Rac regulates the interaction of fascin with protein kinase C in cell migration

Maddy Parsons[1,*] and Josephine C. Adams[2,3,*]

[1]Randall Division of Cell and Molecular Biophysics, King's College London, Guy's Campus, London SE1 1UL, UK
[2]Department of Cell Biology, Lerner Research Institute, The Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA
[3]Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, The Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA

*Authors for correspondence (e-mail: adamsj@ccf.org; maddy.parsons@kcl.ac.uk)

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Summary

Fascin is an actin-bundling protein that is low or absent in normal epithelia; its upregulation correlates with poor prognosis in many human carcinomas. We have recently demonstrated in mouse xenograft models that fascin contributes to tumour development and metastasis through its dual actin-bundling and active PKC-binding activities. Rac was implicated as a regulator of fascin-dependent colon carcinoma cell migration in vitro. Here, we tested the hypothesis that Rac regulates the interaction of fascin with active PKC. The major conventional PKC in colon carcinoma cells is protein kinase Cγ (PKCγ). Endogenous PKCγ, fascin and Rac1 colocalised at lamellipodial margins of migrating cells. Colocalisation of fascin and PKCγ depended on Rac activity, and inhibition of Rac decreased PKCγ activity in cell extracts but not in vitro. Fluorescence resonance energy transfer/fluorescence lifetime imaging microscopy uncovered that fascin and PKCγ interact in protrusions and filopodia of migrating cells. Mechanistically, the interaction depended on phosphorylated fascin, active PKCγ and active Rac, but not on active Cdc42. The activity of Rac on the fascin/PKC complex was mediated in part by Pak. Elucidation of this novel pathway for regulation of the fascin/PKCγ complex in migrating carcinoma cells suggests novel targets for therapeutic intervention in metastasis.

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Key words: Cell protrusions, Filopodia, Migration, Signalling, Rac, Pak

Introduction

Fascin is an actin-bundling protein expressed by normal mesenchymal, neuronal and dendritic cells that contributes to the assembly of cortical cell protrusions and filopodia (reviewed by Kureishy et al., 2002; Adams, 2004). Most normal epithelia are negative for fascin; however, upregulation of fascin is frequently associated with a poor prognosis in human carcinomas and is implicated in metastasis (Pelosi et al., 2003; Hashimoto et al., 2004; Hashimoto et al., 2006; Yoder et al., 2005; Zigeuner et al., 2006; Puppa et al., 2007). We have recently demonstrated that fascin expression by human colon carcinoma cells contributes to assembly of filopodia, focal adhesion dynamics and cell migration in culture, and tumour development and metastasis in vivo (Hashimoto et al., 2007). We also found that the role of fascin in carcinoma migration is dependent on Rac activity. Rac GTPase is a well-established regulator of actin polymerisation that, via its effector p21-activated kinase, activates Lim kinase to inactivate cofilin (Machesky and Hall, 1997; Hartwig et al., 1995; Yang et al., 1998). These mechanisms impact on the availability of F-actin, which is a prerequisite for F-actin bundling by fascin (Edwards and Bryan, 1995; Adams and Schwartz, 2000).

Both Rac and Cdc42 are upstream regulators of the organisation of fascin and F-actin in protrusions and ruffles in cells adherent to thrombospondin 1 (Adams and Schwartz, 2000). Within the N-terminal actin-binding domain of fascin, residue Ser39 (S39) is a target for phosphorylation by protein kinase Cα (PKCα) (Ono et al., 1997). S39-phosphorylated fascin does not bundle actin, yet interacts with the regulatory domain of active PKCα and contributes to myoblast migration on fibronectin through this interaction (Anilkumar et al., 2003). The migratory and metastatic capacity of fascin-positive human colon carcinoma cells depend on both the actin-bundling and the active PKC-binding activities of fascin (Hashimoto et al., 2007). However, whether Rac or Cdc42 regulate the phosphofascin/active PKC interaction is unknown. We have investigated this issue and report here direct evidence for specific regulation of the fascin/PKCγ complex by Rac via a Pak-dependent process.

