Focal adhesion kinase–dependent activation of the early endocytic protein Rab5 is associated with cell migration

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© Cecilia Arriagada†, Patricio Silva‡§, Martial Millet‡, Luis Solano†, Carolina Moraga†, and © Vicente A. Torres‡¶
From the †Institute for Research in Dental Sciences, Faculty of Dentistry, Universidad de Chile, Santiago 8380000, Chile, the §Faculty of Health Sciences, Universidad Central de Chile, Santiago 8380000, Chile, and the ¶Advanced Center for Chronic Diseases, Universidad de Chile, Santiago 8380000, Chile
Edited by Alex Toker

Focal adhesion kinase (FAK) is a central regulator of integrin-dependent cell adhesion and migration and has recently been shown to co-localize with endosomal proteins. The early endocytic protein Rab5 controls integrin trafficking, focal adhesion disassembly, and cell migration and has been shown to be activated upon integrin engagement by mechanisms that remain unclear. Because FAK is a critical regulator of integrin-dependent signaling and Rab5 recapitulates FAK-mediated effects, we evaluated the possibility that FAK activates Rab5 and contributes to cell migration. Pulldown assays revealed that Rab5-GTP levels are decreased upon treatment with a pharmacological inhibitor of FAK, PF562,271, in resting A549 cells. These events were associated with decreased peripheral Rab5 puncta and a reduced number of early endosome antigen 1 (EEA1)–positive early endosomes. Accordingly, as indicated by FAK inhibition experiments and in FAK-null fibroblasts, adhesion-induced FAK activity increased Rab5-GTP levels. In fact, expression of WT FAK and FAK/Y180A/M183A (open conformation), but not FAK/Arg454 (kinase-dead), augmented Rab5-GTP levels in FAK-null fibroblasts and A549 cells. Moreover, expression of a GDP-bound Rab5 mutant (Rab5/S34N) or shRNA-mediated knockdown of endogenous Rab5 prevented FAK-induced A549 cell migration, whereas expression of WT or GTP-bound Rab5 (Rab5/Y79L), but not Rab5/S34N, promoted cell migration in FAK-null fibroblasts. Mechanistically, FAK co-immunoprecipitated with the GTPase-activating protein p85α in a phosphorylation (Tyr397)–dependent manner, preventing Rab5-GTP loading, as shown by knockdown and transfection recovery experiments. Taken together, these results reveal that FAK activates Rab5, leading to cell migration.

Cell adhesion and migration are tightly regulated by the formation and disassembly of integrin-containing macromolecular complexes known as focal adhesions (FAs).2 Focal adhesion kinase (FAK) is a central regulator of FA dynamics, and it has been implicated in many cellular processes, such as cell adhesion, survival, and migration (1). FAK is a 125-kDa protein comprised of an N-terminal 4.1-ezrin–radixin–moesin domain, a central kinase domain, and a C-terminal domain that contains the FA-targeting site (2). Upon integrin engagement, FAK undergoes autophosphorylation on Tyr397, creating a binding site for SH2-containing proteins such as Src, Grb2, and p85α (1, 3). Intriguingly, recent studies have shown that integrins can signal from endocytic compartments and that FA proteins, including FAK, co-localize with endocytic proteins, which is required for integrin-dependent signaling (4–6).

Compelling evidence indicates that deregulated endocytic trafficking is a recurrent mechanism that contributes to the acquisition of malignant traits in tumor cells, including enhanced cell migration, invasion, and metastasis (reviewed in Refs. 7, 8). This is particularly observed in a group of small GTPases involved in endosome/vesicle trafficking, the Rabs, because several members of this family are up-regulated in cancer (7). From this group, the early endocytic protein Rab5 plays critical roles in cell adhesion and migration because it is activated in migrating cells, promoting FA disassembly (9–11), integrin endocytosis and recycling (12, 13), and Rac1 activation (14, 15), among other events (reviewed in Refs. 16, 17). Early studies showed that engagement of integrin β1 triggers Rab5-GTP loading, leading to tumor cell migration and invasion in vitro and in vivo (13). The mechanisms underlying activation of Rab5 upon integrin engagement have not been explored, although it has been shown to depend on phosphorylation events (14, 18). Specifically, Src-dependent phosphorylation of pro-caspase-8 on Tyr380 creates a docking site for SH2-containing proteins involved in cell migration, such as p85α (18–20). In addition to its role as the regulatory subunit of PI3K, p85α depicts GTPase-activating protein (GAP) activity toward Rab5, decreasing Rab5-GTP levels, Rab5-dependent trafficking, and early endosome localization (21, 22). Thus, phosphorylation of pro-caspase-8 is followed by sequestration of p85α, precluding Rab5-GTP hydrolysis and promoting cell migration (13, 18).

