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Ezrin is an Epac1-anchoring protein that couples receptor activation to Rap1 signalling

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Ezrin is an Epac1-anchoring protein that couples receptor activation to Rap1 signalling

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Abstract

Epac is a cAMP-regulated exchange factor for the small GTPase Rap, which is involved in various cAMP-dependent processes including cell adhesion and secretion. Here we show that Epac1 is particularly abundant at the apical surface of epithelial cells. In search for an apical anchor protein for Epac1 we identified the membrane-cytoskeleton linker protein Ezrin as an Epac1-interacting protein. Indeed Epac1 colocalised with Ezrin at the apical membrane of polarised epithelial cells and at the apical, lumenal surface of kidney tubule epithelial cysts grown in collagen gels. The interaction between Ezrin and Epac1 was dependent on the Ezrin FERM domain and was promoted by receptor activation, suggesting that Epac1 interacts with the open conformation of Ezrin. For Epac1, the N-terminal 49 amino acids are both necessary and sufficient for apical localisation. Importantly, either deletion of Ezrin by RNA interference or disruption of the Epac-Ezrin interaction with the N-terminal 49 amino acids displaced Epac1 from the apical membrane and inhibited Rap1 activation following β-adrenergic receptor stimulation. From these results we conclude that Ezrin is an anchor protein for Epac1 that regulates the apical localisation of Epac1 and couples β-adrenergic receptor stimulation to Rap1 signalling.

Epac1 (exchange protein directly activated by cAMP) is a widely expressed guanine nucleotide exchange factor (GEF) for the small GTPase Rap1 and Rap2[1-4]. The N-terminal regulatory region of the protein consists of a DEP (Dishevelled, Pleckstrin, Egl-10) domain, involved in membrane localization and a cAMP-binding domain. The cAMP-binding domain maintains Epac in an auto-inhibited state in the absence of cAMP[5]. The cAMP analogue 8-pCPT-2'-O-Me-cAMP (007) that specifically activates Epac but not protein kinase A[6], has allowed a number of biological processes to be assigned to Epac1. Most notably, Epac1 has been implicated in integrin-mediated cell adhesion[7], E-cadherin[8,9] and VE-cadherin-mediated cell junction formation[10-13], regulation of sodium proton exchange activity[14] and regulation of secretion[15,16]. The presence of several protein or lipid interaction domains in Epac1 indicates that Epac1 is recruited to specific regions in the cell. Indeed Epac1 is reported to be located in mitochondria[17], in the perinuclear region[18], in cell-cell junctions[8] and in the brush border of kidney tubules[14].
Ezrin is a member of the ERM (Ezrin Radixin Moesin) family of proteins, which link integral plasma membrane proteins with the actin cytoskeleton[19]. ERM proteins possess an N-terminal FERM (band 4.1 ERM) domain and a C-terminal domain which includes an actin binding domain and residues that mediate an intra-molecular interaction with the FERM domain which prevents interaction with the membrane and F-actin[20]. Ezrin is regulated by phosphorylation and by phospholipid binding, which contribute to release of auto-inhibition and thus promote its crosslinking function[21-23]. Ezrin is expressed principally at the apical domain of epithelial cells, where it enhances microvilli formation[24-26]. Ezrin is also involved in the regulation of adherens junctions and focal adhesions and membrane ruffles and consequently plays a role in adhesion and migration[27,28]. In addition to stabilising membrane-actin interactions, Ezrin also functions as a scaffold for cell signalling, associating with the β-adrenergic receptor and PKA (protein kinase A) and thus bringing together a source of cAMP with its target kinase[29,30]. By linking up with PKA-regulated membrane channels, such as NHE3, Ezrin targets this signalling complex to its effector proteins[31]. A recent report showed that Epac also regulates NHE3 function[14].
We show here that Ezrin binds to Epac1 and is responsible for the targeting of Epac1 to the apical membrane. Binding of Epac1 requires the active conformation of Ezrin. Indeed, receptor stimulation induces this association. Furthermore, the Epac-Ezrin interaction is required to mediate β-adrenergic receptor-dependent activation of Rap. From these results we conclude that Ezrin is an anchor protein for Epac1, that regulates the apical localisation of Epac1 and couples β-adrenegic receptor stimulation to Rap1 signalling.

Results

Apical localisation of Epac1

We showed previously that Rap1 is activated by the Epac1-specific cAMP-analogue 007 in the ovarian carcinoma cell line OvCar3[6]. We therefore examined the expression of Epac1 in these cells. We observed that the monoclonal antibody 5D3, which was raised against Epac1, recognized a double band of the predicted size of Epac1. These bands were absent in cells treated with siRNA against Epac1 (Fig. 1A). Two additional Epac1 siRNAs also suppressed

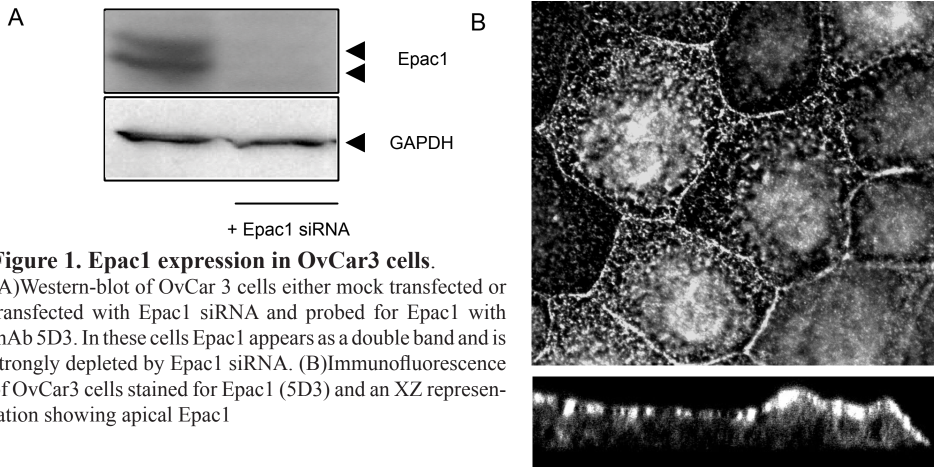

Figure 1. Epac1 expression in OvCar3 cells. (A)Western-blot of OvCar 3 cells either mock transfected or transfected with Epac1 siRNA and probed for Epac1 with mAb 5D3. In these cells Epac1 appears as a double band and is strongly depleted by Epac1 siRNA. (B)Immunofluorescence of OvCar3 cells stained for Epac1 (5D3) and an XZ representation showing apical Epac1

Epac1 expression (not shown). Moreover, a polyclonal antibody raised against Epac1, α-Epac1-1, recognized the same two bands (not shown). Confocal analysis of OvCar3 cells showed that Epac1 was located predominantly in punctate structures and at sites of cell-cell contact, a staining that was abolished after treating the cells with Epac1 siRNA (Fig. 1B upper panel, and data not shown). Interestingly, Z-stack analysis of OvCar3 cells demonstrated a predominantly apical plasma membrane localisation of Epac1, suggesting that the punctate structures represented microvilli (Fig 1B lower panel).
To study the apical localisation of Epac1 further, we used Madin Darby Canine Kidney cells (MDCK), which develop a polarised 'cobblestone' morphology with distinct apical and basal-lateral domains. Since MDCK cells do not express detectable levels of Epac1, we stably expressed human Epac1 in these cells by retroviral transduction[8]. The localisation of Epac1 in these cells was similar to that observed in OvCar3 cells (Fig. 2A). Z-stack analysis of cells co-stained for Epac1 and the tight junction marker ZO-1 showed that Epac1 is located predominantly at the apical surface. This result was corroborated by immunoelectron microscopy (EM) showing that the majority of Epac1 is located in microvilli at the apical surface of the cell. In addition to the apical localisation, Epac1 was found in the perinuclear region, including the Golgi (Fig. 2B), particularly in less polarized cells. We conclude that in polarized epithelial cells, the majority of Epac1 is present in microvilli at the apical plasma membrane.

