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ORIGINAL ARTICLE

Beta-arrestins operate an on/off control switch for focal adhesion kinase activity

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Abstract

Focal adhesion kinase (FAK) regulates key biological processes downstream of G protein-coupled receptors (GPCRs) in normal and cancer cells, but the modes of kinase activation by these receptors remain unclear. We report that after GPCR stimulation, FAK activation is controlled by a sequence of events depending on the scaffolding proteins β-arrestins and G proteins. Depletion of β-arrestins results in a marked increase in FAK autophosphorylation and focal adhesion number. We demonstrate that β-arrestins interact directly with FAK and inhibit its autophosphorylation in resting cells. Both FAK–β-arrestin interaction and FAK inhibition require the FERM domain of FAK. Following the stimulation of the angiotensin receptor $AT_{1A}R$ and subsequent translocation of the FAK–β-arrestin complex to the plasma membrane, β-arrestin interaction with the adaptor AP-2 releases inactive FAK from the inhibitory complex, allowing its activation by receptor-stimulated G proteins and activation of downstream FAK effectors. Release and activation of FAK in response to angiotensin are prevented by an AP-2-binding deficient β-arrestin and by a specific inhibitor of β-arrestin/AP-2 interaction; this inhibitor also prevents FAK activation in response to vasopressin. This previously unrecognized mechanism of FAK regulation involving a dual role of β-arrestins, which inhibit FAK in resting cells while driving its activation at the plasma membrane by GPCR-stimulated G proteins, opens new potential therapeutic perspectives in cancers with up-regulated FAK.

Keywords G-protein-coupled receptors · Beta-arrestin · β-Arrestin · AP-2 · FAK · G proteins

Abbreviations

AP-2	Adaptor protein 2
$AT_{1A}R$	Angiotensin II type 1 receptor
β-arr	β-Arrestin
BRET	Bioluminescence resonance energy transfer
FA	Focal adhesion
FERM	4.1, Ezrin, radixin, moesin
GFP	Green fluorescent protein
MEF	Mouse embryonic fibroblast
SiRNA	Small interfering RNA
WT	Wild type
YFP	Yellow fluorescent protein

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Introduction

Focal adhesion kinase (FAK), a highly conserved non-receptor tyrosine kinase [1], is a key signalling mediator downstream of integrins, receptor tyrosine kinases, and G-protein-coupled receptors (GPCRs) [2–5]. FAK, which controls cell adhesion, polarity, motility, proliferation, and survival [6, 7], plays a critical role during development and its deletion in mice results in early embryonic death [8–12]. FAK is also involved in human diseases such as cardiac hypertrophy and cancer [13, 14]. Increased FAK expression in tumours [7] promotes their progression and metastasis formation [14, 15]. FAK activation may also serve as a tumour cell adaptive resistance mechanism [16]. There is, therefore, considerable interest in targeting FAK with therapeutic agents, inhibitors of FAK kinase activity being currently under investigation in phase I–II clinical trials for various cancers, such as non-small-cell lung cancer, ovarian cancer, and mesothelioma [14, 16, 17]. Functional outputs of FAK rely on both kinase-dependent and kinase-independent functions, on its subcellular localization and on interaction with phospholipids and protein partners [14, 18, 19]. Indeed, in addition to its catalytic properties, FAK is also a scaffolding protein [15, 18–20] with kinase-independent functions [14, 21].

Subcellular pools of FAK at various places within the cell including the plasma membrane, lamellipodia, or the nucleus are engaged in distinct multimolecular signalling complexes with specific biological functions [6, 22]. FAK is also enriched at focal adhesions (FA), which are cellular structures connecting the actin cytoskeleton and the extracellular matrix (ECM), regulating their turnover and integrin-mediated cell adhesion [19, 22, 23]. Molecular mechanisms initiating FAK activation depend on cell context, and remain to be fully characterized in most cases; their identification might provide important new therapeutic avenues.

The main effect attributed to FAK kinase activity is the autophosphorylation of Tyr^{397}, which is positioned between the N-terminal 4.1, ezrin, radixin, moesin (FERM), and the central kinase domains [6]. Phospho-Y397-FAK (pY397-FAK) binds Src-family kinases (SFKs) [5], which mediate most FAK-associated kinase activities including the phosphorylation of FAK on $Tyr^{576/577}$ (pY576/577), Tyr^{861} (pY861), and Tyr^{925} (pY925) which are important for FAK catalytic activity, for interaction with signalling partners and for the subcellular localization of FAK [5, 24]. In the cytoplasm of quiescent cells, intramolecular interaction between FERM and kinase domains prevents Tyr^{397} accessibility and blocks the catalytic site maintaining FAK in the inactive state [25–28]. Plasma membrane recruitment, which involves the FERM domain, is most often required for FAK activation; FAK dimerization also being important for its autophosphorylation [29]. However, the molecular mechanisms triggering release of constitutive FAK auto-inhibition remain incompletely understood [18, 19].

FAK is activated in response to several GPCR ligands [3, 4] such as vasopressin [30], angiotensin II (AngII) [31], and gastrin [32] which also elicit FA formation in smooth muscle and colon carcinoma cells, respectively [33, 34]. Several heterotrimeric G proteins can mediate FAK activation [3, 34, 35] which in turn promote cell proliferation, migration, and tumour progression downstream of activated GPCRs [3, 34, 36]. So far, however, the molecular mechanisms leading to FAK activation by G-protein downstream of stimulated GPCRs have remained elusive.

β-Arrestin 1 and 2 (β-arr1 and β-arr2) are ubiquitous proteins that were originally identified as negative regulators of GPCR function [37]. Indeed, β-arrs uncouple receptors from cognate G proteins and mediate their endocytosis, by bridging GPCRs with clathrin and the adaptor protein AP-2 [38, 39]. Through their scaffolding properties β-arrs also control a broad range of cellular functions, including cytoskeletal rearrangement, cell proliferation, polarity, motility, and apoptosis [40–42], which are also regulated by FAK [6, 7]. β-arrs were also suggested to regulate cell spreading and FA dynamics [43]. Thus FAK and β-arrs can be activated by the same receptors, operate in the same subcellular compartments, and share signalling partners, such as Src-family kinases, PI3-Kinases, and PTEN phosphatase [6, 18, 19, 44–46]. In the present work, we have identified a direct FAK–β-arr interaction and have investigated the cross-talk between FAK and β-arrs. We report that β-arrs regulate both basal and GPCR-stimulated FAK activity. In the absence of stimulation, β-arrs interact with FAK in the cytoplasm, preventing its autophosphorylation, maintaining the kinase inactive, and negatively regulating FA number. Following GPCR activation, however, the β-arr/FAK complex is recruited to stimulated receptors at the plasma membrane. Subsequently, β-arrs interaction with AP-2 releases FAK from β-arrs enabling G-protein-dependent activation of FAK.

Materials and methods

Reagents and antibodies

AngII and arginine vasopressin (AVP) were from Sigma. Barbadin (IUPAC: 3-amino-5-(4-benzylphenyl)-3H,4H-thieno[2,3-d]pyrimidin-4-one, Life Chemicals, F0745-0322) was solubilized in 100% DMSO. All assays using Barbadin were carried out using tips and plates coated with Sigmacote. Antibodies used were: anti-pY397 FAK (clone EP21060Y), anti-FAK (Clone 4.47) from Millipore; anti-FAK (C20) from Santa Cruz; anti-β-arrestin1/2 (D2H49), anti-β-arrestin2 (C16D9), anti-rabbit (DA1E) IgG, anti-pY118-paxillin, anti HA (C29F4), anti-pY397 FAK, anti-pY576/577 FAK, anti-pY925 FAK from Cell Signaling Technology; anti-HA (3F10), anti-Myc (9E10) and anti-GFP from Roche; anti-Paxillin (clone 349) from BD Biosciences; anti-vinculin from Sigma; anti-β-arrestin1 (E274) from Abcam and anti-pY861 FAK (26H16L4) from Invitrogen.

Plasmids

Myc-β-arr1/2 [47], GST-β-arr1/2, Flag-β-arr1/2, His_6-TAT-HA-β-arr2 [45], β-arr1/2-GFP [48], β-arr2ΔAP-2-GFP, GFP-β-arr2-C1, GFP-β-arr2-C1 (ΔAP-2Δclat) [49], β-arr1/2-GFP10, [50], His_6-TAT-HA-FLNA (23–24) [51], YFP-AP2 [52], β-arr2-Rluc [53], and GFP10-β-arr2-RlucII [54] constructs were described previously. SNAP-Flag-V2R [55] was a gift from Dr. R. Jockers (Institut Cochin, Paris) and Thomas Roux (Cisbio, France), and HA-AT_{1A}R and Flag-AT_{1A}R-RlucII plasmids [56] were gifts from M. G. Caron (Duke University, USA) and S. A. Laporte (McGill University, Canada), respectively. FAK-pCMV2, FAK-Nter-pCMV2, and GFP-FAK vectors were provided by Dr J-A Girault (UMR-S839, France). RlucII-FAK was generated by subcloning RlucII cDNA upstream of rat FAK in pCMV2 (*AgeI–KpnI*). HA-FAK and HA-FAK truncations were generated by introducing the HA-Tag 5′ to the corresponding FAK-derived sequence in pCMV or pCMV2 vectors. FAK-RlucII was constructed by exchanging GFP10 and FAK cDNA in the pcDNA3.1-GFP10-RlucII plasmid (*Nhe1–Kpn1*). PCR-generated FAK, FAK-Nter (1–402), and FAK-ΔNter (403–1055) were cloned downstream of the Gal4 transactivation domain in pGAD-GE to generate Gal4AD-FAK, Gal4AD-FAK-Nter, and Gal4AD-FAK-ΔNter plasmids. Gal4BD-β-arr2 was constructed by subcloning rat β-arr2 cDNA into pGBT9 (*EcoRV–Sal1*). Human β-arr2ΔAP-2-GFP10 and mCherry-β-arr2ΔAP-2 (β-arr2-R395A), HA-DRY/AAY-AT_{1A}R [57] and RlucII-FAKY397F were generated by site-directed mutagenesis (Stratagene). Details of all primers used are provided in Supplementary Table 1.

Cell culture and transfections

HEK-293, HEK-AT_{1A}R [58], HEK-V2R [59] cells and wild-type and β-arr1/$2^{-/-}$ (DKO) MEFs were maintained in DMEM supplemented with 10% foetal bovine serum and penicillin/streptomycin. HEK-AT_{1A}R and HEK-V2R cells were supplemented with 0.2 mg/ml Geneticin. HEK-derived cell lines were transfected using Genejuice (Novagen), MEF cell lines using Fugene HD (Promega). On-TARGET plus Smartpool siRNAs from Dharmacon were used to individually target β-arr1 (L-011971) or β-arr2 (L-007292), the 5′-ACCUGCGCCUUCCGCUAUG-3′ siRNA to target both β-arrs simultaneously [60]. A Non-targeting pool (D-001810-10-20) or the 5′-UAGCGACUAAACACAUCA A-3′ sequence was used as control. 60–70% confluent cells were transfected with the indicated siRNAs at a final concentration of 250 nM using DharmaFECT1 (Thermo Scientific).