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This article contains Figs. S1–S5.

1 To whom correspondence should be addressed: Institute for Research in Dental Sciences, Faculty of Dentistry, Universidad de Chile, Olivos 943, Independencia, Santiago 8380000, Chile. E-mail: vatorres@med.uchile.cl.

2 The abbreviations used are: FA, focal adhesion; FAK, focal adhesion kinase; GAP, GTPase-activating protein; MEF, mouse embryonic fibroblast; GEF, guanine nucleotide exchange factor; Ctrl, control; WCL, whole-cell lysate(s).
Because FAK phosphorylation on Tyr\textsuperscript{397} is a central event in cell adhesion and migration, p85α interacts with phosphorylated FAK on Tyr\textsuperscript{397} via its SH2 domains (3), and because sequestration of p85α is a mechanism that accounts for sustained Rab5 activation (18), we hypothesized that FAK is involved in Rab5 activation during integrin-mediated cell adhesion. In this work, we report that FAK stimulates Rab5 activity, leading to increased cell migration, as shown in models involving integrin-dependent activation of FAK and expression of FAK mutants in A549 cells and by reconstitution experiments in FAK-null fibroblasts. Mechanically, FAK formed a complex and sequestered p85α in a phosphorylation-dependent manner, maintaining elevated Rab5-GTP levels. Accordingly, Rab5 activity was required for FAK-induced cell migration.

**Results**

**Inhibition of FAK decreases Rab5-GTP levels in non-stimulated and spreading cells**

Studies from our group and others have shown that ligation of integrin β\textsubscript{1} is followed by a time-dependent increase in Rab5-GTP levels (9, 13). Because FAK is a central effector of downstream integrin engagement (1) and because Rab5 stimulates cellular responses that are reminiscent of those initiated by FAK, such as FA assembly and disassembly (9), Rac1 activation (14, 15), persistent cell migration (9, 11), and matrix metalloproteinase activation (9, 23), we hypothesized that FAK is an upstream regulator of Rab5 that promotes GTP loading via an intermediate regulator yet to be identified. To address this hypothesis, A549 cells, which are known to adhere to fibronectin via integrin α\textsubscript{v}β\textsubscript{3} (9, 13), were treated with the small molecule PF562,271, which specifically inhibits FAK autophosphorylation on Tyr\textsuperscript{397} (24), and Rab5-GTP levels were measured by pulldown, as reported previously (9, 18). As expected, PF562,271 caused a dose-dependent decrease in FAK autophosphorylation in A549 cells (Fig. S1A), with a 70% reduction at a concentration of 1 μM (Fig. 1A). Interestingly, FAK inhibition with PF562,271 was associated with a substantial decrease in Rab5-GTP levels compared with control cells (Fig. 1B). These effects seemed to be selective for Rab5, because PF562,271 did not induce any fluctuations in Rab11-GTP levels (Fig. S1B), nor association of Rab4 with GTP-bound Rab5 (data not shown). Previous studies showed that Rab5-GTP loading is associated with relocation of early endosomes (9, 18). Accordingly, PF562,271-mediated reduction of Rab5-GTP levels was associated with decreased early endosome number but not size, as judged by using the effector of Rab5 and early endosome-specific marker EE1 (Fig. 1, C and D). Importantly, these fluctuations were not associated with changes in total Rab5 or EE1 levels (Fig. S1C and Fig. 1B). Furthermore, PF562,271 decreased staining of Rab5-positive puncta at the cell periphery (Fig. 1, E and F), which is in agreement with previous reports showing activation-dependent relocation of Rab5 (9, 18). Intriguingly, PF562,271 did not affect the size or number of Rab5-positive structures (Fig. S1D) or the localization of other related Rab proteins (Fig. S1E). These observations suggest that basal Rab5-GTP levels are partially maintained by FAK in a phosphorylation-dependent manner and that these events are associated with peripheral localization of early endosomes.