Results

Colocalisation of PKCγ with fascin in colon carcinoma cells is Rac dependent

Initial experiments established that PKCγ is the predominant conventional PKC isoform in human colon carcinoma cells (see supplementary material Fig. S1). These cells express Rac1 (Hashimoto et al., 2007). Of the other members of the Rac family, Rac2 is restricted to haematopoietic cells (Shirsat et al., 1990) and antibodies specific for Rac3 are unavailable. In this study we will refer to 'Rac1' if reagents of defined specificity were used, and otherwise to 'Rac'. Because the relationship between PKCγ and fascin was unknown, we examined the localisations of endogenous Rac1, fascin and PKCγ in SW480 cells randomly migrating on laminin (LN) surfaces. This experimental setup correlates excellently with carcinoma cell behaviour in vivo (Hashimoto et al., 2007). Fascin, Rac1 and PKCγ were each present in the cell body and colocalised at protrusive tips and lamellipodial edges (Fig. 1A,B; insets therein of control cells at higher magnification). Using previously determined conditions for pharmacological inhibition of PKC or Rac in SW480 cells (Hashimoto et al., 2007), inhibition of

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PKC activity by bisindolylmaleimide I (BIM) was found to reduce both fascin and PKCγ staining at membrane protrusions (Fig. 1A), whereas Rac1 remained detectable at cell edges (Fig. 1B, arrows). By contrast, application of the Rac inhibitor NSC23766 (Gao et al., 2004), which blocks Rac activation [thereby reducing the pool of Rac-GTP (Hashimoto et al., 2007) (supplementary material Fig. S2)], resulted in loss of all three proteins from lamellipodial edges (Fig. 1A,B and insets). These results demonstrate that endogenous PKC activity impacts on the cellular localisation of fascin in migrating human colon carcinoma cells, and suggest a role for Rac upstream of both fascin and PKCγ.

Rac regulates PKCγ kinase activity by an indirect mechanism

To establish whether the mechanism of Rac-dependent PKCγ relocalisation involved PKCγ kinase activity, we treated cells migrating on LN with BIM, NSC23766 or solvent control, and examined PKCγ activity by immunoblot. Both pharmacological treatments significantly reduced PKCγ activity (Fig. 1C, quantified in 1E). Phorbol ester activation of conventional PKC reversed the effect of Rac inhibition, further supporting the concept that PKC is activated downstream of Rac (Fig. 1C,E). To examine whether the effect of NSC23766 was direct, we added the inhibitors in vitro upon immunoprecipitation of PKCγ. Whereas BIM still inhibited

