Reciprocal regulation of PKA and Rac signaling

Verena A. Bachmann[a,b], Anna Riml[a,b], Roland G. Huber[b,c], George S. Baillie[d], Klaus R. Liedl[b,c], Taras Valovka[a,b], and Eduard Stefan[a,b,1]

Institutes of [a]Biochemistry and [c]Theoretical Chemistry and [b]Center for Molecular Biosciences Innsbruck, University of Innsbruck, A-6020 Innsbruck, Austria; and [d]Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary, and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Edited* by Susan S. Taylor, University of California, San Diego, La Jolla, CA, and approved April 5, 2013 (received for review September 12, 2012)

Activated G protein-coupled receptors (GPCRs) and receptor tyrosine kinases relay extracellular signals through spatial and temporal controlled kinase and GTPase entities. These enzymes are coordinated by multifunctional scaffolding proteins for precise intracellular signal processing. The cAMP-dependent protein kinase A (PKA) is the prime example for compartmentalized signal transmission downstream of distinct GPCRs. A-kinase anchoring proteins tether PKA to specific intracellular sites to ensure precision and directionality of PKA phosphorylation events. Here, we show that the Rho-GTPase Rac contains A-kinase anchoring protein properties and forms a dynamic cellular protein complex with PKA. The formation of this transient core complex depends on binary interactions with PKA subunits, cAMP levels and cellular GTP-loading accounting for bidirectional consequences on PKA and Rac downstream signaling. We show that GTP-Rac stabilizes the inactive PKA holoenzyme. However, β-adrenergic receptor-mediated activation of GTP-Rac–bound PKA routes signals to the Raf-Mek-Erk cascade, which is critically implicated in cell proliferation. We describe a further mechanism of how cAMP enhances nuclear Erk1/2 signaling: It emanates from transphosphorylation of p21-activated kinases in their evolutionary conserved kinase-activation loop through GTP-Rac compartmentalized PKA activities. Sole transphosphorylation of p21-activated kinases is not sufficient to activate Erk1/2. It requires complex formation of both kinases with GTP-Rac1 to unleash cAMP-PKA–boosted activation of Raf-Mek-Erk. Consequently GTP-Rac functions as a dual kinase-tuning scaffold that favors the PKA holoenzyme and contributes to potentiate Erk1/2 signaling. Our findings offer additional mechanistic insights how β-adrenergic receptor-controlled PKA activities enhance GTP-Rac–mediated activation of nuclear Erk1/2 signaling.

signal transduction | cross-talk

Signal transduction cascades coordinate the plethora of extracellular stimuli into biological responses within cells. The specificity of receptor-initiated signaling responses is encoded by spatial and temporal dynamics of downstream signaling networks (1). These networks, initiating from e.g., the G protein-coupled receptor (GPCR) superfamily and receptor tyrosine kinases (RTK), tightly regulate signaling pathways at several critical points via feedback loops and cross-talk among other pathways (2–5). A large number of GPCR signaling cascades uses cAMP as an intracellular second messenger (3, 6). In response to hormone binding to distinct GPCRs, cAMP is produced and binds to its canonical effector, the cAMP-dependent protein kinase A (PKA). cAMP binding to the PKA regulatory subunits (R) induces dissociation of the tetrameric PKA holoenzyme, resulting in active PKA catalytic subunits (PKAc; Fig. 1*C*) (7, 8). To ensure substrate specificity, PKA is tethered to distinct subcellular compartments through physical interaction with A-kinase anchoring proteins (AKAPs; refs. 9 and 10). It has been long regarded that the GPCR-cAMP-PKA signaling axis participates, among others, in the regulation of cell growth, differentiation, and motility (9–13). Such fundamental cellular functions are controlled by mitogenic signals that are transmitted through cascades involving crucially regulated mitogen-activated protein kinases (MAPK) like Erk1/2 (14). It has been described that the Rho GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) participates in the regulation of transformation, growth, and survival of tumor cells at least partially by controlling extracellular signal-regulated kinase (Erk)1/2 activation (15, 16). So far, several functionally diverse means have been illustrated how hormone-triggered cAMP pulses participate in the regulation and transmission of mitogenic signals originating from RTK via the Raf-Mek-Erk pathway (11–13). At different stages of RTK-mediated signal transmission, these pathways interact to regulate proliferation. Cross-talk originates from trimeric G protein switching [at the GPCR level: shown for the β-2 adrenergic receptor (β2AR)], through different mode of actions of cAMP-PKA on G proteins like Ras-related protein 1 (Rap1) and inhibitory G protein alpha i (Gαi) and by modulation of the proto-oncogene Raf1 (13, 17–20). In addition, scaffolding proteins like AKAP-Lbc, phosphorylation of phosphodiesterases and phosphatases contribute to this cross-talk related to MAPK signaling (21–23). Moreover, the mentioned β2AR pathway (3, 6) leading to cAMP-PKA activation has been implicated in malignant cell growth in a mouse model of ovarian carcinoma (24). Here, we examine a unique mechanism how cAMP-activated PKA is involved in the regulation of Erk1/2 activities. We report that Rac1, a member of the Rho GTPase family (25), contains AKAP properties and, thus, show direct interactions with PKA R subunit type IIβ (RIIβ) in vitro and in vivo. Our cell-based studies demonstrate that complex formation of active GTP-Rac1 and PKA increase inactive PKA complexes but does not directly affect Rac1 GTPase activity. However, we unveil that βAR-activated PKA phosphorylates the main Rac-effectors, p21-activated kinases (PAKs), which leads to GTP-Rac1–dependent elevation of downstream signaling to Erk1/2. We describe a mechanism through which PKA, a key component of cAMP-GPCR cascades, participates in the regulation of RTK-activated Rac-PAK-Erk1/2 signaling to nuclear transcription factors.

Results

PKA RII Subunits Form Protein Complexes with Rac1 in Vitro and in Vivo. We performed a systematic screen using a "Venus" yellow fluorescent protein (YFP) protein-fragment complementation assay (PCA) in mammalian cells to identify transient protein: protein interactions emanating from PKA R subunits with downstream components of RTK and GPCR cascades (18). We identified interaction of PKA RIIβ with the small Rho family GTPase Rac1 (Fig. 1*A*). The PKA homodimer RIIβ:RIIβ was restricted to the cytosol, but both the Rac1:Rac1 homodimer and the identified Rac1:RIIβ complex were primarily localized to the plasma membrane (Fig. 1*B*). This observation highlights physical connection between PKA and Rac1, which are key effectors of canonical receptor cascades, e.g., of the GPCR and RTK family, respectively (Fig. 1*C*). We confirmed direct protein:protein interaction of Rac1:RIIβ by using two independent in vitro tests: First, we confirmed

Author contributions: E.S. designed research; V.A.B., A.R., R.G.H., and E.S. performed research; G.S.B., K.R.L., and T.V. contributed new reagents/analytic tools; V.A.B., R.G.H., and E.S. analyzed data; and E.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: eduard.stefan@uibk.ac.at.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1215902110/-/DCSupplemental.