Immunocytochemistry

MEF cells seeded on Labtek chamber slides (Thermo Scientific) and siRNA-treated HEK-AT_{1A}R cells seeded on collagen-coated 12 mm coverslips were fixed with 4% PFA in PBS for 20 min, permeabilized with 0.2% triton-X-100 in PBS for 5 min, and incubated with blocking buffer (PBS/5% BSA) for 60 min at room temperature (RT). Primary-antibody incubation was performed overnight at 4 °C (pY397-FAK 1:150, Paxillin 1:300, Vinculin 1:300) in blocking buffer. Samples were incubated with appropriate Alexa fluor-conjugated IgG (H + L) antibodies (1:500) (Invitrogen) for 60 min at RT and mounted using Prolong-Antifade Mounting medium-containing DAPI (Invitrogen). Images were acquired using an inverted Leica spinning-disk microscope (63× objective) with a CoolSnap HQ2 (Photometrics) CCD camera controlled by Metamorph 7 software. For live cell imaging, HEK-293 cells were seeded on 35 mm glass bottom dishes (Ibidi) and transfected with HA-AT_{1A}R, GFP-FAK, and mCherry-β-arr2ΔAP-2 for 24 h. Samples (60% confluent) were washed once with DMEM without phenol red and imaged in the same buffer. The stage of the inverted Leica spinning disk microscope was kept at 37 °C during the experiment and images acquired at 40×. Samples were visualized using laser excitation at 491 and 561 nm, and emission filters set at 506–545 nm for GFP and 573–637 nm for mCherry. Representative images were prepared using ImageJ (https://rsb.info.nih.gov/ij/). Icy Bio-imaging software was utilized to quantify focal adhesion number and fluorescence intensity. The pY397-FAK/paxillin images were analysed using the Spot detector plugin [61] set to threshold and filter FA of a minimum size of 5 µm (25 pixels) to quantify the number and fluorescence intensity of FA per cell.

Western blotting and co-immunoprecipitation

Samples were lysed at 4 °C with ice-cold lysis buffer (ICLB), as described [8], and protein concentration determined using Pierce BCA protein assay kit. For western blotting, 30–100 µg of proteins was used. For immunoprecipitations (500 µg proteins) and co-immunoprecipitations (1–1.2 mg protein for cells, 9 mg for rat brain), lysates were pre-cleared with Protein A/G Plus agarose beads (PA/GPAB, Santa Cruz Biotechnology) and incubated with appropriate antibodies overnight at 4 °C. Immunoprecipitated proteins (IP) were collected with PA/GPAB, washed five times with ICLB, and eluted in Laemmli Buffer for the experiments using cell lysates. For the co-immunoprecipitation experiments from brain lysates, the immunoprecipitated proteins were collected with PA/GPAB, washed once with ICLB containing 300 mM NaCl and once with ICLB (150 mM NaCl) before being eluted in Laemmli Buffer. Lysates or IP were resolved using SDS-PAGE gels, blotted on nitrocellulose membranes, and incubated with appropriate antibodies (1:1000) overnight at 4 °C. Samples were revealed by enhanced chemiluminescent detection system (ECL, Thermo Scientific) after incubation with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and visualized using a chemiluminescent reader LAS-3000 (Fuji Lifesciences).

Quantification was performed using Image J (https://rsb.info.nih.gov/ij/).

GST pull down

Freshly prepared recombinant GST, GST-β-arr1, and GST-β-arr2 proteins [45, 51, 62] bound on Glutathione Sepharose4B beads (GE Amersham) (10 µg) were incubated with 100–500 µg of cell lysate proteins for 2 h at 4 °C. The beads were washed three times with ice-cold lysis buffer [8] containing 300 mM NaCl, two times with ICLB and resuspended in Laemmli Buffer before SDS-PAGE.

Kinase assay

Recombinant His_6-TAT-HA-β-arr2 or His_6-TAT-HA-FLNA(23–24) proteins were prepared as above. The kinase assays were performed in kinase buffer containing 20 mM Tris-Base pH 7.5, 150 mM NaCl, 25 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM Na_2VO_4, and 5 mM β-mercaptoethanol. GST-FAK (Active Motif) was incubated on ice for 10 min with either PBS (ctrl) or increasing amount of His_6-TAT-HA-β-arr2 corresponding to molar β-arr2/FAK ratio of 0.5; 1.3 and 3.3 (Supplementary Fig. 2a) or with a molar β-arr2/FAK and FLNA(23–24)/FAK ratio of 3.3 (Fig. 3a). An aliquot was taken from the reaction Mix (0) when 100 µM ATP (Abcam) was added to initiate the assay, which was performed at 30 °C for another 10 min. 2×Laemmeli buffer was added to terminate the reaction and samples processed by SDS-PAGE. Each single time point monitored the autophosphorylation of 50 ng (0.344 pmol) of GST-FAK in a 10 µl volume.

Yeast two-hybrid assay

The assay was performed using the HF7c yeast reporter strain as described [62].

BRET

BRET is a proximity assay for proteins situated at a respective distance of 10 nM or less. Two proteins of interest are fused to the BRET donor, a luciferase, or to the BRET acceptor, a fluorescent protein. Upon addition of the luciferase substrate, the non-radiative energy emitted by the enzyme is transferred to the fluorescent protein, which emits fluorescence at a specific wavelength. BRET-1 experiments were conducted with the Renilla luciferase (Rluc) and the yellow fluorescence protein (YFP) as BRET donor and acceptor, using coelenterazine h as substrate. In BRET-2 experiments, an optimized version of the luciferase (RlucII) and that of the GFP (GFP10) fluorescent proteins were used in the presence of Coelenterazine 400a as substrate. BRET saturation assays were performed as described [62]. Briefly, HEK-293 cells transfected with a constant amount of RlucII-FAK (BRET donor) plasmid and increasing amounts of β-arr1/2-GFP10 (BRET acceptor) plasmid were seeded on poly-ornithine (30 µg/ml) coated white 96-well optiplates (Perkin Elmer) at a density of 20,000 cells per well 24 h post-transfection. The next day, BRET readings were performed in HBSS buffer using the Mithras LB 940 (Berthold Technologies). GFP-10-associated fluorescence was first measured to quantify in each well the amount of BRET acceptor in each well; after the addition of the luciferase substrate Coelenterazine 400a (Interchim, 2.5 µM final), both RlucII (410 nm) and GFP10 (515 nm) signals were measured simultaneously. BRET signal is the ratio of light emitted at 515 nm over the light emitted at 410 nm. Values are means ± s.e.m. of three independent experiments. Saturation curves were plotted as described [62] using GraphPad Prism. Single-point BRET experiments (using RlucII-FAK/β-arr2-GFP10 or β-arr2-Rluc/YFP-AP2) were conducted to monitor the effects of AngII or AVP stimulation over time. In these experiments, HEK-293 cells were co-transfected with the appropriate receptor plasmids (HA-AT_{1A}R or SNAP-V2R-Flag) and serum-starved for 6 h in DMEM before stimulation. AngII or AVP were added for the indicated time at 37 °C and BRET measurements were performed immediately as described above. In BRET 1 experiments (using β-arr2-Rluc/YFP-AP2 as donor and acceptor, respectively), YFP-associated fluorescence was first measured; then, after the addition of Coelenterazine h (Interchim, 5 µM final), both Rluc (485 nm) and YFP (530 nm) signals were measured simultaneously. The BRET ratio signal is the ratio of light emitted at 530 nm over the light emitted at 485 nm. Values are means ± s.e.m. of three independent experiments. Inverse BRET is described in the Supplemental Methods section.

Statistical analyses

Data are mean ± s.e.m. Statistical analyses were performed using Graphpad Prism with either unpaired two-tailed Student's *t* test or ANOVA with Bonferroni post hoc tests for significance comparison as appropriate. Data was considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ respectively.

Results

pY397-FAK is enhanced in β-arr deficient cells

Comparison of FAK autophosphorylation on Tyr^{397} in serum-deprived wild-type (wt) and β-arr1/2 knock-out (DKO) mouse embryonic fibroblasts (MEFs) [63] showed an increase in basal autophosphorylated FAK (pY397-FAK) in cells lacking β-arrs (Fig. 1a). FAK autophosphorylation returned to a level close to that observed in wt-MEFs upon expression of Flag-tagged β-arr1 or β-arr2 (Supplementary Fig. 1a), suggesting that β-arrs downregulate FAK autophosphorylation. Since the tyrosine kinase Src binds to pY397-FAK and then phosphorylates FAK on $Tyr^{576/577}$, Tyr^{861} and Tyr^{925}, we also compared the phosphorylation of these specific residues in wt and DKO cells (Supplementary Fig. 1b). In DKO MEFs, the phosphorylation of $Tyr^{576/577}$ and Tyr^{925} was increased compared to wt cells, similarly to that of Tyr^{397}, whereas the phosphorylation of Tyr^{861} was unchanged.

Since β-arrs were reported to control cell spreading [43] and proteins implicated in cell adhesion [64], we examined whether enhanced FAK autophosphorylation might also occur in DKO MEFs held in suspension. Because FAK basal autophosphorylation is markedly decreased in suspended cells, FAK was immunoprecipitated from lysates of serum-deprived cells in suspension to enrich the signal (Fig. 1b). Again basal pY397-FAK was increased in DKO compared to wt-MEFs. These results indicate that β-arrs inhibit basal FAK autophosphorylation independently of extracellular stimuli, in both non-adherent and adherent MEFs. Paxillin, an FAK-interacting protein localized to focal adhesions (FA), and pY397-FAK were co-localized in FA of wt and DKO MEFs (Fig. 1c); Paxillin and pY397-FAK-containing FA were three times more abundant in DKO than in wt MEFs, the average intensity of pY397-FAK labelling in FA being markedly enhanced in the absence of β-arrs (Fig. 1d–f). These observations support the hypothesis that β-arrs maintain a low basal pY397-FAK in MEFs and negatively regulate FA number.

FAK and β-arrestins are interacting partners

We next examined whether β-arrs and FAK might associate in the same molecular complex. Co-immunoprecipitation experiments from lysates of transfected HEK-293 cells showed that HA-FAK co-immunoprecipitated GFP-tagged β-arr1 or β-arr2 (Fig. 2a) and, conversely, that both Myc-tagged β-arrs co-immunoprecipitated HA-FAK (Fig. 2b). These results confirm recent data, showing that FAK and β-arr1 can be found in the same molecular complex [65], and also suggest that β-arr2 interacts with FAK. We confirmed this observation by showing that FAK and β-arr2 specifically interact in a two-hybrid assay (Fig. 2c) and that purified recombinant β-arr1 or β-arr2 pulled down both transfected HA-FAK and endogenous FAK from lysates of HEK-293 cells (Fig. 2d, e). Importantly, endogenous FAK could also be coimmunoprecipitated with endogenous β-arrs from rat brain lysate (Fig. 2f). Bioluminescence resonance energy transfer (BRET) saturation experiments [62] were also used to monitor the proximity of these proteins in living cells. A constant amount of a plasmid encoding the fusion protein between FAK and Renilla Luciferase 2 (RlucII, the BRET donor) and increasing amounts of plasmids encoding either β-arr1 or β-arr2 fused upstream of the GFP variant GFP10 (the BRET acceptor) were transfected in HEK-293 cells. Hyperbolic curves indicative of specific FAK/β-arr proximity (< 10 nm) were obtained in attached cells compared to linear bystander non-specific BRET observed with GFP_{10} as control BRET acceptor (Fig. 2g; Supplementary Fig. 2a). In addition, similar BRET50 values were calculated for saturation with β-arr1 or β-arr2, indicating that both β-arr isoforms display a similar propensity to be in close proximity with FAK. Similar results were obtained in cells maintained in suspension prior to BRET measurements (Fig. 2h). The specificity of BRET between FAK and β-arrs was further confirmed by an inverse BRET experiment, in which constant amounts of BRET acceptor were expressed in the presence of increasing concentrations of the BRET donor. Supporting specific interaction, the BRET signal decreased with increasing concentrations of RLucII-FAK (Supplementary Fig. 2b). These data indicate that β-arrs and FAK interact under basal conditions even in the absence of cell adhesion and that the basal inhibition of FAK autophosphorylation in wt MEFs, compared to β-arr DKO-MEFs, might be caused by this interaction.

