin cytoskeletal rearrangements (de Rooij, Zwartkruis et al. 1998, Gloerich, Ponsioen et al. 2010). The two Epac proteins, Epac1 and Epac2, are differentially expressed, with Epac1 being more abundant in the central nervous system, ovaries and uterus, and Epac2 in the pancreas and adrenal gland in mammals (Gloerich and Bos 2010). Both are multidomain proteins containing an N-terminal regulatory region comprising of one (in the case of Epac1) or two (in the case of Epac2) cyclic nucleotide binding (CNB) domains and a DEP domain. The catalytic region at their C-terminus harbors the CDC25-homology domain (CDC25-HD) for exchange activity, a Rap/Ras exchange motif (REM) and a Ras association (RA) domain (Figure 1). Both proteins assume a closed auto-inhibitory conformation, which is relieved upon cAMP binding (Rehmann, Das et al. 2006). In the case of Epac1, the allosteric activation by cAMP is accompanied by a very rapid relocalization of the protein from the cytosol to the PM. This translocation is mediated by the DEP domain, which directly interacts with phosphatidic acid at the membrane (Figure 3D). A polybasic stretch encompassing residue R82 in the DEP domain is responsible

for this binding (Figure 2) (Ponsioen, Gloerich et al. 2009, Consonni, Gloerich et al. 2012). This interaction is required for proper functioning of Epac1 as deletion of the DEP domain or mutation of R82 to a non-polar amino acid inhibits cAMP-induced, Epac1 mediated cell adhesion (Ponsioen, Gloerich et al. 2009). Amide hydrogen and deuterium mass spectrometry studies on Epac2 elucidated the possible mechanisms of regulation of its cAMP-induced DEP domain-mediated tethering to the plasma membrane. Upon cAMP signaling, some regions of the DEP domain become more solvent exposed, resulting in reorientation of the β-hairpin containing the essential R82 residue and in this way allowing plasma membrane anchoring (Li, Tsalkova et al. 2011, Consonni, Gloerich et al. 2012). Interestingly, the cAMP-binding domain is located at the same position as the C-terminal helical extension of the DEP domain (important for its function) of the R7 family of RGS proteins and Dvl. So far, no additional interacting proteins have been identified for Epac DEP domains. It should be noted that, currently, the function of the DEP domain of Epac2 is elusive as the DEP domain does not seem to be required for localization of this protein to the plasma membrane (Li, Asuri et al. 2006, Consonni, Gloerich et al. 2012).

P-Rex DEP domain in intra-molecular interactions

PI(3,4,5)P3-dependent Rac exchanger (P-Rex) proteins act as GEFs for the small GTPase Rac. They both contain a catalytic Dbl-homology (DH) domain at their N-terminal adjacent to a pleckstrin homology (PH) domain required for binding to PI(3,4,5)P3, followed by two tandem DEP domains and two tandem PDZ domains. At their C-terminus they comprise an inositol polyphosphate 4-phosphatase (IP4P) domain needed for binding to protein phosphatase 1α (PP1α) (Figure 1) (Hill, Krugmann et al. 2005, Pandiella and Montero 2013). P-Rex is likely to be in an auto-inhibited state in the cytosol and translocates to the membrane where it binds to Gβγ subunits and PI(3,4,5)P3 via its DH and PH domains after activation by PI(3,4,5)P3 (Figure 3E) (Pandiella and Montero 2013). P-Rex has recently attracted much attention as it was found to play a role in metastatic diseases. Indeed, P-Rex1 was found to increase cell proliferation in breast cancer cells and to induce migration and invasion of prostate cancer cells by activating Rac (Qin, Xie et al. 2009, Sosa, Lopez-Haber et al. 2010). Interestingly, the DEP domain mediates intra-molecular interactions with the PDZ and the IP4P domains. Such binding is needed for association with Gβγ and for Gβγ-induced GEF activity (Urano, Nakata et al. 2008).

Pleckstrin DEP domain in auto-inhibition

Pleckstrin is another DEP domain-containing protein family. Pleckstrin is one of the main substrates for protein kinase C (PKC) in platelets and comprises two pleckstrin homology (PH) domains for protein or lipid interactions and a central DEP domain (Figure 1) (Kharrat, Millevoi et al. 1998). The structure of Pleckstrin DEP domain is more divergent than that of DEP domains in other DEP domain-containing proteins, as it comprises an additional β-strand positioned after the β-hairpin and an additional helix located towards the end of the DEP domain, the latter of which is thought to be important for increasing protein mobility and facilitating protein-protein interactions (Ma, Brass et al. 1997, Civera, Simon et al. 2005). The DEP domain of Pleckstrin does not directly mediate membrane anchoring of the

protein but it interacts intra-molecularly with the N-terminal PH domain. Such interaction is relieved only after phosphorylation by PKC, which allows activation and membrane localization of the protein (Figure 3F) (Civera, Simon et al. 2005). This finding suggests a function for DEP domains in auto-inhibitory domain interactions.

Concluding remarks and future directions

DEP domains are globular proteins domains that, despite having diverse mechanisms of action, generally serve in assisting in the translocation of the cognate protein to the plasma membrane. Often the presence of a specific sequence or exposure of a particular amino acid is pivotal for allowing such accurate spatial regulation of proteins, thus highlighting the importance of sequence context and specificity (Axelrod, Miller et al. 1998, Ponsioen, Gloerich et al. 2009).

The presence of additional domains or folds adjacent to the DEP domain appears important for signaling specificity. The Dvl DEP domain requires a YHEL motif for membrane anchoring during non-canonical signaling and both the PDZ domain and a C-terminal region cooperate with DEP to bind to Fz during canonical signaling (Yu, Rual et al. 2007, Tauriello, Jordens et al. 2012). Similarly, the DEP domain of RGS9 needs a DHEX helical fold for membrane localization (Cheever, Snyder et al. 2008, Masuho, Wakasugi-Masuho et al. 2011). Conformational changes also play an important role in the regulation of DEP domain-mediated signaling. This is the case for Epac and possibly P-Rex, which require a conformational change triggered by cAMP and PI(3,4,5)P3 respectively in order to become active and PM localized (Rehmann, Das et al. 2006, Pandiella and Montero 2013). Additional levels of regulation might be involved, such as post-translational modifications and of domain-domain interactions (Civera, Simon et al. 2005, Urano, Nakata et al. 2008). This illustrates the diversity of DEP domain-mediated regulation of signaling but also emphasizes the fact that the DEP domain is not simply a localization device, but it also affects protein function by being itself subject to regulation.

As our understanding of how DEP domains function and regulation is increasing, it is becoming clear that they use many ways to perform their task in a variety of cellular events (Figure 3).

Further identification of all the factors and regulatory mechanisms that are involved in such regulation will help to understand how DEP domains achieve such signaling specificity despite being so structurally similar. Importantly, the ability of protein domains to have different functional properties by providing diverse binding interfaces is more and more appreciated as a recurrent theme in the control of signaling in time and in space.

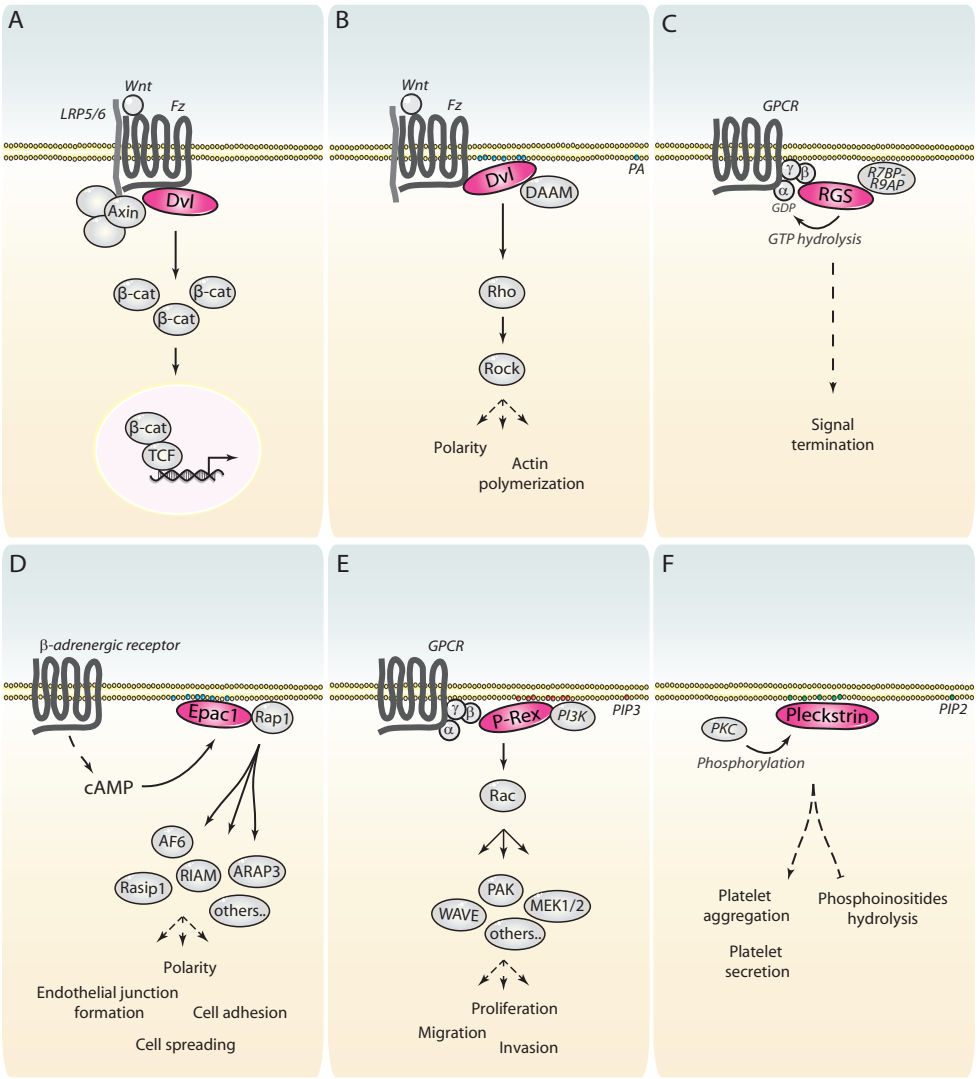

Figure 3: Diversity of pathways regulated by DEP domain-containing proteins
DEP domain-contaning proteins shown in pink. A) Dishevelled (Dvl) during canonical Wnt signaling. The DEP domain of Dvl binds to the G protein coupled receptor (GPCR) Frizzled (Fz) and allows formation of a signaling complex that results in β-catenin translocation to nucleus and subsequent transcription of Wnt target genes. B) Dvl during non-canonical planar cell polarity Wnt signaling. By interacting with phosphatidic acid at the membrane (shown in green), the DEP domain of Dvl mediates cytoskeletal changes and polarity via Dishevelled associated activator of morphogenesis (DAAM) and subsequent downstream activation of Rho–ROCK signalling. C) Regulators of G protein signaling (RGS). To promote GPCR signal termination, RGS proteins associate with their cognate receptor aided by their DEP domain and promote GTP hydrolysis of the Gα subunit. D) Epac. In response to cAMP, Epac1 translocates to the plasma membrane where it binds to phosphatidic acid via its DEP domain. This results in activation of plasma membrane-located Rap and downstream effectors and of Rap-mediated cellular processes such as cell polarity, cell adhesion, cell spreading and cell-cell junction formation. E) PI(3,4,5)P3-dependent Rac exchanger (P-Rex). After activation by PI(3,4,5)P3 (PIP3), P-Rex translocates to the PM where it binds to Gβγ subunits and PIP3. This results in Rac activation and subsequent activation of cell migration, proliferation and invasion. F) Pleckstrin. Phosphorylation by PKC is required for its localization to the PM, where it binds to PI(4,5)P2 (PIP2). Downstream signaling then results in induction of platelet aggregation and secretion and inhibition of phosphoinositides hydrolysis.