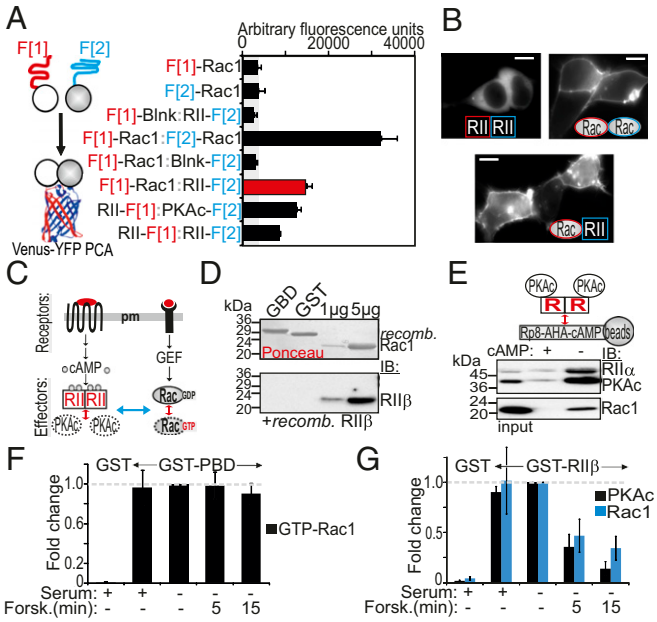

Fig. 1. PKA regulatory subunits form complexes with Rac1 in vitro and in vivo. (*A*) PCA strategy to capture binary protein complexes in living cells. Fluorometric analysis of transiently transfected HEK293 cells coexpressing indicated protein couples (fluorescence spectroscopy; representative of $n = 3$, ±SEM). (*B*) Fluorescence images of HEK293 cells expressing indicated PCA protein couples. (Scale bars: 10 µm.) (*C*) Schematic representation of ligand-activated receptor pathways (GPCR, RTK). Blue arrow indicates physical interaction (pm, plasma membrane). (*D*) Far western blot illustrates direct binding of RIIβ to Rac1 (representative of $n = 3$). (*E*) cAMP precipitation of PKA complexes using Rp-8-AHA-cAMP agarose resin from total brain lysates of mice (representative of $n = 3$). (*F*) GTPase measurements of endogenously expressed Rac1 following Forskolin exposure (100 µM; $n = 5$, ±SEM; HEK293). (*G*) GST pulldown of Rac1 and PKAc following general cAMP elevation (Forskolin, 100 µM; $n = 4$, ±SEM; HEK293).

direct interaction in a far Western blot analysis by using the recombinant full-length and untagged proteins RIIβ, Rac1 and, serving as negative controls, the recombinant geminin binding domain (GBD) and glutathione S-transferase (GST) (Fig. 1*D*). Second, we showed direct interaction of recombinant RIIβ and Rac1 in cAMP precipitation assays (Fig. S1*A*). To analyze whether the Rac1:R-subunit complex exists under physiological conditions, we applied total brain lysates of mice to Rp-8-AHA-cAMP–coupled agarose beads (precipitates R:PKAc holoenzymes). Isolated PKA complexes coprecipitate endogenous Rac1 (Fig. 1*E*). Additionally, we isolated Rac1:R-subunit complexes from the ovarian cancer cell line OVCAR3 and HeLa cells, respectively (Fig. S1*B*).

Impact of PKA Activation on GTPase Activity and RII:Rac1 Complex Formation. Upon exposure to mitogenic stimuli, Rho GTPases are converted to active GTP-bound forms that interact and activate downstream effectors like the p21-activated kinases (PAK1–6) (26, 27). We used purified PAK binding domain (PBD; exclusive binding site for GTP-Rac1) to determine the GTP loading of Rac1. Treatment of HEK293 cells with Forskolin, which triggers general cAMP elevation and PKA activation, had no significant impact on the GTPase activity of endogenous Rac1 (Fig. 1*F*). We confirmed this observation with ectopically expressed Rac1 variants: wild-type, dominant negative (T17N) and constitutively active (Q61L) Rac1-YFP (ref. 28; Fig. S1 *C* and *D*). We next precipitated endogenous cellular proteins by using GST and GST-RIIβ from HEK293 cells following treatment with Forskolin. As predicted, we detected a decrease of affinity between GST-RIIβ and PKAc. Next, we confirmed binding of endogenous Rac1 to GST-RIIβ. Upon cAMP elevation, we observed a decrease of GST-RIIβ:Rac1 complexes, suggesting that Rac1 interacts preferentially with the inactive PKA holoenzyme (Fig. 1*G* and Fig. S1*E*).

