Signaling of the direction-sensing FAK/RACK1/PDE4D5 complex to the small GTPase Rap1

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We recently reported that a complex between focal adhesion kianse (FAK) and the molecular scaffold RACK1 controlled nascent integrin adhesion formation and cell polarization, via peripheral recruitment of the cAMP-degrading PDE4D5 isoform. Here we review and extend these studies by demonstrating that the FAK/RACK1/PDE4D5 'direction-sensing' complex likely functions by signaling, via the guanine nucleotide exchange factor EPAC, to its small GTPase target Rap1. Specifically, activating EPAC suppresses polarization of squamous cancer cells, while, in contrast, modulating PKA, the other major cAMP effector, has no effect. Moreover, FAK-deficient malignant keratinocytes re-expressing a FAK mutant that cannot bind to RACK1, namely FAK-E139A,D140A, display elevated Rap1 that is linked to impaired polarization. Thus, it is likely that the FAK/RACK1/PDE4D5 complex signals to keep Rap1 low at appropriate times and in a spatially-regulated manner as cells first sense their environment and make decisions about nascent adhesion stabilization and polarization. RACK1 is abundantly expressed in both normal and malignant keratinocytes, while FAK and PDE4D5 are both elevated in the cancer cells, suggesting that the FAK/RACK1/PDE4D5/Rap1 signaling axis may contribute to FAK's well documented role in tumor progression.

Cell Polarization and Nascent Adhesion Structures: RACK1

Cell polarization requires the differential localization of specific protein complexes within cells, as well as the correct spatial distribution of structures like Golgi and the microtubule-organizing center. Understanding the signals that regulate spatial events is important, since polarization and directional migration are key to many physiological processes such as embryonic development, inflammatory responses, wound healing and during cancer cell invasion. Although important for directional migration, the mechanisms underlying assembly and spatial distribution of signaling complexes as cells first contact ECM remain to be fully defined. Previous studies have identified protein complexes that regulate polarization in a number of contexts. For example, integrin-induced activation of Cdc42 controls the ability of astrocytes to polarize in response to a wound made in a confluent cell monolayer, by leading to the activation and recruitment of the mPar6/PKC complex.[1,2]

Cell polarization and directional migration also require the coordinated and dynamic regulation of the actin cytoskeleton, critically at structures close to the protruding edge and cell-substratum interface as cells move forward. Mass spectrometry studies have identified proteins that can bind transiently to focal adhesion components, and demonstrated the existence of nascent adhesion structures, originally called Spreading Initiation Centers (SICs). These are actin-based structures that form transiently as

cells first make contact locally with the ExtraCellular Matrix (ECM; reviewed in ref. 3). Exaggerated versions of SICs form in close proximity to the cell-substratum interface as suspended cells are plated onto ECM, and so first make contact with ECM components. Therefore, SICs probably represent nascent or 'first point of contact', structures that assemble and disassemble rapidly as cells adhere and move around on ECM components. SICs have been described to contain ribosomal RNA and RNA-binding proteins, which contribute to cell spreading[3] and are clearly distinct from mature focal adhesions. In our recent work, we described nascent adhesive structures that may be similar to the previously described SICs, and these are distinguished from other focal adhesion structures by containing the signaling scaffold protein Receptor for Activated C Kinase 1 (RACK1; reviewed in ref. 4), which is not generally found at mature adhesions.[3,4]

RACK1: A Complex with FAK Important for Nascent Adhesion Assembly

RACK1 is a widely expressed WD-repeat protein that was originally identified as a protein that could bind phorbol ester-activated PKC isoforms, notably PKCβII.[5-8] However, RACK1 is now known to regulate diverse cellular processes such as protein translation, cytokinesis, cell adhesion and migration. RACK1 is a multi-functional scaffold protein that binds to a wide variety of proteins, impacting on multiple signal transduction pathways via compartmentalization, so bringing together key signaling components in time and space.[8-10] Amongst the increasing repertoire of RACK1 binding partners are Src and Abl tyrosine kinases,[11,12] β1-integrin cytoplasmic domains,[13] the p85 regulatory sub-unit of PI 3-kinase,[14] a number of protein tyrosine phosphatases, including PTP1B[13,15-17] and PTPmu,[17] PLCγ,[18] dynamin,[10] p120Ras-GAP,[19] HIF1α[20] and others (reviewed in ref. 8 and 21). Notable amongst RACK1 binding partners is the cAMP-degrading phosphodiesterase PDE4D5, which binds to RACK1 via two well-defined sites, one within its isoform-specific amino-terminal region