Because integrin ligation promotes both FAK autophosphorylation on Tyr\textsuperscript{397} (1) and Rab5 activation (9, 13), we next evaluated whether adhesion-induced Rab5-GTP loading required FAK. To this end, A549 cells were allowed to adhere and spread onto fibronectin-coated surfaces for 2 h, and the effects of PF562,271 were analyzed. First, as anticipated, suspended cells showed minimal levels of phospho-Tyr\textsuperscript{397} FAK, which were further inhibited by PF562,271. However, cell adhesion to fibronectin was followed by a substantial increase in phospho-Tyr\textsuperscript{397} FAK levels, which were inhibited by PF562,271 (Fig. 2, A and B). Then Rab5 activity was measured in suspended and adherent cells, and, in agreement with previous studies (13), Rab5-GTP levels were substantially increased during adhesion to fibronectin (Fig. 2C). Interestingly, adhesion-driven Rab5 activation depended on FAK phosphorylation because PF562,271 decreased Rab5-GTP levels in adherent but not in suspended cells (Fig. 2, C and D).

FAK inhibition experiments suggest that both basal and adhesion-induced activation of FAK are required for Rab5-GTP loading. These findings were further supported by genetic approaches because expression of WT FAK and FAK/Y180A/M183A (open conformation, constitutively active FAK), but not FAK/Arg\textsuperscript{345} (kinase-dead mutant of FAK), which were readily autophosphorylated on Tyr\textsuperscript{397} (Fig. 3, A and B), increased endogenous Rab5-GTP levels in A549 cells (Fig. 3, C and D). These observations were confirmed in FAK-null mouse embryonic fibroblasts (FAK–/– MEFS), because Rab5-GTP levels were substantially decreased in FAK–/– MEFS compared with control FAK+/+ MEFS (Fig. 3, E–H). Most importantly, reconstitution of FAK–/– MEFS with GFP–FAK, but not GFP–FAK/Arg\textsuperscript{345} (Fig. 3, E and F), restored Rab5-GTP loading to levels comparable with those observed in FAK+/+ MEFS (Fig. 3, G and H). Collectively, these results confirm the finding that FAK activity is necessary for Rab5 activation in nonstimulated and adhering cells and that it may constitute an unanticipated mechanism involved in FAK-driven cell migration.

**Rab5 is relevant for FAK-driven cell migration**

Several studies have shown the relevance of Rab5 in different aspects of cell migration (reviewed in Ref. 17), and although much evidence has been focused on downstream events triggered by Rab5, a few studies are available regarding upstream regulation leading to Rab5 activation in the context of cell migration. Because FAK is a critical regulator of cell migration, and because Rab5-GTP levels were shown to be increased by FAK (Figs. 1–3), we evaluated whether FAK-induced cell migration required Rab5 activation. To this end, A549 cells were co-transfected with FAK and Rab5/S34N (the GDP-bound, inactive mutant of Rab5). As expected, and in agreement with previous studies (9), expression of WT FAK promoted a significant increase in cell migration, whereas expression of Rab5/S34N decreased basal cell migration by 35% (Fig. 4, A and B). Most importantly, co-transfection of Rab5/S34N along with WT FAK inhibited FAK–induced cell migration to levels that were below those observed in control cells (Fig. 4B). In agreement with these observations, FAK−/− MEFS
FAK-driven Rab5 activation

Depicted decreased cell migration compared with control FAK+/+ MEFs (Fig. S2A), whereas expression of Rab5/Q79L (the GTP-bound, active mutant of Rab5) and, to a lesser extent, WT Rab5, but not Rab5/S34N, promoted cell migration in FAK/H11002 MEFs (Fig. 4, C and D). The requirement of Rab5 for FAK-induced cell migration was also confirmed in Rab5 knockdown experiments because shRNA-mediated targeting of endogenous Rab5 decreased the extent of cell migration induced by FAK (Fig. S2B). Collectively, these data suggest that Rab5 is a novel downstream player in FAK-driven cell migration.