β-arr inhibits FAK catalytic activity and FA number

In vitro kinase assays with purified recombinant proteins were performed to investigate the effect of β-arr2 on FAK autophosphorylation. GST-FAK autophosphorylation was inhibited up to 70% with His6-HA-β-arr2 (Fig. 3a) in a dose-dependent manner (Supplementary Fig. 3a). In control experiments, incubation with carboxyterminal regions of filamin A (His6-HA-FLNA 23–24) [51] had no effect on FAK autophosphorylation (Fig. 3a). These results indicate that β-arr2 interacts directly with FAK and inhibits its autophosphorylation.

Next, we investigated whether the constitutive inhibition exerted by β-arrs on FAK also affects its stimulation by a GPCR. The amount of either or both β-arrs was modulated in HEK-AT_{1A}R cells, which stably express the angiotensin receptor AT_{1A}R, using appropriate siRNAs (Supplementary Fig. 3b, c). Under basal conditions, the decrease of either or both isoforms significantly enhanced endogenous pY397-FAK (Fig. 3b), suggesting that both β-arr1 and β-arr2 inhibit basal FAK catalytic activity as observed in MEFs (Fig. 1). After a short (2 min) stimulation of HEK-AT_{1A}R cells with AngII, both pY397-FAK and FAK phosphorylation on Tyr-576/577 were enhanced (Supplementary Fig. 4a). When β-arr1 and β-arr2 expression was inhibited independently or simultaneously, the amount of endogenous pY397-FAK observed after 2 min stimulation of the AT_{1A}R by AngII

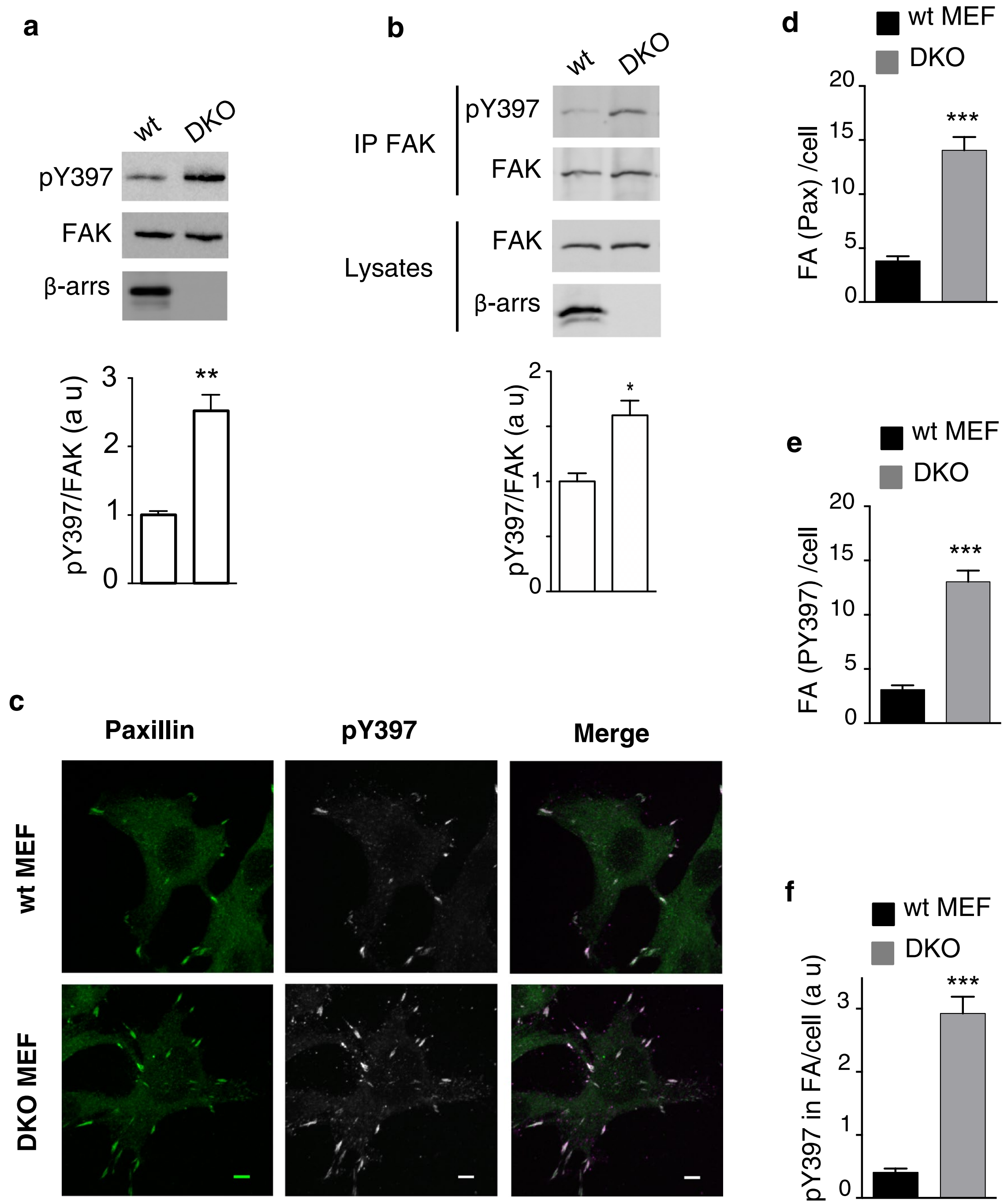


Fig. 1 β-arrs regulate FAK autophosphorylation and focal adhesion number. **a** Adherent wt and β-arr1$^{-/-}$/β-arr2$^{-/-}$ (DKO) MEF cells were serum starved, lysed, and analysed for FAK-pY397, FAK, and β-arrs by immunoblotting. The mean ± s.e.m. of pY397/FAK values, calculated from three independent experiments, was normalized to the value obtained in wt MEFs. (**P value < 0.01, t test). **b** Immunoblotting for FAK-pY397 and total FAK was performed after IP FAK from lysates of serum-deprived MEF cells held in suspension for 60 min at 37 °C. Quantification (lower panel, *P < 0.1, t test) was performed as in **a**. **c** Confocal images of serum-starved MEF cells fixed and stained for paxillin (green) and pY397-FAK (grey). Merge is shown as paxillin (green) and pY397-FAK (magenta). Scale bar = 5 μm. FA number per cell indicated by paxillin (**d**) or pY397-FAK staining (**e**) and pY397-FAK intensity staining in focal adhesions (FA) per cell (**f**) were quantified in MEFs from **c**. For each condition, 35–40 cells from different coverslips were quantified (***P < 0.001, t test)

was further increased compared to control siRNA-treated cells (Fig. 3b).

Stimulation of $AT_{1A}R$ was reported to regulate actin cytoskeleton remodelling [51, 66–68]. The activation of FAK by AngII in HEK-$AT_{1A}R$ induced cell spreading and the formation of stress fibres associated with an increased number of FAs where FAK was colocalized with vinculin (Supplementary Fig. 4b). Following down-modulation of β-arr1, β-arr2, or both, under basal conditions, the number of FA per cell was doubled compared to cells treated with control siRNA and further increased after AngII stimulation (Fig. 3c). These changes reflect both the increase of the number of FAs per cell (Fig. 3d) and the content of pY397-FAK in FAs (Fig. 3e). In conclusion, the above results demonstrate that β-arr content modulates FAK autophosphorylation and FA number under both basal and stimulated conditions.

FAK N-ter is required for FAK/β-arr interaction and FAK inhibition

To identify the domain of FAK involved in β-arr-binding, we used a two-hybrid assay and found that the N-terminal fragment of FAK (Nter, 1–402) (Fig. 4a), which contains the FERM domain, interacted with β-arr2 (Fig. 4b), whereas the deletion of this fragment (FAK-ΔNter, Fig. 4a) resulted in loss of interaction (Fig. 4b). Co-immunoprecipitation experiments comparing β-arr1 and β-arr2 confirmed that both isoforms could interact with FAK-Nter (Fig. 4c). In addition, the presence of excess FAK-Nter in a BRET assay using FAK-RLucII as donor at minimal saturating concentration of the BRET acceptor β-arr2-GFP_{10}, decreased the BRET signal by approximately 50% (Fig. 4d), indicating that the FAK-N-ter fragment competed with the full-length FAK for its interaction with β-arr2.

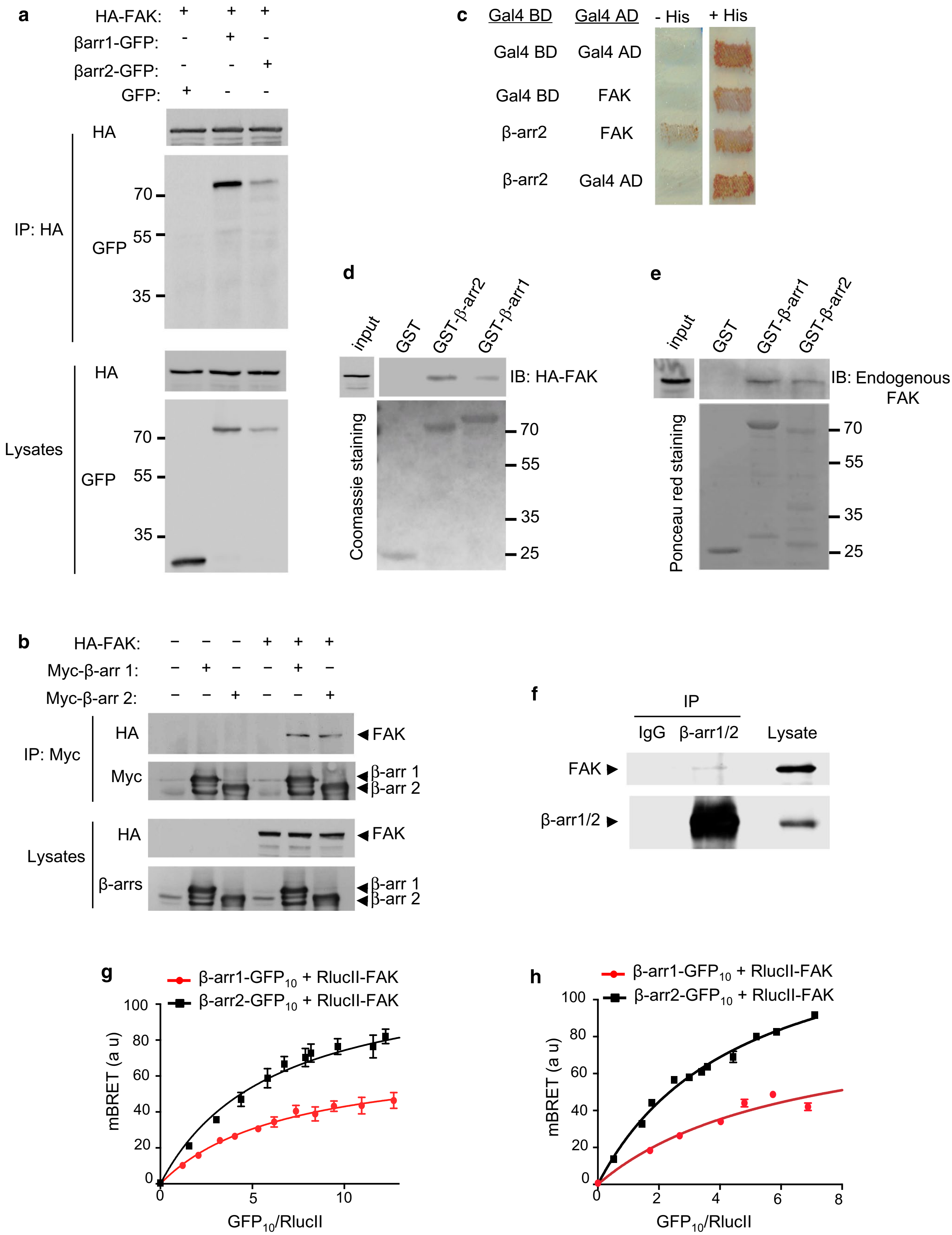
Since β-arrs interact with the N-terminal of FAK, this region should be required for the basal inhibition of FAK autophosphorylation by β-arrs. To test this hypothesis, HEK-293 cells treated with control siRNA or siRNA targeting both β-arr1 and β-arr2 were transfected with full-length HA-FAK or FAK deleted of residues 1–375 [HA-FAK-(376–1055)], and their basal autophosphorylation was monitored after immunoprecipitation (Fig. 4e). In control cells, the autophosphorylation of HA-FAK-(376–1055) was increased compared to HA-FAK, confirming the expected lack of FAK autoinhibition of its catalytic activity, which is normally exerted by the FERM domain [26, 69]. Down-modulation of β-arrs resulted in increased basal pY397-HA-FAK (as shown for endogenous FAK in Fig. 3b), but did not change the autophosphorylation of HA-FAK-(376–1055), indicating that the truncation of residues 1–375, which contain the FERM domain of FAK, renders FAK insensitive to β-arr basal inhibition in intact cells.