and the other within its conserved catalytic unit.[22,23] A number of these RACK1 protein interactions influence integrin function, and integrin-dependent cell migration and chemotaxis.[13,15,16] RACK1 binds to the integrin effector protein Focal Adhesion Kinase (FAK) and RACK1 tyrosine phosphorylation by c-Abl is proposed to mediate insulin-like growth factor1-dependent regulation of FAK.[24]

FAK is present and co-localizes with RACK1, at nascent protrusive structures that form as Mouse Embryo Fibroblasts (MEFs) spread on ECM components.[4,25] FAK is a pivotal signal integrator operating at focal adhesions (its biological functions are depicted in **Fig. 1A**) and is required for cell spreading, optimal integrin-dependent cell migration and integrin-induced signaling that promotes proliferation and survival.[26-32] FAK's role in assembly of actin-based structures that mediates cell spreading includes the binding and regulation of the Arp2/3 actin nucleation complex.[25] Nascent lamellipodia, which originate at the tips of SICs, do not form in FAK-deficient cells or in cells in which FAK cannot be tyrosine phosphorylated after integrin engagement.[4,25] Thus, FAK links integrin signaling directly to the actin assembly machinery, and we have implicated the FAK FERM domain in this process.[4,25]

In more recent work, we identified that the FAK FERM domain, specifically residues E139 and D140, which is required for direct binding of FAK to RACK1. Unlike the previously identified complex between FAK and Arp3, where binding is regulated by integrin induced FAK-Y397 phosphorylation, the FAK/RACK1 complex does not change under these conditions. Using Squamous Cell Carcinoma (SCC) cells, in which FAK could be genetically deleted by tamoxifen treatment, we found that FAK is required for both cell spreading and RACK1 localization to nascent adhesions close to the cell-substratum interface as imaged by Total Internal Reflection Fluorescence microscopy (TIRF).[4] Small patches of FAK/RACK1 co-localization are also evident at nascent adhesive structures in fully spread cells. Disruption of this interaction through expression

of an effecter mutant of FAK, namely FAK-E139A,D140A, results in profound changes in cellular morphology reminiscent of non-polarized cells that lack cytoplasmic actin filaments (**Fig. 1B** and reviewed in ref. 4). Moreover, RACK1 localization is restricted to the cytoplasm and the FAK-E139A,D140A-expressing cells are unable to efficiently form nascent adhesion structures when compared to FAK-wt-expressing SCC cells (**Fig. 1C**; solid arrows point to nascent adhesions in FAK-wt cells and broken arrows point to cytoplasmic RACK1 in FAK-E139A,D140A cells). These data indicate that the FAK/RACK1 complex is spatially regulated at nascent adhesions.

FAK/RACK1: A "Direction-Sensing Complex"