Recruitment of p85α by FAK prevents Rab5 inactivation

To obtain insights into the mechanisms involved in FAK-mediated Rab5 activation, we searched for putative guanine nucleotide exchange factors (GEFs) and GAPS that could potentially be associated with FAK. In this group of regulators, which included the Rab5 GEFs RIN2 and ALS2 and the Rab5 GAP p85α, only p85α was found to undergo relocalization within early endosomes upon FAK inhibition with PF562,271 (Fig. S3). This observation was interesting because p85α, which is commonly described as the regulatory subunit of PI3K, is known to interact with FAK in a phosphorylation-dependent manner (3), and it is known to depict GAP activity toward Rab5 (21, 22).

Figure 1. FAK inhibition decreases basal Rab5-GTP levels. A and B, A549 cells were treated with DMSO (Ctrl) or 1 μM PF562,271 (PF), and whole-cell lysates were prepared for Western blot analysis (A) and R5BD pulldown assays (B). Top panels, representative Western blot images are shown for phospho-Tyr397–FAK (pY397-FAK), total FAK, β-actin, Rab5-GTP, and total Rab5. The graphs indicate the quantification of pTyr397–FAK versus total FAK and Rab5-GTP versus total Rab5, respectively, as obtained from scanning densitometric analysis. Data represent the average of four independent experiments (mean ± S.E.; **, p < 0.01). C, EEA1-positive early endosomes were visualized by immunofluorescence and confocal microscopy using a rabbit polyclonal antibody and 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining in cells treated with either DMSO or 1 μM PF271. Representative images are shown. Scale bar = 10 μm. D, early endosome number and size were quantified from data obtained as in C by using ImageJ software. Data were obtained from quantification of 40 cells per condition (mean ± S.E.; ***, p < 0.001; n.s., nonsignificant). E, Rab5 localization in A549 cells treated with DMSO or 1 μM PF562,271 was analyzed by immunofluorescence and confocal microscopy using a specific mAb against Rab5. Arrowheads indicate peripheral Rab5 localization. Representative images are shown. Scale bar = 10 μm. F, left panel, intensity profiles for Rab5 localization were obtained by tracing a line from the nuclei to the cell periphery and plotted with ImageJ software. Right panel, quantification of peripheral Rab5, obtained by measuring the relative fluorescence intensity within the proximal 5 μm next to the cell periphery (inset square, left panel). Data were obtained by averaging 15 cells per condition (mean ± S.E.; *, p < 0.05). A.U., arbitrary units.
Alternatively, the requirement of p85α of FAK-null fibroblasts, we confirmed that FAK and p85α Akt pathway (events are independent of activation of the conventional PI3K/Akt pathway (Fig. 1). A549 cells affected active Akt levels, suggesting that these

and observed that neither FAK inhibition nor Rab5 depletion in

recruitment of p85α with respect to total FAK (B) and Rab5-GTP with respect to total Rab5 (D), as obtained from scanning densitometric analysis. Data represent the average of six independent experiments (mean ± S.E.; *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., nonsignificant).