Analyses of Binding Regions of RIIβ on Rac1 and Related GTPases. To map the interaction sites on Rac1, we performed a peptide spotting experiment of Rac1b to confirm interaction in vitro and to determine specific amino acids required for RIIβ binding (18). We identified two potential binding sites located at the C terminus of Rac1, in regions distinct from GTPase-activity sites ("switch regions"; Fig. 2*A* and Fig. S1*F*). In the Rac1 structure, we highlight two binding sites (BD1 and BD2) located in close vicinity to permit protein:protein interaction with RIIβ (Fig. 2*B, Left*). We allocated an amphipathic helix motif (BD2) almost matching with a described consensus site for RII-binding domains (RIIBD) found in other AKAPs [Fig. 2*B, Right*; red brackets and red F (Phenylalanine) indicate the difference; ref. 29]. Structural and detailed biochemical analyses have specified that amino acids 1–45 of RIIβ cover the primary determinants for binary protein:protein interaction of R subunits with AKAPs (30, 31). We confirmed that the first 45 amino acids of RIIβ are sufficient to interact with full-length Rac1 (Fig. 2*C*). Next, in a GST-pulldown experiment with GST and GST-BD2 hybrid proteins, we precipitated endogenous RIIβ from HEK293 cells independent from altering cAMP levels (Fig. 2*D*). Next, we tested conserved members of the Rho GTPase family for AKAP properties (Fig. S2*A*) (32). We observed that substitutions of amino acids in the nonpolar region of BD2 are sufficient to decrease the affinity between GST-RIIβ and peptide mutants of Rac1/2/3. Surprisingly, we detected interaction of human RIIβ at least with the *Saccharomyces cerevisiae* Rho GTPase Rho1 (Fig. 2*E*). To further characterize the RII binding sites in Rac1, we performed alanine substitution scanning of BD1 and BD2 to identify key amino acids responsible for the interaction (Fig. S2 *B* and *C*). Following structural examination of BD1 in Rac1 and based on the alanine substitution experiment, we generated the GTP-Rac1 mutants Q61L-A1 (H104A, P106A) and Q61L-A2 (T108A, P109A). Moreover, we disrupted the C-terminal amphipathic helix of BD2 of GTP-Rac1 and generated the Q61L-P mutant (I173P). Exchange of I173P abolished binding of Q61L-P to both GST-RIIβ and GST-PBD. In contrast, A1 mutations of BD1 in GTP-Rac1 (Q61L-A1) had no impact on PBD binding but showed a slight reducing effect on complex formation of GTP-Rac1:RIIβ (Fig. 2*F*). These results support the notion that both binding domains of Rac1 are involved in the formation of cellular complexes with RIIβ.

GTP-Rac1 Stabilizes the PKA Holoenzyme. To get insights whether PKA RIIβ subunits have different affinities for the mentioned Rac1-YFP variants, we performed GST-pulldown experiments. We observed preferential binding of GST-RIIβ to cellularly expressed GTP-Rac1 (Q61L) (Fig. 3*A* and Fig. S3*A*). This result underlines that PKA preferentially binds to cellular GTP-Rac1 complexes that exist bound to downstream effectors like PAKs. First, we evaluated the effect of GTP-Rac1 overexpression on PKA activity. We transiently overexpressed indicated variants of Rac1 in the osteosarcoma cell line U2 (U2OS) and in HEK293 cells, which both stably express the *Rluc*-PCA based PKA reporter (Fig. 3*B*; ref. 33). Exclusively, the overexpression of GTP-Rac1 (Q61L) increased the inactive complex of RIIβ:PKAc in transient transfections in both cell lines significantly approximately 50% (Fig. 3*B*). Following the analyses of the impact of GTP-Rac1 on PKA signaling, we set the focus on investigating the influence of PKA activities on Rac1 downstream signaling to the nucleus.

cAMP-Dependent Regulation of GTP-Rac1 Signaling to Erk1/2. It has been reported that Rac1 participates to promote the Raf-Mek-Erk cascade (16, 34–36). To investigate the role of GTP-Rac1 in Erk1/2 activation, we used HEK293 cells, which show no increase of Erk1/2 phosphorylation in response to Forskolin alone (18). We confirmed observations that exclusively the overexpression of the constitutive active GTP-Rac1 variant activates Erk1/2 (16) (Fig. 3*C*). Activation of Erk1/2 causes activation

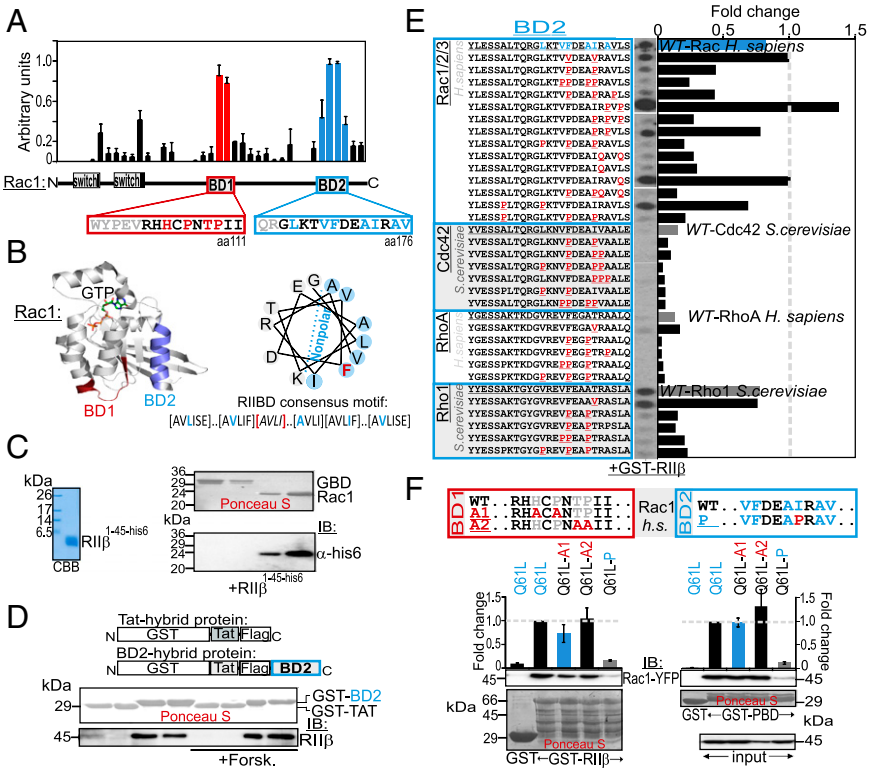

Fig. 2. Analyses of binding regions of RIIβ on Rac1 and related GTPases. (*A*) Colored bars point to the sequences of binding domains (BD1/BD2) obtained by dot blot analyses for RIIβ subunits in the modular structure of Rac1 (n = 3, ±SEM). (*B*) Structure of Rac1 (PDB ID code 1E96) illustrating the localization of BD1, BD2, and GTP. RIIBD consensus motif of AKAPs (29) and helical wheel projection of the amphipathic BD2 of Rac1. (*C*) Coomassie Brilliant Blue (CBB) staining of RIIb$^{1\text{-}45\text{-}his6}$ and a far western blot experiment. (*D*) GST pulldown of cellular RIIβ using indicated GST hybrid proteins (±100 μM Forskolin, 15 min; shown are independent samples). (*E*) Dot blot analysis of Rho GTPases peptides. Exchanged amino acids are indicated with red letters (average of n = 2). (*F*) Interaction studies of indicated GTP-Rac1 variants in biochemical GST pulldown assays (±SEM, n = 4).