While typical apical-basolateral epithelial polarity is frequently lost in cancer cells, polarization and directional migration towards extracellular stimuli (and perhaps host cells) likely plays a role in cancer invasion and metastatic spread. However, little is known about protein complexes specifically involved in direction-sensing and how invasive cancer cells turn and polarize, towards extracellular stimuli. We found a role for the FAK/RACK1 complex in cell invasion and wound induced cell polarity. In particular, FAK is needed for wound-induced polarization of SCC cells and chemotactic invasion through 3D matrix gels.[4] We believe this represents a novel "direction-sensing complex" that is essential for the formation of nascent adhesion structures which permit cells to sense and polarize towards chemotactic factors. As the FAK/RACK1 complex was also evident in fully spread cells, we used time-lapse microscopy to monitor RACK1 position in live cells. This demonstrated that cells are constantly generating RACK1 containing membrane "blebs", which can stabilize and these transient structures presumably act as environmental sensors; the pattern of their stabilization and turnover, i.e., their dynamic regulation controls cell polarization and migration in response to directional cues (Movies can be viewed in Sup. Information in ref. 4).
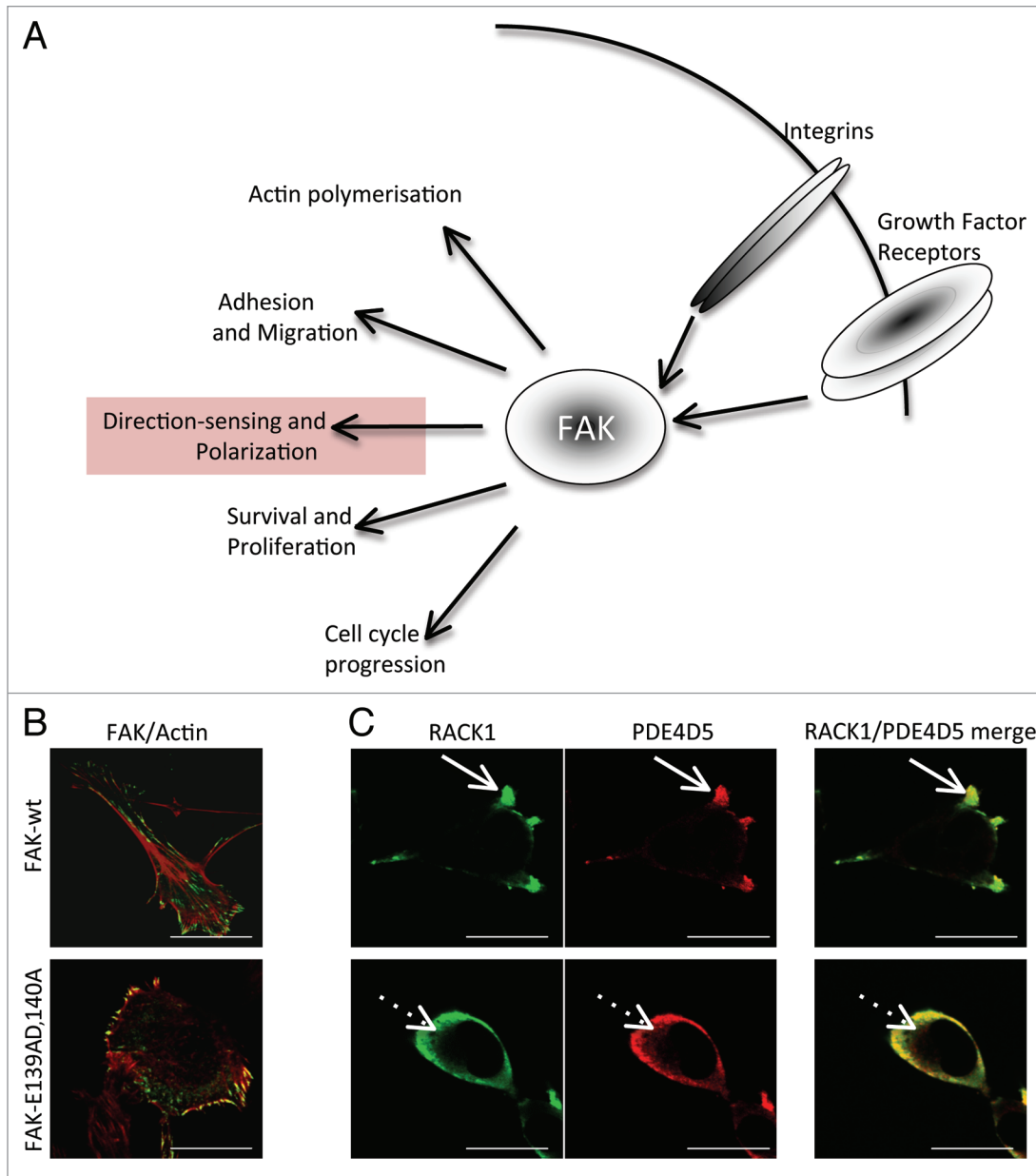
Figure 1. (A) Schematic showing the recognized cellular functions of FAK, highlighting the direction-sensing and polarization functions we describe here. (B) Images show comparison of cell shape and actin filaments in FAK-deficient SCC cells re-expressing either FAK-wt or the FAK/RACK1 binding impaired mutant FAK-E139A,D140A. (C) The co-staining of RACK1 and PDE4D5 is shown in protrusive nascent adhesions as FAK-wt SCC cells are plated on to FN for 15 min (solid arrows). By comparison, both RACK1 and PDE4D5 are cytoplasmic in FAK-E139A,D140A-expressing cells (broken arrows). Scale bars 20 μm.