G proteins mediate FAK activation upon AT1AR stimulation

Since GPCRs concurrently signal through β-arrs and activate FAK, we next investigated whether and how the constitutive inhibitory effect of β-arrs on FAK activity is actually released under conditions of FAK stimulation. Stimulated GPCRs, including the $AT_{1A}R$, can elicit signalling pathways through both G proteins and β-arrs [70]. The $AT_{1A}R$-DRY/AAY receptor mutant lacks functional coupling with G proteins, but maintains β-arr recruitment and downstream signalling [57, 71]. Contrasting with $AT_{1A}R$, agonist-activated $AT_{1A}R$-DRY/AAY failed to promote endogenous FAK autophosphorylation above basal levels (Fig. 5a), whereas they both promoted the expected comparable recruitment and activation of β-arrs [57] as shown with GFP_{10}-β-arr2-RlucII, a BRET-based β-arr2 translocation biosensor [54] (Fig. 5b). Consistently, the AngII-derived DVG peptide, a β-arr-biased agonist of the $AT1_{1A}R$ [56, 72], failed to stimulate FAK autophosphorylation (Fig. 5c), at a concentration that elicits maximal β-arr translocation in response to AngII (Fig. 5d) [54]. These data indicate that increased FAK autophosphorylation by AngII requires the activation of G-proteins downstream of the $AT1_{1A}R$.

β-arr/AP-2 interaction upon AngII stimulation releases FAK

The potentiation of FAK activation following down-modulation of β-arrs, under both basal and GPCR-stimulated conditions (Fig. 3), suggests that full GPCR-dependent activation of FAK requires both the role of G proteins and the simultaneous release of the constitutive inhibitory effect of β-arrs. Co-immunoprecipitation experiments from lysates of HEK-$AT_{1A}R$ transfected with HA-FAK and GFP-β-arr2, indeed showed a marked decrease (50%) in the amount of FAK co-immunoprecipitated with β-arr2 upon $AT_{1A}R$ stimulation, compared to unstimulated conditions (Fig. 6a), demonstrating that FAK is released from β-arr2 following AngII treatment. Importantly, no autophosphorylated FAK was contained in the fraction co-immunoprecipitated with β-arr2 from lysates of both non-stimulated and AngII-stimulated cell lysates (Fig. 6a) and a FAK mutant that cannot autophosphorylate (FAKY397F) showed similar BRET saturation with β-arr2 than wild-type FAK (Supplementary Fig. 5a), both RlucII-FAK and RlucII-FAK mutant being expressed at a level similar to endogenous FAK (Supplementary Fig. 5b). These results indicate that non-autophosphorylated FAK is maintained inactive by its interaction with β-arr2 under basal conditions and that the complex dissociates following AngII treatment (Fig. 6a). We then investigated how β-arr–FAK complex dissociation may occur. BRET experiments were conducted using FAK-RLucII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βarr2-GFP: - - +
GFP: + - -
IP: HA
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Gal4 BD
Gal4 AD
- His
+ His
Gal4 BD Gal4 AD
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β-arr2 FAK
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HA-FAK: − − − + + +
Myc-β-arr 1: − + − − + −
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IP: Myc
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IP
IgG
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g
β-arr1-GFP10 + RlucII-FAK
β-arr2-GFP10 + RlucII-FAK
mBRET (a u)
100
80
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5
10
GFP10/RlucII
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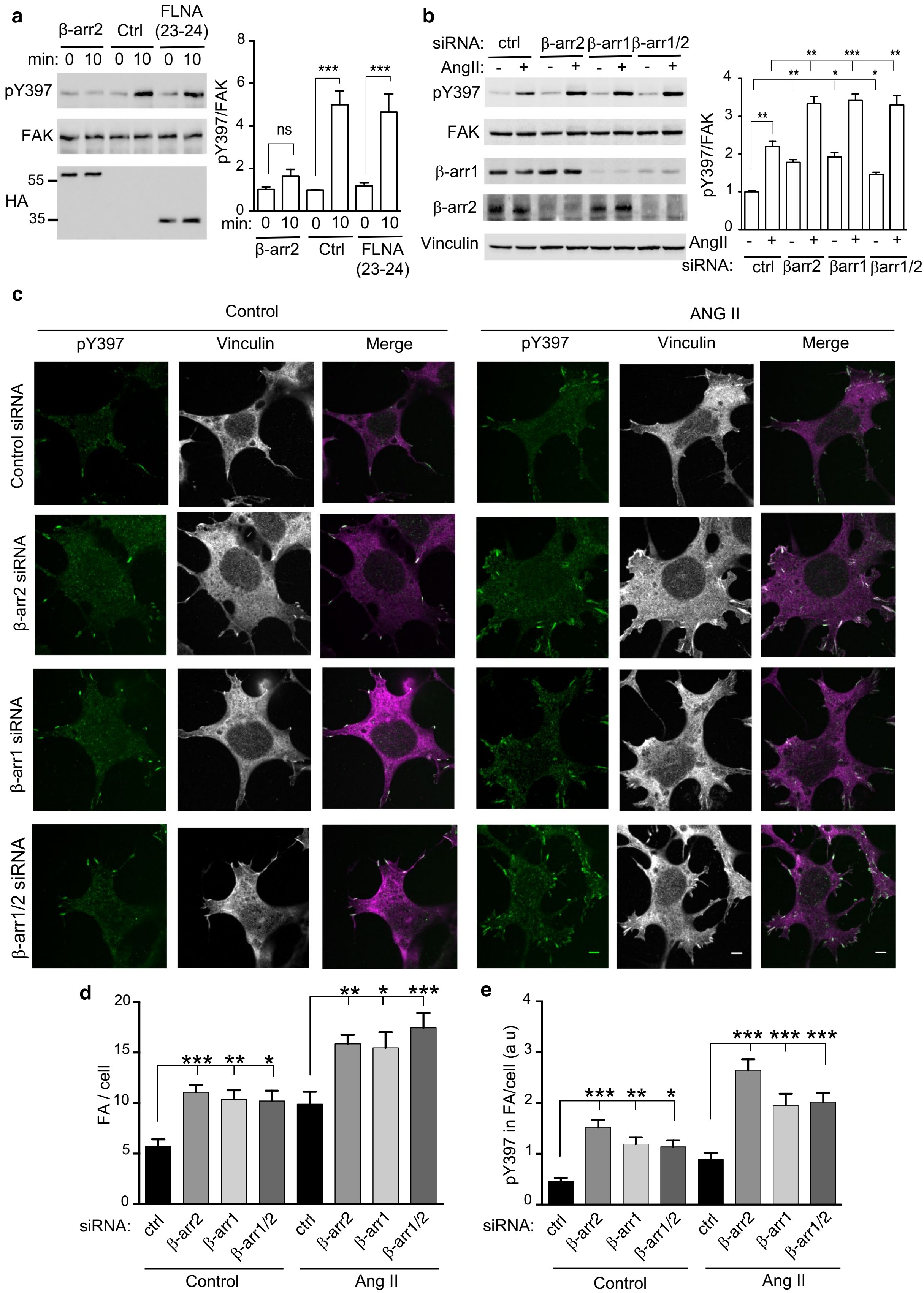
β-arr1-GFP10 + RlucII-FAK
β-arr2-GFP10 + RlucII-FAK
mBRET (a u)
100
80
60
40
20
0
0
2
4
6
8
GFP10/RlucII

◂**Fig. 2** FAK interacts with β-arrs. **a** IPs with HA antibodies were performed on lysates of HEK-293 cells transfected with HA-FAK and GFP or GFP-β-arr1 or GFP-β-arr2. Lysates and IP were immunoblotted for GFP-β-arrs and HA-FAK. **b** IPs with anti-myc antibodies were performed on lysates of HEK-293 transfected with HA-FAK, Myc-β-arr1, or Myc-β-arr2. Lysates and IP were immunoblotted for β-arrs and HA-FAK. **c** Yeast reporter strain HF7c was transformed with the indicated plasmids. Growth in the absence of histidine indicates interaction between β-arr2 and FAK fusion proteins. **d** Purified GST, GST-β-arr1, or GST-β-arr2 bound to glutathione-agarose beads (Coomassie staining) were incubated with HEK-293 lysates transfected with HA-FAK and immunoblotted with an anti-HA antibody. Input is 5% of cell lysate. **e** Purified GST, GST-β-arr1, or GST-β-arr2 bound to glutathione-agarose beads (Ponceau red staining) were incubated with HEK-293 lysates and immunoblotted for endogenous FAK. Input is 5% of cell lysate. **f** IP with rabbit anti-β-arr1/2 antibodies or rabbit IgG were performed on rat brain lysates. Lysates and IP were immunoblotted for FAK and β-arrs. BRET saturation experiments were performed on adherent HEK-293 cells (**g**) or HEK-293 in suspension (**h**) after co-transfection with a constant amount of plasmid for RlucII-FAK and increasing concentrations of β-arr1-GFP_{10} or β-arr2-GFP_{10} plasmids. mBRET values (β-arr1-GFP_{10}: red circles; β-arr2-GFP_{10}: black squares) are means ± s.e.m. of three independent experiments (three replicates for each condition per experiment). $BRET_{50}$ values were 6.89 ± 1.38 and 6.0 ± 1.09 for β-arr1-GFP_{10} and β-arr2-GFP_{10}, respectively (**g**)