of nuclear transcription factors like Elk1. We observed that ectopic expression of GTP-Rac1 further enhances activation of gene transcription by Erk1/2-mediated Elk1 phosphorylation (Fig. 3*D*). Next, we tested the impact of cAMP elevation on Erk1/2 activity. We observed that cAMP elevation enhances Erk1/2 activation exclusively in the presence of GTP-Rac1 to above threefold (Fig. 3*E*). We tested the GTP-Rac1 mutants presented in Fig. 2*F* for interference with signaling to Erk1/2. We observed that the GTP-Rac1 mutant Q61L-A1 prevented Forskolin-mediated Erk1/2 activation. This observation underlines that BD1 in Rac1 participates to permit cAMP-PKA–mediated Erk1/2 activation (Fig. 3*F* and Fig. S3*B*). However, to confirm that PKA kinase activity accounts for potentiating Erk1/2 phosphorylation in the presence of GTP-Rac1, we pretreated cells with the selective PKA inhibitor KT5720. PKA inhibition prevents the Forskolin-mediated potentiation of Erk1/2 phosphorylation (Fig. S3*C*). To analyze whether GPCR cascades linked to cAMP production participate in the regulation of GTP-Rac1 signaling to Erk1/2, we tested the involvement of the β2AR that, among other vital cellular functions, has also been linked to aberrant proliferation (3, 6, 24). As already known, the β-adrenergic agonist Isoproterenol (Iso) induces a transient increase of Erk1/2 phosphorylation in HEK293 cells stably expressing the β2AR. Isoproterenol-triggered activation of ERK1/2 is mediated via pathways that are sensitive to both the PKA inhibitor H89 and Gαi inhibitor pertussis toxin (37). In addition, it has been described that GPCR-bound β-arrestin participates in signaling to Erk1/2 (38). Isoproterenol treatment of HEK293-β2AR cells transiently overexpressing GTP-Rac1 further potentiated Erk1/2 phosphorylation (Fig. 3*G*). These data support the notion that β2AR provoked cAMP release and subsequent activation of GTP-Rac1–bound PKA further promotes activation of Erk1/2 as illustrated with the blue arrows in the scheme.

PKA Activities Affect p21-Activated Kinase Signaling to Erk1/2. First, we confirmed other studies that Rac1 is no direct target for PKAc phosphorylation, using the PKA substrate RhoA as positive control (39) (Fig. 4*A*). We aimed to identify the target of PKA phosphorylation, which links GTP-Rac1 to Erk1/2 activities. In Fig. 3*A*, we demonstrate that cellular GTP-Rac1 complexes have the highest affinity for the PKA holoenzyme. The key effector and conserved interacting partner of active GTP-Rac1 are PAKs: PAK1–6 (26, 27, 40). It has been described that PAK1 is phosphorylated by PKA (41). Sequence alignment and phosphorylation prediction highlight the existence of a PKA consensus site in the evolutionary conserved activation loop of PAKs (Fig. 4*B*). This highly conserved site can be found in the activation loop of PKAc subunit as well (Thr197). In both cases, autophosphorylation has been confirmed upon kinase activation (42, 43). In Fig. 4*B*, we highlight structural conservation. Just recently Park et al. (44) showed that PKAc subunits form protein complexes with its substrate PAK4. That is why we hypothesized that physical association of GTP-Rac1 with both kinases, PKA and PAKs, accounts for the observed GTP-Rac– and PKA-dependent Erk1/2 activation. First, we confirmed the possibility of physical interaction of PKAc subunits with PAK1 in dot blot analyses. We identified two preferential stretches of interaction close to the PKA consensus site for phosphorylation in the ultimate C terminus of PAK1 (Fig. S3 *D* and *E*). These results indicate that interaction of PKAc:PAK might be involved in stabilizing the interaction of the PKA holoenzyme with Rac1 variants (Figs. 1*G*, 2*F*, and 3 *A* and *B*). Next, in two independent cell systems, we tested whether PKA activation causes PAK phosphorylation, which has been described to modulate Raf-Mek-Erk signaling (16, 34–36): First, we show that both overexpression of PKAc and Forskolin treatment elevates PAK1-Thr423 and PAK2-Thr402 phosphorylation (Fig. S4*A*). Next, we analyzed whether type II PAKs (PAK4–6) are targets of PKA as well. Upon general cAMP elevation and following activation of β2AR (Iso), we observed significant elevations of PAK4-Ser474 phosphorylation in the absence and presence of indicated Rac1 versions. Overexpression of GTP-Rac1 causes basal levels of PAK4-Ser474 phosphorylation, which can be further raised by PKA activation (Fig. 4*C*). However, PKA-mediated PAK4 phosphorylation is not sufficient to promote downstream Erk1/2 activation. These data highlight that PKA activities in the