Epac1 interacts with the apical membrane-cytoskeleton linker protein, Ezrin
To identify possible apical anchor proteins for Epac1 we performed a yeast 2-hybrid screen of a human placenta cDNA library using full length Epac1 as bait. This screen identified Ezrin and Radixin as Epac1-interacting proteins (9 and 5 colonies respectively). Ezrin and Radixin are members of the Ezrin-Radixin-Moesin (ERM) family of proteins, that exist either in a closed, auto-inhibited state whereby a C-terminal region interacts with the FERM domain, or in an open conformation which permits association with interacting proteins (see introduction). Importantly, Ezrin is predominantly localized in apical membranes[32] and thus is a prime candidate for being the apical anchor protein for Epac1. Co-immunoprecipitation experiments were performed to test whether Epac1 interacts with

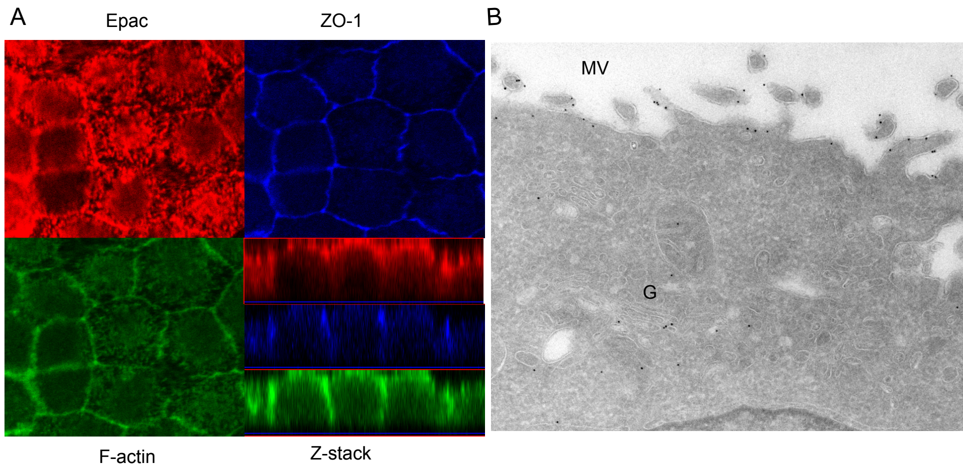

Figure 2. Apical Epac1 expression in polarised MDCK cells.
(A) Immunofluorescent micrographs of confluent MDCK cells stably expressing Epac1 stained with anti-Epac1 monoclonal antibody (5D3), showing a predominantly apical localisation of Epac1. Tight junctions are labelled with anti-ZO-1 polyclonal antibodies and Alexa-488 phalloidin to label filamentous actin. XZ representations are shown in the lower right panel. (B) Transmission electron microscopy image of MDCK-Epac1 cells labelled with a-Epac1-1. The microvilli (MV) and the Golgi aparatus (G), which show most Epac1 labelling are indicated.

Ezrin *in vivo*. HA-tagged Epac1 was transfected into 293T cells together with flag-tagged forms of either full-length Ezrin or with the N-terminal 492 amino acids of Ezrin (Ezrin-N3) lacking the auto-inhibitory C-terminus (Fig. 3A). Immunoprecipitation of Ezrin-N3, resulted in co-precipitation of Epac1 (Fig. 3B). In contrast, full-length Ezrin only weakly associates with Epac1 suggesting that Epac1 interacts preferentially with the FERM domain-containing N-terminal region of Ezrin and not with auto-inhibited Ezrin. Similarly, Epac1 also interacted with Radixin-N3 but not full-length Radixin (not shown). To confirm that the endogenous proteins interact as well, we examined the interaction of Epac1 and Ezrin in ACHN human kidney carcinoma cells. Indeed, Ezrin co-immunopreciptates with Epac1 (Fig. 3C). S1P induces activation of the small GTPase Rho, and the subsequent synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2)[33], which promotes Ezrin association with the plasma membrane and acquisition of an open conformation[23,34]. We therefore tested whether stimulation of ACNH cells with S1P would result in an increased association between Epac1 and Ezrin. Indeed, co-immunoprecipitation of Ezrin by Epac1 was enhanced when ACNH cells were stimulated with sphingosine-1-phosphate (S1P) (Fig. 3C). Co-immunoprecipitation of endogenous Epac1 and Ezrin was also observed in OvCar3 cells following S1P stimulation (not shown). We conclude from these results that Epac1 interacts with the open conformation of Ezrin *in vivo*. These results further imply that the interaction is regulated by stimuli that activate Ezrin.

Epac1 contains a number of domains that mediate protein-protein interactions, nucleotide exchange and membrane localisation (Fig. 3D). We therefore examined a series of deletion mutants of Epac1 to identify the region of interaction with Ezrin. We observed that deletion of the N-terminal 49 amino acids (Epac1-Δ49) strongly reduced co-immunoprecipitation with Ezrin-N3 (Fig. 3E), suggesting that Ezrin interacts with the N-terminal 49 amino acids of Epac1.