4

The PDZ domain of the guanine nucleotide exchange factor PDZGEF directs its binding to phosphatidic acid during brush border formation

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Manuscript submitted

PDZGEF is a guanine nucleotide exchange factor for the small G protein Rap. It was recently found that PDZGEF contributes to establishment of intestinal epithelial polarity downstream of the kinase Lkb1. By binding to phosphatidic acid enriched at the apical membrane, PDZGEF locally activates Rap2a resulting in induction of brush border formation via a pathway that includes the polarity players TNIK, Mst4 and Ezrin. Here we show that the PDZ domain of PDZGEF is essential and sufficient for targeting PDZGEF to the apical membrane of polarized intestinal epithelial cells. Inhibition of PLD and consequently production of phosphatidic acid inhibitis targeting of PDZGEF to the plasma membrane. Furthermore, localization requires specific positively charged residues within the PDZ domain. We conclude that local accumulation of PDZGEF at the apical membrane during establishment of epithelial polarity is mediated by electrostatic interactions between positively charged side chains in the PDZ domain and negatively charged phosphatidic acid.

Introduction

The protein family of the Ras-like small GTPase Rap controls a variety of cellular pathways linked to cell adhesion, cell spreading and endothelial junction control (Bos 2005, Pannekoek, van Dijk et al. 2011, Ross, Post et al. 2011, Post, Pannekoek et al. 2013). Recently, an additional role for Rap in the regulation of brush border formation during intestinal epithelial polarity has been identified (Gloerich, ten Klooster et al. 2012). Rap proteins are kept under control by guanine nucleotide exchange factors (GEFs) that promote their active GTP-bound conformation and by GTPases accelerating proteins (GAPs) that induce their inactive state (Bos, Rehmann et al. 2007). PDZGEFs are one of such Rap-specific exchange factors. They are characterized by the presence of a PDZ domain from which they derive their name and by a CDC25 homology domain (CDC25-HD) at their C-terminus present in all GEFs for Ras-like GTPases (de Rooij, Boenink et al. 1999). Moreover, they encompass one or two N-terminal cyclic nucleotide binding (cNBD) domains, even though they are unable to interact with cAMP or cGMP, a Rap/Ras exchange motif (REM) and Ras associating (RA) domain (Figure 1a) (Kuiperij, de Rooij et al. 2003). PDZGEFs have been recently identified as the GEFs responsible for the activation of Rap2a during brush border formation (Gloerich, ten Klooster et al. 2012). Establishment of intestinal epithelial polarity requires the formation of a polarization complex composed of the protein kinase Lkb1, the pseudo-kinase STRADα and the adaptor protein Mo25 (Baas, Kuipers et al. 2004, ten Klooster, Jansen et al. 2009). This results in establishment of apico-basal polarity and asymmetric distribution of polarity landmarks, such as accumulation of PtdIns(4,5)P2 at the apical membrane. Subsequent localization of phospholipase D (PLD) leads to local generation of phosphatidic acid (PA) and recruitment of PDZGEF. The following signaling cascade leads to Rap2a-mediated brush border formation (Gloerich, ten Klooster et al. 2012).
Here we investigate which domain of PDZGEF is required for the apical recruitment of PDZGEF by PA during epithelial cell polarization. We find that the PDZ domain mediates apical localization of PDZGEF by directly binding to PA at the membrane. Moreover, we show that basic residues within the PDZ domain are needed for this interaction.

Materials and methods

Antibodies, DNA constructs, cell culture and transfections
The anti-HA (12CA5) antibody was produced in-house. PDZGEF1 (RapGEF2) and PDZGEF2 (RapGEF6) were cloned C-terminal to a CFP or Citrine YFP tag in a pcDNA3 vector or an HA tag in a pmt2 vector using the Gateway system (Invitrogen). All mutants were generated by site-directed mutagenesis. Ls174T-W4 cells were mainted in RPMI medium supplemented with 10% tetracycline free fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 1.4 mM L-glutamine (all from Lonza). Cells were transfected with expression plasmids using Xtreme gene 9 (Roche).

Fluorescence microscopy
For confocal live-imaging, 1 day after transfection cells were seeded overnight in glass-bottomwells (WillCo Wells) and stimulated overnight with doxycycline (1 µg/ml) or treated with

the PLD1 inhibitor CAY10593 (1 μM) (Cayman Chemicals) as indicated and examined in L-15 Leibovitz medium (Invitrogen) at 37 °C. Images were acquired on an inverted Zeiss LSM510 confocal microscope equipped with 63× magnification objective lens (N.A. 1.4; Leica).

Protein–Lipid Overlay Assays
HEK293T cells were transfected with HA tagged PDZ domain of PDZGEF and then lysed in buffer containing 50 mM Tris•HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM MgCl2, and protease and phosphatase inhibitors. Lysates were then incubated overnight with protein G Sepharose beads and anti-HA antibody. The bound proteins were eluted with 3× HA peptide (250 μg/mL) in BC300 buffer [20 mM Tris•HCl (pH 7.9), 20% glycerol, and 300 mM KCl] after washing, and protein recovery was determined by Western blotting. Eluted proteins were incubated with nitrocellulose membranes spotted with a variety of lipids as per manufacturer's instructions (PIP strips; Echelon Biosciences). Bound protein was detected using anti-HA antibody and visualized by Odyssey Infrared Imaging (Li-Cor).

Results

The PDZ domain of PDZGEF binds to phosphatidic acid
To visualize association of PDZGEF with PA, we employed a previously used assay (Gloerich, ten Klooster et al. 2012). Briefly, HEK293T cells were transfected with the PtdIns(4,5)P2-producing enzyme PIP5Kα, which results in accumulation of PtdIns(4,5)P2 on endocytic vesicles. Such confined production of PtdIns(4,5)P2 can be readily visualized with the RFP-tagged PH domain of PLCδ (van den Bout and Divecha 2009). The subsequent vesicular recruitment of PLD results in a local increase of PA and as a consequence accumulation of PDZGEF (Sciorra, Rudge et al. 1999, Hodgkin, Masson et al. 2000, Gloerich, ten Klooster et al. 2012). To determine which domain is responsible for the interaction of PDZGEF1 with PA, we analyzed a series of deletion mutants (Figure 1a). Of the mutants tested, only a PDZGEF1 mutant lacking its PDZ domain (ΔPDZ) was unable to accumulate at PA-enriched vesicles in PIP5Kα–stimulated cells (Figure 1b-d), suggesting that the PDZ domain is responsible for the interaction. Indeed, the PDZ domain of PDZGEF1 (PDZ) localizes at the endocytic vesicles of PIP5Kα- transfected cells (Figure 2a). We next investigated whether PA was indeed responsible for this interaction as previously observed for the full length PDZGEF protein (Gloerich, ten Klooster et al. 2012). We therefore treated the cells with CAY10593, an inhibitor of the PA-generating enzyme PLD1. We observed that the interaction between the PDZ domain of PDZGEF and PA was abolished (Figure 2b). This indicates that the PDZ domain interacts with PA. To confirm that the PDZ domain directly binds to PA, we carried out protein-lipid overlay assays. For this, several lipids spotted onto nitrocellulose membrane were incubated with HA tagged PDZ domain of PDZGEF1 isolated from HEK293T cells. Indeed, we detected binding of the PDZ domain to PA as well as to several other negatively charged lipids (Figure 2c). Similar results were obtained when the PDZ domain of the related family member PDZGEF2 was used (Figure S1). Although true specificity is lacking, these results show that the PDZ domain of PDZGEF can bind to PA.

Basic residues within the PDZ domain are required for binding to PA
Since positively charged residues are able to interact specifically with negatively charged phospholipids such as PA at the membrane, we next investigated whether basic residues

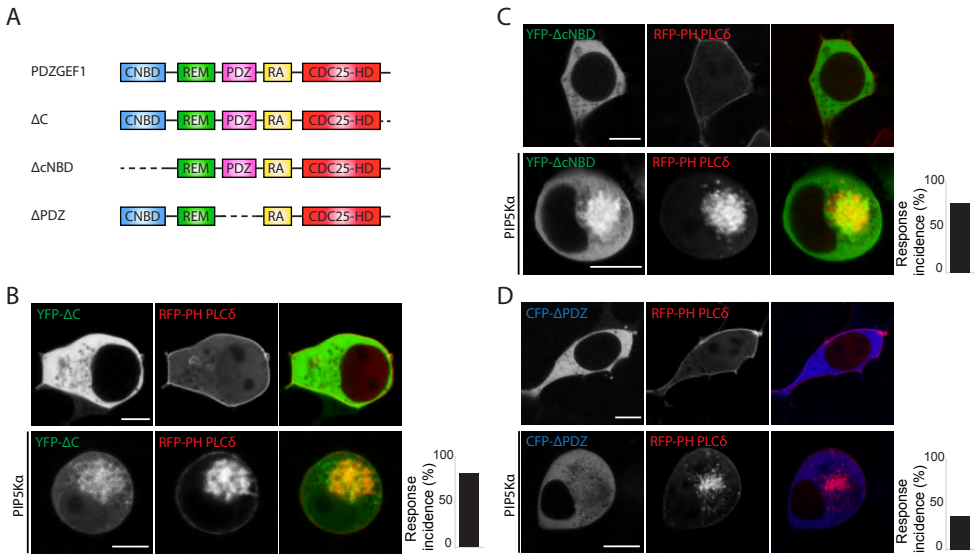

Figure 1: Characterization of the domain repsonsible for binding to phosphatidic acid
A) Domain architecture of PDZGEF mutants. cNBD: cyclic nucleotide binding domain; REM: Rap/Ras exchange motif; PDZ: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), Zonula occludens-1 protein (Zo-1); RA: Ras association domain; CDC25-HD: CDC25 homology domain. B), C) Mutants of PDZGEF either lacking the C-terminus (YFP- ΔC) or the cNBD domain (YFP- ΔCNBD) transfected in HEK293T cells are unable to associate with PA on vesicles in PIP5Kα-stimulated cells. RFP-PH PLCδ confirms production of PtdIns(4,5)P2 by PIP5Kα which then recruits the PA-producing enzyme PLD. D) Lack of the PDZ domain (CFP-ΔPDZ) abolishes association of the protein with PA on PIP5Kα-generated vesicles in HEK293T cells. Representative images are shown. The bar graphs show the percentage of cells showing vesicular localization of the protein in the presence of PIP5Kα (8/10 cells for YFP-ΔC, 9/12 cells for YFP-ΔCNBD and 4/11 cells for CFP-ΔPDZ). Scale bars: 10 μm.

within the PDZ domain contribute to apical membrane targeting of PDZGEF (Kooijman, Tieleman et al. 2007). Mutational analysis revealed that the positively charged lysine 428 and arginine 429 residues present in the PDZ domain are needed for binding to PA. Indeed, mutation of both of these residues to a non-polar alanine (KRA) abolished accumulation of PDZGEF1 to PA-enriched vesicles in PIP5Kα-stimulated cells (Figure 3a).
In order to determine which positively charged side chain contributes the most to the interaction with PA, single residue mutants were analyzed. Replacement of lysine 428 with an alanine (KA) decreased the ability of PDZGEF to interact with PA on endocytic vesicles (Figure 3b). Likewise, mutation of arginine 429 to alanine (RA) inhibited in part the localization of PDZGEF1 to PIP5Kα-generated PA-rich vesicles (Figure 3c).
The above results suggest that the positively charged lysine 428 and arginine 429 contribute to the apical accumulation of PDZGEF, but both residues are needed for protein localization and function.