and β-arr2-GFP_{10} at the minimal saturating concentration of BRET acceptor in HEK-293 cells expressing $AT_{1A}R$. Treatment of the cells with AngII induced a reduction of the BRET signal (Fig. 6b) reflecting the release of FAK from its complex with β-arr2. Upon recruitment to activated GPCRs, the carboxyterminal tail of β-arr2 is tilted, unmasking binding sites for the β2-adaptin subunit of AP-2 and for clathrin allowing β-arr2 interaction with AP2 [49, 73, 74]. Accordingly, BRET experiments with βarr2-Luc and YFP-AP2 showed that the initial increase of FAK autophosphorylation in response to the stimulation of the $AT_{1A}R$, which peaks at 2–5 min (Supplementary Fig. 4, Fig. 6e, Fig. 7b, coincides with an increased interaction between β-arr2 and AP-2 (Supplementary Fig. 6). To examine whether the agonist-promoted dissociation of the FAK–β-arr2 complex could be functionally connected with β-arr2/AP-2 interaction, we took advantage of a β-arr2 mutant (β-arr2ΔAP-2) with an Alanine substitution of the Arginine-395 that blocks its interaction with β2-adaptin but not recruitment to GPCRs [49, 74, 75]. BRET experiments showed that β-arr2ΔAP-2 interacted with FAK similarly to the wt β-arr2 under basal conditions (Fig. 6b and Supplementary Fig. 7a) and that β-arr2ΔAP-2 was recruited to the AT1AR like the wt β-arr2 upon Ang II treatment (Supplementary Fig. 7b). Live immunofluorescence confocal microscopy of HEK-293 cells transfected with GFP-FAK and mCherry-β-arr2ΔAP-2 (Fig. 6c) further indicated that FAK and β-arr2ΔAP-2 were expressed diffusely in the cytoplasm in absence of stimulation, and that they accumulated simultaneously at the plasma membrane, where they were co-localized following AngII treatment (Fig. 6c). We next compared FAK release and activation in the presence of β-arr2ΔAP-2 or wt β-arr2. Although the basal BRET measured with FAK and β-arr2ΔAP-2 constructs was unchanged, stimulation with AngII failed to induce the same BRET change observed with wt β-arr2 (Fig. 6b). A plausible interpretation of these findings is that the interaction of β-arr2 with AP-2, after the translocation of the β-arr2/FAK complex to activated receptors, would produce a conformational change in the complex, which would release FAK from β-arr2. To validate this hypothesis, co-immunoprecipitation experiments were performed using lysates of control or AngII-stimulated HEK-$AT_{1A}R$ cells expressing HA-FAK and β-arr2-GFP or β-arr2ΔAP-2-GFP. Compared to basal conditions, stimulation of the $AT_{1A}R$ did not change the amount of FAK, which was co-immunoprecipitated with β-arr2ΔAP-2 as opposed to the marked decrease of FAK co-immunoprecipitated with wild-type β-arr2 (Fig. 6d). These results support the hypothesis that the interaction of β-arr2 with AP-2 induces the dissociation of the β-arr2/FAK complex and the release of FAK after AngII treatment. We next examined whether the release of FAK from β-arr2, consequently to its interaction with AP-2, would also relieve the constitutive inhibition of β-arr2 on FAK. FAK autophosphorylation was monitored in HEK-$AT_{1A}R$ expressing comparable levels of β-arr2-GFP or β-arr2ΔAP-2-GFP (Fig. 6e). Agonist-promoted endogenous FAK autophosphorylation was markedly decreased (> 50%) at all time points in cells expressing β-arr2ΔAP-2-GFP, compared to cells containing β-arr2-GFP. Moreover, Barbadin (β-arrestin/β2-adaptin interaction inhibitor), a recently characterized small molecule, which inhibits GPCR endocytosis by specifically blocking the interaction of β-arrs with AP-2 [76], abolished the AngII-promoted BRET change, without affecting basal BRET (Fig. 7a; Supplementary Fig. 8a, b). Consistent with our hypothesis and recapitulating the effect observed in cells expressing β-arr2ΔAP-2 (Fig. 6e), preincubation with Barbadin markedly decreased the level of endogenous FAK autophosphorylation induced by $AT_{1A}R$ stimulation (Fig. 7b). To determine whether the interaction between β-arr and AP-2 regulates FAK activation downstream of additional GPCRs, we performed similar experiments in HEK-293 cells stably expressing the V2 vasopressin receptor (HEK-V2R). Vasopressin (AVP) induced a strong autophosphorylation of FAK in these cells with a peak between 2 and 5 min (Fig. 7c) that was correlated with an increased β-arr2/AP-2 interaction (Supplementary Fig. 6) similarly to what was observed in response to AngII stimulation (Figs. 4, 6e, 7b and Supplementary Fig. 6). Barbadin strongly inhibited too the endogenous activation of FAK in response to AVP treatment in HEK-V2R (Fig. 7c). Thus, the role of β-arr and of its interaction with AP-2 in the activation of FAK is not limited to the $AT_{1A}R$. Noteworthy, 15 min after stimulation, FAK autophosphorylation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β-arr2
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FLNA (23-24)
min: 0 10 0 10 0 10
pY397
FAK
55
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min: 0 10 0 10 0 10
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AngII: - + - + - + - +
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◂**Fig. 3** β-arrs regulate FAK catalytic activity and focal adhesion number. **a** GST-FAK was incubated with PBS (Ctrl) or equivalent amounts of purified HA-β-arr2 or HA-FLNA (23–24) for 10 min on ice. Aliquots were taken at the initiation (0) of the autophosphorylation assay and after 10 min incubation at 30 °C and immunoblotted for pY397, FAK and HA. Data represent mean ± s.e.m. of pY397-FAK/FAK normalized to the control (0) time point set to 1, from five independent experiments. **b** HEK-$AT_{1A}R$ cells were transfected with control, β-arr1, β-arr2 or β-arr1/2 siRNA, serum starved and left untreated or stimulated with 100 nM AngII for 2 min. Lysates were immunoblotted with the indicated antibodies. Data on the right panel represent means ± s.e.m. of pY397-FAK/FAK values from five independent experiments normalized to unstimulated cells transfected with control siRNA and set to 1. $*P<0.05$; $**P<0.01$; $***P<0.001$; one-way ANOVA, Bonferroni. Values obtained for β-arr siRNA-treated cells (targeting either isoform or both) were not significantly different either in basal or stimulated conditions. **c** HEK-$AT_{1A}R$ cells prepared as in **b** and seeded on collagen-coated coverslips, were untreated or stimulated with 100 nM AngII for 5 min and immunostained for pY397-FAK (green) and Vinculin (grey) with the merge shown as pY397-FAK/green and Vinculin/magenta. Scale bar = 5 µm. **d**, **e** For each condition, the number of FA per cell and the pY397-FAK intensity staining in FA per cell were quantified for 20–30 cells from different coverslips. **a**–**e** $*P<0.05$; $**P<0.01$; $***P<0.001$; one-way ANOVA, Bonferroni

was still sustained in response to AngII, whereas it markedly decreased in response to AVP (Fig. 7b, c). To determine whether this difference in the kinetic of FAK dephosphorylation was correlated with the interaction between FAK and β-arr, we compared co-immunoprecipitation between β-arr2 and FAK in HEK-$AT_{1A}R$ and HEK-V2R cells after 15 min of receptor stimulation. In co-immunoprecipitation experiments from AngII-stimulated HEK-$AT_{1A}R$ cell lysates, the amount of FAK co-immunoprecipitated with β-arr2 remained markedly decreased compared to unstimulated conditions (Supplementary Fig. 9a) reflecting FAK release from β-arr2. Under the same conditions, the amount of FAK co-immunoprecipitated with β-arr2 from AVP-stimulated HEK-V2R cell lysates was comparable to that obtained in basal conditions (Supplementary Fig. 9b). These results confirm that the level of FAK autophosphorylation is correlated to its interaction with β-arr. Phosphorylation of paxillin downstream of activated FAK mediates the reorganization of the cytoskeleton, FAs' turnover, and cell movement [77]. To further demonstrate that the regulation of FAK by β-arrs impacts important downstream signalling, we determined paxillin phosphorylation in response to $AT_{1A}R$ stimulation. Similarly to pY397-FAK, paxillin phosphorylation was markedly decreased in cells expressing β-arr2ΔAP-2 (Fig. 6e) or preincubated with Barbadin (Fig. 7b). Similar and even larger effects were also observed in HEK-V2R cells pretreated with Barbadin (Fig. 7c). These results demonstrate that the regulation of FAK by β-arrs has important downstream signalling effects in cells.

Saturation of coated pit-associated AP-2 and clathrin with the C-terminal tail of β-arr2 (amino acids 317–410; C1) inhibits β-arr interaction with AP-2 and GPCR endocytosis [49]. Endogenous FAK autophosphorylation in response to AngII treatment was decreased by 50% in cells expressing this C1 peptide fused to GFP (GFP-C1), compared to GFP-transfected cells, indicating a dominant negative effect on FAK activation. Mutation of the AP-2 and clathrin-binding sites within the C1 sequence (GFP-C1mut) restored the level of pY397-FAK to that observed in cells expressing GFP, indicating that binding to AP-2 and clathrin is essential for GFP-C1 dominant negative effect (Supplementary Fig. 10).

Taken together, our data indicate that the GPCR-dependent activation of FAK in complex with β-arrs at steady state results from the synergistic termination of the constitutive inhibition exerted by β-arrs and the G-protein-dependent activation of FAK released from the complex with β-arrs.

Discussion

We report that β-arrs are essential regulators of FAK activity under both basal and GPCR-stimulated conditions. We uncovered a mechanism whereby β-arrs appear to play a dual role in the regulation of FAK activation: they inhibit FAK autophosphorylation under basal conditions while promoting localized FAK activation by G proteins through their recruitment to activated receptors (Fig. 8).

In the cytoplasm of resting cells, β-arrs are associated with FAK. The observation of increased basal FAK autophosphorylation in cells with no or reduced expression of β-arrs, together with the observation that only non-autophosphorylated FAK interacts with β-arr2, support a model where the constitutive association of these proteins maintains FAK inactive and prevents its autophosphorylation (Fig. 8). This model is also supported by the direct inhibition of FAK catalytic activity by β-arr2 in vitro.

Our data reveal an unknown role for β-arrs as FAK inhibitors. In non-stimulated cells, cytoplasmic FAK is maintained inactive by intra-molecular contacts between the FERM and the catalytic domains [25–28]. We found that the FAK N-terminus, which contains the FERM domain, is sufficient for the association with β-arr and necessary for the β-arr-dependent basal inhibition of FAK. Under basal conditions, β-arrs could stabilize the interaction between FERM and kinase domains preventing FAK "opening" (Fig. 8). Bound β-arrs may also mask Tyr^{397}, which is involved in the initial activation of FAK (Fig. 8). FIP200, another FAK protein inhibitor [78], acts differently from β-arr, since it binds directly to the kinase domain of FAK [79].