How does FAK/RACK1 Complex Control Direction-Sensing?: Recruitment of PDE4D5

A key question was why inhibiting FAK binding to the enzymatically-inert scaffold protein RACK1 blocked stabilization of transient nascent adhesion structures and caused profound direction sensing and polarization defects. We addressed the nature of the sequestered cargo delivered by FAK-bound RACK1 to nascent adhesions, and found a key role for the RACK1 cargo—a particular PDE4 family isoform, namely PDE4D5,[23] known to bind to RACK1 through a well-characterized interaction involving its isoform specific N-terminal region.[33,34] Inhibition of the FAK/RACK1 complex blocked the co-recruitment of RACK1 and PDE4D5 to nascent adhesions (also shown here in **Fig. 1C**) and to the leading edge of polarizing cells.[4] Moreover, a complex exists containing FAK, RACK1 and PDE4D5 that requires FAK's association with RACK1 and RACK1's association with PDE4D5, and this complex is necessary for nascent adhesion stabilization and wound-induced polarization.[4] Our work showed that when the FAK-RACK1 complex is perturbed, neither RACK1 nor PDE4D5 can localize to nascent protrusions or to the leading edge of wounded cells. However, the

question remained as to the nature of PDE4D5-induced or suppressed, signals that are permissive for stabilization of nascent adhesion, cell polarization and directional migration.

FAK/RACK1/PDE4D5: Signaling via PKA or EPAC?

It is now well established that cAMP signaling is compartmentalized in cells with gradients of cAMP interpreted by sequestered populations of the two cAMP effectors, Protein Kinase A (PKA) and EPAC.[34] Sub-populations of spatially constrained PDEs form and shape these gradients at defined locales, thereby determining the activation threshold for specific signaling complexes that incorporate either or both, PKA and EPAC.[34] The role of EPAC can usefully be inferred by using the selective EPAC agonist, 8-CPT-2OMe-cAMP (CPT-cAMP), which is unable to activate PKA.[35] We found that CPT-cAMP treatment of FAK-wt cells elicited a profound loss of wound-induced polarization (~71% to ~31%), similar to the polarization displayed by FAK-deficient cells (~27%, data not shown) or in cells where FAK and RACK1 can no longer interact (~28%) (FAK-E139A,D140A termed E139 in **Fig. 2A**), suggesting that the cAMP polarization phenotype regulated by the FAK/RACK1/PDE4D5 complex may be determined by the action of EPAC rather than PKA (**Fig. 2A**). We also noted that CPT-cAMP did not induce any further loss of polarization in FAK-E139 cells, implying the FAK/RACK1 disrupting mutation and CPT-cAMP influence the same signaling effector, namely EPAC. Consistent with this, we now report that the selective PKA agonist, N6-Bnz-cAMP,[35] had no effect on polarization, even although it visibly induced phosphorylation of the transcription factor substrate of PKA, i.e., CREB (cAMP-regulatory binding protein; **Fig. 2B**, right part). Furthermore, loss of polarization in cells challenged with the cAMP analogue, 8-Brm-cAMP, which can activate both EPAC and PKA, was not attenuated by the selective PKA inhibitor, KT5720 (**Fig. 2A**). This occurred under circumstances where the PKA-mediated phosphorylation of CREB was clearly inhibited by KT5720 (**Fig.**

2B). Thus, it appears that elevated cAMP exerts its inhibitory effect on polarization through an action mediated by EPAC, rather than by PKA.

RACK1 Mediates Signaling from FAK to the Small GTPase Rap1

EPAC exerts actions via its role as a guanine-nucleotide exchange factor for the Rap small GTP binding proteins.[35] We found that challenge of FAK-wt cells with CPT-cAMP (the EPAC agonist) increased Rap1 activation, as judged by increased GTP loading, providing evidence that CPT-cAMP does indeed activate EPAC in these SCC cells (**Fig. 2C**, right part). Importantly, we also found that the activity of Rap1 in FAK-E139A,D140A cells was increased when compared to FAK-wt cells (**Fig. 2C**, left part), while there was no change in Cdc42 activity that is often associated with polarity (not shown). Quantification shown is a combination of multiple experiments and demonstrated a consistent 1.8 fold increase in Rap1 activity in FAK-E139A,D140A cells vs. FAK-wt cells. As this FAK-E139A,D140A mutant fails to sequester the PDE4D5/RACK1 complex and stabilize nascent adhesions (or SICs) during cell spreading, our data imply that in cells expressing this FAK/RACK1 binding-impaired FAK mutant protein—cAMP levels to rise unchecked in the vicinity of FAK at the periphery of cells as they sense their environment. In this way, EPAC and its downstream target Rap1, would become constitutively activated. This uncontrolled activity of EPAC/Rap1 is linked to the loss of ability to stabilize nascent adhesive structures, and consequentially impaired cell spreading and responses to directional cues that are needed for cells to polarize.