The PDZ domain localizes PDZGEF at the apical membrane during brush border formation via electrostatic interactions

We next validated the role of the PDZ domain in membrane targeting of PDZGEF during establishment of epithelial polarity. For this, we examined the localization of the PDZ domain in polarized cells. Ls174T-W4 intestinal epithelial cells were used for their ability to form a brush border upon doxycycline stimulation, which results in activation of the pseudo-kinase

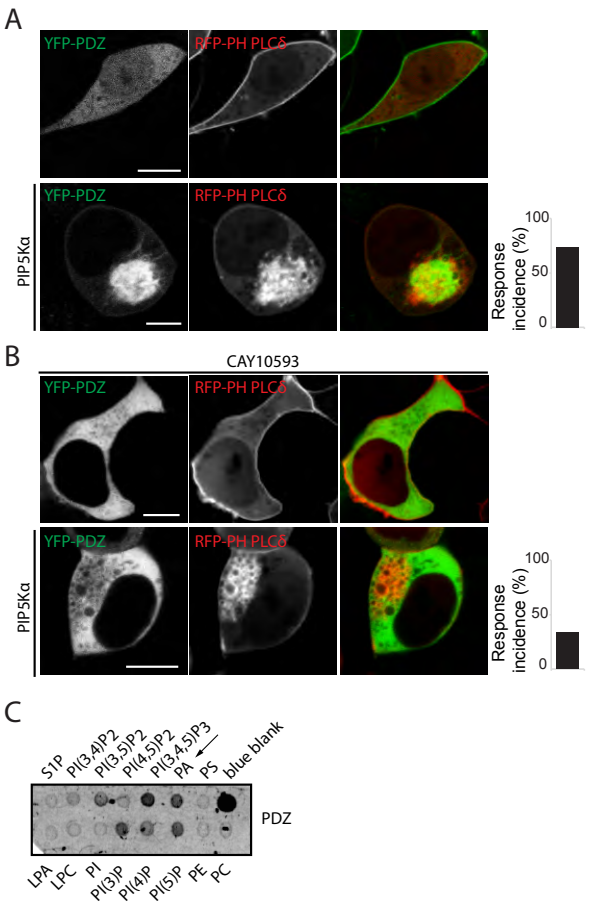

Figure 2: The PDZ domain of PDZGEF binds to phosphatidic acid
A) A mutant of PDZGEF consisting only of the PDZ domain (YFP-PDZ) transfected in HEK293T cells shows vesicular localization in the presence of PIP5Kα. B) Treatment of HEK293T cells with the PLD1 inhibitor CAY10593 impairs association of the PDZ domain of PDZGEF (YFP-PDZ) with locally generated PA on PIP5Kα–generated vesicles. Representative images are shown. The bar graphs show the percentage of cells showing vesicular localization of the protein in the presence of PIP5Kα (8/11 cells for CFP-PDZ and 4/12 cells for CFP-PDZ in presence of CAY10593). Scale bars: 10 µm. C) Protein-lipid overlay assay of HA tagged PDZ domain of PDZGEF1 (PDZ) isolated from HEK293T cells using PIP strips containing 100pmol/spot of the following lipids: lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylinositol (PI), phosphatidylinositol 3-phosphate [PI(3)P], PI(4)P, phosphatidylinositol 5-phosphate [PI(5)P], phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2], phosphatidylinositol 1,2-bisphosphate [PI(4,5)P2], phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], phosphatidic acid (PA), or phosphatidylserine (PS). Arrow indicates binding to PA.

STRADα and subsequent establishment of polarity in individual cells by the kinase Lkb1 (Baas, Kuipers et al. 2004). Live-imaging of single cells showed that, upon doxycycline stimulation, the PDZ domain of PDZGEF1 (YFP-PDZ) relocated to the brush border at the apical membrane, as also indicated by a similar accumulation pattern of the actin-binding probe LifeAct-Ruby (Figure 4a). Such apical redistribution in doxycycline stimulated cells was lost following treatment with the PLD1 inhibitor CAY10593 (Figure 4b), thus indicating that the PDZ domain interacts with PA at the brush border.

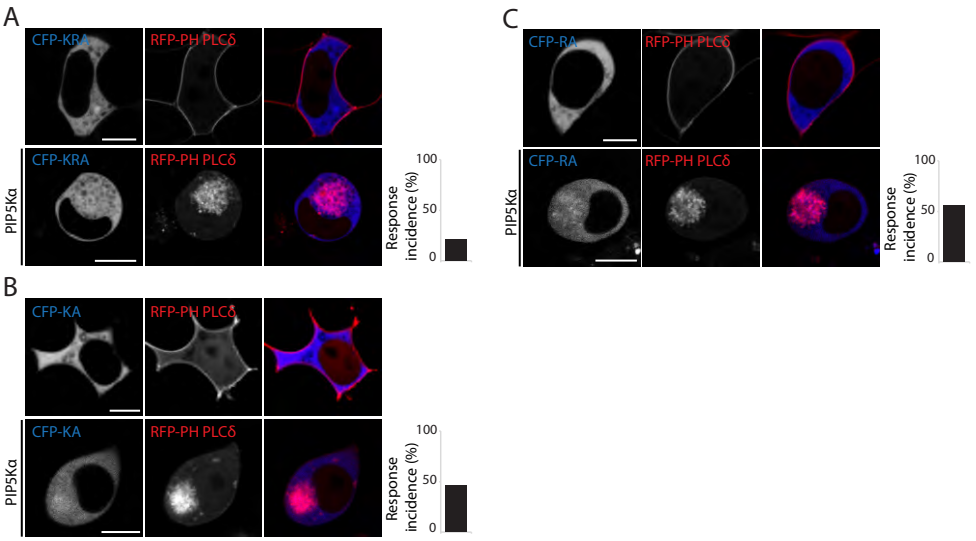

Figure 3: Positive residues within the PDZ domain are required for binding to PA
A) Mutation of lysine 428 and arginine 429 within the PDZ domain to a non-polar alanine (CFP-KRA) impairs interaction with PA on PIP5Kα-generated vesicles in HEK293T cells. B), C) Substitution of either lysine 428 or arginine 429 alone with alanine (CFP-KA and CFP-RA respectively) results in impaired ability of the protein to associate with vesicular PA in HEK293T cells. The bar graphs display the percentage of cells showing protein localization on PIP5Kα-generated vesicles (3/11 cells for CFP-KRA, 5/11 cells for CFP-KA and 6/11 cells for CFP-RA). Representative images are shown. Scale bars: 10 µm.

We next tested whether the positively charged amino acids within the PDZ domain also contribute to such polarized localization of PDZGEF. The KRA mutant was unable to accumulate to the brush border of doxycycline-stimulated cells (Figure 4c). Thus, the PDZ domain mediates localization of PDZGEF at the apical membrane by binding to PA via basic residues during intestinal epithelial polarity.

Conclusion

We have recently shown that Rap2a activity is needed downstream of Lkb1 for induction of intestinal epithelial brush border formation via a pathway that employs its effector TNIK, the kinase Mst4 and Ezrin (Gloerich, ten Klooster et al. 2012). PDZGEF plays an important role in this as it mediates activation of Rap2a at the apical membrane. We find that the PDZ domain of PDZGEF directly binds to PA, enriched at the apical membrane during epithelial cell polarization. Moreover, we show that positively charged side chains within the PDZ domain are involved in such interaction, since their mutation to a non-polar amino acid abolishes apical localization of PDZGEF. Thus, this study provides further insights into the mechanisms by which PDZ domains mediate protein-lipid interactions.
PDZ domains usually interact with short motifs at the C-terminus of proteins (Lee and Zheng 2010). Phosphorylation events or disulphide bonds formation contribute to the specificity of PDZ-mediated interactions (Mauceri, Gardoni et al. 2007, Mishra, Socolich et al. 2007, Chen, Pan et al. 2008). The importance of PDZ domains as lipid-binding in addition to protein-binding modules in cellular signaling has recently become more and more appreciated.

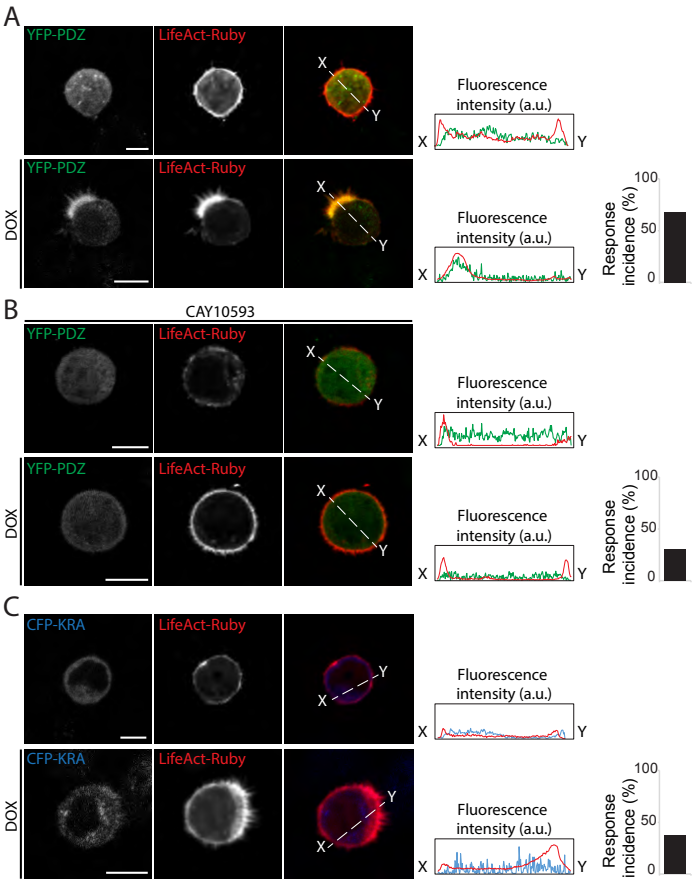

Figure 4: The PDZ domain localizes PDZGEF at the apical membrane during brush border formation via electrostatic interactions
A) Live-cell imaging of Ls174T-W4 cells transfected with YFP-PDZ and the actin binding probe LifeAct-Ruby with and without stimulation with doxycycline. B) Ls174T-W4 cells treated with the PLD1 inhibitor CAY10593 do not show accumulation of YFP-PDZ to the brush border of doxycycline-stimulated cells. C) The double mutant CFP- KRA is not able to localize at the brush border of Ls174T-W4 cells after doxycycline treatment. Representative images are shown. The profiles represent the fluorescence intensity across the cell as indicated by the dotted line. The bar graphs show the percentage of cells showing brush border localization of the protein following doxycycline treatment (8/12 cells for YFP-PDZ, 3/10 cells for YFP-PDZ in presence of CAY10593 and 4/11 cells for CFP-KRA). Scale bars: 10 µm.

Indeed, PDZ domains have been shown to regulate a variety of cellular processes by binding mainly to the signaling lipid phosphatidylinositol (Zimmermann, Meerschaert et al. 2002, Wu, Feng et al. 2007, Gallardo, Ivarsson et al. 2010). A recent study also demonstrated that the PDZ domains of the Drosophila Par-3 homolog Bazooka directly bind to PA, and that this interaction influences establishment of polarity landmarks (Yu and Harris 2012). Our results demonstrate that PDZ domains can interact with PA also in mammalian cells.

Aknowledgements:

We thank R. Wedlich-Söldner for the LifeAct-Ruby construct and members of our laboratory for helpful discussions. This work is supported by Chemical Sciences (S.V.C.) and the Netherlands Genomics Initiative (J.L.B.) of the Netherlands Organization for Scientific Research (NWO).