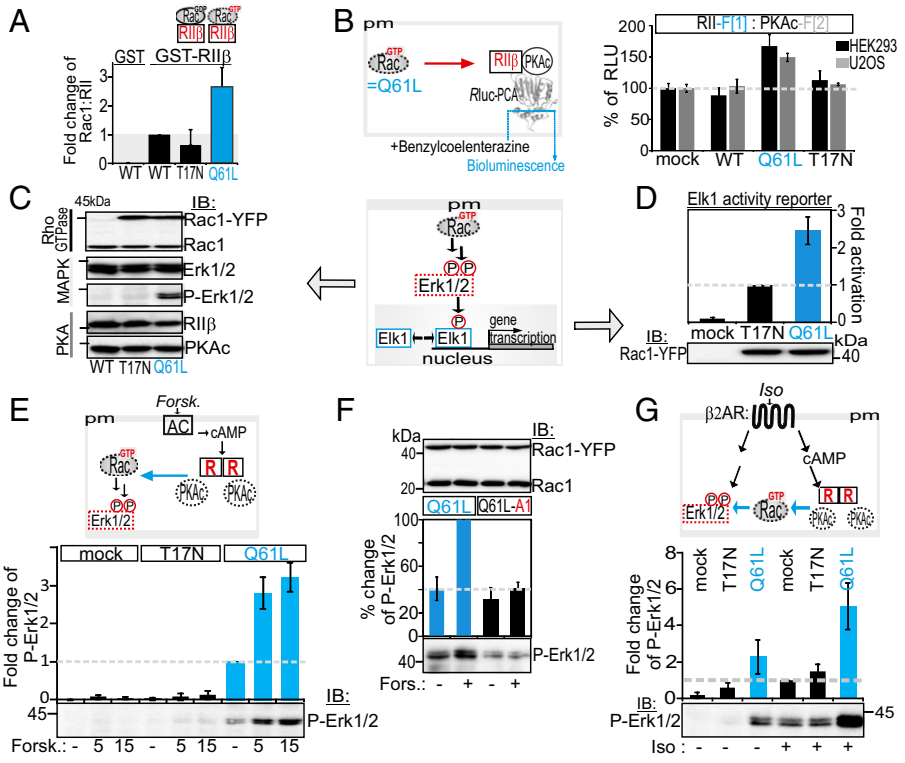

Fig. 3. Reciprocal regulation of PKA and GTP-Rac1 signaling. (*A*) Complex formation of GST-RIIβ and Rac1-YFP variants (±SEM, $n = 3$, normalized on the input). (*B*) Impact of indicated Rac1 versions on the formation of the RIIβ:PKAc complex in HEK293 and U2OS cells stably expressing the *Rluc*-PCA PKA sensor (representatives of $n = 3$, ±SEM). (*C*) Impact of overexpression of Rac1 versions on protein abundance and Erk1/2 phosphorylation. Scheme highlights GTP-Rac1 signaling to Erk1/2. (*D*) Effect of transient overexpression of indicated Rac1 versions on transcription factor Elk1 activation ($n = 5$, ±SEM, Elk1 activity luciferase reporter assay). (*E*) Effect of Rac1 version overexpression on Forskolin-mediated Erk1/2 phosphorylation ($n = 3$, ±SEM). (*F*) Effect of GTP-Rac1 (Q61L, Q61L-A1) overexpression on Forskolin-mediated Erk1/2 phosphorylation ($n = 5$, ±SEM). (*G*) Impact of Rac1 overexpression and Isoproterenol treatment on Erk1/2 phosphorylation (β2AR-HEK293; $n = 3$, ±SEM).

presence of GTP-Rac1 further enhance PAK phosphorylation, which elevates GTP-Rac:PAK signaling to Erk1/2 (Fig. S4*B*). Next, we tested GTP-Rac1 mutants for interference with signaling to PAK4 and Erk1/2. We detected that basal enhancements of Erk1/2 and PAK4-Ser474 phosphorylations are comparable in the presence of Q61L or Q61L-A1. However, we observed that compared with GTP-Rac1 (Q61L), the mutant Q61L-A1 significantly reduced Isoproterenol-mediated Erk1/2 and PAK4-Ser474 phosphorylation. This observation underlines that BD1 of GTP-Rac1 participates to boost β2AR-controlled and cAMP-PKA–mediated PAK4 and Erk1/2 phosphorylation (Fig. 4*D*). Therefore, we conclude that PAK activities are controlled also through a third condition: First, PAKs need to be phosphorylated in the activation loop. Second, PAKs need to bind GTP-Rac1 to allow signal transmission to Erk1/2. Third, our data support the notion that GTP-Rac1–compartmentalized and cAMP-activated PKA participates in phosphorylating PAKs in their activating loops, thereby contributing to the progression of Rac-PAK-Erk signaling. Further, we revealed an additional connection from βAR cascades to Erk1/2 activities. We present evidence that β2AR-controlled cAMP-PKA activation participates in GTP-Rac1:PAK–initiated Erk1/2 phosphorylation. It has been described that in an ovarian carcinoma mouse model, activation of the β2AR-PKA signaling axis contributes to tumor growth (24). In another study, it has been shown that PAK4 activities regulate ovarian cancer cell proliferation, migration, and invasion (45). Therefore, we decided to analyze one human ovarian cancer cell line (OVCAR3) from this study (45) for the involvement of βAR-PKA and PAK4/6 signaling in Erk1/2 activation and proliferation. First, we demonstrate basal levels of PAK4-Ser474 and PAK6-Ser560 phosphorylation in OVCAR3 cells. Upon cAMP elevation with Forskolin and Isoproterenol, we confirmed elevation of PAK4/6 phosphorylation (Fig. 4*E* and Fig. S4*C*). Isoproterenol activates selectively endogenously expressed βAR, which led to activation of Erk1/2 and Elk1 (Fig. 4*E*). PKA inhibition prevented Isoproterenol-initiated PAK4/6, Erk1/2, and Elk1 phosphorylation (Fig. 4*E*). These findings link βAR pathways to the activation of the proliferation relevant PAK-Erk-Elk1 cascade. Next, we performed cellular proliferation assays (18 h) to test how far βAR activities and scaffolding complexes like RII:Rac1 are relevant for proliferation. Activation of βAR cascades showed a significant enhancement of proliferation of OVCAR3 cells (Fig. 4*F*, doubling time of 4 d; ref. 46). In addition, we tested membrane permeable AKAP:PKA disrupting peptides (Fig. S4*D*), which significantly reduced proliferation (Fig. 4*F*). The peptides (in the used concentration of 10 μM) showed no impact on the formation of the PKA–holoenzyme complex (Fig. S4*E*). These results support the notion that βAR-controlled PKA activities contribute to proliferative effects by active participation in the GTP-Rac:PAK signaling axis leading to Erk1/2 phosphorylation and activation of the nuclear transcription factor Elk1. Overall, our observations highlight a bidirectional function of the Rac1:PKA complex. GTP-Rac1 enriches and compartmentalizes inactive PKA complexes through binary interaction with RII subunits. However, the three-part complex of PKA:*GTP-Rac1*:PAKs acts as compartmentalized modulator of Erk1/2 activities that is controlled first by GTP loading, second through physical interaction with both kinases (PKA and PAK), third through PAK activities, and last but not least through βAR-triggered cAMP-PKA activities (Fig. 4*G*).