Final Thought

Thus, we previously defined a novel "direction sensing complex" that comprises of FAK, RACK1 and PDE4D5. This is needed for cells to stabilize nascent actin-based adhesive structures and to polarize in response to directional cues. This work identified a new function for the FAK FERM domain, and a physiological function for the enigmatic RACK1-PDE4D5

complex. Indeed, both FAK and RACK1 are molecular scaffolds that can bind independently to a wide range of intracellular proteins, yet these two scaffold proteins also bind to each other, with FAK dictating the spatial distribution of RACK1 and one of its clients, PDE4D5. The assembly of this complex presumably regulates local gradients of cAMP. Here we show that this likely regulates EPAC-dependent activation of the small GTPase Rap1. This new work 'joins up' a line of research articles that show the EPAC-Rap1 signaling axis has an important role in integrin adhesion dynamics, migration and polarity.[35-37] We interpret our results and these previous findings to imply that Rap1's role in cell polarization requires both GTPase activation and inactivation to be tightly regulated in space and time, and that the FAK/RACK1/PDE4D5 complex serves to keep Rap1 transiently inactive at appropriate times in the vicinity of nascent adhesions as they form and stabilize. The FAK mutant protein that would result in an inability to depress Rap1 activity in the vicinity of nascent adhesions as they form causes lack of stabilization (depicted in model in **Fig. 3A**).

The spatially determined FAK/RACK/ PDE4D5-EPAC/Rap1 signaling axis may well contribute to FAK's role in promoting the invasive cancer phenotype, that we showed previously by tissue-specific FAK deletion studies in skin and breast cancer models.[38,39] Several studies have also implicated release of cAMP gating, PDE enzymes and PDE4 in particular, in cancer cell invasion and migration of a number of cell types.[40-42] Hence the direction-sensing FAK/RACK1/PDE4D5 complex, via signaling to EPAC and Rap1 in space and time, is a likely contributor to the invasive cancer phenotype. In this regard, we note that while RACK1 is abundantly and equivalently expressed in both murine normal and malignant keratinocytes (SCC cells) (**Fig. 3B**), both FAK and PDE4D5 are elevated in SCC cells, showing that the pathway potential is elevated during progression to the malignant phenotype.