Supplementary information

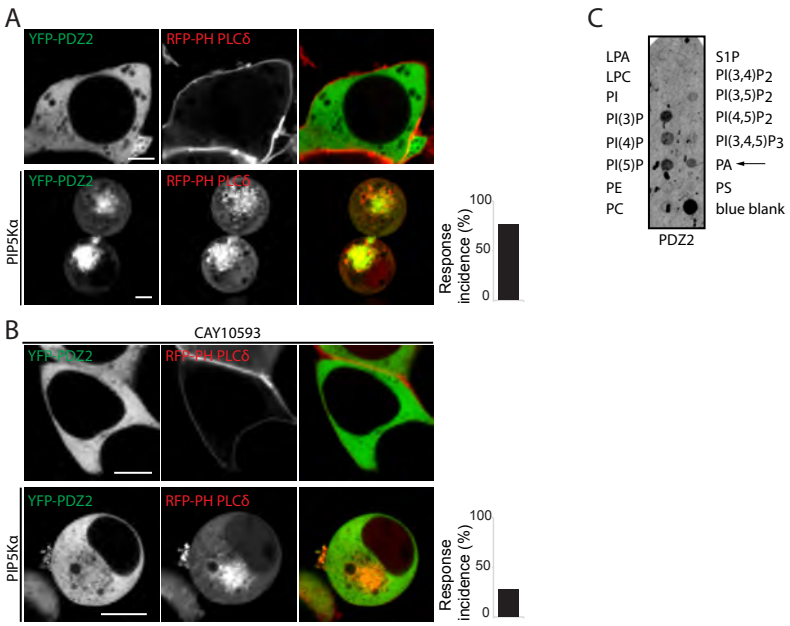

Figure S1: The PDZ domain of PDZGEF binds to phosphatidic acid
A) In HEK293T cells the PDZ domain of PDZGEF2 (YFP-PDZ2) localizes at vesicles in the presence of PIP5Kα. B) The PLD1 inhibitor CAY10593 impairs the ability of the PDZ domain of PDZGEF2 (YFP-PDZ2) to interact with PA on PIP5Kα–generated vesicles in HEK293T cells. Representative images are shown. The bar graphs show the percentage of cells showing vesicular localization of the protein in the presence of PIP5Kα (9/12 cells for CFP-PDZ2 and 3/11 cells for CFP-PDZ2 in presence of CAY10593). Scale bars: 10 µm. C) Protein-lipid overlay assay of HA tagged PDZ domain of PDZGEF2 (PDZ2) isolated from HEK293T cells using PIP strips containing 100pmol/spot of the following lipids: lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylinositol (PI), phosphatidylinositol 3-phosphate [PI(3)P], PI(4)P, phosphatidylinositol 5-phosphate [PI(5)P], phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2], phosphatidylinositol 1,2-bisphosphate [PI(4,5)P2], phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], phosphatidic acid (PA), or phosphatidylserine (PS). Arrow indicates binding to PA.

5

LIM domain only 7 negatively regulates cAMP-Epac1 signaling

Sarah V. Consonni, Lars A. T. Meijer, Patricia M. Brouwer, On Ying A. Chan, Johannes L. Bos

Manuscript in preparation

Epac1 is a cAMP-responsive guanine nucleotide exchange factor for the small G protein Rap. By binding to diverse anchors at distinct cellular compartments, Epac1 transduces cAMP signals into the cell through activation of Rap. Here we performed SILAC-based mass spectrometry in order to identify Epac1-interacting proteins. Of the several hits, we further characterized the LIM domain-containing protein Lmo7. Lmo7 interacts with the catalytic domain of Epac1 and prevents Epac1 translocation to the plasma membrane. This interaction is sensitive to an inhibitor of calcium/calmodulin-dependent protein kinase II, suggesting that the binding is mediated by phosphorylation. Moreover, Lmo7 expression inhibits cAMP-induced Epac1-mediated Rap1 activation in vivo. From these results we conclude that Lmo7 acts as a negative regulator of cAMP-Epac1 signaling most likely by preventing Epac1 translocation to the plasma membrane.

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Introduction

Rap is a member of the Ras family of small G proteins. As other G proteins, it switches between an inactive GDP bound state and an active GTP bound state. Such a cycle is orchestrated by several guanine nucleotide exchange factors (GEFs), which exchange GDP for the more abundant GTP thereby activating the small G protein, and by several GTPase-activating proteins (GAPs), which inactivate it by inducing GTP hydrolysis. One of the GEFs responsible for activating small G proteins of the Rap family is Epac1. Epac1 is directly regulated and activated by the second messenger cAMP (de Rooij, Rehmann et al. 2000). Upon cAMP binding, Epac1 undergoes a conformational change which induces not only its activation, but also its translocation to the plasma membrane (PM) (Ponsioen, Gloerich et al. 2009). This cAMP-dependent relocalization of Epac1 is mediated by binding of the DEP domain of Epac1 to phosphatidic acid and is one of the mechanisms of translocation required for activation of PM-located Rap (Gloerich, Ponsioen et al. 2010, Consonni, Gloerich et al. 2012). Another manner is the interaction of the N-terminal 49 amino acid residues of Epac1 with activated Ezrin, Radixin, Moesin (ERM) proteins (Gloerich, Ponsioen et al. 2010, Ross, Post et al. 2011).
Additional membrane anchors and subcellular localizations of Epac1 have been described (Gloerich and Bos 2010). For instance, Epac1 is present at the nuclear pore complex where RanBP2 inhibits its activity by binding to its catalytic CDC25 homology domain (Gloerich, Vliem et al. 2011). These and other reported localizations link Epac1 to various cellular processes, such as endothelial cell-cell junction modulation, myocardial contraction and synaptic potentiation (Lohse, Engelhardt et al. 2003, Kaneko and Takahashi 2004, Pannekoek, van Dijk et al. 2011).
Lim domain only 7 (Lmo7) is an alternatively spliced modular protein with reported tissue specificity (Putilina, Jaworski et al. 1998, Furuya, Tsuji et al. 2002, Lindvall, Blomberg et al. 2005). It is composed of a calponin homology (CH) domain for binding to actin, a central PDZ domain for protein localization and a LIM domain at the C-terminus (Lindvall, Blomberg et al. 2005, Dedeic, Cetera et al. 2011). LIM domains, whose name derives from LIN-11, Isl1 and MEC-3 where they were firstly identified, are usually 55 amino acids long with 8 highly conserved zinc-binding residues important for defining two zinc finger modules. Such highly stable structure is able to mediate protein-protein interactions by building tandem contacts with the interacting protein (Kadrmas and Beckerle 2004, Zheng and Zhao 2007).
Here we identify Lmo7 as an interactor of Epac1. By sequestering Epac1 at cytoplasmic vesicular compartments, Lmo7 acts as a negative regulator of cAMP-Epac signaling. This interaction is regulated by phosphorylation, possibly by calcium/calmodulin-dependent protein kinase II (CamKII).

Materials and Methods

Reagents, antibodies and DNA constructs
8-pCPT-2'-O-Me-cAMP (007) and 8-pCPT-2'-O-Me-cAMP-AM (007-AM) were from Biolog Life Sciences. The CamKII inhibitor KN-93 and the inactive compound KN-92 were from Sigma-Aldrich. The following antibodies were used: anti-Rap1 (Santa Cruz), anti-GFP (Roche), anti-Epac1 5D3 monoclonal antibody (Cell Signaling), anti-HA antibody (Covance), anti-Lmo7 antibody (Sigma-Aldrich) and the anti-Flag antibody (Sigma-Aldrich). ON-TARGET plus

SMARTpool siRNAs directed against Lmo7 and scrambled control siRNAs were from Thermo Scientific Dharmacon. Lmo7 constructs were kindly provided by A. Mull (University of Chicago, USA). All mutants were generated by site-directed mutagenesis.

Generation of stable cell lines
To generate retrovirus particles, Phoenix ecotropic packaging cells were transfected with pBabe-puro-1S (EV) and pBabe-puro-1S-Epac1 (Epac1). Twenty-four hours prior to infection, the HeLa target cells were electroporated with an expression plasmid for the ecotropic receptor. HeLa cells were infected by adding an equal volume of culture medium containing retrovirus particles to the refreshed HeLa culture medium and the addition of polybrene to a final concentration of 5 µg/ml. The medium was refreshed 24 hours after infection and replaced by medium containing 1 µg/ml puromycin 48 hours after infection to allow selection of cells expressing the desired constructs. Monoclonal colonies were isolated 9 days after infection and screened for expression by immunoblot analysis. Clone 1 of the Epac1 cells was selected for its relatively low expression of 1S-tagged Epac1 and clone 43 of the EV cells was selected for its similar proliferation rate.

Cell culture
All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 1.4 mM L-glutamine (all from Lonza).
For SILAC experiments, HeLa cells were cultured for 6 cell doublings in SILAC DMEM high glucose without L-arginine and L-lysine (PAA) supplemented with 73 mg/l L-lysine (Lys0) (Sigma-Aldrich) and 42 mg/l L-arginine (Arg0) (Sigma-Aldrich) for the 'Light' condition and 73 mg/l 13C615N2 L-lysine (Lys8) (Isotec) and 42 mg/l L-arginine (Arg0) for the 'Heavy' condition. For both conditions the media were supplemented with 10% dialyzed FBS (Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin and 1.4 mM L-glutamine.

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Rap activity assay
Cells were stimulated with 1 µM of 007-AM for 15 minutes when indicated or treated or not with the CamKII inhibitor KN-93 for 2 hours. Next, Rap activity was assayed as described previously (van Triest and Bos 2004). Briefly, cells were lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 10% glycerol, 2 mM MgCl2 and protease and phosphatase inhibitors. Lysates were cleared by centrifugation and active Rap was precipitated with a GST fusion protein of the Ras-binding domain (RBD) of RalGDS precoupled to glutathione-sepharose beads. Bound proteins were eluted in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting.

Co-immunoprecipitations
Immunoprecipitations were carried out with lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2, and protease and phosphatase inhibitors. Cell lysates were cleared by centrifugation and incubated with protein A agarose beads (GE Healthcare) coupled to the indicated antibody. Bound proteins were eluted after washing in Laemmli buffer and analyzed by SDS-PAGE and Western blotting with the indicated antibodies.

Fluorescence microscopy

For confocal live-imaging, 1 day after transfection cells were seeded overnight in glass-bottomwells (WillCo Wells). 2 hours before imaging in L-15 Leibovitz medium (Invitrogen) at 37 °C cells were treated or not with the CamKII inhibitor KN-93 for 2 hours as indicated. MDCK and HL-1 cells were grown on 12 mm glass cover slips and fixed with 3.8% formaldehyde. Images of live and fixed cells were acquired on an inverted Zeiss LSM510 confocal microscope equipped with 63× magnification objective lens (N.A. 1.4; Leica).

Affinity purifications, protein digestion and sample preparation
Per each condition of the EV versus Epac1 SILAC-based affinity purifications 5 × 15 cm dishes of adherent and confluent cultures of HeLa cells were washed with ice-cold PBS and lysed in Buffer 1. Lysates were cleared by centrifugation and protein concentrations in the supernatant were determined using the BIO-RAD Protein Assay (BIO-RAD). In separate reaction tubes for each condition, equal amounts of supernatant were incubated with Strep-Tactin Superflow for 15 minutes. After washing with Buffer 2, proteins were eluted by incubating with 50 mM D-biotin in Buffer 2 for 15 minutes. Elution fractions were digested according to the filter-aided sample preparation (FASP) method (Wisniewski, Zougman et al. 2009). Briefly, samples were incubated with 20 mM DTT for 15 minutes at 50 °C. Formaldehyde cross-linked samples were incubated for 20 minutes at 95 °C to reverse the cross-links. Next, samples were bound onto Microcon YM-10 filters (Millipore) with a 10 kDa molecular weight cut-off and incubated with 50 mM IAA for 5 minutes. After several washing steps, proteins were digested using Lys C (Wako) at an enzyme to protein ratio of 1:50 followed by incubation overnight at room temperature. Finally, peptides were eluted using 0.5 M NaCl and desalted using C18 StageTips as previously described (Rappsilber, Mann et al. 2007). Prior to LC-MS/MS analysis, peptides were eluted from the StageTips, dried in vacuo and reconstituted in 5% formic acid.

Mass spectrometry
Peptides were subjected to a reversed phase nano-LC-MS/MS analysis consisting of an Agilent 1200 series HPLC system (as described before (Raijmakers, Berkers et al. 2008)) connected to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). The HPLC system was equipped with a 20 mm × 100 µm ReproSil-Pur 120 C18-AQ (3 µm bead size, Dr. Maisch) trapping column (packed in-house) and a 400 mm × 50 µm ReproSil-Pur 120 C18-AQ (3 µm bead size, Dr. Maisch) analytical column (packed in-house). Mobile phase solvents consisted of 0.1 M acetic acid in double distilled water (Solvent A) and 0.1 M acetic acid in 80% acetonitrile (Solvent B). Samples were loaded onto the trapping column and washed with 100% Solvent A for 10 min at a flow rate of 5 µl/min. Subsequently, peptides were eluted using a 110 minute multi-segment linear gradient of 10-100% Solvent B. During the elution gradient, the flow rate was passively split from 0.60 mL/min to 100 nL/min. Nanospray was achieved using a distally gold-coated fused silica emitter (360 µm outer diameter, 20 µm inner diameter (i.d.), 10 µm tip i.d.; pulled and coated in-house) set to an ion spray voltage of 1.7 kV.
The LTQ Orbitrap Velos instrument was operated in positive ion mode and in data-dependent acquisition mode to automatically switch between MS and MS/MS. Survey full-scan MS spectra were acquired from 350 to 1,500 m/z in the Orbitrap analyzer with a resolution of 60,000 at 400 m/z after accumulation to a target value of 500,000 in the linear ion trap (LTQ). Charge state screening was enabled and precursors with an unknown charge state or a charge state of 1 were excluded from fragmentation. The five most intense precursor

ions at a threshold of above 500 counts were fragmented by collision-induced dissociation (CID) at a target value of 5,000. The normalized collision energy was set to 35% and dynamic exclusion was enabled (exclusion list size 500, exclusion duration 90 s). The 445.120025 ion was used as lock mass with a target lock mass abundance of 0% for internal mass calibration.