Discussion

Rac1 belongs to the Rho GTPase family of small GTP-binding proteins. Prominent members of this family Rho, Cdc42, and Rac emerge to regulate a diverse array of cellular events, including control of the reorganization of the cytoskeleton, cell growth, and activation of diverse protein kinases (25). We now report that Rac1 contains AKAP properties and directly interacts with PKA RII subunits. Complex formation of RII:Rac1 is not static; it depends on cellular GTP loading, bound Rac effectors, and cAMP elevation, and it accounts for bidirectional consequences on signal transmission. We describe a mode of regulation that is complementary to the regulation by guanine exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine dissociation inhibitors. We show that cAMP elevation has no direct impact on Rac1 GTPase activities and, therefore, GTP loading. First, our data reveal that complex formation of cellular GTP-Rac1 with PKA RII subunits stabilizes the inactive PKA holoenzyme. Second, we show

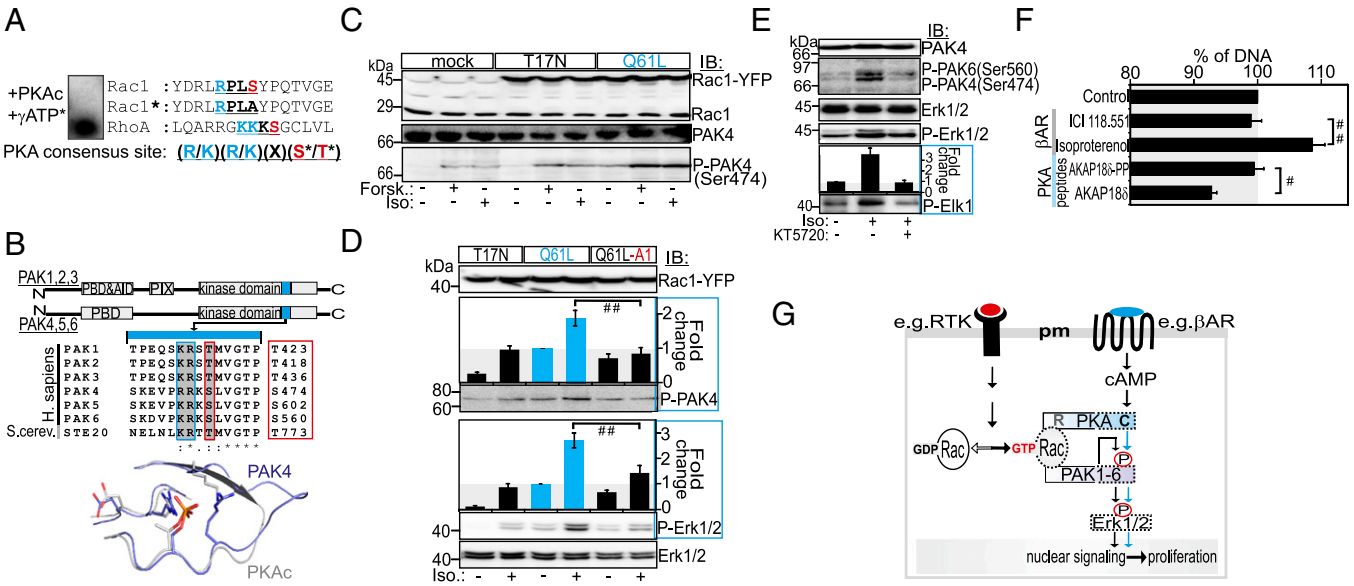

Fig. 4. βAR-PKA axis participates in PAK signaling. (*A*) PKA phosphorylation of indicated peptides. (*B*) Modular structure of PAKs with potential PKA phosphorylation site. Structural alignment of PAK4 and PKAc activation loops (PDB ID codes: 2CDZ/1BKX) with indication of Ser474 and Thr197. (*C*) PAK4 phosphorylation upon overexpression of Rac1-versions (Iso, 10 μM; Forskolin, 50 μM; 15 min, HEK293). (*D*) Effect of GTP-Rac1 (T17N, Q61L, Q61L-A1) overexpression on Iso-mediated Erk1/2 and PAK4-Ser474 phosphorylation ($n \geq 4$, ±SEM, 5 min, 10 μM). Statistical significance was assessed by using a paired Student *t* test ($^{\#\#}P < 0.01$). (*E*) Impact of PKA inhibition (KT5720: 1 h, 5 μM) and Iso exposure (15 min, 1 μM) on PAK4/PAK6 phosphorylation (±SEM, $n = 3$, OVCAR3). (*F*) Proliferation assay of OVCAR3 cells following 18 h of Iso (1 μM), ICI118.551 (2 μM; β2AR antagonist), and AKAP18δ peptides (10 μM) exposure ($n = 6$, ± SEM). Statistical significance was assessed by using a paired Student *t* test ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$). (*G*) Signals from e.g., activated RTK and βAR cascades converge on the Rac:PKA complex leading to modulation of the MAP kinase Erk1/2. GTP-Rac interacts with its main effectors p21-activated kinases (PAK1-6), but it stabilizes the inactive PKA-holoenzyme (R:C). βAR-controlled cAMP elevation promotes PKA–mediated phosphorylation of GTP-Rac1–bound and GTP-Rac1–activated PAK that contributes to elevations of Erk1/2 phosphorylation (P) that, in turn, enhances nuclear signaling leading to proliferation.