Methods

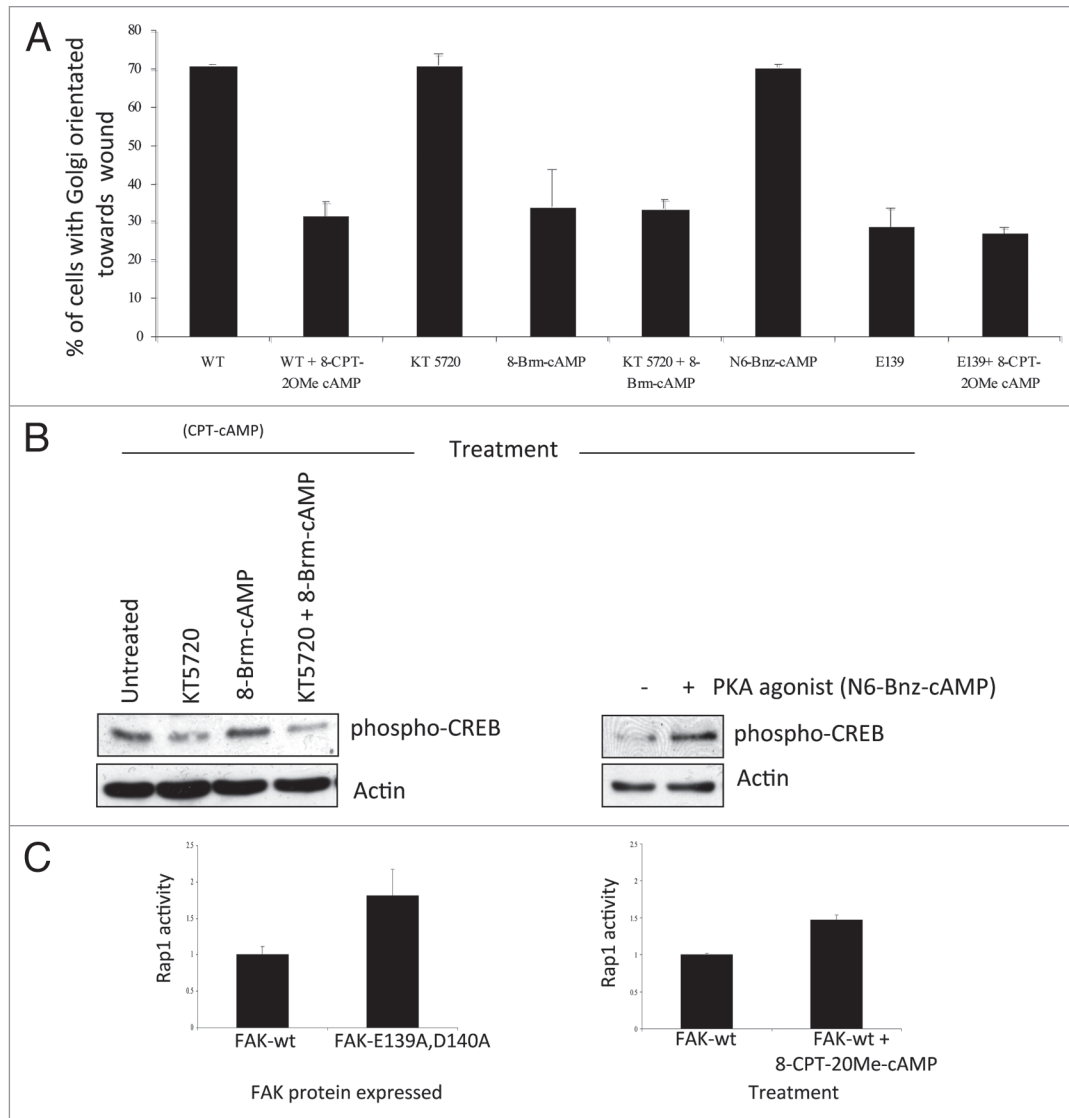**Materials.** Antibodies for immunoblotting and immunocytochemistry were

Figure 2. (A) FAK-wt or FAK-E139A,D140A cells (as indicated) were plated on FN for 2 hours and wounded in the presence of 8-CPT-2OMe cAMP (CPT-cAMP; 10 μM), KT 5720 (1 μM), 8-Brm-cAMP (10 μM), KT 5720 + 8-Brm-cAMP or N6-Bnz-cAMP (10 μM). After 1.5 hours cells were fixed and stained with the Golgi marker anti-GM130, TRITC phalloidin and DAPI, to assess polarization in response to the wounded areas. The percentage of each cell type with the Golgi orientated to the wound was calculated by counting 100 cells in 3 experiments and is shown in the graph. (B) FAK-wt cells were treated with KT 5720 (1 μM) and/or N8 = Brm-cAMP (left parts) and with 6-Bnz-cAMP (10 μM) for 30 min then immunoblotted using anti-phospho-CREB and actin antibodies as probes. (C) FAK-wt cells with or without CPT-cAMP (10 μM for 3.5 hrs, time point chosen to maintain consistency with cell polarization experiments, conditions detailed above; left part) or FAK-wt and FAK-E139A,D140A cells (right part), were harvested and a Rap1 assay performed as described in Materials and Methods. Quantification of Rap1-GTP normalized to total Rap1 from three separate experiments is shown.

as follows: anti-FAK (Transduction Laboratories, 610088); anti-actin (Sigma, A4700); anti-pCREB (Cell Signaling Technology, 9196); and anti-PDE4D5 (described previously in ref. 4). Anti-mouse and anti-rabbit IgG-peroxidase conjugated secondary antibodies were from New England Biolabs (7076, 7074), anti-sheep IgG-peroxidase conjugated secondary antibodies were obtained from Invitrogen and TRITC-phalloidin was obtained from Sigma. Human fibronectin (FN) and reduced growth factor Matrigel were obtained from Becton Dickinson, (354230). Triton X100, TRITC-phalloidin and 4-OHT were obtained from Sigma.