We showed that GPCR-dependent activation of FAK is controlled by an ordered sequence of events that depends on both β-arr and G-protein activation. We found that β-arr and FAK translocate simultaneously to the plasma membrane where they co-localize upon AngII treatment. The

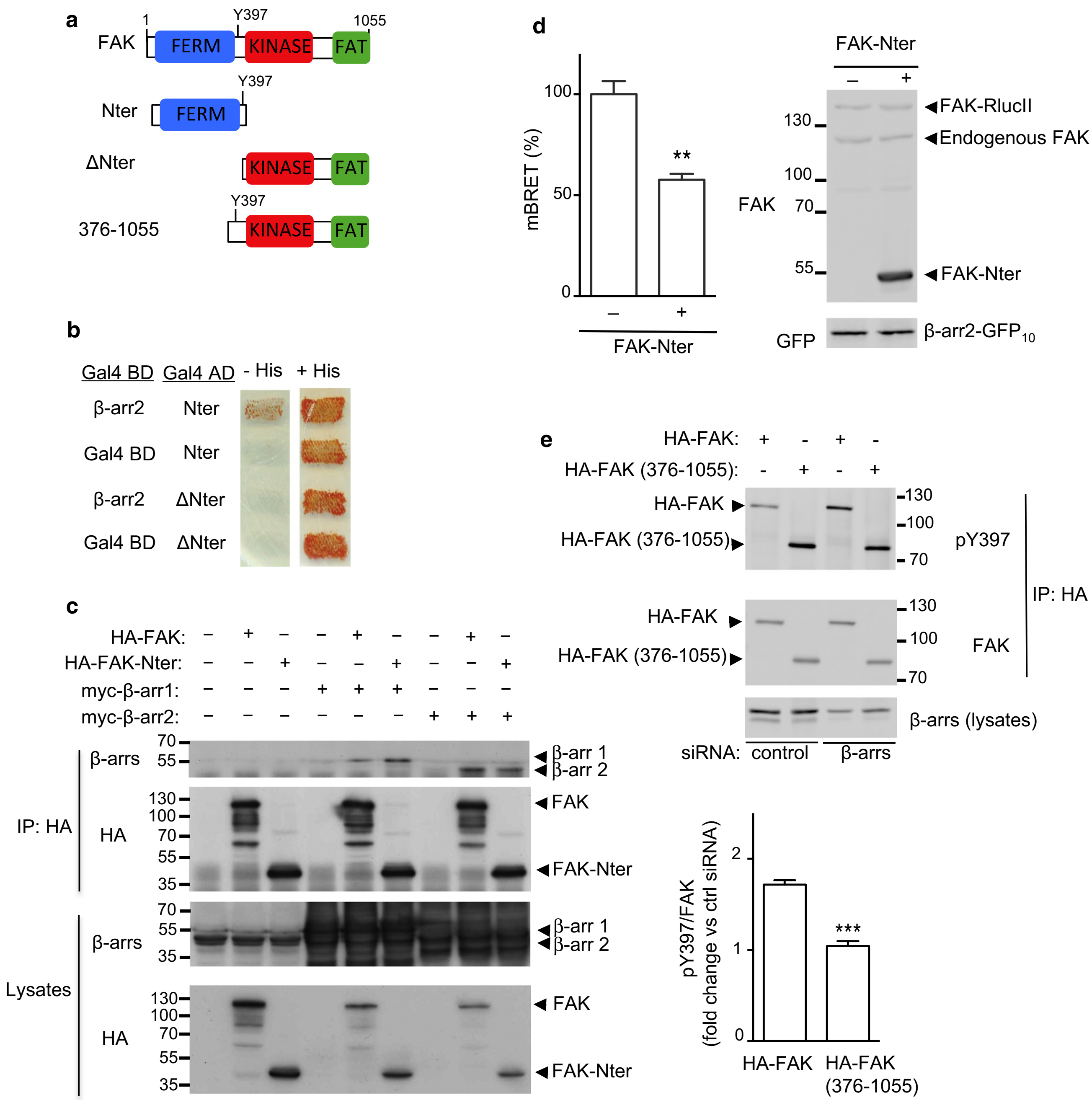


Fig. 4 FAK N-terminal domain is necessary for β-arr interaction and FAK inhibition. **a** Schematic of FAK truncations used in **b**–**e**. FAK-Nter (1–402) and FAK-ΔNter (403–1055). **b** HF7c yeast reporter strain was transformed with the indicated plasmids. In the absence of histidine, β-arr2 elicited growth with FAK-Nter but not with FAK-ΔNter. **c** IPs were performed with anti-HA antibodies on lysates of HEK-293 cells transfected with the indicated plasmids. Lysates and IP were immunoblotted with anti-β-arr and anti-HA antibodies. **d** HEK-293 cells were co-transfected with plasmids coding for β-arr2-GFP_{10}, FAK-RlucII, and FAK-Nter at a 3:1 FAK-Nter/FAK-RlucII or empty vector/FAK-RlucII ratio. Graph represents mean ± s.e.m of BRET values from three independent experiments (three replicates per experiment), normalized to the BRET observed in cells transfected without FAK-Nter, and set to 100% (**$P < 0.01$, *t* test). Samples were lysed and immunoblotted using anti-FAK and anti-GFP antibodies (right panel). **e** HEK-293 cells were treated with indicated siRNA for 24 h followed by transfection with HA-FAK or HA-FAK (376–1055) plasmids for another 24 h and serum starved. IP performed with anti-HA antibodies on the lysates were immunoblotted for pY397-FAK and FAK, and lysate for β-arrs. Data represent the fold increase of pY397-FAK/FAK or pY397-FAK (376–1055)/FAK (376–1055) in the β-arr knockdown condition normalized to their respective control siRNA condition. Mean ± s.e.m. of three independent experiments are shown (***$P < 0.001$, *t* test)

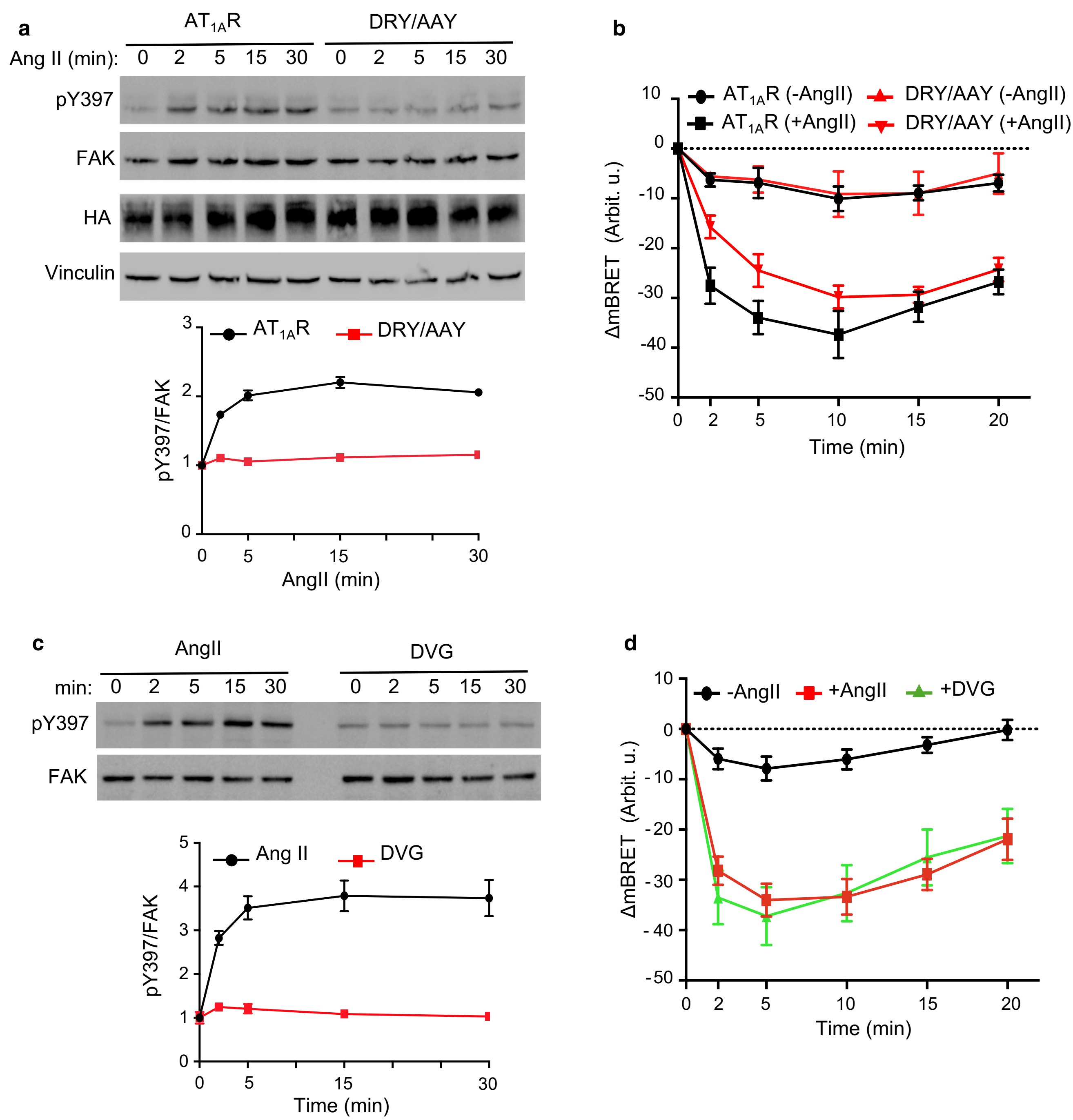
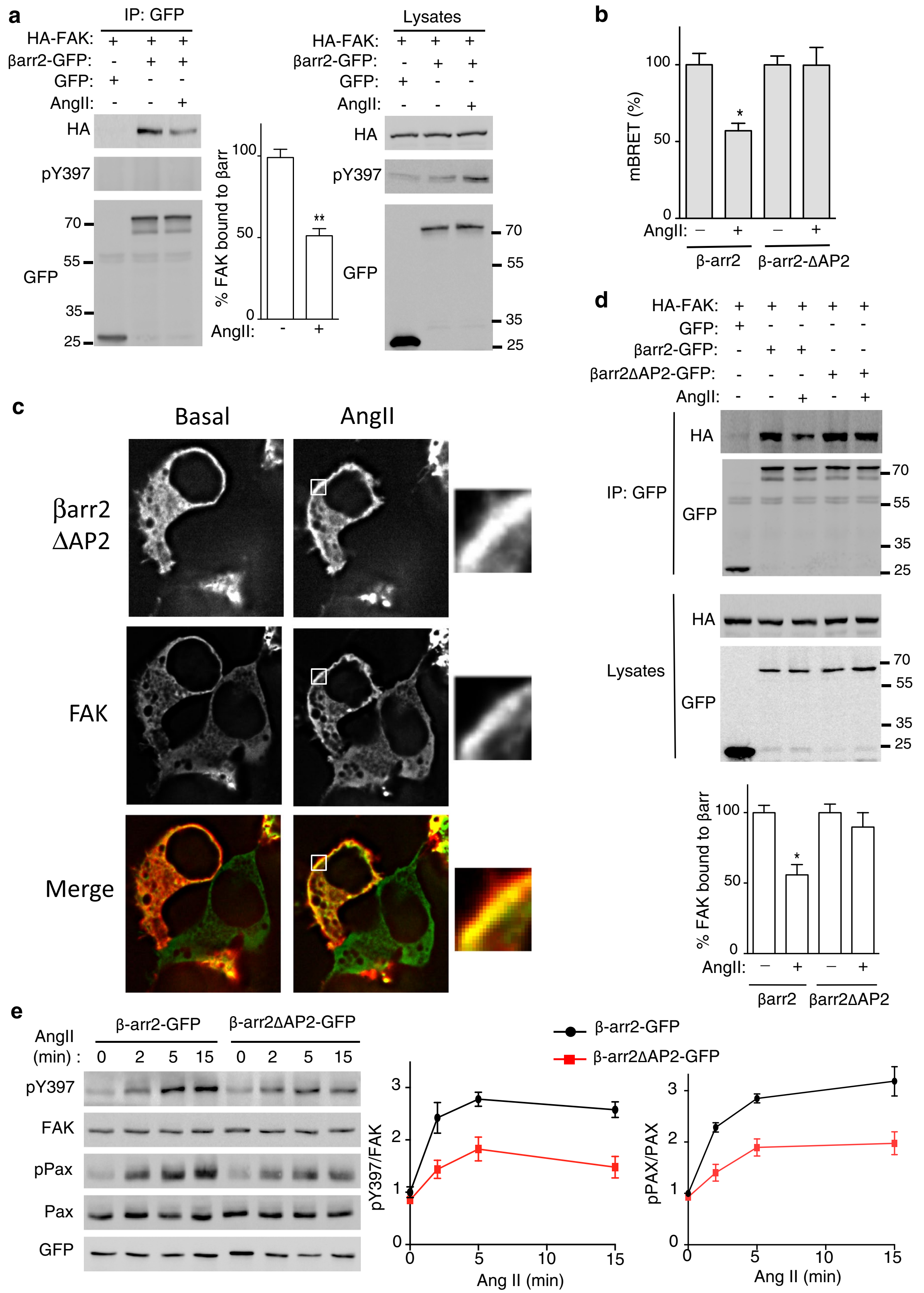