that active GTP-Rac1 augments Erk1/2 phosphorylation, which causes subsequent activation of the transcription factor Elk1. Third, we demonstrate that the activity of the GTP-Rac1:PAK signaling axis leading to activation of Erk1/2 can be directly controlled through GTP-Rac1–compartmentalized PKA activities. Fourth, we have revealed that in the ovarian cancer cell line OVCAR3, the proliferation-relevant βAR-PKA signaling axis (24, 47) is linked to Rac-PAK-Erk1/2-mediated activation of the transcription factor Elk1. Overall our findings disclose a unique crossroad of frequently targeted receptor cascades (RTK, βAR) that integrates cAMP responses and GTPase activities spatially and temporally, leading to modulation of the crucial Raf-Mek-Erk signaling axis (11–16). It has been described that PKA activities regulate GEFs and GAPs that change the GTP loading of Rac1, resulting in changes of signaling related to morphological alterations of the cytoskeleton (48–50). These processes are distinct from the mechanism we describe here, where Rac-anchored PKA activities directly contribute to the activation of downstream effectors of GTP-Rac signaling leading to Erk1/2 activation. Another GTPase, which we have identified in the same screen to be a binary interaction partner of PKA RII subunits, is a component of the trimeric G protein complex Gαi, although without AKAP features (18). In contrast to Rac1:PKA, complex formation of RII subunits with Gαi was observed in response to cAMP elevation. Indeed, mechanistically different, the appearance of cAMP-RII:Gαi elevates GPCR-mediated downstream signaling leading, among others, to Erk1/2 activation. Rac1 is not the first small GTPase with AKAP properties. The GTPase Rab32 is classified as an AKAP and targets PKA activities to the mitochondrion. Interestingly, another scaffolding protein with AKAP properties, WAVE-1, directs actin reorganization by relaying signals from the GTPase Rac to downstream effectors. Overall several AKAPs (like AKAPlbc or AKAP220) group PKA, other Rho GTPases, and their regulator molecules together, thereby regulating small GTPase activities affecting cytoskeleton reorganizations (9, 10, 48, 51). Several means have been described how the second messenger cAMP alters signaling through the Ras-Raf-Erk cascade positively or negatively. However, it is still a controversy how cell type-dependent components of the cAMP machinery (PKA, AKAPs, Epac, PDEs) contribute to these opposed consequences on Erk1/2 activation that lead to cell growth and/or aberrant proliferation (11–13, 17–23, 52, 53). Here, we present a mechanism how GPCR and cAMP-mediated PKA activation regulates GTP-Rac1 signaling via Erk1/2 to nuclear transcription factors. The detailed mechanism of Rac1-mediated Erk1/2 activation has been described: GTP-Rac1 endorses Raf-Mek-Erk signaling by PAK-mediated phosphorylation of Raf at Ser338 or of Mek at Ser298, which promotes interaction between Erk and Mek (16, 34–36). A link between PKA activities and Rho GTPase-PAK1 signaling has been discovered more than a decade ago (41). Just recently, it has been depicted that PKAc subunits form a protein complex with PAK4 (44), which we confirmed with PAK1 (Fig. S3 *D* and *E*). First, this observation is one possible explanation how cellular GTP-Rac1 stabilizes the PKA holoenzyme by improved affinities of cellular GTP-Rac:PAKs for RIIβ:PKAc (Fig. 3 *A* and *B*). Second, this data underlines the concept of compartmentalization of PKA through interaction with the GTP-Rac1:effector complex. In agreement with Park et al. (44), we show that PAK4 is a target of PKA activity by phosphorylating the conserved kinase activation loop (Fig. 4). We link GTP-Rac1–compartmentalized PKA activities, initiated directly by adenylyl cyclase or βAR activation, to the phosphorylation of PAK isoforms. This PKA phosphorylation event contributes to sustained elevations of Erk1/2 activities that implicates enhanced proliferation. Of note in this work is that sole transphosphorylation of PAK4 in its activation loop is not sufficient to promote Erk1/2 activation (Fig. S4*B*). It requires the complex formation of compartmentalized and PKA-phosphorylated PAK4 with GTP-Rac1, which acts as an active kinase-tuning scaffold [in positive (PAKs) or negative manner (PKA)] to unleash cAMP-PKA–controlled Erk1/2 activation (Fig. 4*G*), which can be prevented by introducing mutations into BD1 of GTP-Rac1 (Fig. 4*D*). PAKs are key effectors central to numerous

physiological processes whereby PAK deregulation has been implicated in oncogenesis (26, 44, 45). It has been described that PAK4 activities regulate cancer cell proliferation, migration, and invasion. Besides the abundance of PAK4, the phosphorylation of PAK4-Ser474 has been determined as a crucial factor in cancer progression (45). In this context, our research highlights a unique route how βAR-provoked cAMP fluxes might participate, besides PKA-mediated Erk1/2 modulation, in further diverse functions of the miscellaneous Rac-effectors PAKs. Given that PAKs activities are considered as marker for the prognosis of different types of cancer, we would like to note that besides PAKs abundance and its phosphorylation status, the GTP-Rac:PKA complex and βAR activities need to be considered (16, 44, 45, 54). The disclosure of the involvement of the βAR-PKA cascade in regulation of Rac-PAK–mediated Erk1/2 activities offers an explanation how cAMP fluxes contribute to cell growth in a cell-dependent manner.

Materials and Methods

Description of antibodies, protein purification, kinase assays, and far Western blots are in *SI Materials and Methods*. The *Renilla* luciferase-based PCA assay has been described in detail previously (33). PCA assays, SPOT synthesis and overlay experiments, cAMP-agarose precipitations and phosphorylation assays have been described (18) and are in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Klaus Bister for critical discussions, generous support, and for providing the GBD protein; Antonio Feliciello for comments on the manuscript; Klaus Hahn for Rac1 and PBD expression constructs; Ruth MacLeod and Michael Beyermann for dot blot membranes; Alain Zeimet and Heidi Fiegl for OVCAR3 cells; Michi Ausserlechner for access to the imaging platform; Gabi Reiter for management support; and Sonja Geisler for technical assistance. The interaction screen was initiated in the laboratory of Stephen Michnick (Université de Montréal), and E.S. thanks Stephen Michnick for his generous support. This work was supported by Austrian Science Fund Grant P22608 (to E.S.) and P24251 (to T.V.) and Junior Researcher Support (University of Innsbruck) (to E.S.).