Data analysis
Raw mass spectrometric SILAC samples data were analyzed with the MaxQuant software package (version 1.0.13.12) (Cox and Mann 2008, Cox, Matic et al. 2009). The false discovery rate (FDR) was set at 1% for proteins and peptides and the minimum required peptide length was set at 6 amino acids. MS/MS spectra were searched against the IPI human database (version 3.52) using Mascot (version 2.2.04). Quantification of the data was performed as previously described (Cox and Mann 2008). Plots were generated using the MaxQuant module Perseus.

Results

Epac1 interacting proteins

To gain further insights into the complex spatial regulation of Epac1, we made use of stable isotope labeling by amino acids in cell culture (SILAC)-based system. This allows identification of specific interacting proteins by quantifying peptides originating from protein complexes that are isolated from two different cell populations using a single low-stringency purification step. Figure 1a shows a schematic diagram of the experimental workflow (Blagoev, Kratchmarova et al. 2003, Vermeulen, Hubner et al. 2008).
We were able to identify several Epac1-specific interactors (Figure 1b) most of which are related to the cytoskeleton according to their gene ontology terms. Among these are Moesin (MSN), a member of the Ezrin, Radixin, Moesin (ERM) family of proteins, and RanBP2, proteins that were previously identified as Epac1 interacting proteins (Gloerich, Ponsioen et al. 2010, Gloerich, Vliem et al. 2011), thus confirming the previously observed interactions.

Lmo7 binds to Epac1

A potentially interesting cytoskeleton-related protein is LIM domain only 7 (Lmo7). In epithelial cells, Lmo7 connects the nectin-AF6 and E-cadherin-catenin cell adhesion systems through the interaction with the filamentous actin (F-actin) binding proteins AF6 and α-actinin, thereby acting as a stabilizer (Ooshio, Irie et al. 2004). Interestingly, AF6 has been identified as a Rap1 effector and has been implicated in promoting adherens junction formation (Hoshino, Sakisaka et al. 2005, Zhang, Rehmann et al. 2005). This provides a potential link between Lmo7 and the Epac1-Rap signaling node.
To confirm the binding between Epac1 and Lmo7, HEK293T cells were transfected with Flag-Epac1 and GFP-tagged Lmo7. Epac1 co-immunoprecipiated with Lmo7, both in the absence or presence of the Epac-selective cAMP agonist, 8-CPT-2'OMe-cAMP-AM (007-AM) (Figure 2a). In contrast, Flag-Epac2 did not interact with GFP-Lmo7, indicating that the binding is specific for the Epac1 isoform (Figure 2b). To further explore the binding between Epac1 and Lmo7, we examined their cellular localization. Epac1 resides in the cytoplasm and translocates to the PM after stimulation with cAMP or cAMP analogues to activate PM-localized Rap (Left panel, Figure 2c) (Ponsioen, Gloerich et al. 2009, Consonni, Gloerich et al. 2012).

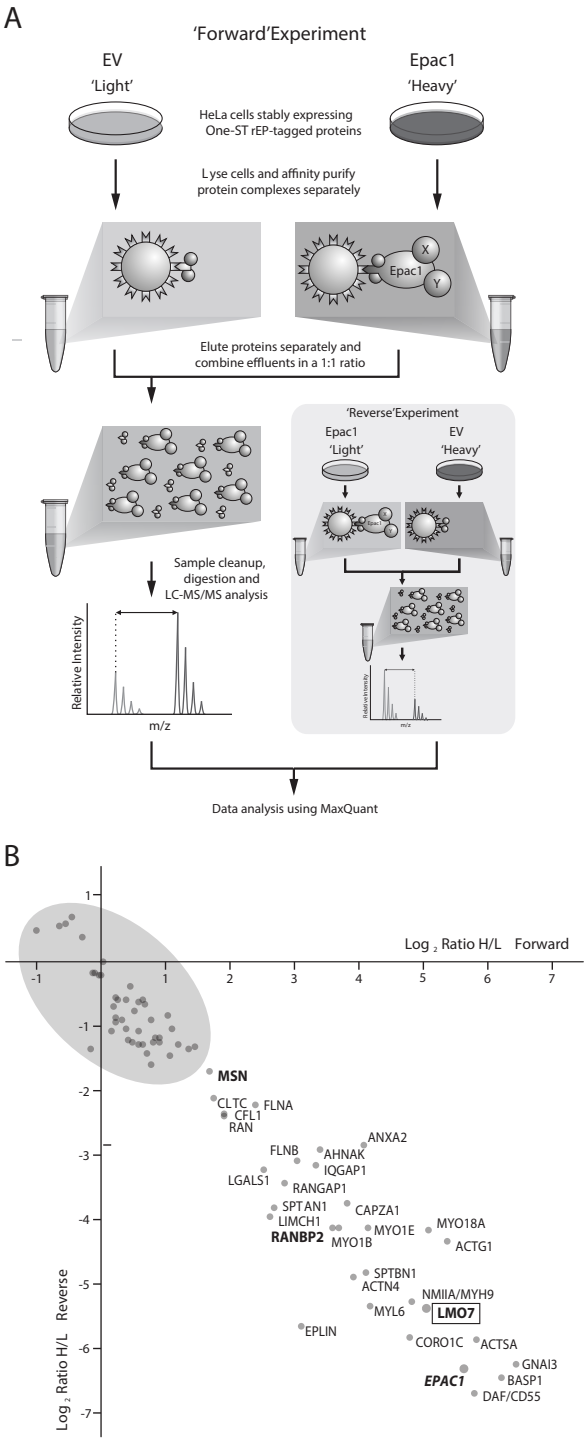

Figure 1: Epac1 interacting proteins
A) Schematic overview of the stable isotope labeling by amino acids in cell culture (SILAC)-based workflow. In the 'Forward' experiment, HeLa cells stably expressing the 1S-tag only (empty vector (EV)) are cultured in medium containing normal ('Light') amino acids. HeLa cells stably expressing Epac1 are cultured in medium containing stable isotope labeled ('Heavy') analogues of these amino acids. Next, cells are lysed and Epac1 and its interacting proteins (X and Y) are purified separately from the EV using Strep-Tactin material. Subsequently, Epac1 complexes and the EV are eluted using biotin, pooled in a 1:1 ratio, digested, and ultimately subjected to liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis. Finally, the LC-MS/MS data are analyzed using the MaxQuant software package and abundance ratios between the Epac1 and EV samples are calculated. In parallel, an experimental replicate is performed, termed 'Reverse' experiment, in which the cell culture conditions are swapped compared to the 'Forward' experiment. Thus, the Epac1 cells are cultured in 'Light' medium and the EV cells in 'Heavy' medium (light grey inset). B) Scatter plot in which the log2 values of the calculated protein ratios in the 'Forward' experiment are plotted against those in the 'Reverse' experiment. Proteins with ratios of around 1:1 (0 on a logarithmic scale), which represent background proteins, are present in the light grey oval. Epac1 is indicated in bold italic. Lmo7 is found to be an Epac1-specific interactor and indicated in bold and boxed. RanBP2 and Moesin (MSN), previously confirmed to be both genuine Epac1-interacting proteins, are indicated in bold (Gloerich et al. 2010, Gloerich et al. 2011).

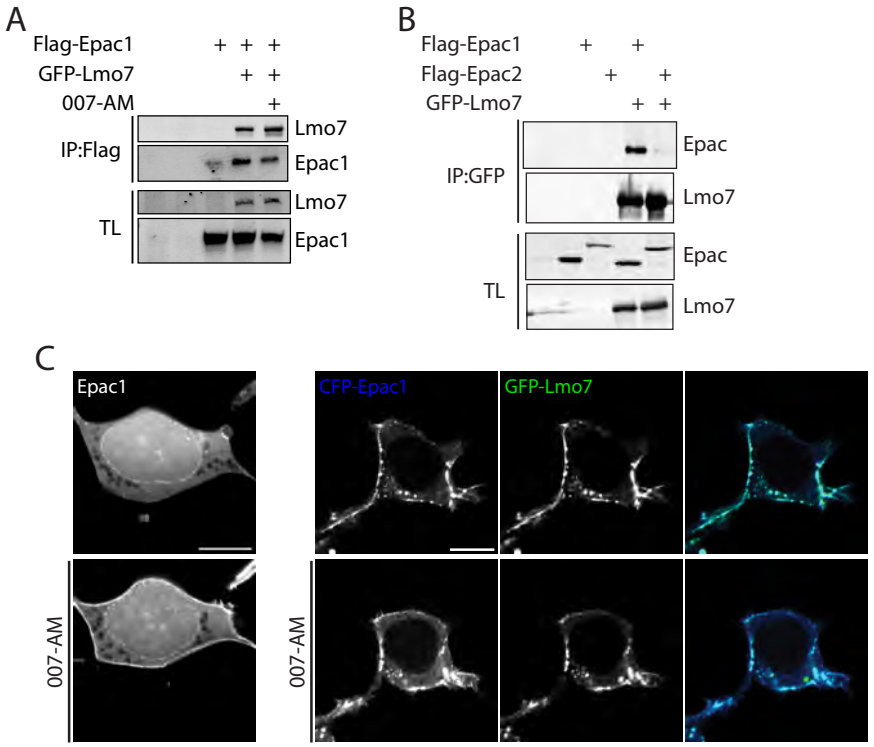

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Figure 2: Lmo7 is a novel interactor of Epac1
A) Co-immunoprecipitation of Flag-Epac1 and GFP-Lmo7 in HEK293T cells before and after stimulation with 1 µM 007-AM. B) Co-immunoprecipiation from HEK293T cells showing binding of GFP-Lmo7 to Flag-Epac1 but not to Flag-Epac2. C) Left panel: Live-imaging of HEK293T cells showing translocation of Epac1 to the PM upon stimulation with 1 µM 007-AM. Right panel: Live-imaging of HEK293T cells showing co-localization of CFP-Epac1 and YFP-Lmo7 before and after treatment with 1 µM 007-AM. Representative images are shown. Scale bars: 10 µm.

Lmo7 is localized at cytoplasmic vesicles and at the cell periphery like other LIM domain-containing proteins (Tree, Shulman et al. 2002, Ooshio, Irie et al. 2004). When CFP-Epac1 and GFP-Lmo7 are co-expressed in HEK293T cells, Epac1 shows complete accumulation at the sites where Lmo7 is present (Right panel, Figure 2c). In contrast, a CFP-tagged empty vector did not show co-localization with GFP-Lmo7, thus confirming that this was not due to bleed-through of fluorescence emission (Supplementary Figure 1). A similar punctate localization pattern was observed in different cell types (Supplementary Figure 2). When Epac1 was activated by treating the cells with 007-AM, it remained bound to Lmo7 and its translocation to the PM was abolished (Figure 2c). The same results were obtained with a splice variant of Lmo7 that differs in its tissue expression patterns (Supplementary Figure 3). Taken together, these results indicate that Lmo7 interacts with Epac1 and is able to inhibit cAMP-induced translocation of Epac1 to the PM.