Generation of FAK-deficient SCC cell lines. K14*Cre*ER^T2/FAK*flox/flox* mice were subjected to chemical carcinogenesis and cells generated as described previously in reference 4. The cells were harvested by trypsinization and subcultured. To delete *fak*, cells were treated with 4-hydroxy (OH)-tamoxifen at a concentration of 10 μM for 24 hours. FAK-deficient (-/-) SCC cells expressing FAK mutants (wt or FAK-E139A,D140A) were generated by amaxa nucleofection kit v, programme P20, using the pWZL-FAK expression constructs outlined before.[4]

Immunocytochemistry and cell polarization assays. For immunocytochemistry, cells were grown on glass coverslips, rinsed in TBS, fixed in 3.7% formaldehyde-PBS, 100 mM K-Pipes pH 6.8, 10 mM EGTA,
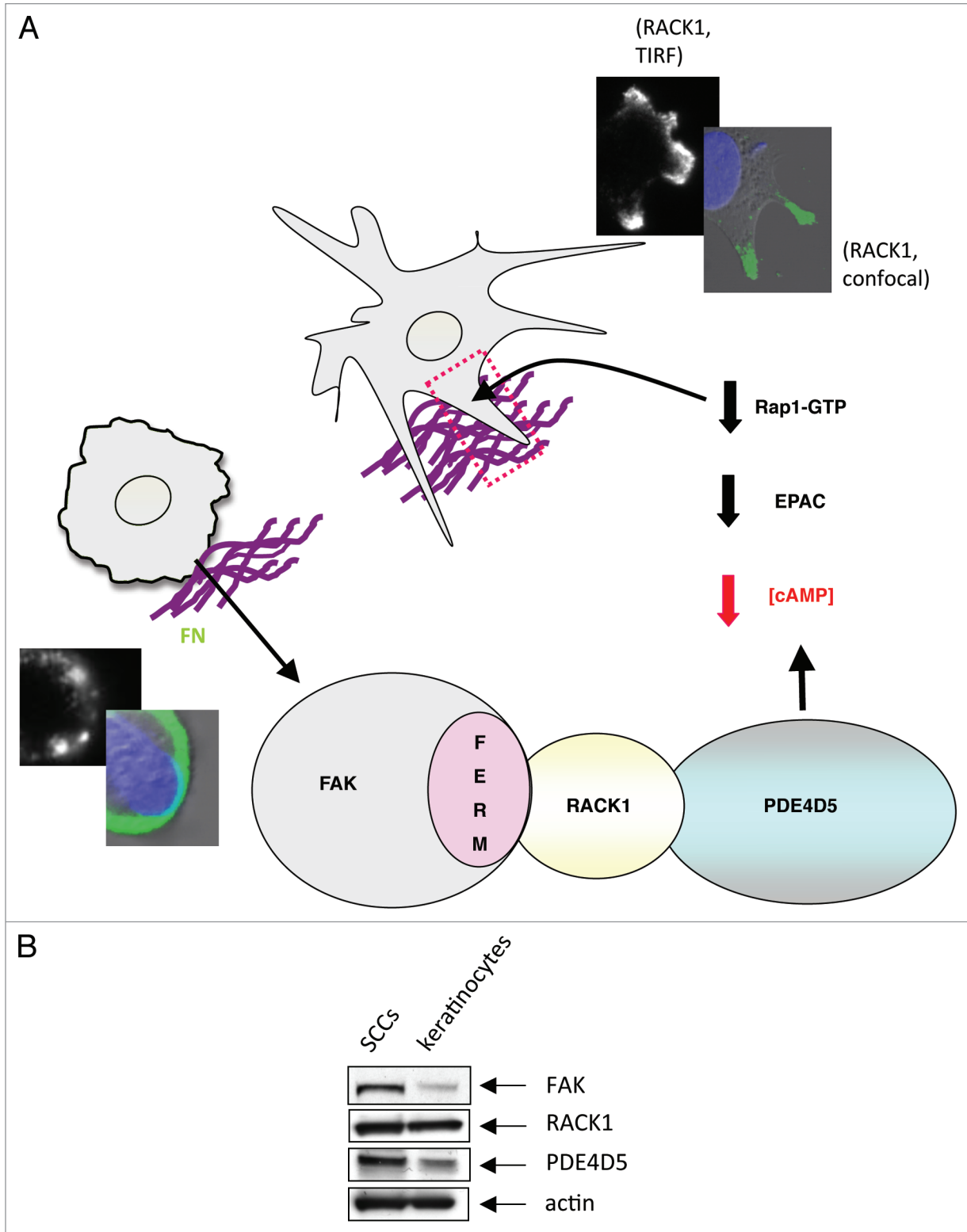
Figure 3. (A) Model outlining how our data explains the role of the FAK/RACK1/PDE4D5 complex in keeping cAMP levels low in the vicinity of nascent peripheral adhesion structures as these form and sense their environment. In our model, this is required for a sub-set of nascent adhesions to stabilize, typically along one cell edge, so inducing a polarized phenotype. The FAK/RACK1-mediated recruitment of PDE4D5 signals to Rap1, likely via EPAC, to keep Rap1 appropriately suppressed in space and time so as to permit nascent adhesion stabilization, spreading and polaization. This is in keeping with our earlier data recently published in reference 4. The edges of cells expressing FAK-wt (top images showing stabilized protrusive structures) and FAK-E139A,D140A (lower images) are shown, including TIRF images (black and white) taken at the cell-substratum interface. (B) Expression levels of FAK, RACK1 and PDE4D5 were determined in lysates from normal and malignant keratinocytes (SCCs) which were immunoblotted using anti-FAK, anti-RACK1, anti-PD4D5 and anti-actin antibodies as probes.