1. Scott JD, Pawson T (2009) Cell signaling in space and time: Where proteins come together and when they're apart. *Science* 326(5957):1220–1224.
2. Bhola NE, Grandis JR (2008) Crosstalk between G-protein-coupled receptors and epidermal growth factor receptor in cancer. *Front Biosci* 13:1857–1865.
3. Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3(9):639–650.
4. Schäfer B, Gschwind A, Ullrich A (2004) Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* 23(4):991–999.
5. Delcourt N, Bockaert J, Marin P (2007) GPCR-jacking: From a new route in RTK signalling to a new concept in GPCR activation. *Trends Pharmacol Sci* 28(12):602–607.
6. Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. *Nat Rev Cancer* 7(2):79–94.
7. Taylor SS, et al. (2008) Signaling through cAMP and cAMP-dependent protein kinase: Diverse strategies for drug design. *Biochim Biophys Acta* 1784(1):16–26.
8. Zhang P, et al. (2012) Structure and allostery of the PKA RIIβ tetrameric holoenzyme. *Science* 335(6069):712–716.
9. Malbon CC, Tao J, Wang HY (2004) AKAPs (A-kinase anchoring proteins) and molecules that compose their G-protein-coupled receptor signalling complexes. *Biochem J* 379(Pt 1):1–9.
10. Wong W, Scott JD (2004) AKAP signalling complexes: Focal points in space and time. *Nat Rev Mol Cell Biol* 5(12):959–970.
11. Gerits N, Kostenko S, Shiryaev A, Johannessen M, Moens U (2008) Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: Comradeship and hostility. *Cell Signal* 20(9):1592–1607.
12. Dumaz N, Marais R (2005) Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft für Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. *FEBS J* 272(14):3491–3504.
13. Stork PJ, Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol* 12(6):258–266.
14. Cuevas BD, Abell AN, Johnson GL (2007) Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene* 26(22):3159–3171.
15. Sahai E, Marshall CJ (2002) RHO-GTPases and cancer. *Nat Rev Cancer* 2(2):133–142.
16. Wang Z, et al. (2010) Rac1 is crucial for Ras-dependent skin tumor formation by controlling Pak1-Mek-Erk hyperactivation and hyperproliferation in vivo. *Oncogene* 29(23):3362–3373.
17. Baillie GS, et al. (2003) beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi. *Proc Natl Acad Sci USA* 100(3):940–945.
18. Stefan E, et al. (2011) PKA regulatory subunits mediate synergy among conserved G-protein-coupled receptor cascades. *Nat Commun* 2:598.
19. Häfner S, et al. (1994) Mechanism of inhibition of Raf-1 by protein kinase A. *Mol Cell Biol* 14(10):6696–6703.
20. Cook SJ, McCormick F (1993) Inhibition by cAMP of Ras-dependent activation of Raf. *Science* 262(5136):1069–1072.
21. Smith FD, et al. (2010) AKAP-Lbc enhances cyclic AMP control of the ERK1/2 cascade. *Nat Cell Biol* 12(12):1242–1249.
22. Hoffmann R, Baillie GS, MacKenzie SJ, Yarwood SJ, Houslay MD (1999) The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. *EMBO J* 18(4):893–903.
23. Nika K, et al. (2004) Haematopoietic protein tyrosine phosphatase (HePTP) phosphorylation by cAMP-dependent protein kinase in T-cells: Dynamics and subcellular location. *Biochem J* 378(Pt 2):335–342.
24. Thaker PH, et al. (2006) Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nat Med* 12(8):939–944.
25. Jaffe AB, Hall A (2005) Rho GTPases: Biochemistry and biology. *Annu Rev Cell Dev Biol* 21:247–269.
26. Molli PR, Li DQ, Murray BW, Rayala SK, Kumar R (2009) PAK signaling in oncogenesis. *Oncogene* 28(28):2545–2555.
27. Zhao ZS, Manser E (2005) PAK and other Rho-associated kinases—effectors with surprisingly diverse mechanisms of regulation. *Biochem J* 386(Pt 2):201–214.
28. Kraynov VS, et al. (2000) Localized Rac activation dynamics visualized in living cells. *Science* 290(5490):333–337.
29. Hundsrucker C, et al. (2010) Glycogen synthase kinase 3beta interaction protein functions as an A-kinase anchoring protein. *J Biol Chem* 285(8):5507–5521.
30. Kinderman FS, et al. (2006) A dynamic mechanism for AKAP binding to RII isoforms of cAMP-dependent protein kinase. *Mol Cell* 24(3):397–408.
31. Alto NM, et al. (2003) Bioinformatic design of A-kinase anchoring protein-in silico: A potent and selective peptide antagonist of type II protein kinase A anchoring. *Proc Natl Acad Sci USA* 100(8):4445–4450.
32. Wennerberg K, Der CJ (2004) Rho-family GTPases: It's not only Rac and Rho (and I like it). *J Cell Sci* 117(Pt 8):1301–1312.
33. Stefan E, et al. (2007) Quantification of dynamic protein complexes using Renilla luciferase fragment complementation applied to protein kinase A activities in vivo. *Proc Natl Acad Sci USA* 104(43):16916–16921.
34. King AJ, et al. (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature* 396(6707):180–183.
35. Sundberg-Smith LJ, Doherty JT, Mack CP, Taylor JM (2005) Adhesion stimulates direct PAK1/ERK2 association and leads to ERK-dependent PAK1 Thr212 phosphorylation. *J Biol Chem* 280(3):2055–2064.
36. Eblen ST, Slack JK, Weber MJ, Catling AD (2002) Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Mol Cell Biol* 22(17):6023–6033.
37. Daaka Y, Luttrell LM, Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* 390(6655):88–91.
38. DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* 69:483–510.
39. Ellerbroek SM, Wennerberg K, Burridge K (2003) Serine phosphorylation negatively regulates RhoA in vivo. *J Biol Chem* 278(21):19023–19031.
40. Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 367(6458):40–46.
41. Howe AK, Juliano RL (2000) Regulation of anchorage-dependent signal transduction by protein kinase A and p21-activated kinase. *Nat Cell Biol* 2(9):593–600.
42. Steichen JM, et al. (2012) Structural basis for the regulation of protein kinase A by activation loop phosphorylation. *J Biol Chem* 287(18):14672–14680.
43. Zenke FT, King CC, Bohl BP, Bokoch GM (1999) Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. *J Biol Chem* 274(46):32565–32573.
44. Park MH, et al. (June 18, 2012) p21-Activated kinase 4 promotes prostate cancer progression through CREB. *Oncogene*, 10.1038/onc.2012.255.
45. Siu MK, et al. (2010) p21-activated kinase 4 regulates ovarian cancer cell proliferation, migration, and invasion and contributes to poor prognosis in patients. *Proc Natl Acad Sci USA* 107(43):18622–18627.
46. Ligr M, et al. (2011) Expression and function of androgen receptor coactivator p44/Mep50/WDR77 in ovarian cancer. *PLoS ONE* 6(10):e26250.
47. Cole SW, Sood AK (2012) Molecular pathways: Beta-adrenergic signaling in cancer. *Clin Cancer Res* 18(5):1201–1206.
48. Logue JS, Whiting JL, Tunquist B, Langeberg LK, Scott JD (2011) Anchored protein kinase A recruitment of active Rac GTPase. *J Biol Chem* 286(25):22113–22121.
49. Schlegel N, Waschke J (2009) VASP is involved in cAMP-mediated Rac 1 activation in microvascular endothelial cells. *Am J Physiol Cell Physiol* 296(3):C453–C462.
50. Birukova AA, et al. (2007) Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. *Exp Cell Res* 313(11):2504–2520.
51. Howe AK, Baldor LC, Hogan BP (2005) Spatial regulation of the cAMP-dependent protein kinase during chemotactic cell migration. *Proc Natl Acad Sci USA* 102(40):14320–14325.
52. Gloerich M, Bos JL (2010) Epac: Defining a new mechanism for cAMP action. *Annu Rev Pharmacol Toxicol* 50:355–375.
53. Baillie GS, Houslay MD (2005) Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes. *Curr Opin Cell Biol* 17(2):129–134.
54. Kumar R, Gururaj AE, Barnes CJ (2006) p21-activated kinases in cancer. *Nat Rev Cancer* 6(6):459–471.