Epac1 CDC25-HD domain directly interacts with Lmo7 LIM domain
In order to gain more insight into the regulation of the binding between Epac1 and Lmo7,

we examined which regions of Epac1 and Lmo7 interact with each other. We found that the catalytic region of Epac1 was able to co-immunoprecipitate with Lmo7 from HEK293T cells similar to the full length protein (Figure 3a). Accordingly, the catalytic region of Epac1 also assumed a vesicular subcellular localization in cells co-transfected with Lmo7 (Figure 3b). Since LIM domains of other LIM domain-containing proteins have been shown to mediate protein-protein interactions (Feuerstein, Wang et al. 1994, Schmeichel and Beckerle 1994,

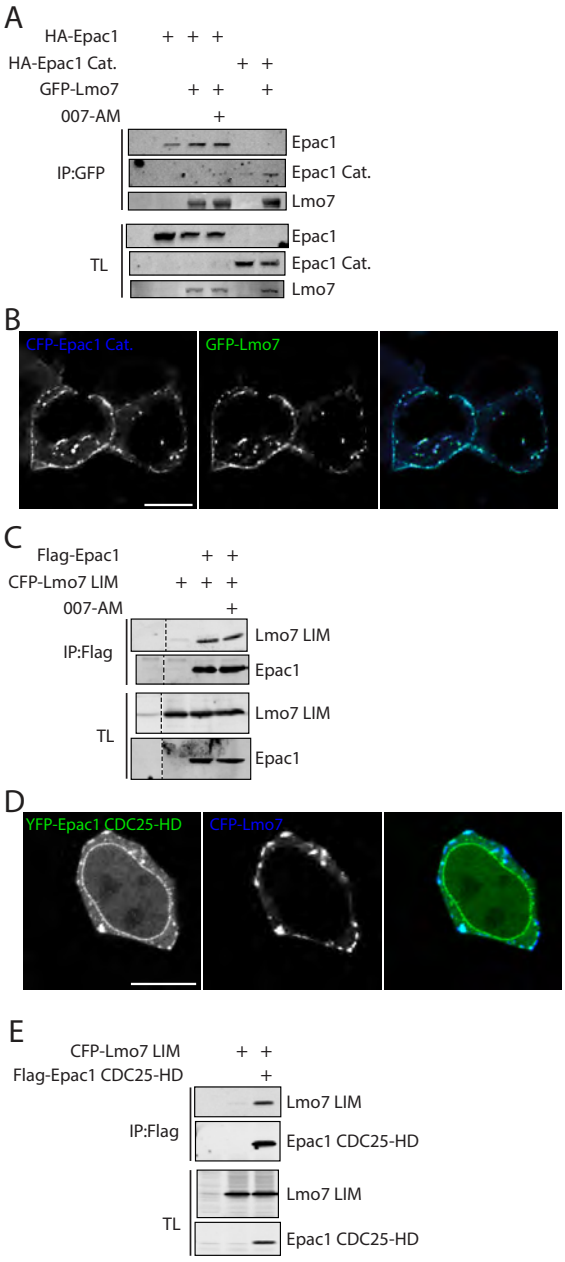

Figure 3: Epac1 CDC25-HD directly interacts with Lmo7 LIM domain.
A) Co-immunoprecipitation of HA-Epac1 and HA-Epac1 catalytic region (Epac1 Cat.) with GFP-Lmo7 in HEK293T cells before and after stimulation with 1 µM 007-AM. B) Live-imaging of HEK293T cells showing accumulation of CFP-Epac1 catalytic region (Epac1 Cat.) at the sites where GFP-Lmo7 is present. C) Co-immunoprecipitation of Flag-Epac1 with CFP-Lmo7 LIM domain in HEK293T cells before and after stimulation with 1 µM 007-AM D) Immunofluorescence of YFP-Epac1 CDC25 homology domain (CDC25-HD) and GFP-Lmo7 in HEK293T cells. Representative images are shown. Scale bars: 10 µm. E) Co-immunoprecipitation of Flag-Epac1 CDC25 homology domain (CDC25-HD) with GFP-Lmo7 in HEK293T cells.

Tree, Shulman et al. 2002, Zheng and Zhao 2007), we next investigated whether the LIM domain of Lmo7 is responsible for binding to Epac1. Co-immunoprecipitation of cells transfected with Flag-Epac1 and a CFP- LIM domain showed direct binding between Epac1 and the LIM domain (Figure 3c). Furthermore, the CDC25 homology domain of Epac1 directly interacts with the LIM domain of Lmo7 and also localizes at the sites where Lmo7 is present in cells (Figure 3d, e). We conclude that Lmo7 sequesters Epac1 by binding to its catalytic domain via its LIM domain.

Binding of Lmo7 negatively affects Epac1-Rap signaling

Our results thus far suggest that Lmo7 negatively regulates the function of Epac1, either by preventing its translocation to the PM or by direct inhibition as a consequence of its interac-

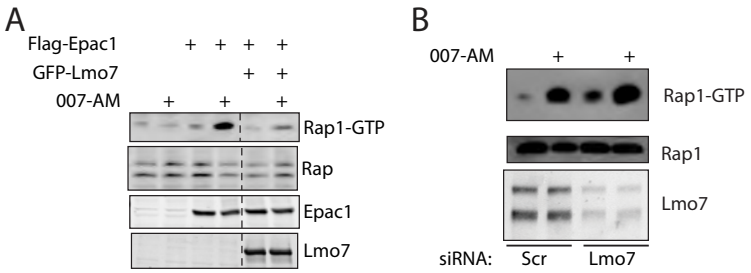

Figure 4: Lmo7 negatively affects Epac1-Rap signaling.
A) Pull-down of Rap-GTP from HEK293T transfected with Epac1 and Lmo7. The ability of Epac1 to increase Rap-GTP levels in response to 007-AM when Lmo7 is co-transfected is reduced. The bottom blots show expression levels of the transfected constructs. B) Pull-down of Rap-GTP from MDA-MD231 cells stably expressing Epac1 upon stimulation with 1 µM 007-AM 60 hours after transfection with control (siScr) or Lmo7 siRNA. The bottom blot shows efficiency of Lmo7 knockdown.

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tion with the CDC25 homology domain of Epac1. To test whether Lmo7 inhibits Epac1 function as a GEF for Rap1 in vivo, we performed Rap-GTP pulldown experiments. Activation of Epac1 by stimulation with 007-AM results in increased Rap1-GTP levels. This was markedly reduced upon co-transfection of Lmo7 (Figure 4a). Correspondingly, knockdown of Lmo7 in MDA-MB231 cells stably expressing Epac1 resulted in an increase in the amount of active Rap under both basal and 007-AM treated conditions (Figure 4b). This strongly supports the notion that Lmo7 is a negative regulator of Epac1.

Phosphorylation events regulate the interaction between Epac1 and Lmo7

Recently, a quantitative global phosphoproteomics study found Lmo7 to have a regulated phosphorylation profile after activation of the Epac1-Rap signaling (Meijer, Zhou et al. 2013). Two novel phosphorylation sites on Lmo7 were found to be up-regulated and both sites are predicted to be regulated by Calcium/calmodulin-dependent protein kinase II (CaMKII). BLAST alignment of mammalian Lmo7 proteins shows complete conservation of the sequence context of the found phosphosites, suggesting that these phosphorylation events are important for protein regulation and function. In addition, Epac1 has been reported to lead to the activation of CamKII via a Rap/PLCε-mediated pathway (Oestreich, Malik et al. 2009, Mangmool, Shukla et al. 2010). We therefore examined whether CamKII participates in the regulation of the Epac1/Lmo7 interaction using the specific CamKII inhibitor KN-93 (Rokolya and Singer 2000). Treatment of HEK293T cells expressing CFP-Epac1 and

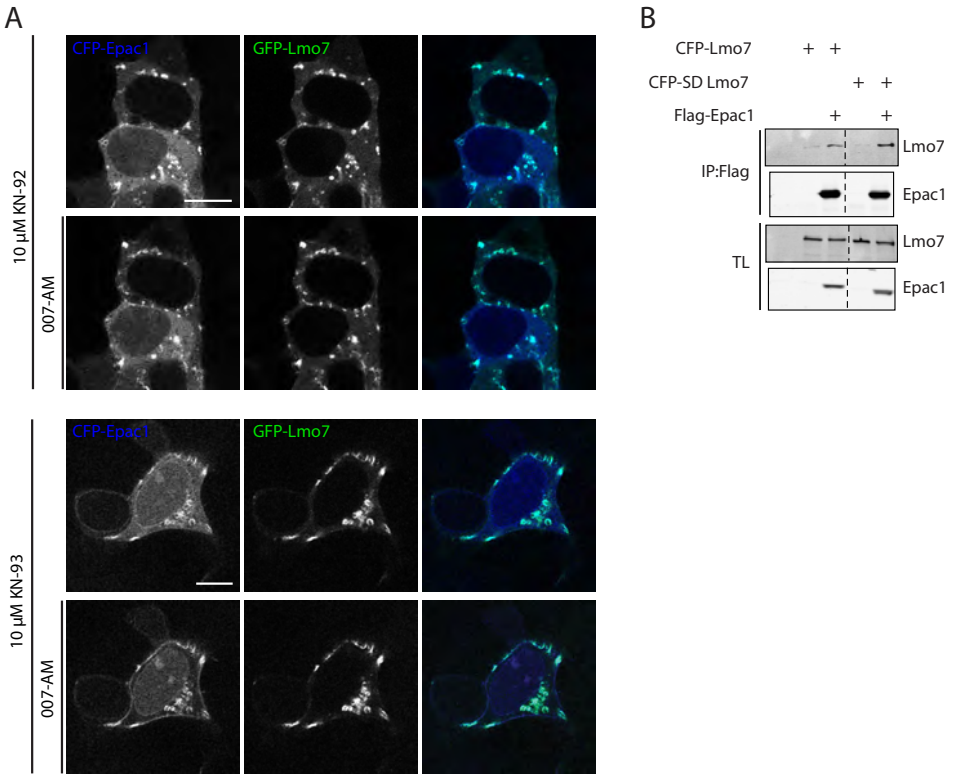

Figure 5: CamKII regulates the interaction between Epac1 and Lmo7.
A) Top panel: Representative live-imaging of CFP-Epac1 and GFP-Lmo7 in HEK293T cells treated with 10 µM of the inactive compound KN-92 for 2 hours, before and 10 minutes after stimulation with 1 µM 007-AM. Bottom panel: Representative live-imaging of CFP-Epac1 and GFP-Lmo7 in HEK293T cells treated with 10 µM of the CamKII inhibitor KN-93 for 2 hours, before and 10 minutes after stimulation with 1 µM 007-AM. Scale bars: 10 µm. B) Co-immunoprecipitation between Lmo7 phospho-mimicking mutant (SD Lmo7) and Epac1 from HEK293T cells.

GFP-Lmo7 with KN-93 at least partially restores the translocation of Epac1 to the PM. This re-localization is not observed when the related inactive compound KN-92 is used (Figure 5a). In the absence of Lmo7, KN-93 did not per se affect Epac1 localization nor its cAMP-induced translocation (Supplementary Figure 4). Together these results show that KN-93 inhibits the interaction of Lmo7 with Epac1, thus strongly suggesting that phosphorylation is required for their binding.
To further validate the contribution of Lmo7 phosphorylation to the binding between Epac1 and Lmo7, we generated Lmo7 phosphorylation mutants. Phospho-mimicking mutations (serine to aspartate mutations, SD Lmo7) enhanced the ability of Lmo7 to bind to Epac1 (Figure 5b), suggesting that phosphorylation is needed for Epac1/Lmo7 interaction.