1 mM MgCl$_2$, 0.2% triton x100 (Sigma), washed twice in TBS 0.1% triton x100 and blocked in TBS 0.1% BSA (Sigma, A7906) and incubated with primary antibody overnight. Primary antibody incubation was followed by several washes with TBS containing 0.1% triton x100 and subsequent incubation with FITC-labelled or Texas Red-labelled secondary antibodies. Actin stress fibres were visualized after staining with TRITC-phalloidin. For polarization assays, cells were plated on FN at a density of 2.5 x 10^6 cells per well on a 12 well dish for 2 hours. Cells were then wounded using a pipette tip, washed twice in PBS and twice in complete medium, left in complete medium for 1.5 hours and fixed, as above. To investigate the role of EPAC and PKA in cell polarity assays, cells were incubated in the presence of either 10 μM 8-CPT-2MeO-cAMP (Sigma, C8988), 10 μM 8-Brm-cAMP (BioLog, B022), 1 μM KT5720 (Sigma, K3761) or 10 μM N6-Bnz-cAMP (BioLog, B009) for the entire duration of the experiment as indicated above.

Immunoblotting. Cells were washed twice with PBS and lysed in RIPA lysis buffer (50 mM Tris-HCL at pH 7.4, 150 mM sodium chloride, 0.1% SDS and 1% deoxycholate) with inhibitors (10 mM pyrophosphate, 100 mM sodium fluoride, 1 mM PMSF, 10 μg ml^{-1} aprotinin, 100 mM sodium orthovanadate, 10 mg ml^{-1} leupeptin and 10 mg ml^{-1} benzamidine; all purchased from Sigma). Clarification was by high speed centrifugation (16,000x g at 4°C for 15 min). Cell lysate (10–20 mg protein, as measured by Micro BCA Protein Assay Kit, was supplemented with SDS sample buffer (Tris at pH 6.8, 20% glycerol, 5% SDS, β-mercaptoethanol and bromo-phenol blue) separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with specific antibodies.

Rap1 activity assays. 0.5 x 10^6 cells were plated on a 90 mm tissue culture dish in the presence of serum and left to grow for 48 hours. Cells were then harvested and Rap1 activity measured using an Ez detect Rap1 activation kit (Perbio, 89872) as per manufacturers instructions. Samples were resolved on a 4–12% Nupage gel (Invitrogen, NPO322BOX) and western blotted for both active and total Rap1. For analysis, densitometry measurements were taken using Image J software and the percentage of activated Rap1 calculated relative to total Rap1.

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