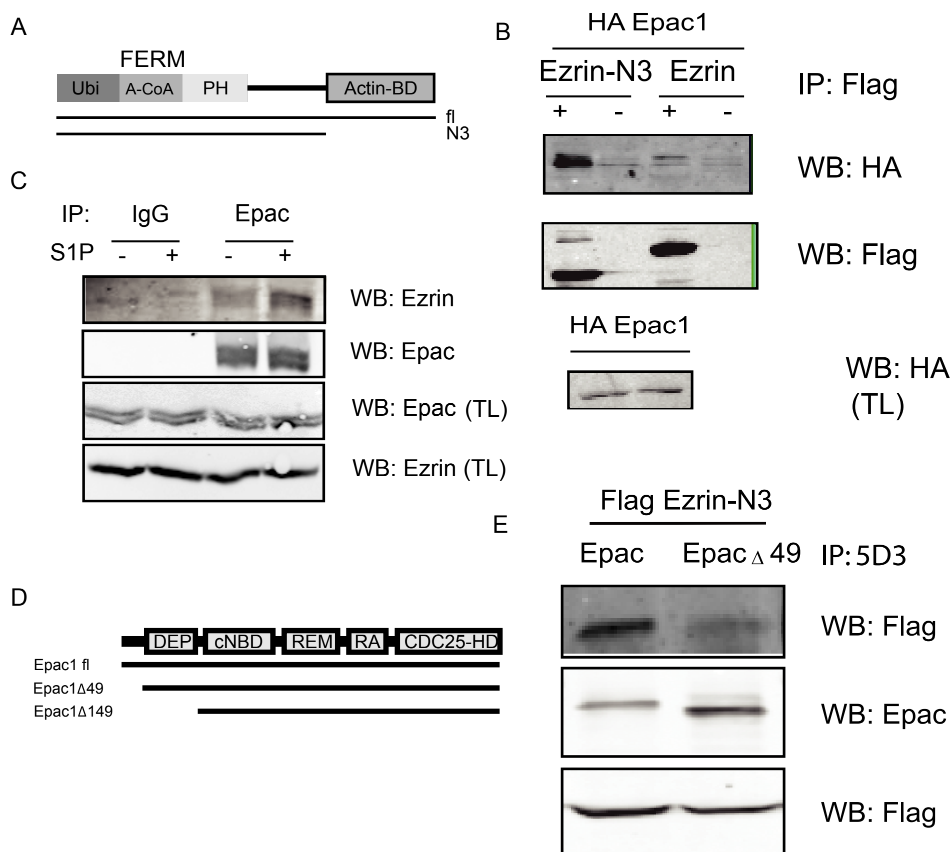

Figure 3. Epac1 interacts with ezrin in vitro.
(A) Domain structure of Ezrin, showing the N-terminal FERM domain, which consists of a ubiquitin-like fold, acyl Co-A binding region and a pleckstrin homology (PH) domain. The C-terminal contains an actin-binding domain and residues required for interaction with the N-terminal. (B) Anti-HA western blot (top panel) shows co-immunoprecipitation of flag-Ezrin with HA-Epac1 from lysates of 293T cells. Flag-tagged Ezrin-N3 but not flag-tagged full length Ezrin precipitates HA-Epac1 using beads coupled to anti-flag antibodies (+) but not beads coupled to anti-myc control antibody (-). Western blots also show that equal amounts of flag-tagged Ezrin were precipitated (middle panel) and HA-Epac1 was equally present in the total lysates, which were subsequently divided for the flag and control IP's (lower panel). (C) Co-immunoprecipitation of endogenous Ezrin with anti-Epac1 antibodies (5D3) from lysates of ACHN cells stimulated with sphingosine 1-phosphate (S1P), but not from unstimulated cells (upper panel). Epac1 was equally precipitated by 5D3 antibodies, but was not precipitated by control anti myc antibodies (IgG). Ezrin and Epac1 were present equally in the cell lysates (lower two panels). (D) Domain structure of Epac1: DEP domain also contributes to membrane targeting, nucleotide binding domain (NBD), Ras Exchange Motif (REM), Ras association (RA) domain and CDC25 homology catalytic domain. The deletion mutants used in this study are also shown. (E) Anti-flag blot showing co-immunoprecipitation of flag-Ezrin-N3 by myc-tagged full length Epac1 (Epac), but less by myc-tagged Epac1Δ49 (EpacΔ49). The lower panel shows approximately equal expression of Ezrin-N3 in original lysates.

The N-terminal tail of Epac1 is required for co-localization with ezrin at the apical plasma membrane.

We next investigated whether Epac1 colocalizes with Ezrin in the apical membrane. We observed that transiently expressed Myc-tagged Epac1 colocalises with Ezrin at punctate structures (Fig. 4A, upper panels). We also examined co-localisation of these proteins in cells grown in 3-dimensional collagen gels, which allows a more developed level of apical-basolateral polarisation. Under these conditions, stably expressed Epac1 shared an almost

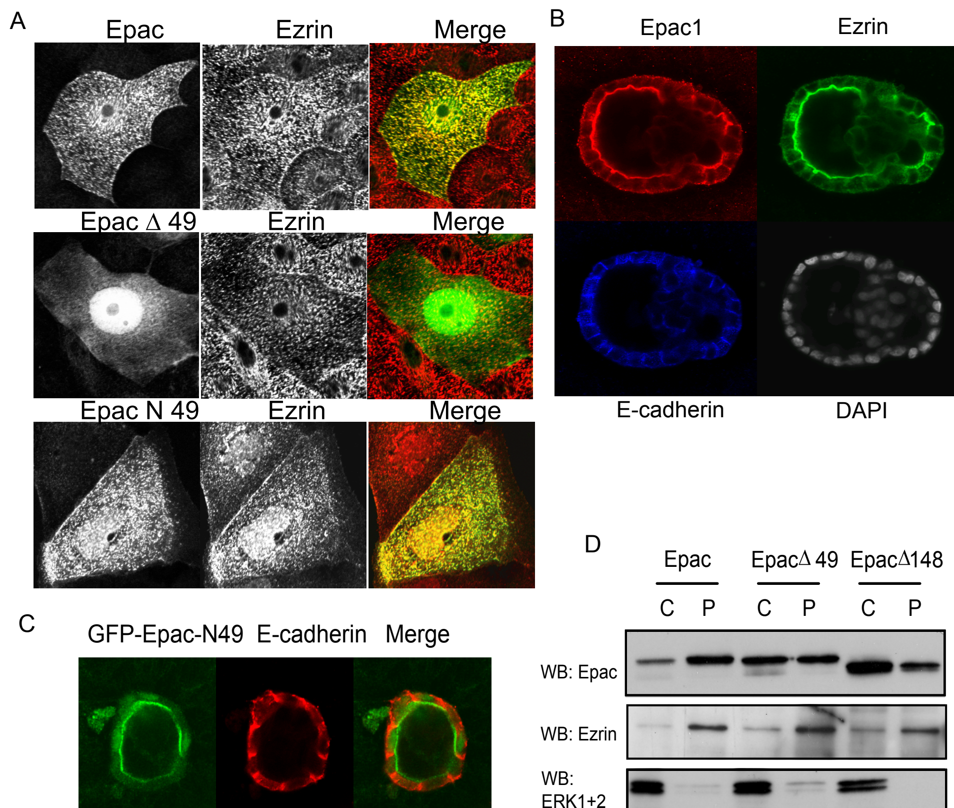

Figure 4. Ezrin colocalisation with Epac1.