Discussion

Using mass spectrometry to identify interacting proteins for Epac1, we identified a number potential interacting proteins for Epac1. Most of these proteins have cytoskeleton-related

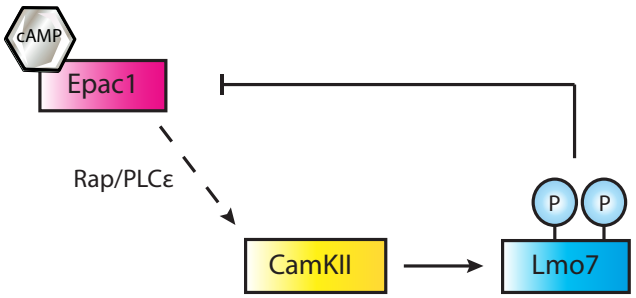

Figure 6: Model for Epac1-Lmo7 negative feedback pathway.
Summarizing model. Activated Epac1 results in CamKII activation via a Rap/PLCε pathway (Oestreich and Wang 2007, Oestreich 2009). Phosphorylated Lmo7 by the action of CamKII can then sequester Epac1 by binding to its CDC25-HD domain, thereby inactivating its GEF activity.

functions, compatible with the role of Epac1 as a GEF for Rap proteins, small GTPases that regulate actin-mediated events, like cell adhesion. Here we evaluated one of these proteins, Lmo7. We confirmed that Lmo7 interacts with Epac1 by co-immunoprecipitation and furthermore we show that Lmo7 sequesters Epac1 to vesicular compartments. The LIM domain of Lmo7 interacts with Epac1 and is responsible for the sequestration of Epac1 to cytosolic compartments. As translocation to the plasma membrane is essential for proper functioning of Epac1 (Ponsioen, Gloerich et al. 2009), this result already predicts that Lmo7 is a negative regulator of Epac1. Indeed, depletion of Lmo7 from MDA-MB231 cells enhances cAMP-induced, Epac1-mediated activation of Rap1. We are currently investigating whether binding of Lmo7 influences downstream effects that require Epac1-mediated activation of Rap1 at the PM such as cell adhesion. The LIM domain interacts with the catalytic region of Epac1, which could imply that Lmo7 directly inhibits catalytic function. We were unable to isolate stable protein to test this directly in vitro, but, interestingly, LIM domains are zinc finger domains that share similarities to domains in RanBP2 that were shown to bind to and inhibit Epac1 catalytic activity (Gloerich, Vliem et al. 2011).

We previously observed by phosphoproteomics that 007 could induce the phosphorylation of the LIM domain of Lmo7 at CamKII consensus sequence sites. Furthermore, the CamKII inhibitor KN-93 inhibited the interaction between Epac1 and Lmo7 and increased cAMP-induced Epac1 translocation. This strongly suggests that phosphorylation events mediate the interaction. Although we were unable to monitor the phosphorylation independently due to the lack of phospho-specific antibodies, a phosphorylation-mimicking mutant of Lmo7 exhibits increased interaction with Epac1. These results suggest that cAMP-induced phosphorylation of the LIM domain of Lmo7, presumably by CamKII, traps Epac1 in a compartment where it is unable to activate Rap1 and thus may constitute a negative feedback loop that controls Epac1 activity (Figure 6). Interestingly, Epac1 has been shown to regulate CamKII activation through Rap and PLCε in cardiomyocytes. In these cells, Epac1 regulates calcium-mediated contraction via CamKII (Oestreich, Wang et al. 2007, Oestreich, Malik et al. 2009). Since both Epac1 and Lmo7 are highly expressed in cardiac tissues, this negative feedback may operate in these cells and may be involved in the regulation of excitation-contraction coupling or may be necessary to dampen the signal after stress or exercise.

Acknowledgements

We thank A. Mull for the Lmo7 constructs and members of our laboratory for helpful discussions. This work is supported by Chemical Sciences (S.V.C.) and the Netherlands Genomics Initiative (J.L.B.) of the Netherlands Organization for Scientific Research (NWO).

Supplemetary information

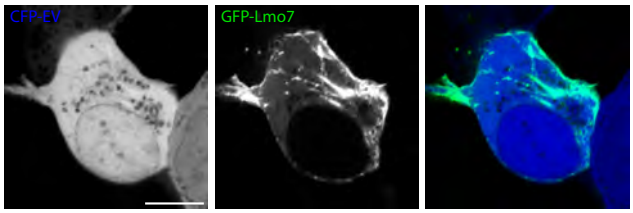

Figure S1:
Representative imaging of CFP-tagged empty vector (EV) and GFP-Lmo7 in HEK293T cells showing distinct localization. Scale bars: 10 µm.

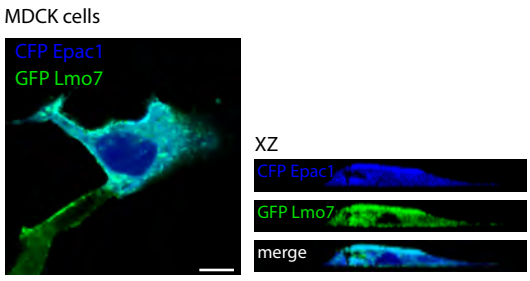

Figure S2:
Representative imaging of CFP-Epac1 and GFP-Lmo7 in fixed MDCK cells with side view (XZ plane) shown on the right panel. Scale bars: 10 µm.

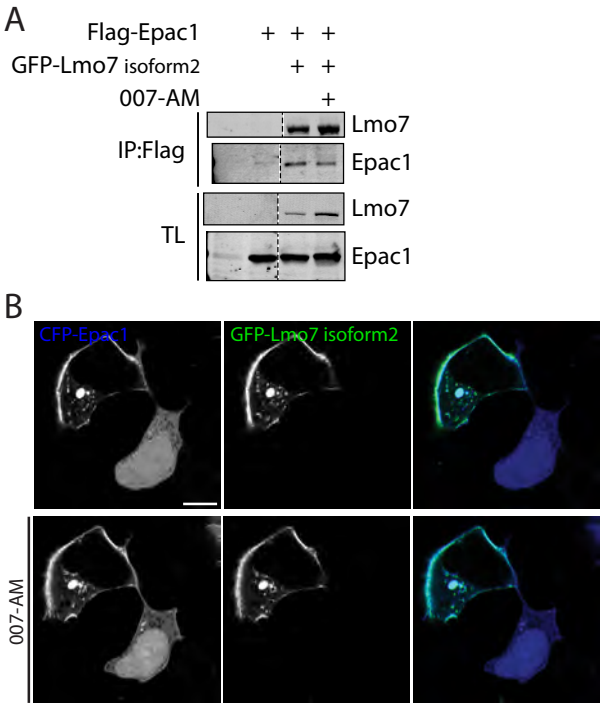

Figure S3:
A) Co-immunoprecipitation of Flag-Epac1 with GFP-Lmo7 isoform 2 in HEK293T cells before and after stimulation with 1 µM 007-AM. B) Representative live-imaging of CFP-Epac1 and GFP-Lmo7 isoform 2 in HEK293T cells showing complete accumulation of Epac1 to the sites where Lmo7 is present before and 10 minutes after stimulation with 1 µM 007-AM.

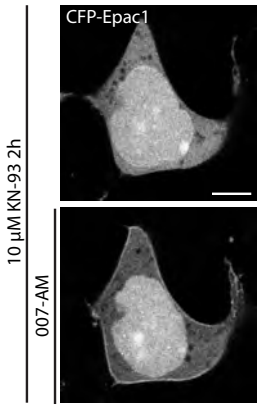

Figure S4:
Representative live-imaging of CFP-Epac1 in HEK293T cells treated with 10 µM of the CamKII inhibitor KN-93 for 2 hours, before and 10 minutes after stimulation with 1 µM 007-AM.

6

Summarizing Discussion

Signal transduction pathways initiate with the binding of a ligand to its cognate receptor in response to extracellular cues. A cascade of signaling events then leads to a cellular response, such as changes in cell morphology, cell fate or transcriptional activation. In order to achieve the desired response, signaling pathways need to be regulated. The modular nature of effectors assures signal specificity, as each domain has determined binding capabilities or catalytic functions. In addition, modifications such a phosphorylation events or allosteric changes ensure that the system can be switched on or off and thus regulated. Compartmentalization is also crucial, as a variety of cellular responses are triggered in response to stimuli only by a limited set of effectors. For instance, G protein coupled receptor signaling makes use of scaffolding complexes to restrict signaling to a particular cellular compartment.

In this thesis we have studied the different mechanisms of regulation of Rap guanine nucleotide exchange factors (GEFs) in time and in space. We have investigated how such modular proteins contribute to Rap signaling efficiency by specific anchoring and cellular targeting mechanisms. We focused on the exchange factors Epac1 and PDZGEF that serve as a paradigm for spatial and temporal control of signaling proteins.

In Chapter 2 we provided insight in the molecular mechanism of regulation of Epac1. We had previously reported that the Rap exchange factor Epac1 is regulated both in time and in space by cAMP (Ponsioen, Gloerich et al. 2009). cAMP binding induces a major conformational change which liberates the substrate binding site in Epac1 (Rehmann, Das et al. 2006). This also results in Epac1 translocation to the plasma membrane (PM) and allows activation of Rap proteins at this compartment. We also found that the DEP domain of Epac1 was responsible for this translocation (Ponsioen, Gloerich et al. 2009). We now show that phosphatidic acid (PA) is the membrane anchor and that the arginine 82 (R82) residue within the DEP domain mediates this binding. A single point mutation of the R82 residue completely abolishes translocation of Epac1 and binding to PA. Importantly, similar to the translocation of Epac1 to the plasma membrane, the binding of Epac1 to PA in vitro is cAMP dependent. We conclude that cAMP induces a conformational change in Epac1 and in the DEP domain that results in exposure of the critical R82 residue and binding to PA. Moreover, we find that the DEP domain of Epac1 is unique in allowing such membrane targeting. Indeed, Epac2, the other isoforms of Epac1, does not translocate to the PM in response to cAMP nor does it bind to PA (discussed in the Addendum Chapter 2). This provides insights into the diversity of regulation and function of the DEP domains.

In Chapter 3 we review the mechanisms of regulation of DEP domains. The DEP domain is best known for its function in allowing membrane anchoring in Dishevelled and RGS proteins (Ballon, Flanary et al. 2006). Together with our finding that the DEP domain of Epac1 is subject to regulation and mediates binding specifically to PA, DEP domains are becoming one of the prime example of a regulated protein domain in signal transduction. DEP domains all share a core structure consisting of an α-helical core and a β-hairpin arm. Superimposition of the DEP domain of Epac1 and Dishevelled (Figure 1) illustrates such similarity but also denotes small differences such as the different orientation of the critical residue required for membrane anchoring. It is therefore the few dissimilarities at the structural level that allow (i) DEP domains to be subject to diverse regulatory mechanisms and (ii) DEP domain-containing proteins to achieve signal specificity.

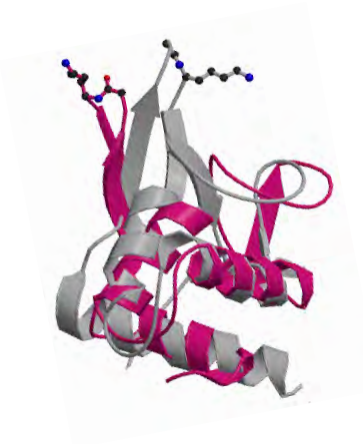

Figure 1: Structure of Epac and Dishevelled DEP domains.
The DEP domain of Epac is shown in grey and the DEP domain of Dishevelled is shown in pink. The residues required for membrane binding (arginine 82 for Epac1 and lysine 434 for Dishevelled1) are represented as ball-and-stick.

In Chapter 4 we describe how another protein module mediates membrane anchoring of the exchange factor PDZGEF during establishment of epithelial cell polarity. We had previously reported that the RapGEF PDZGEF, by binding to PA enriched at the apical membrane, is responsible for activating Rap2a during establishment of brush border formation (Gloerich, ten Klooster et al. 2012). We now show that the PDZ domain of PDZGEF directs binding to PA via electrostatic interactions. Indeed, positively charged side chains in the PDZ domain are required for binding to PA and for localizing PDZGEF at the apical membrane during bush border formation. We demonstrate that the PDZ domain is a lipid-binding module capable of interacting with phospholipids in vitro and in mammalian cells. This provides further insights into the mechanisms by which PDZ domains mediate protein-lipid interactions and into role of PDZ domains in the spatial control of signaling.