Figure 2. FAK inhibition prevents adhesion-induced activation of Rab5. A549 cells were either left in suspension (Susp.) or allowed to adhere to fibronectin-coated plates (2 μg/ml) for 2 h in the presence of DMSO (Ctrl) or 1 μM PF562,271 (PF). A–D, cell extracts were prepared and used for subsequent whole-cell lysate analysis (A and B) or RSBD pulldown assays (C and D). A and C, representative Western blot images are shown for phospho-Tyr397–FAK (pY397–FAK), total FAK, β-actin, Rab5-GTP, and total Rab5. B and D, quantification of pY397–FAK with respect to total FAK (B) and Rab5-GTP with respect to total Rab5 (D), as obtained from scanning densitometric analysis. Data represent the average of six independent experiments (mean ± S.E.; *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., nonsignificant).

microscopy; FAK and p85α co-localized at the cell periphery in a PF562,271-dependent manner (Fig. 5C). Similar results were obtained with different combinations of antibodies that recognized endogenous FAK and p85α (Fig. S4). These data indicate that recruitment of p85α by FAK requires phosphorylation on Tyr397, which parallels the up-regulation of Rab5-GTP levels by FAK. It is of note that, because p85α is a critical regulator of the PI3K/Akt pathway, we measured phospho-Thr308–Akt levels and observed that neither FAK inhibition nor Rab5 depletion in A549 cells affected active Akt levels, suggesting that these events are independent of activation of the conventional PI3K/Akt pathway (Fig. S2C). Finally, by using the alternative model of FAK-null fibroblasts, we confirmed that FAK and p85α associate in a phosphorylation-dependent manner because FAKζ/ζ–MEFs reconstituted with WT FAK, but not FAK/Arg454 showed higher co-immunoprecipitation of FAK and p85α (Fig. 5, D and E).

Then, to evaluate the role of p85α in Rab5 activation by FAK, we performed pulldown assays in co-expression experiments with WT FAK and p85α. In accordance with our observations (Fig. 3), co-transfection of GFP-FAK with an empty vector (mCherry alone) did not affect FAK-induced Rab5 activation (Fig. 6A). However, exogenous expression of p85α prevented FAK-induced Rab5 activation, as shown in co-transfection experiments using mCherry–p85α and GFP-FAK (Fig. 6A). Alternatively, the requirement of p85α in FAK-induced Rab5 activation was evaluated in siRNA-mediated knockdown experiments. In agreement with previous observations (Fig. 1), FAK inhibition with PF562,271 decreased Rab5-GTP levels in siRNA control cells; however, these effects were not observed upon siRNA-mediated targeting of endogenous p85α (Fig. 6B). In fact, sole knockdown of endogenous p85α caused an increase of Rab5-GTP levels, which is in agreement with earlier reports showing augmented Rab5 activity in p85α-deficient cells (21, 25). Nevertheless, under these conditions, FAK inhibition did not decrease elevated Rab5-GTP levels in siRNA-p85α cells (Fig. 6B). Taken together, these results indicate that p85α is involved in FAK-driven Rab5 activation.

Discussion

Several studies have shown that the small GTPase Rab5 promotes cell adhesion and migration via different mechanisms that include enhancing integrin trafficking, FA turnover, and Rac activation (9–11, 14, 15) (reviewed in Refs. 16, 26). However, leaving aside downstream signaling stemming from Rab5, upstream regulators accounting for increased Rab5 activity during cell adhesion and migration have remained largely unknown. Here we identify FAK as an unanticipated, indirect activator of Rab5 that promotes cell migration via increased Rab5-GTP loading. By using pharmacological and genetic approaches, we show that FAK activity is required for both maintenance of Rab5-GTP levels under basal conditions in resting cells and adhesion-induced Rab5-GTP loading. FAK-driven activation of Rab5 was associated with peripheral and enlarged early endosomes, which is in accordance with earlier studies indicating that Rab5 is necessary for maintenance of the number of early and late endosomes (27). A similar outcome has been documented previously, showing that Rab5-GTP levels increase upon phosphorylation of pro-Caspase-8 on Tyr380, leading to relocalization of Rab5 to the cell periphery, hence affecting the trafficking of cargo from early to late endosomes (18–20).