(A) Confocal micrographs of MDCK cells transiently transfected with myc-tagged full length Epac1, myc-tagged Epac1Δ49 (lacking the N terminal 49 residues) and CFP fused to the N-terminal 49 amino acids of Epac1 (CFP-Epac1-N49). Cells were labelled with anti-Ezrin antibodies. Myc-tagged Epac1 proteins are labelled with anti-Epac1-1 polyclonal antibodies. CFP fluorescence reveals CFP-Epac1-N49 localisation. Note, cells were fixed 40 h after transfection and seeding on coverslips and are therefore quite flat. (B) Epac1 expressing MDCK cells grown in a 3-dimensional collagen gel and stained with anti-Epac1 polyclonal antibody, anti-Ezrin monoclonal antibody, anti E-cadherin rat monoclonal antibody (DECMA) and DAPI to label nuclei. (C) Confocal micrographs of MDCK cells stably expressing GFP fused to the N-terminal 49 residues of Epac1 (GFP-Epac1-N49), showing an apical distribution. E-cadherin staining (DECMA) is also shown. (D) Fractionation of MCF-7 cells expressing full length or deletion mutants of Epac1 showing that both the N49 tail and DEP domain contribute to membrane localisation of Epac1. C: cytosol, P: particulate fraction. Erk1/2 and Ezrin are localised predominantly in the cytosol and particulate fractions respectively.

identical distribution with Ezrin at the apical lumenal surface of MDCK epithelial cell cysts (Fig. 4B), supporting the notion that these proteins interact. Interestingly, non polarised clusters of cells showed a disordered distribution of both Ezrin and Epac1. In agreement with the failure of Epac1-Δ49 to interact with Ezrin, this mutant did not show the punctate microvillar distribution and did not co-localise with Ezrin. Instead, Epac1-Δ49 showed a cytoplasmic and nuclear distribution, while the distribution of Ezrin remained punctate (Fig. 4A, middle panel). Interestingly, a fusion protein of CFP with the isolated N-terminal 49 amino acids of Epac1 (CFP-Epac1-N49) did colocalise with Ezrin at microvilli (Fig. 4A, lower panel). Similarly, GFP-Epac1-N49 also localised to the lumen of epithelial cysts (Fig. 4C). The microscopy findings were confirmed by cell fractionation experiments; deletion of N49 resulted in an increase in Epac1 present in the cytosolic fraction. Deletion of an extended region of the N terminus of Epac1 which includes the DEP domain (Epac1Δ148) resulted in a further increase in cytosolic Epac1, confirming a previous report that the DEP domain contributes to the membrane localisation of Epac1 (Fig. 4D and[2]). Together, these results show that the majority of Epac1 colocalizes with Ezrin and that the N-terminal region of Epac1 is responsible for the colocalization.

Ezrin targets Epac1 to the apical membrane.

The above findings indicate that Ezrin targets Epac1 to the apical plasma membrane. To test this further, we treated OvCar3 cells with siRNA to deplete Ezrin and examined the localisation of Ezrin and Epac1 by immunofluorescence. The efficiency of Ezrin knockdown varied between cells producing a mosaic of Ezrin expression. We observed that in cells where Ezrin was strongly depleted, Epac1 is localised to the nucleus (Fig. 5 upper panels). We also examined the effect of simultaneous depletion of Ezrin, Radixin and Moesin on Epac1 distribution. Western blot analysis confirmed that all three proteins were depleted by at least 50% (not shown). We observed a similar effect on knockdown of Ezrin and mislocalisation of Epac1 as when Ezrin alone was depleted (Fig. 5 lower panels). From these results we conclude that indeed Ezrin is required for the apical localisation of Epac1 and suggest that in OvCar3 cells, Radixin (and Moesin) do not contribute significantly to this localisation.

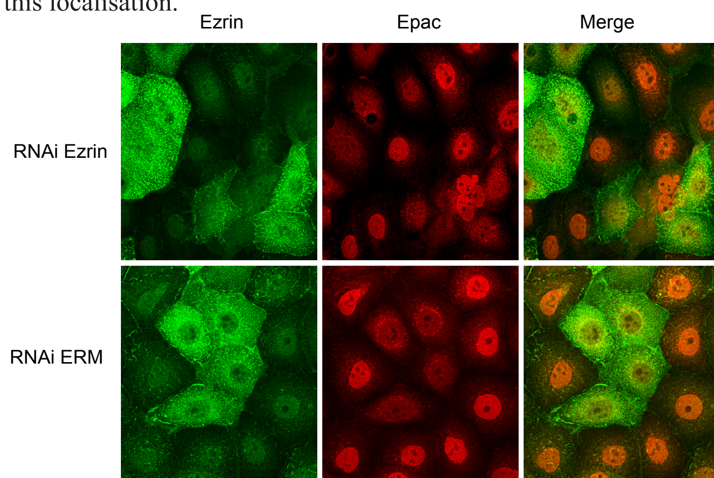

Figure 5. Ezrin is required for Epac1 localisation and function. Confocal microscopy of OvCar3 cells labelled with anti-Epac1 and anti-Ezrin antibodies following treatment with siRNAi oligos for Ezrin (upper panels) and Ezrin, Radixin and Moesin combined (lower panels). Note that in Ezrin depleted cells, the corresponding localisation of Epac1 is disrupted, resulting in strong nuclear localisation.

Apical targeting of Epac1 is required for efficient Rap1 activation.
To determine whether the apical localisation of Epac1 was important for its function in activating Rap, we expressed deletion mutants of Epac1 in A14 NIH 3T3 fibroblasts and examined Rap1 activation using 'pull-down' assays. Overexpression of full length Epac1 was sufficient to induce HA-Rap1 activation in the absence of additional cAMP-inducing stimulation. We observed that deletion of N49 from Epac1 strongly reduced its capacity to activate Rap1. Deletion of N148, which also includes the DEP domain, completely abolished the capacity of Epac1 to activate Rap1 (Fig. 6A). Thus, the ability of Epac1 to activate Rap1 correlated with its localisation to the plasma membrane (compare with Fig. 4D). Previous studies showed that deletion of the N-terminal 148 amino acids of Epac1 does not affect the regulation of Epac1 activity *in vitro*[2]. Taken together, these findings suggest that localisation of Epac1 via its N-terminal 49 amino acids are required for efficient activation of Rap1 in cells.