Fig. 5 FAK autophosphorylation in response to AngII stimulation is mediated by G protein-dependent pathways. **a** HEK-293 cells were transfected with HA-AT$_{1A}$R or HA-AT$_{1A}$R (DRY/AAY), serum starved, and stimulated with 100 nM AngII for the indicated times. Lysates were immunoblotted for pY397-FAK, FAK, HA, and vinculin. Data shown (bottom panel) represent mean ± s.e.m of pY397-FAK/FAK values [HA-AT$_{1A}$R: black circles, HA-AT$_{1A}$R (DRY/AAY): red squares] normalized to untreated cells transfected with the HA-AT$_{1A}$R and set to 1 from three independent experiments. **b** Real-time BRET measurement of HEK-293 cells co-transfected with GFP$_{10}$-β-arr2-RlucII and HA-AT$_{1A}$R (black symbols) or HA-AT$_{1A}$R (DRY/AAY) (red symbols), left untreated (− AngII, black circles and red base down-triangles), or stimulated with 100 nM AngII (+ AngII, black squares and red base up-triangles). Graph represents agonist-induced ΔmBRET mean values (mBRET value − mBRET value at time 0) from three independent experiments (3–8 replicates for each condition per experiment). **c** Following serum deprivation, HEK-AT$_{1A}$R cells were stimulated with 100 nM AngII or with 10 μM of the β-arr-biased ligand DVG for the indicated time. Lysed samples were immunoblotted for pY397-FAK, and FAK. Quantification (AngII: black circles, DVG: red squares) was performed from three independent experiments as in **a**. **d** Real-time BRET measurement of HEK-293 cells co-transfected with GFP$_{10}$-β-arr2-RlucII and HA-AT$_{1A}$R, left untreated (− AngII, black circles), or stimulated with 100 nM AngII (+ AngII, red squares) or 10 μM DVG (+ DVG, green triangles). Graph represents ΔmBRET mean values from three independent experiments (4–6 replicates for each condition per experiment)


a
IP: GFP
HA-FAK: + + +
βarr2-GFP: - + +
GFP: + - -
AngII: - - +
HA
pY397
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% FAK bound to βarr
100
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AngII: - +
Lysates
HA-FAK: + + +
βarr2-GFP: - + +
GFP: + - -
AngII: - - +
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pY397
GFP
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Basal
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HA-FAK: + + + + +
GFP: + - - - -
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βarr2ΔAP2-GFP: - - - + +
AngII: - - + - +
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% FAK bound to βarr
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AngII: – + – +
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AngII (min) :
β-arr2-GFP
β-arr2ΔAP2-GFP
0 2 5 15 0 2 5 15
pY397
FAK
pPax
Pax
GFP
β-arr2-GFP
β-arr2ΔAP2-GFP
pY397/FAK
Ang II (min)
pPAX/PAX
Ang II (min)

◂**Fig. 6** Interaction of β-arr2 with AP-2 upon AngII stimulation releases FAK from β-arr2 and its constitutive inhibition over FAK. **a** HEK cells were transfected with HA-FAK and GFP or β-arr-GFP, serum starved and left unstimulated or treated with 100 nM AngII (10 min). IPs were performed using anti-GFP antibodies, and both IPs and lysates were immunoblotted for HA-FAK, pY397-FAK, and GFP. Data represent mean ± s.e.m. of the ratio of co-immunoprecipitated HA-FAK to immunoprecipitated GFP normalised to GFP in the lysate, and normalised to untreated cells set to 100% from three independent experiments (**$P < 0.01$, *t* test). **b** HEK-293 cells co-transfected with HA-$AT_{1A}R$, RlucII-FAK, and β-arr2-GFP_{10} or β-arr2ΔAP-2-GFP_{10} were untreated or stimulated with 1 μM AngII for 15 min and BRET measured. Graph represents mean ± s.e.m. of BRET normalized to BRET in untreated cells transfected with β-arr2-GFP_{10} and set to 100% from three independent experiments (4–6 replicates for each condition per experiment). *$P < 0.05$; one-way ANOVA, Bonferroni. **c** Live cell imaging of GFP-FAK and mCherry-β-arr2ΔAP-2 upon AngII treatment. Confocal images of HEK-293 cells transfected with HA-$AT_{1A}R$, GFP-FAK (grey), and mCherry-β-arr2ΔAP-2 (grey) were acquired in absence of stimulation (basal) and 5 min after the addition of 100 nM AngII. Merged images are shown as GFP-FAK in green and mCherry-β-arr2ΔAP-2 in red. **d** HEK-$AT_{1A}R$ cells were transfected with indicated plasmids, processed as in **a** and both IPs and lysates were immunoblotted for HA-FAK and GFP-β-arrs. Three independent experiments were quantified as in **a**. *$P < 0.05$, one way ANOVA. **e** HEK-$AT_{1A}R$ cells transfected with β-arr2-GFP or β-arr2ΔAP-2-GFP were serum starved and unstimulated or stimulated with 100 nM AngII for the indicated time. Lysates were immunoblotted as indicated. Data represent mean ± s.e.m of pY397-FAK/FAK and phospho-Paxillin/Paxillin values (β-arr2-GFP: black circles, β-arr2ΔAP-2-GFP: red squares), normalized to untreated β-arr2-GFP-transfected cells and set to one from three independent experiments

translocation of the β-arr/FAK complex to activated receptors promotes the interaction between β-arr and AP-2. This contact releases FAK from β-arrs and enables its activation by proximal receptor-stimulated G proteins (Fig. 8). The functional significance of this regulation is demonstrated by the downstream effect on paxillin phosphorylation. Thus, we have identified an essential role of β-arrs in which the interaction of β-arrs with AP-2 is involved in the release of an inhibitory control mode of β-arrs over a downstream effector system. Our study also deciphers the respective contribution of β-arrs and G proteins to FAK activation and the delineation of the molecular mechanism of their coordinated action involving AP-2.

Reported models of FAK activation and stress fibres and/or FA formation downstream of GPCRs involve RhoA GTPases [33, 66–68] and potential cross-talk between RhoA and β-arrs has also been documented in different contexts [45, 66, 80]. For example, RhoA participates in the β-arr-dependent regulation of PTEN downstream of the lysophosphatidic acid receptor [45, 80]. The Rho exchange factor Rgnef and $G\alpha_{13}$ also concur to recruit and activate FAK at the plasma membrane, downstream of CCK2 receptors [32]. FAK clustering was proposed to contribute to its activation [14, 18, 19]; β-arrs might thus also promote its autophosphorylation, by accumulating FAK in proximity to activated GPCRs. FAK activation requires both the release of the autoinhibition exerted by the FERM domain and the trans-phosphorylation by nearby FAK catalytic domains [19]. Both events can be achieved via the FERM domain-dependent recruitment of FAK to β-integrins, growth-factor receptors, and/or plasma membrane-associated lipids [14, 18, 19]. In this context, β-arrs, by forming a bridge between the FERM domain and the activated $AT_{1A}R$, might also contribute to increase proximal local concentrations of FAK, thus promoting subsequent trans-phosphorylation.

FAK is regulated by several GPCRs and most activated GPCRs recruit β-arrs. In the present study, we report that the β-arr2-mediated control of FAK activity is modulated by both the $AT_{1A}R$ and the V2R. It appears that, whereas the activation rate of FAK autophosphorylation by these receptors is similar, the duration of FAK activation may vary depending of the receptor, since FAK autophosphorylation was sustained in response to AngII but more transient in response to AVP, this difference being correlated to the amount of β-arr2 bound to FAK in each case. Multiple mechanisms might be involved in this phenomenon, such as the duration of G-protein coupling with the receptor, which depends on desensitization mechanisms, or the specific G protein that the receptors are preferentially coupled to. Whereas V2R is principally coupled to Gs, $AT_{1A}R$ is coupled to Gq; downstream effectors of either G protein might contribute to FAK re-association with β-arrs. Specific investigations will be required to address this issue. Other GPCRs might also activate FAK in a β-arr-dependent manner. Indeed, a β-arr1/STAM1 (Signal-transducing Adaptor Molecule 1) complex was recently found to modulate FAK downstream of the GPCR CXCR4 [65]. On the other hand, β-arr-dependent FAK regulation could also occur downstream of integrins, since β-arrs interact with filamin, an integrin-binding protein, to regulate cytoskeleton remodelling [51].

We found that Barbadin, a newly identified AP-2 binding small molecule that prevents β-arrestin interaction with AP-2 without interfering with its recruitment to activated GPCRs [76] inhibits FAK activation. Barbadin may, therefore, represent an interesting tool for the development of FAK inhibitors downstream of GPCRs.