In Chapter 5 we describe another targeting mechanism for Epac1. To gain further insights into the complex spatial regulation of Epac1, we made use of mass spectrometry to identify novel binding partners for Epac1. Our screen identified the LIM-domain only 7 (Lmo7) protein as a strong interactor of Epac1. We confirm the interaction and find that Lmo7 sequesters Epac1 by binding to its catalytic domain via its LIM domain. Interestingly, LIM domains are cysteine-rich zinc-binding domains very similar to domains in RanBP2 that were shown to bind to and inhibit Epac1 catalytic activity (Gloerich, Vliem et al. 2011). Possibly, proteins make use of similar mechanisms for regulating cellular signaling in response to different stimuli and in different cellular contexts. From our study we find that the Epac1/Lmo7 interaction results in inhibition of Epac1 activity and in a negative feedback loop. We put forward the hypothesis that such a loop may be employed in the context of cardiac contraction. Indeed, Epac1 has been shown to regulate CamKII-mediated contraction via regulation of calcium levels and to potentiate cardiomyocites contraction during stress or exercise (Ruiz-Hurtado, Morel et al. 2013). Moreover, since both Epac1 and Lmo7 are highly expressed in cardiac tissues, such a negative feedback loop may regulate excitation-contraction coupling or may be necessary to dampen the signal after stress or exercise. Additionally, we find that this binding is regulated by phosphorylation events. Interestingly, the LIM domain-containing Prickle protein (Drosophila) was shown to bind to Dishevelled (Dsh) upon phosphoryla-

tion of Prickle (Daulat, Luu et al. 2012). This Prickle/Dsh interaction blocks the recruitment of Dsh by Fz to the cortex, which is required for PCP signaling, thus resulting in a negative feedback loop mechanism (Tree, Shulman et al. 2002). This might point out a common mode of action of LIM domain-containing proteins as negative regulators of cell signaling. Our results provides further insights into the complex control of cAMP signaling and into the spatial regulation of Epac1.

In conclusion, in this thesis we have described novel aspects of the complex regulation in time and space of exchange factors involved in Rap signaling. We found that similar mechanisms are used by GEFs to control signaling. Moreover, we highlighted how few critical differences can determine signal specificity and enable GEFs to control different aspects of Rap function.
We broadened our understanding of the regulation of exchange factors which will contribute to the unraveling of the control of signal transduction networks in time and space.

S

Summary:

English summary
Nederlands samenvatting
Riassunto in Italiano

English Summary

(also for the outsider)

The cell is the smallest biological unit of all living organisms. In multicellular organisms, like humans, different cell types exist, each adapted to a particular function. In order to survive, cells make use of a complex communication network that regulates their differentiation, growth and specific function.

In order to communicate, cells secrete chemical messengers, such as hormones or neurotransmitters. Chemical messengers bind to specific protein receptors on the surface of the recipient cell. Via a process known as signal transduction, receptors then transmit the information to signal proteins inside the cell that, by relaying the signal to other specialized proteins, affect and alter cellular behavior or gene expression.

An interesting group of signal proteins is the Ras family of proteins. This family includes proteins involved in the control of gene expression, cell proliferation and survival. Ras is the archetype of the family and mutated in 15% of all human cancers. Rap is a close relative that is involved in the control of cell adhesion, migration and polarity. Rap is under tight control of a specific set of signal proteins called guanine nucleotide exchange factors (abbreviated GEFs) that are responsible for its activation. GEFs usually comprise conserved parts, called domains, which have specific structure and function. In this thesis we investigate and describe how these GEFs are in turn regulated in time and in space, with particular focus on the GEFs Epac1 and PDZGEF.

Following the first introductory chapter, in chapter 2 we describe the mechanism behind the regulation of the GEF Epac1 at the molecular level. In response to hormones, Epac1 activates Rap at the outer compartment of the cell, the plasma membrane. Here we find that Epac1 localizes at the plasma membrane by interacting via its DEP domain with the lipid phosphatidic acid. In chapter 3 we focus on the DEP domain, also present in other GEFs proteins, as a prime example of a regulated protein domain in signal transduction. In chapter 4 we investigate the regulation of another GEF, namely PDZGEF. Similarly to Epac1, PDZGEF also activates Rap at the plasma membrane by binding to phosphatidic acid but during a specific cellular process that results in cellular polarization. We find that the PDZ domain of PDZGEF is responsible for interacting with phosphatidic acid. In chapter 5 we describe a different mechanism of regulation of Epac1 by the protein Lmo7. We show that Lmo7 negatively affects Epac1 by inhibiting its localization to the plasma membrane and we unravel the molecular mechanisms and regulation of this interaction. Finally, in chapter 6 we summarize the data obtained and discuss the contribution of the findings towards the understanding of regulation of signal transduction networks by GEFs.

Samenvatting in het Nederlands
(ook voor niet-ingewijden)

De cel is de kleinste biologische eenheid van alle levende organismen. In meercellige organismen, zoals mensen, bestaan verschillende celtypes, elk aangepast om een bepaalde functie uit te voeren. Om te overleven maken cellen gebruik van een complex communicatienetwerk dat hun differentiatie, groei en specifieke functie regelt.
Om te communiceren scheiden cellen chemische boodschappers uit, zoals hormonen of neurotransmitters. Chemische boodschappers binden aan specifieke eiwit receptoren op het oppervlak van de ontvangende cel. Via een proces dat bekend staat als signaaltransductie, geven deze receptoren dan de informatie door aan andere gespecialiseerde eiwitten die het cellulaire gedrag of genexpressie veranderen.
Een interessante groep van signaal eiwitten is de Ras-familie van eiwitten. Deze familie omvat eiwitten die betrokken zijn bij de controle van genexpressie, cel proliferatie en overleving. Ras is het archetype van de familie en is gemuteerd in 15 % van alle menselijke kankers. Rap is een Ras-familielid dat betrokken is bij de controle van celadhesie, migratie en polariteit. Rap staat onder strenge controle van een specifieke set van signaal eiwitten genaamd guanine nucleotide exchange factoren (afgekort GEFs) die verantwoordelijk zijn voor de activering van Rap. Deze GEFs bevatten gewoonlijk geconserveerde delen, genaamd domeinen, die een specifieke structuur en functie hebben. In dit proefschrift onderzoeken en beschrijven we hoe deze GEFs op hun beurt geregeld worden in tijd en plaats, met bijzondere aandacht voor de GEFs Epac1 en PDZGEF.
Na het eerste inleidende hoofdstuk, beschrijven we in hoofdstuk 2 het mechanisme achter de regulering van de GEF Epac1 op moleculair niveau. In reactie op hormonen activeert Epac1 Rap in het buitenste compartiment van de cel, het plasmamembraan. We zien dat Epac1 lokaliseert aan het plasmamembraan door interactie via haar DEP-domein met de lipide fosfatidezuur in het membraan. In hoofdstuk 3 richten we ons specifiek op het DEP-domein, welke ook aanwezig is in andere GEFs, als een typisch voorbeeld van een gereguleerd eiwitdomein in signaaltransductie. In hoofdstuk 4 onderzoeken we de regulatie van een andere GEF, namelijk PDZGEF. Net als Epac1 activeert PDZGEF Rap aan het plasmamembraan door binding aan fosfatidezuur, maar wel tijdens een specifiek cellulair proces dat resulteert in cel polarisatie. Wij vinden dat het PDZ-domein van PDZGEF verantwoordelijk is voor de interactie met fosfatidezuur. In hoofdstuk 5 beschrijven we een ander mechanisme van regulering van Epac1, namelijk door het eiwit Lmo7. We zien dat Lmo7 een negatieve invloed op Epac1 heeft door remming van de lokalisatie aan het plasmamembraan en we ontrafelen de moleculaire mechanismen en regulering hiervan. Tenslotte vatten we in hoofdstuk 6 verkregen gegevens samen en bediscussiëren de bijdrage van de bevindingen aan het begrip van de regulatie van signaaltransductie netwerken door GEFs.

Riassunto in Italiano
(anche per non esperti in campo scientifico)

La cellula è la più piccola unità biologica di tutti gli organismi viventi. Negli organismi multicellulari, come gli esseri umani, esistono diversi tipi di cellule, ciascuno con una particolare funzione. Per sopravvivere, le cellule fanno uso di una sofisticata rete di comunicazione che regola la crescita cellulare, il differenziamento e le specifiche funzioni di ciascun tipo di cellula.
Le cellule comunicano per mezzo di specifici messaggeri chimici, ad esempio ormoni o neurotrasmettitori. I messaggeri chimici riconoscono e si legano a specifiche proteine recettoriali sulla superficie della cellula bersaglio. Attraverso un processo noto come trasduzione del segnale, i recettori trasmettono le informazioni ad una serie di proteine segnalatrici all'interno della cellula che, trasmettendo il segnale ad altre proteine specializzate, a loro volta influenzano il comportamento cellulare o l' espressione genetica.
Un interessante gruppo di proteine segnalatrici è la famiglia delle proteine Ras. Questa famiglia comprende proteine coinvolte nella regolazione dell' espressione genetica, la proliferazione e la sopravvivenza cellulare. Ras è l' archetipo della famiglia ed è mutato nel 15% di tutti i tumori umani. Rap è un parente stretto, coinvolto nel controllo di adesione, migrazione e polarità delle cellule. Rap è sotto stretto controllo di specifiche proteine segnalatrici chiamate fattori di scambio dei nucleotidi guaninici (GEFs in breve) che sono responsabili della sua attivazione. Solitamente i GEFs sono costituiti da parti conservate, chiamate domini, che hanno una struttura e funzione specifica. In questa tesi viene studiato e descritto come questi GEFs vengono a loro volta regolati, con particolare attenzione sui GEFs Epac1 e PDZGEF.
Dopo il primo capitolo a carattere introduttivo, nel capitolo 2 viene descritto il meccanismo della regolazione del GEF Epac1 a livello molecolare. In seguito alla secrezione di ormoni, Epac1 attiva Rap nel compartimento più esterno della cellula, la membrana plasmatica. Questo studio dimostra come Epac1 si localizzi sulla membrana plasmatica tramite il suo dominio DEP interagendo con l' acido fosfolipidico. Nel capitolo 3 viene descritto il dominio DEP, presente anche in altri GEFs, come primo esempio di un dominio di proteine che viene regolato durante la trasduzione del segnale. Nel capitolo 4 indaghiamo la regolazione di un altro GEF: PDZGEF. Analogamente a Epac1, PDZGEF attiva Rap sulla membrana plasmatica legandosi all'acido fosfatidico durante il processo di polarizzazione cellulare. In questo studio troviamo che il dominio PDZ di PDZGEF è responsabile dell'interazione con l'acido fosfolipidico. Nel capitolo 5 viene descritto un diverso meccanismo di regolazione di Epac1 da parte della proteina Lmo7. Lo studio dimostra che Lmo7 influisce negativamente su Epac1 inibendo la sua localizzazione sulla membrana plasmatica. Inoltre, vengono spiegati i meccanismi molecolari e la regolazione di questa interazione. Infine, nel capitolo 6, vengono riassunti e discussi i dati ottenuti.

R

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CV

Curriculum Vitae

Curriculum Vitae

Sarah Valeria Consonni was born on May 12, 1986 in Mariano Comense (CO), Italy. She completed her high school studies at the Liceo Scientifico `Marie Curie´ in Meda (MB), Italy in June 2005.

After receiving her diploma, she studied Biotechnology (applied Molecular Biology) at the University of Aberdeen, Scotland, UK. She graduated with a Bachelor of Sciences (1st class Honours) in July 2009. During her studies in Aberdeen she completed two internships, one in the lab of Prof. Ian Booth in the Department of Microbiology, and one in the lab of Dr. Andy Schofield in the Department of Cancer Research, both at the Institute of Medical Sciences in Aberdeen, Scotland, UK.

From January 2010 she has been working as a Ph.D. student in the lab of Prof. dr. Johannes L. Bos in the Department of Molecular Cancer Research at the University Medical Center Utrecht, The Netherlands. The results of her work are discussed in this thesis.

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Publications

Publications

Consonni S.V., Gloerich M., Spanjaard E., Bos J.L.
cAMP regulates DEP domain-mediated binding of Epac1 to phosphatidic acid at the plasma membrane.
PNAS 2012; 109 (10): 3814-3819

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The PDZ domain of the guanine nucleotide exchange factor PDZGEF directs its binding to phosphatidic acid during brush border formation.
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Consonni S.V., Meijer L.A.T., Brouwer P.M., Chan O.Y.A., Bos J.L.
LIM domain only 7 negatively regulates cAMP-Epac1 signaling.
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Dankwoord - Thanks - Ringraziamenti

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