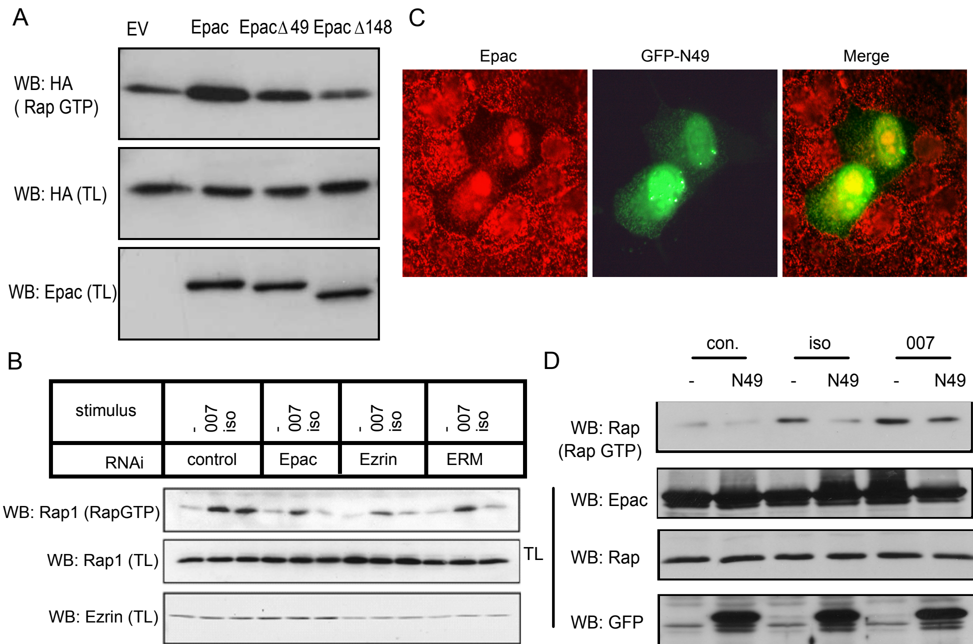

Figure 6. Ezrin-Epac1 interaction is required for Rap1 activation.
(A) Deletion of the N-terminal 49 amino acids and DEP domain of Epac1 impair their capacity to activate Rap. Empty vector, full length myc-Epac1, myc-Epac1-Δ49 and myc-Epac1-Δ148 were transfected into A14 NIH 3T3 cells together with HA-Rap1. Precipitated active HA-Rap1, and expression of HA-Rap1 and myc-Epac1 constructs in total cell lysates are shown. (B) Pull-down assay of active Rap1 from OvCar3 cells treated with siRNA duplexes for Epac1, Ezrin and Ezrin, Radixin and Moesin combined or control oligos. Cells were treated with 007 (100 uM) for 15 minutes, isoproteronol (100 uM) for 2 minutes or buffer control. The upper panel shows the amount of active Rap1 precipitated from cells. The middle panel shows that equivalent levels of Rap1 were present in cell lysates. Ezrin levels were reduced both by Ezrin and by pooled ERM siRNA oligos (Lower panel).
(C) Confocal microscopy showing the displacement of Epac1 in cells transiently expressing GFP-Epac1-N49.
(D) Pull down assay of activate Rap from MDCK-Epac1 cells with or without stable expression of GFP-Epac1-N49 and stimulated with 007 (100 uM, 15 minutes) or isoproteronol ('iso', 100 uM, 2 minutes). Rap, Epac1 and GFP-Epac1-N49 expression in total lysates are also shown.

Ezrin is required for efficient activation of Rap1 by Epac.
Ezrin is reported to associate both directly and indirectly with the β-adrenergic receptor (β-AR), one of the stimuli that activate Rap1 through Epac[7]. We therefore hypothesized that Ezrin brings Epac1 into the proximity of β-AR-induced local increases in cAMP concentrations and is thus required for efficient coupling of β-AR signalling to Rap1. To test this hypothesis, we examined the effect of RNAi-mediated depletion of Ezrin on the activation of Rap1 by β-AR stimulation. Stimulation of OvCar3 cells with isoproteronol to activate β-AR results in activation of Rap1 in OvCar3 cells and also induced Rap1 activation in cells which had been treated with scrambled siRNA. However, Rap1 activation induced by isoproteronol treatment was inhibited in cells depleted of Ezrin (Fig. 6B). Rap1 activation induced by the Epac-specific analog 007 was also inhibited by Ezrin RNAi, although to a lesser extent. Simultaneous depletion of Ezrin, Radixin and Moesin had a similar effect on Rap1 activation as depletion of Ezrin alone. These findings suggest that Ezrin couples adrenergic receptor signalling to Epac-Rap1 activation. Since the effect of Ezrin depletion on Rap1 activation could also be due to more general disruption of microvilli, we examined the effect of inhibiting the Ezrin-Epac1 interaction using the N-terminal 49 amino acids of Epac1, which we predicted would function as a competitive inhibitor of the interaction. Indeed, when overexpressed, GFP-Epac1-N49 results in the release of Epac1 from the microvilli (Fig. 6C). In addition, GFP-Epac1-N49 potently inhibited isoproteronol-induced Rap1 activation and also induced moderate inhibition of 007-induced Rap1 activation (Fig. 6D). We conclude from these experiments that Ezrin-dependent targeting of Epac1 to the apical plasma membrane is required for efficient coupling of β-AR stimulation to Epac-Rap1 signalling.

Discussion

We show here that Epac1 is localised predominantly on microvilli at the apical plasma membrane of various epithelial cell lines. Furthermore, we identified the apical membrane-cytoskeleton cross-linking protein, Ezrin, as a binding partner of Epac1. Ezrin is a member of the Ezrin-Radixin-Moesin (ERM) family of proteins that contain a C-terminal actin binding domain and an N-terminal FERM domain, which interacts with integral membrane proteins. ERM proteins exist either in a closed, auto-inhibited state whereby a C-terminal region interacts with the FERM domain, or in an open conformation which permits association with interacting proteins. The conclusion that Epac1 interacts with Ezrin is based on a number of observations. Firstly, Ezrin binds to Epac1 in a yeast two hybrid screen. Secondly, endogenous Epac1 co-immunoprecipates with endogenous Ezrin. Thirdly, Epac1 and Ezrin co-localize at the apical site of cells. Fourthly, the N-terminal 49 amino acids of Epac1 are required both for apical localisation and for interaction with Ezrin. Finally, knock-down of Ezrin results in a release of Epac1 from the apical surface. We therefore concluded that indeed Ezrin is a *bona fide* interaction partner of Epac1 that targets Epac1 to the apical plasma membrane. An apical localisation of Epac1 was previously described in proximal tubes of the kidney[14], a result confirmed by us using our antibodies (data not shown). This implies that the apical localisation is physiologically relevant.
We mapped the region required for Epac1 binding to the first 492 amino acids of Ezrin,

which contains the FERM domain. This region is also required for interaction with other Ezrin binding partners, such as EBP50/NHERF, ICAM-2, CD44 and FAK[35-37]. Further deletions of either the N or C termini disrupted interaction with Epac1 – presumably due to disruption of the tertiary structure of the FERM domain. Epac1 did not bind to full length Ezrin, suggesting that the interaction was suppressed by the C-terminal auto-inhibitory region. Indeed, addition of the C-terminal region of Radixin inhibits Epac1 association with the N-terminal part of Radixin (not shown). Consistent with a requirement for an open Ezrin conformation, the interaction between endogenous Epac1 and Ezrin was induced upon stimulation with sphingosine-1-phosphate (S1P), which via Rho activation induces PIP2 synthesis, which has been shown to promote Ezrin conformational activation. This indicates that Epac1 activity is subject to dual regulation – firstly by agonists that induce increases in intracellular cAMP and thus Epac1 activation and secondly, by agonists that regulate Ezrin conformational activation and thus Epac1 targetting.