Src recruited to pY397 can phosphorylate FAK on Tyr^{576}, Tyr^{577}, Tyr^{861}, and Tyr^{925}. As expected, the absence of β-arrs in DKO MEFs, which results in increased pY397, also promotes phosphorylation of $Tyr^{576/577}$ and Tyr^{925}. $Tyr^{576/577}$ are located in the central kinase domain of FAK and their phosphorylation is required for full catalytic activity of FAK, whereas pY925 acts as a docking site for growth-factor-receptor-bound protein 2 (Grb2), which permits signalling to the Ras-ERK cascade and regulates FAK localization at FA [5, 24]. The phosphorylation of Tyr^{861}, which may have multiple functions and has mostly been characterized as an

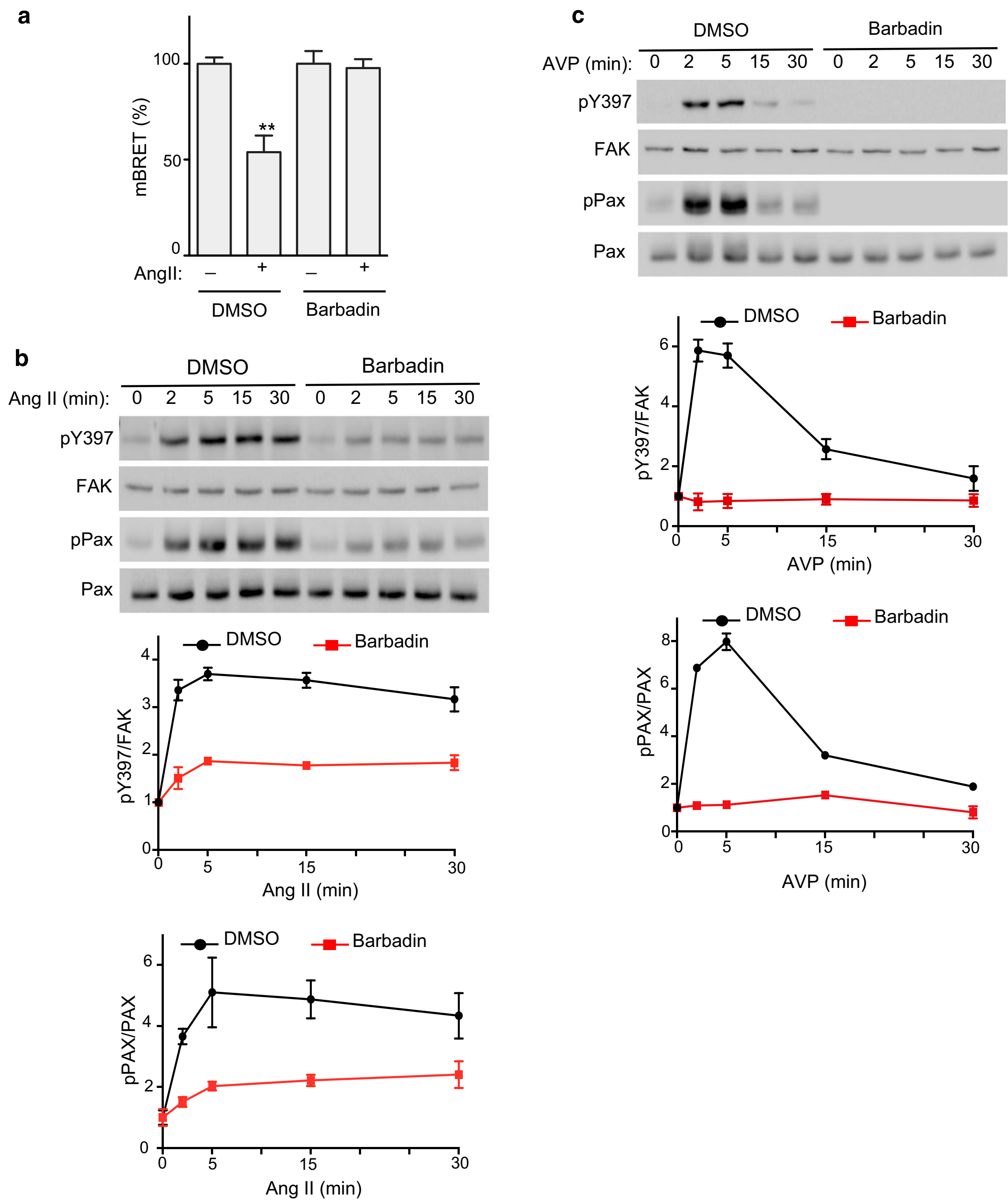

Fig. 7 Inhibition of β-arr2–AP-2 interaction downregulates FAK activation in response to AngII stimulation. **a** HEK-293 cells co-transfected with HA-$AT_{1A}R$, RlucII-FAK, and β-arr2-GFP_{10} plasmids were pre-treated with DMSO or 50 µM Barbadin for 10 min and untreated or stimulated with 1 µM AngII (15 min) before BRET measurements. Graph represents mean ± s.e.m. of BRET values normalized to the value obtained in control cells (DMSO) left untreated and set to 100% from three independent experiments (3–6 replicates for each condition per experiment). $^{**}P < 0.01$; one-way ANOVA, Bonferroni. HEK-$AT_{1A}R$ (**b**) or HEK-V2R (**c**) cells were serum starved overnight, pre-treated with DMSO or 50 µM Barbadin for 10 min and stimulated with 100 nM AngII (**b**) or 100 nM AVP (**c**) for the indicated time. Lysates were immunoblotted for pY397-FAK, FAK, phospho-Paxillin, and Paxillin. Data calculated from three independent experiments, represent mean ± s.e.m of pY397-FAK/FAK and phospho-Paxillin/Paxillin values (DMSO: black circles, Barbadin: red squares) normalized to untreated cells and set to one

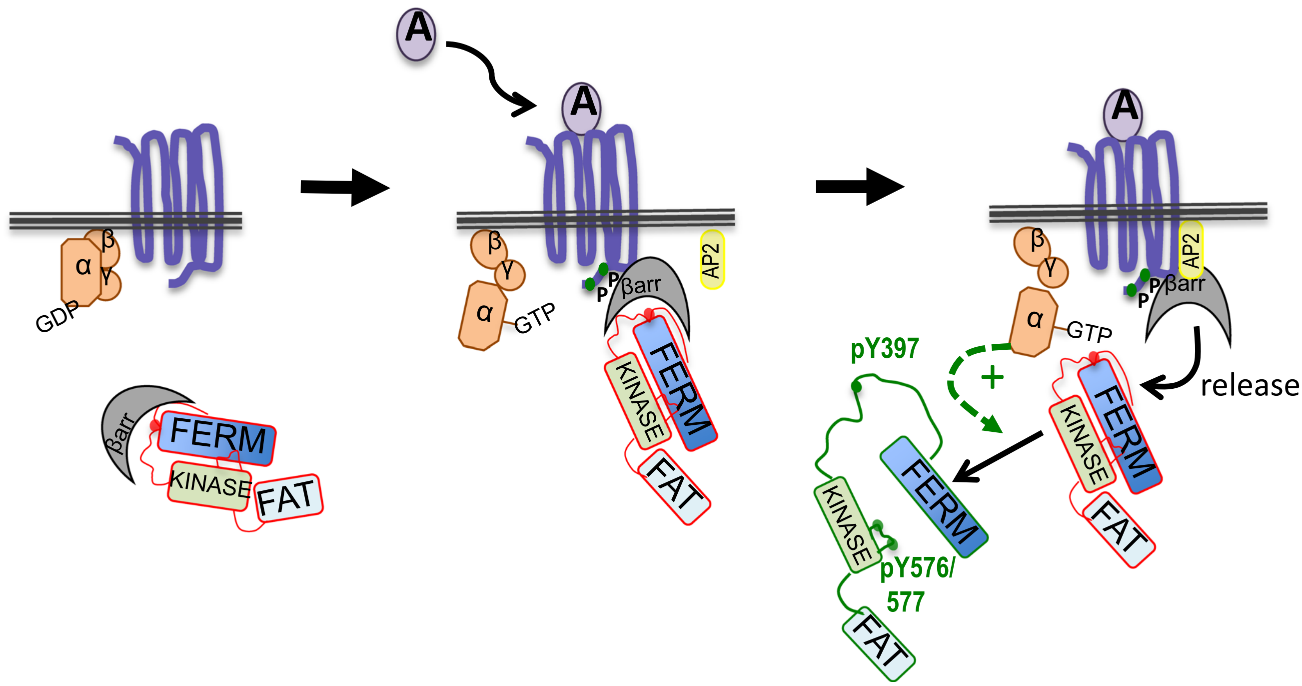

Fig. 8 Schematic model for the regulation of FAK activity by β-arrs under basal and GPCR-stimulated conditions. Under basal conditions, both β-arr1 and β-arr2 form molecular complexes with a pool of non-autophosphorylated FAK in the cytoplasm, inhibiting its catalytic activity and negatively regulating both the amount of pY397-FAK in FA and FA number. Agonist (A)-mediated GPCR activation triggers G-protein activation (illustrated by GDP to GTP exchange); the receptors are then phosphorylated (P) by GRKs (not shown) and recruit β-arr–FAK complexes. β-arr interaction with AP-2 results in the release of FAK from its complex with β-arr, which relieves the inhibition exerted by β-arr on FAK, followed by FAK activation by the G protein

important regulator of angiogenic response, is unchanged in DKO MEFs compared to wt-MEFs. Alternative mechanisms of Tyr^{861} phosphorylation have been suggested. One of these mechanisms would require Src-SH2 domain (that binds to pY397) but not Src enzymatic activity, suggesting that Src bound to pY397 may bridge FAK with another kinase phosphorylating Tyr^{861} [81]. Other studies reported that phosphorylation of Tyr^{861} can be uncoupled from the one of Tyr^{397} [82–86]. Our results suggest that none of the above mechanisms are regulated by β-arrs in MEFs, since the phosphorylation of Tyr^{861} is unchanged in DKO compared to wt-MEFs. Thus, through the regulation of Tyr^{397} phosphorylation, β-arrs also regulate the phosphorylation of $Tyr^{576/577}$ and Tyr^{925} but not Tyr^{861}.

Ang II promotes the formation of stress fibres and FAs [33, 66, 67], and FAK plays an important role in the turnover of FAs [6]. Here, FAK regulation via β-arrs was correlated with FAK autophosphorylation, paxillin phosphorylation, and FA number in resting and AngII-stimulated cells, extending the spectrum of β-arr impact on cytoskeletal reorganization. β-arrs were reported to promote FA disassembly independently of receptor activation [43] and the β2-adrenergic receptor-dependent regulation of FA involves RhoA and the β-arr2-dependent regulation of its upstream activator p115-RhoGEF [87]. Our results suggest that FAK activation and FA formation require both the initial translocation of the FAK–β-arr complex to the activated receptor and subsequent β-arr interaction with AP-2. Although several different mechanisms involving β-arrs and/or FAK likely converge to regulate FA formation, the control of plasma membrane targeting and activation of protein partners involved in this process by β-arrs may be particularly important.

Both β-arr isoforms interact with FAK, and $BRET_{50}$ values, which reflect the apparent propensity of association with FAK in intact cells, were similar for β-arr1 and β-arr2. In addition, introduction of β-arr1 or β-arr2 in DKO MEFs resulted in pY397-FAK returning to the level observed in wt-MEFs and siRNA-mediated knock-down of either β-arr in HEK caused the same effect on FAK autophosphorylation. Thus, both β-arrs appear to contribute to FAK inhibition, a global decrease of β-arr level below a certain threshold being sufficient to enhance basal or stimulated FAK activation. This hypothesis is supported by the dose-dependent inhibition of GST-FAK autophosphorylation by recombinant β-arr2 in vitro. FAK and β-arrs are expressed at variable levels in adult tissues [1, 44] and the effects of β-arrs on FAK activity likely depend on their respective concentrations. Furthermore, FAK expression and autophosphorylation are increased in several human cancers [7], whereas changes in β-arrs are correlated with cancer progression and clinical outcome [44]. Changes of FAK and β-arr concentration in cancer cells might, thus, impact the regulation of FAK activation, particularly in response to GPCR activation.

In summary, we show that β-arrs are endogenous FAK inhibitors regulating FAK catalytic activity and FA formation under basal conditions. The release of basal β-arr-mediated inhibition of FAK is promoted by the recruitment of β-arr/FAK complexes to GPCRs. The subsequent interaction of β-arr with the AP-2 adaptor liberates FAK, allowing subsequent activation by adjacent active G proteins. β-arrs, therefore, operate an on/off switch resulting in the localized control of FAK activity. Since FAK overexpression plays a critical role in tumour progression and metastasis formation, FAK regulation via β-arrs likely has an important impact on cancer development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical standards The experiments comply with the current laws of France, the country in which they were performed.

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