Intriguingly, previous studies by our group and others showed that Rab5 accelerates the rates of FAK autophosphorylation on Tyr397, which is necessary for FA turnover and tumor cell invasion (9, 28), whereas other studies showed that Rab5 expression and activity are necessary for hypoxia-driven FAK activation (29). These seemingly disparate observations could be reconciled with this study on the basis of the existence of a FAK–Rab5 circuitry that operates in a positive feedback loop that provides fine-tuning of cell adhesion and migration and whose deregulation under conditions such as hypoxia leads to sustained cell migration and invasion, as observed in tumor cells.

The observation that FAK autophosphorylation on Tyr397 was required for Rab5-GTP loading in cells undergoing adhesion to fibronectin but not in suspended cells suggests that β1 and β3 integrins are possible initiators required to activate this signaling cascade. Our data favor the involvement of β1 integrins because, although A549 cells express both integrins (29, 30), PF562,271 failed to decrease Rab5-GTP levels in cells adhered to collagen I (a ligand for both β1 and β3-integrins) or to plates coated with anti-β1 antibodies (Fig. S5). This is in addition to the fact that only β1 has been shown to associate with Rab5 in a complex, promoting its endocytic trafficking (9, 12). Although further research is required to identify the subset of integrins that trigger Rab5-GTP loading via FAK, this will be relevant in the context of tumor cell migration because a preference for one specific integrin dictates different migration responses. Specifically, engagement of α5β1 is associated with
random/fast cell motility, whereas \( \alpha_\beta_3 \)-dependent cell migration is associated with directional/persistent but slow cell migration (31).

Our data show that p85\(\alpha\), which is a Rab GAP, co-immunoprecipitates with phospho-Tyr\(^{397}\) FAK, allowing increased Rab5-GTP levels. This is intriguing because, among Rab5 GAPs and GEFs with reported roles in cell migration, p85\(\alpha\), but not ALS2 or RIN2, were found to relocalize to/from early endosomes. However, we cannot exclude the participation of additional GAPs or GEFs yet to be identified. Our study suggests that FAK-induced Rab5 activation requires sequestration of p85\(\alpha\). Importantly, our data suggest that p85\(\alpha\)’s GAP activity, but not side activation of the PI3K/Akt branch, is involved in FAK-driven Rab5 activation and cell migration because phospho-Thr\(^{308}\)–Akt levels remained constant upon FAK inhibition or Rab5 depletion, and expression of Rab5/S34N increased phospho-Thr\(^{308}\)–Akt levels (Fig. S2D), which is opposite to the effects of this mutant in cell migration. In fact, Akt inhibition did not cause any significant fluctuations in Rab5-GTP levels (Fig. S2E). Hence, we propose that FAK-dependent sequestration of p85\(\alpha\) prevents Rab5 inactivation, allowing increased Rab5-GTP levels (Fig. 6C, proposed model).
A similar mechanism of sequestration was shown several years ago. Specifically, Src-mediated phosphorylation of pro-caspase-8 on Tyr\textsuperscript{380} creates a docking site for SH2 domain–containing proteins, including p85\textsuperscript{α}/H9251 (19, 20), which, upon interaction with caspase-8, is sequestered, precluding Rab5-GTP hydrolysis and promoting relocalization of early endosomes to the cell periphery (18). It remains to be explored whether p85\textsuperscript{α}/H9251 associates with FAK via other sites and that p85\textsuperscript{α}/H9251’s SH2 domains are required for Rab5-GTP loading. Intriguingly, recent studies by our group indicated that recruitment of p85\textsuperscript{α} associates with FAK via other sites and that p85\textsuperscript{α}’s SH2 domains are required for Rab5-GTP loading. A, cell migration was evaluated in Boyden chamber assays, and representative images are shown. B, top panel, quantification of cell migration, obtained by averaging three independent experiments (mean ± S.E.; *, p < 0.05; **, p < 0.01; n.s., nonsignificant). Bottom panel, representative Western blot images of total FAK and Rab5. C and D, FAK\textsuperscript{−/−} MEFs were transfected with plasmids encoding for GFP, GFP-Rab5/WT, GFP-Rab5/S34N (GTP-binding defective mutant of Rab5, inactive), or GFP-Rab5/Q79L (GTPase-deficient mutant of Rab5, active) and used for subsequent cell migration assays or whole-cell lysate analysis; endo, endogenous. C, cell migration was evaluated in Boyden chamber assays, and representative images are shown. D, quantification of cell migration, obtained by averaging three independent experiments (mean ± S.E.; *, p < 0.05; **, p < 0.01; ***; p < 0.001). Data are shown as the percentage of cell migration with respect to control MEFs (FAK\textsuperscript{+/+}, extended data analysis in Fig. S2A). Scale bars = 200 μm.