We showed previously that stimulation of the β-AR results in Epac-mediated activation of Rap1[7]. Interestingly, Ezrin also associates both directly and indirectly via EBP50 with the β-AR[29,38], which after stimulation induces cAMP through activation of adenylate cyclase. We therefore tested whether association of Epac1 with Ezrin is required for an efficient coupling of the β-AR to Epac-Rap1 signalling. We observed that depletion of Ezrin by siRNA inhibited Rap1 activation induced by isoproteronol and to a lesser extent by the Epac1 specific analogous 007. This was unlikely to be a consequence of disruption of the apical membrane due to Ezrin loss, since expression of Epac1-N49, which functions as an interfering peptide and releases Epac1 from the apical site, also inhibited isoproteronol-induced Rap1 activation. Importantly, we observed only a partial inhibition of Rap1 activation when the cells were incubated with 007. Also, when we expressed Epac1 lacking the first 49 amino acids, we observed a residual Rap1 activation, indicating that Epac1 that is not linked to the apical membrane can still activate Rap1 although less efficiently. This decreased efficiency is not due to an intrinsic defect of the Epac1 protein, since deletion of the first 148 amino acids has no apparent effect on the ability of Epac1 to activate Rap1 in vitro[2]. We conclude from these results that the targeting of Epac1 to the apical plasma membrane by Ezrin is required for β-AR-mediated activation of Epac1 and subsequent activation of Rap1.

The localisation of Epac1 at the lumenal surface is consistent with recent reports describing a role for Epac1 in the regulation of cytoplasmic pH via the Na^+/H^+ exchanger (NHE3) at the brush border of proximal tubules[14] and exocytosis in the collecting ducts in the kidney[16]. NHE3 also binds directly to Ezrin[39], suggesting that the Ezrin-Epac1 interaction that we describe here is important for NHE3 regulation. Unfortunately, we were thus far unable to make this connection.

Not all functions of Epac can be attributed to a localisation at the apical site of cells. For instance, we have previously reported that Epac-Rap1 signalling enhances integrin-mediated cell adhesion[7] and the formation of E-cadherin-mediated cell-cell contacts[8,9,40]. Interestingly, Ezrin also localises to and regulates cell-cell contacts in epithelial cells[41] and via binding to FAK, mediates focal adhesion formation[27]. Whether the interaction of Epac with Ezrin plays a role in these processes remains to be investigated. It should be noted however, that Epac1 is found at other locations as well, including the plasma membrane and

the perinuclear region. We did not observe significant co-staining with the mitochondrial marker mitotracker (not shown) a result which is at variance with a previous report[17]. Epac1 has been observed at the nuclear envelope in other cell types cells[42]. These differences may reflect cell type variation, but more specifically, may be a consequence of differences in apical-basolateral polarity and the lack of competition for Epac1 by apically localised or conformationally active Ezrin. Interestingly, deletion of the N-terminus of Epac1, depletion of Ezrin by RNA interference or overexpression of the isolated N-terminus of Epac1 all resulted in the nuclear localisation of Epac1. The factors regulating the nuclear localisation of Epac1 and the intriguing possibility that Epac1 performs a signalling function in the nucleus require further investigation.

The interaction of Epac1 with Ezrin is also interesting with respect to the observation that Ezrin binds PKA[30]. In addition, Epac1 was found to interact with phosphodiesterase 4D3[18]. This intriguing network of interactions suggests that Ezrin may play host to an entire cAMP-dependent signalling pathway, including the activating β-AR. A similar role was recently described for mAKAP, a muscle specific adaptor protein which coordinates a complex between PKA, Epac1 and PDE4D3[18]. Coordinated activation of PKA and Epac1 on AKAPs may turn out to be a common feature in cAMP-dependent signalling.

We have shown here that Ezrin targets Epac1 to the apical plasma membrane of polarised epithelial cells, coupling adrenergic receptor activation with the Rap signalling pathway. This gives insight into the mechanism of Epac1 control. It was shown previously that Epac, through the cAMP binding domain, interacts with the light chain of microtubule associating protein[43]. Further proteins domains that may mediate interactions are the DEP domain and the RA-domain. Although the interaction partners of these domains are currently unclear, one can envision complexity in the regulation of Epac1, a feature that seems to be common for GEFs of small GTPases.

Methods

Constructs and antibodies

To generate vectors containing Epac1 full length (aa 2-881), Epac1Δ148 (aa 149-881), Epac1Δ49 (aa 50-881) and Epac1-N49 (aa 1-49), the corresponding coding sequences of human Epac1 (GI:3978530) were introduced into a donor vector, pDONR201 (Invitrogen) to allow sub-cloning by the "Gateway"-procedure (Invitrogen). The constructs were then recombined in pcDNA3-Flag-His, pcDNA3-Myc-, pcDNA-meGFP-, pcDNA-meCFP- and pcDNA3-meYFP-destination vectors according to standard protocols (Invitrogen). Ezrin was obtained as clone IRALp962A182.1 from the Deutsches Ressourcenzentrum für Genomforschung (RZPD) (Berlin, Germany). Using Gateway adapted PCR, we amplified Ezrin and Ezrin-N3 (residues 1 to 492) and cloned these into pDONR201 and then into pcDNA3-flag as above. pcDNA-meCFP and pcDNA-meYFP were kindly provided by O. Rocks and P. Bastiaens (EMBL, Heidelberg, Germany). Ezrin-GFP was kindly provided by Erik Sahai (Cancer Research UK, London).

The mouse Epac1 monoclonal antibody (mAb) 5D3 and the rabbit Epac1 polyclonal antibody (pAb) (α–Epac1-1) were raised against recombinant Epac1Δ148. Rabbit pAb against ZO-1 was from Zymed. Anti-Rat-E-cadherin mAb (DECMA) was from Sigma. Ezrin was from Becton Dickinson. Anti-flag was from Kodak. Anti-HA monoclonal antibody 12CA5

was purified by our laboratory. Anti-ERK1/2 rabbit pAb was generated by our laboratory. Goat-anti-mouse/rabbit/rat Alexa 405,488,568 and 647 secondary antibodies were from Molecular Probes. Fluorescein and Rhodamine phalloidin was from Sigma.

Cell culture:
MDCK-II, MCF7, 293T and A14 cells were routinely cultured in DMEM provided with 10% FCS, 0.5% glutamine, penicillin and streptomycin (all from BioWhittaker, Belgium) at 37 °C in 6.0 % CO2. OvCar3 cells were from ATCC and were cultured as above in RPMI. ACHN cells were cultured in Eagle's MEM contained 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The MDCK Epac1 cell line was generated and cultured as described before8.

Yeast 2-hybrid screening
A human placenta random primed library was custom-screened with Epac1 full length and Epac1 residues 1-328 (Epac1: RapGEF 3, homo sapiens, GI: 3978530) by Hybrigenics S.A. (Paris, France) as previously described[44].

Transfection.
MCF7 were transfected with the indicated constructs using FuGENE 6 (Roche, Diagnostics). MDCK cells were washed with PBS containing 1 mM EGTA, detached by trypsinisation (PBS + 0.2% trypsin, 1 mM EDTA), resuspended in complete medium at a density of $1X10^7$ cells/ml and transfected with 20ug of the indicated constructs by electroporation (1000F, 260v). A14 cells were transfected with using the calcium phosphate method. Cells were lysed or fixed 40 h after transfection.
For si-RNA transfection of OvCar3 cells, siRNA duplexes were diluted in Optimem (Gibco). Oligofectamine (Invitrogen) was added and the mix added to cells according to manufacturers instructions. siRNA oligos were as follows: Epac1 (No.1 GACCGGAAGTACCACCTTA; No.2 CCATCATCCTGCGAGAAGA; No.3:GCACCTACGTCTGCAACAA) were from Dharmacon, siRNA's against Ezrin (No.1GAACAGACCTTTGGCTTGGAGTTGA; No.2 TGGCCTCCA CTATGTGGATAATAAA; No.3 CCTCAAAGAGTGATGGACCAGCACA), against Radixin (GCCAGGCTACCTGGCTAATGATAGA), and Moesin (TGGCCTCGTATGCTGTCCAGTCTAA) were from Invitrogen. Ezrin siRNA is the mixture of No.1, 2 and 3; ERM siRNAi is the mixture of Ezrin No.1, Radixin and Moesin oligos).

Co-immunoprecipitation
293T cells were transiently transfected with 10 µg of plasmid DNA as indicated in the figure legends. After culturing for 40 h, cells were lysed in 1 ml of 1% Triton X-100 buffer containing additionally 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, aprotinin (1 µg/ml), and pepstatin (1 µg/ml). Cell lysates containing equal amounts of total protein were incubated for 2 h at 4°C with 10 µl of antibody pre-coupled protein A-Sepharose beads (Pharmacia). The beads were washed extensively with lysis buffer and bound proteins were

analysed by SDS-PAGE and Western blotting. For immunoprecipitation of Epac1 from ACHN cells, cells were lysed in a buffer containing 20 mM Tris pH 8.0; 1% Triton X-100, 0.5% Na-DOC; 10 mM EDTA; 150 mM NaCl and protease inhibitors. After clearing the lysate, lysates were incubated in the presence of 1 mM 8-Br-cAMP (MP biochemicals) with anti-Epac1 monoclonal antibody 5D3 and protein A beads for 2 h. 8-Br-cAMP is included in the IP since binding of Epac1 5D3 is dependent on an open (cAMP-bound) conformation (JZ unpublished findings). Precipitates were washed and subjected to SDS-PAGE and Western blotting.

Subcellular fractionation
MCF7 cells were treated with hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 1 mM $NaVO_3$, 1 µM leupeptin, 1 µM aprotinin) and homogenized through a 23G 11/4 microlance**.** Trypan blue staining of the cell lysate showed that more than 90% of the cells were broken. The lysate was centrifuged directly at 100,000g at 4°C for 90 minutes and the cytosol-containing supernatant was removed and SDS-sample buffer added (C). The pellet, containing all non-cytosolic material, was washed in lysis buffer and dissolved in sample buffer (P).

Immunofluorescence of cells cultured in 2-dimensions and in 3-dimensional collagen gels.
Cells were cultured on glass coverslips. Before fixation, medium was removed and cells were washed three times with ice-cold PBS. Fixation was performed with ice-cold methanol for 2 min for OvCar3 cells or with 3.8% formaldehyde for 20 min for MDCK cells, followed by permeabilisation with 0.2% TritonX-100 for 10 min. The samples were incubated with blocking buffer containing 4% goat serum and 0.2% bovine serum albumin (BSA) in PBS for 1 h. Cells were labelled with primary antibody for 2 h followed by washing 3 times with PBS. Alexa-conjugated secondary antibodies were applied for 1h. MDCK cells were cultured in collagen I gels as previously described[45]. Briefly, MDCK-II cells stably expressing Epac1 were suspended in a solution containing in 1% collagen and 3.7g/l $NaHCO_3$ in complete medium and added to 24-well plates and allowed to polymerise at 37°C for 20 min. Complete medium was added and replaced every 3 days. For tubulogenesis experiments, to prepare gels for microscopy, gels were incubated with collagenase (Sigma), (5ug/ml), then fixed for 30 minutes in a solution containing 3.7% formaldehyde, 10 mM PIPES pH 6.8, 0.3 M sucrose, 100 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.1% Triton X-100. Gels were washed, permeabilised further with 0.2% Triton X-100 for 5 min , blocked for 1 h with 10% FBS with 0.01 M glycine in PBS. Gels were incubated overnight in primary antibody, washed 3 times in PBS and then incubated for at least 3 h with secondary antibody. Gels were washed extensively with PBS and mounted between glass slide and cover slip in Immu-mount (Shandon). Images were recorded using a LSM510 laser scanning confocal microscope (Zeiss Microimaging). All pictures were obtained by confocal microscopy.

Electron Microscopy

MDCK Cells were fixed by adding 4% freshly prepared formaldehyde and 0.4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 to an equal volume of culture medium for 10 min, followed by post-fixation in 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 without medium. Cells were stored until further processing in 1% formaldehyde at 4ºC. Processing of cells for ultrathin cryosectioning and immuno-labeling according to the protein A-gold method was done as described[46]. In brief, fixed cells were washed with 0.05 M glycine in PBS, scraped gently from the dish in PBS containing 1% gelatin and pelleted in 12% gelatin in PBS. The cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose and afterwards mounted on aluminum pins and frozen in liquid nitrogen. To pick up ultra thin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used[47].

Rap1 pull down and Western blotting

OvCar3 cells were seeded in 10 cm dishes at 40% confluence the day before treatment with siRNA duplexes as described above. A14 cells were transiently transfected with the constructs as indicated. Active Rap1 was determined by 'pull-down' assay as described previously[48]. In brief, cells were stimulated as described, washed with ice-cold PBS and lysed in a buffer containing 1%NP40, 150 mM NaCl, 50 mM Tris-Cl pH 7.4, 10% glycerol, 2 mM $MgCl_2$ with leupeptin and aprotinin. After clearing by centrifugation, active Rap1 was precipitated with the recombinant Rap1 binding domain (RBD) of RalGDS fused to GST immobilized on glutathione beads. Active Rap1 precipitates and samples of total cell lysate were resolved by SDS-PAGE, transferred to PVDF membrane and probed with the appropriate primary and secondary antibodies.