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**Experimental procedures**

**Materials**

The mouse monoclonal antibodies anti-Rab5 (sc46692), anti-p85α (sc-1637), anti-EEA1 (sc-137130), and anti-FAK (sc1688) were from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies included rabbit polyclonal anti-Tyr\textsuperscript{397} pFAK (3283, Cell Signaling Technology), rabbit monoclonal anti-p85α (Ab191606, Abcam), rabbit monoclonal anti-ALS2 (Ab170896, Abcam), goat polyclonal anti-RIN2 (sc-85890, Santa Cruz Biotechnology), goat polyclonal anti-integrin-β\textsubscript{3} (sc-6627, Santa Cruz Biotechnology), rabbit polyclonal anti-
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**Figure 5.** FAK associates with the Rab GAP p85α in a phosphorylation-dependent manner. A and B, A549 cells were treated with either DMSO or 1 μM PF562,271 (PF), and whole-cell lysates (WCL) were prepared for subsequent immunoprecipitation of endogenous FAK with specific antibodies (see "Experimental procedures" for details). A, representative Western blot images are shown for phospho-Tyr397–FAK (pY397–FAK), total FAK, p85α and β-actin in WCL. B, bottom panel, representative Western blot images of FAK and p85α in immunoprecipitates. Top panel, quantification of p85α co-immunoprecipitating (IP) with FAK, as obtained from three independent experiments (mean ± S.E.; ***, p < 0.001). C, confocal microscopy analysis of endogenous p85α, co-immunoprecipitating with FAK, as obtained from three independent experiments (mean ± S.E.; *, p < 0.05). D and E, FAK Y180A/M183A (open conformation), and FAK/Arg454 (kinase-dead), were kindly provided by Dr. David Schlaepfer (University of California, San Diego) and have been described previously (29). The pEGFP-C1 plasmids encoding for WT FAK and Rab5/S34N (GDP-bound mutant) have been described previously (9). The pGEX-GST-FIP3 plasmid was generated by Dr. Rytis Prekeris (University of Colorado) (33) and was kindly provided by Dr. Francisca Bronfman (Pontificia Universidad Catolica de Chile).

**Cell culture**

A549 lung carcinoma cells were cultured in DMEM (high-glucose) supplemented with 10% FBS and antibiotics. Down-regulation of endogenous Rab5A by shRNA has been described previously, and cells expressing a nonspecific shRNA sequence (plasmid 1864, Addgene) were used as an experimental control (9). MEFs derived from FAK−/− MEFs as well as FAK−/− MEFs reconstituted with GFP, GFP-FAK, or GFP-FAK/Arg454 were provided by Dr. David Schlaepfer and have been described previously (29). MEFs were maintained in DMEM (high-glucose) supplemented with 10% FBS and antibiotics.

**Immunofluorescence**

Immunofluorescence was performed as described previously (9). Briefly, cells were grown for 24 h on glass coverslips. Following each treatment, samples were fixed with 4% formaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 2 min, washed with PBS, and blocked with 5% BSA/

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*GFP (sc-8334, Santa Cruz Biotechnology), and rabbit polyclonal anti-EEA1 (sc-33585, Santa Cruz Biotechnology). Rabbit polyclonal anti-phospho-Thr308-Akt (2965) and mouse monoclonal anti-Akt (2922) were from Cell Signaling Technology. Goat anti-rabbit and goat anti-mouse antibodies bound to horseradish peroxidase and anti-actin antibody (A5316) were obtained from Bio-Rad. Control (sc-37007) and p85α-targeting (sc-36217) siRNA constructs were from Santa Cruz Biotechnology. Lipofectamine 2000 as well as Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies were from Invitrogen. GSH-Sepharose 4B was from GE Healthcare. The small molecule PF562,271, described as an inhibitor of FAK autophosphorylation on Tyr397 (24), was from Cayman Chemical (catalog no. 14870) and was used at 3 μM concentration. The EZ-ECL chemiluminescent product and protein A/G beads were from Pierce.