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Addendum

Cell scattering induces nuclear accumulation of Epac1

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In chapter 5, we showed that the EzB domain (the first 49aa) is required and sufficient for association to Ezrin/Radixin and for localization in microvilli. Indeed, removing the EzB domain (EpacΔ49) abolished the ability of Epac1 to bind Ezrin/Radixin and microvillar localization. Surprisingly, EpacΔ49 accumulates in the nucleus (Fig. 1). This promoted us to investigate whether disruption of microvillar structures, for instance in non-polarized cells, may result in a translocation of Epac to the nucleus. We therefore pretreated MDCK-Epac1 cells with HGF which induces cell scattering and consequently disrupts the structure of the microvilli and determined the localization of Epac. After staining with Epac1 Ab, a clear nuclear signal was observed (Fig 2. A). To confirm this result, we used MDCK cells stably expressing GFP-tagged Epac, and indeed also GFP-Epac translocates to the nucleus upon HGF treatment (Fig 2. B). Since overexpression of RapV12 induces cell spreading[1,2] and consequently, destroys the microvilli, we predicted that overexpression of RapV12 in MDCK-Epac1 cells also resulted in the accumulation of Epac1 in the nucleus. Indeed in all cells that do express RapV12 we observe Epac1 in the nucleus (Fig. 3). From these results we conclude that Epac1 accumulates in the nucleus once the microvillar structure was destroyed. One plausible explanation for the release of Epac1 from the microvilli is that Ezrin/Radixin is inactivated as a prerequisite or a consequence of scattering. However, why Epac translocates into the nucleus is unclear. There are a number of explanations. It may be that Epac always needs to be anchored either to the microvilli, or to the perinuclear region (Chapter 4) and that nuclear translocation is an artifact of overexpression. However, we do see some nuclear staining of Epac1 in OvCar 3 cells which expresses Epac1 endogenously after depleting Ezrin with siRAN oligos (Chapter 4 Fig. 5). Alternatively, the nucleus serves as a sink for Epac1 to keep it away from Rap1. However, more challenging is whether Epac does have a function in the nucleus. It has been reported that Rap1 may be in the nucleus

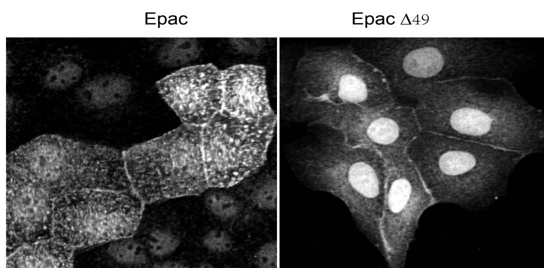
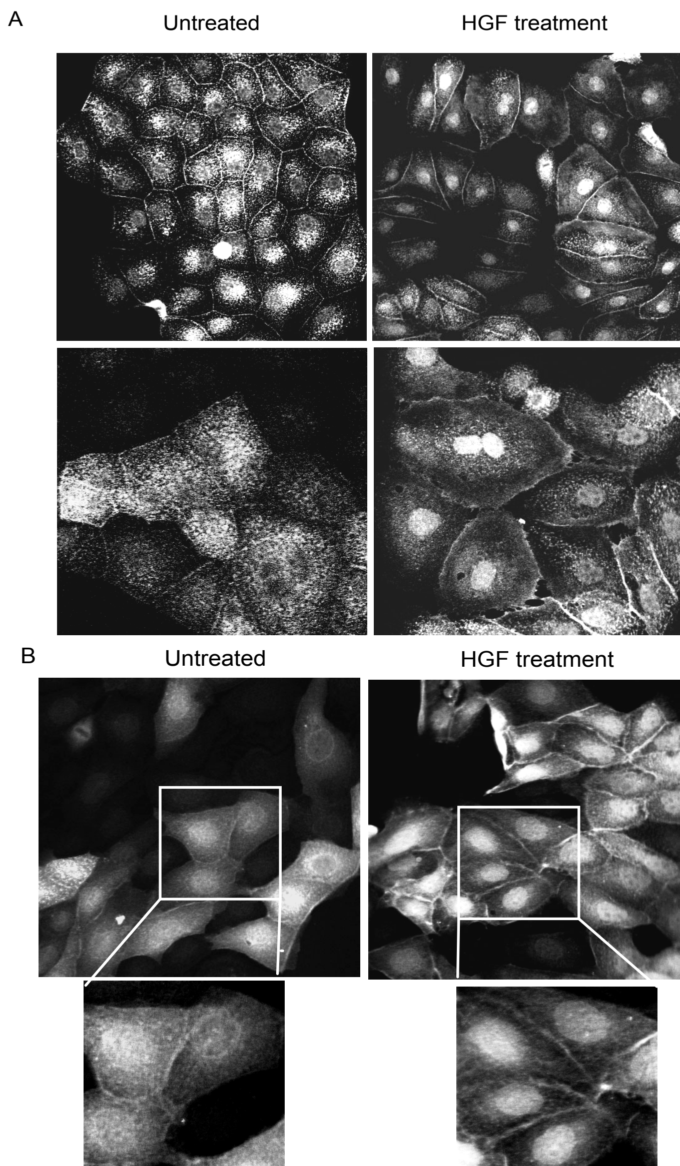


Figure 1. Removing the N-terminus tail induces Epac shifting from microvilli to the nucleus
HA-tagged Epac1 and Epac1Δ49 were transfected into MDCK cells, followed by staining with Epac1 mAb 5D3 (1:500).

Figure 2. HGF induced nuclear accumulation of Epac
MDCK-Epac1 (A) or MDCK-GFP-Epac1 cells (B) were seeded on cover slides and treated with HGF (21ng/ml) for 18h, followed by fixation with 3.8% formaldehyde and labeled with Epac1 pAb (2293) (1:250) (A) or directly rinsed with PBS and mounted on glass slides after fixation with 3.8% formaldehyde (B). In Fig.1 (A), the magnification of upper panel of is 40X; the lower panel is 63X. In Fig1 (B), the lower panel is the enlargement of the upper panel.



as well[3], but whether that is indeed the case remains to be analyzed, particularly since Rap1 needs to be membrane-bound for proper functioning. Alternatively, nuclear Epac1 has a function distinct from Rap1.
How is Epac translocated to the nucleus? Sequence analysis did not reveal a clear nuclear localization signal. Recent mutational analysis of Epac indicated that a region in the catalytic domain (between amino acids 764 and 838) is responsible for the association to the nuclear fraction, but this fraction also contains the perinuclear membranes[4].

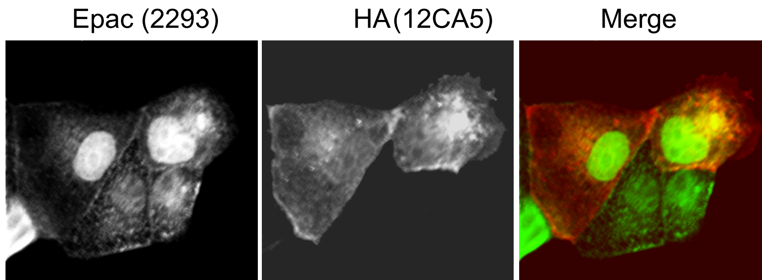


Figure 3. Overexpressed RapV12 induced nuclear accumulation of Epac
MDCK-Epac1 cells were transiently transfected with HA-tagged RapV12, followed by fixation and label with both anti-HA mAb (12CA5 tissue culture supernatant 1:50) and Epac1 pAb (2293 1:250).

In this respect, it is interesting to note that in a yeast two hybrid screen Epac1 was found to interact with RanBP2 (Zhang Z and Bos J.L., unpublished observation), a giant scaffold protein in the nuclear pore which is involved in nuclear translocation. This interaction between Epac1 and RanBP2 may also explain the frequently strong staining of Epac1 with the nuclear envelope (see for instance Fig. 2 of Addendum of Chapter 3.), but may also be instrumental in the translocation of Epac to the nucleus.

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