**Plasmids**

The pEGFP-C1 plasmids encoding for WT FAK, FAK/Y180A/M183A (open conformation), and FAK/Arg454 (kinase-dead), were kindly provided by Dr. David Schlaepfer (University of California, San Diego) and have been described previously (32). The pEGFP-C1 plasmid encoding for WT Rab5 and Rab5/S34N (GDP-bound mutant) have been described previously (9). The pGEX-GST-FIP3 plasmid was generated by Dr. Rytis Prekeris (University of Colorado) (33) and was kindly provided by Dr. Francisca Bronfman (Pontificia Universidad Catolica de Chile).
PBS. Samples were then incubated with primary antibodies for 1 h at 37 °C, washed three times (PBS, 5 min), incubated with secondary antibodies for 1 h, washed three times, and mounted. Samples were visualized with a Nikon C2 confocal microscope (model Eclipse TI), using a 60 objective (Fluorite Plan, numerical aperture 0.5–1.25) unless indicated otherwise. Co-localization was analyzed with Fiji (ImageJ) software using the co-localization plugin.

Transwell migration assay

Boyden chambers were used to perform migration assays (Transwell Costar, 6.5-mm diameter and 8-μm pore diameter). The outer layers of the inserts were precoated with 2 μg/ml fibronectin. A549 cells (5 × 10⁴) were resuspended in serum-free medium and plated onto the top of the chambers. FBS-supplemented medium (10%) was added to the bottom chambers. After 2 h, the inserts were removed and washed. Cells that migrated to the bottom side of the inserts were stained with 0.1% crystal violet in 2% ethanol, and samples were counted in an inverted microscope.

Rab5-GTP pulldown assay

Spread cells underwent a 2-h spreading assay, and suspended cells were incubated for 2 h on ice. Spread and suspended A549 cells were lysed in a buffer containing 25 mM HEPES (pH 7.4), 5 mM MgCl₂, 100 mM NaCl, 1% NP-40, 1 mM DTT, 10% glycerol, and protease inhibitors. Lysates were incubated for 5 min on ice and clarified by centrifugation (10,000 × g, 1 min, 4 °C). Super-
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Proteins were used for pulldown assays with 30 μg of GST-R5BD−precoated GSH beads for each condition. R5BD beads were incubated with recovered supernatant for 15 min at 4 °C in a rotating shaker. Rab5-GTP bound beads were recovered and washed three times with lysis buffer containing 0.01% NP-40. Samples were analyzed by Western blotting. For Rab5-GTP, Rab11-GTP levels were measured in pulldown assays using the GST-FIP3 fusion protein.

**Western blotting**

Cells were washed twice with ice-cold PBS and lysed in 0.2 mM HEPES (pH 7.4), 0.1% SDS, and phosphatase (1 mM Na3VO4) and protease inhibitors for whole-cell lysate analysis. Protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and then Western blotting was performed by blocking with 5% gelatin in 0.1% Tween/PBS, followed by the specific antibodies.

**Immunoprecipitation**

Immunoprecipitations were performed as described previously (29). Briefly, cell extracts were prepared in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, and protease inhibitors by incubating samples on ice (5 min). Samples were centrifuged at 13,000 × g for 1 min at 4 °C, and post-nuclear supernatants (500 μg of total protein) were immunoprecipitated with protein A/G bead–immobilized antibodies for 30 min. FAK was immunoprecipitated with 10 μg of polyclonal antibody. Immunoprecipitated samples were solubilized in Laemmli buffer, boiled, separated by SDS-PAGE, and analyzed by Western blotting as indicated above.

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