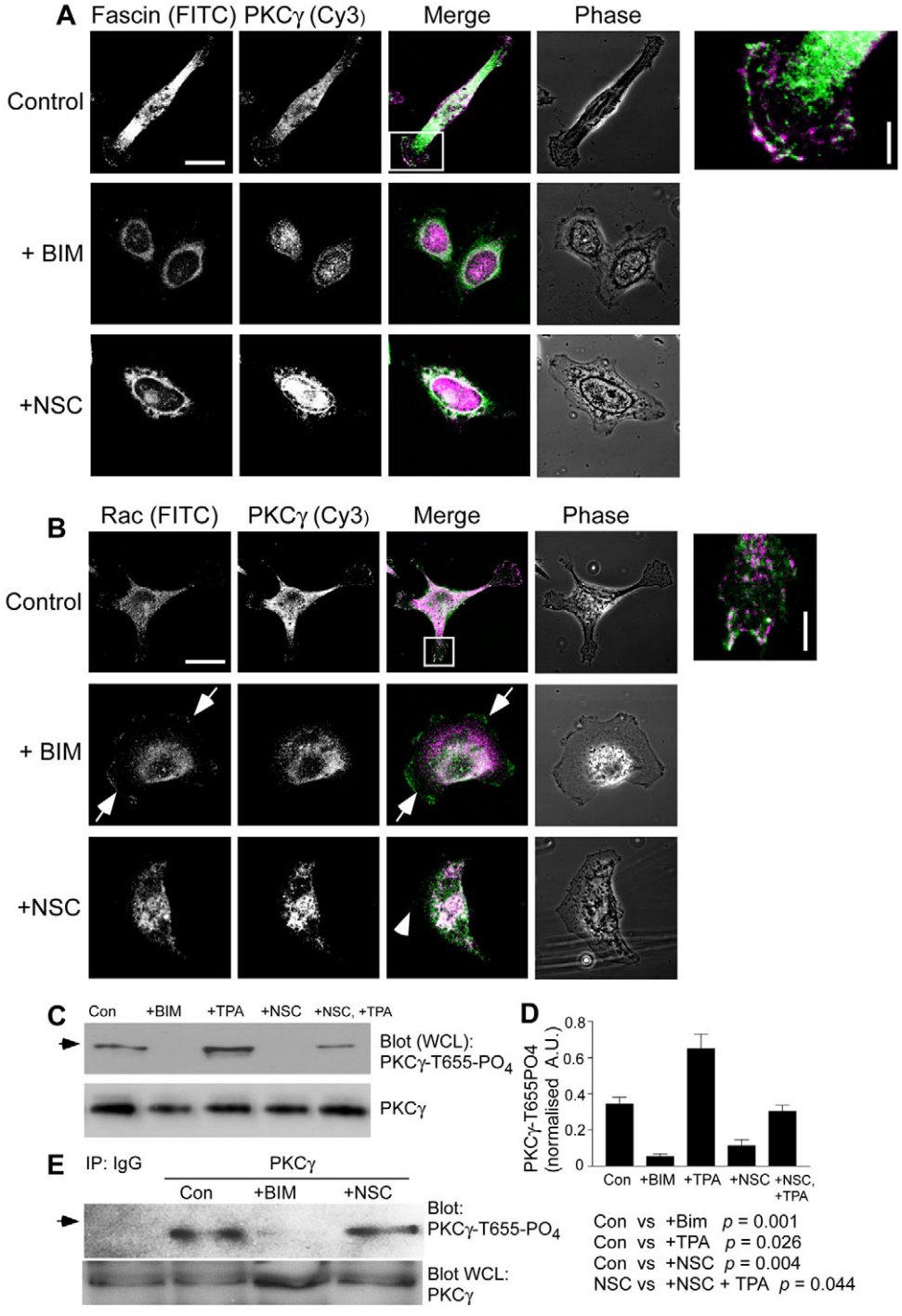

Fig. 1. Colocalisation of fascin and PKCγ is regulated by PKC and Rac activity. (A,B) SW480-Pa cells plated on 15 nM LN for 4 hours in the absence or presence of 1 μM BIM or 100 μM NSC23766 were co-stained with antibodies as stated, and imaged using phase-contrast or confocal microscopy. Scale bars: 10 μm. Insets show higher magnification views of the edges of representative control cells; scale bars: 4 μm. In B, arrows and arrowhead indicate cell edges positive or negative for Rac, respectively. (C,D) PKCγ activation is Rac dependent. SW480-Pa cells were pre-treated with BIM, TPA, NSC23766 or solvent (Con) as described in the Materials and Methods, lysed and analysed by SDS-PAGE and immunoblotting for total PKCγ or PKCγ–T655PO_4 as a reporter of PKCγ activation (C). D shows quantification of results from three independent experiments. Columns represent the mean and bars indicate s.e.m. (E) PKCγ was immunoprecipitated from LN-adherent cells and treated in vitro with 100 μM NSC23766 or 1 μM BIM before activity analysis by immunoblotting. Only BIM treatment decreased PKCγ–T655PO_4. Representative of three independent experiments.

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effectively under these conditions, NSC23766 did not (Fig. 1D). Thus, Rac is functionally upstream of PKCγ and inhibits its kinase activity by an indirect mechanism.

Fascin and active protein kinase Cγ interact in the protrusions of migrating colon carcinoma cells

To examine whether fascin and PKCγ interact in colon carcinoma cells, we turned to fluorescence resonance energy transfer/fluorescence lifetime imaging microscopy (FRET/FLIM), using green fluorescent protein-fascin (GFP-fascin) and PKCγ–monomer red fluorescent protein (PKCγ–mRFP) as the donor and acceptor probes, respectively. This technique allows precise spatial and temporal analysis of protein interactions in intact cells (Parsons et al., 2004; Parsons et al., 2005). First, in order to establish that fascin can interact with PKCγ, serum-starved quiescent SW480 cells were treated with 1 μM 4-β-phorbol-12, 13-dibutyrate (PDBu) to activate PKCγ directly. Interaction of GFP-fascin and PKCγ–mRFP was observed, as measured by time-dependent, statistically significant decreases in GFP donor fluorescent lifetime relative to its lifetime in control cells expressing GFP-fascin alone (Fig. 2A,B). This response was not apparent in control, solvent-treated, co-expressing cells, demonstrating the dependence of the interaction on active PKC (Fig. 2A,B). The role of the S39 PKC-phosphorylation site of fascin in the interaction was established by use of non-phosphorylatable (S39A) and phosphomimetic (S39D) fascin point mutants (reviewed by Kureishy et al., 2002). In cells stimulated by PDBu, GFP-fascin-S39D interacted strongly with PKCγ-mRFP, whereas an interaction of GFP-fascin-S39A was undetectable (Fig. 2C). Thus, active PKCγ interacts with phosphorylated fascin in SW480 cells.

Next, fascin/PKC interactions were analysed in live SW480 cells migrating on LN. To examine the dynamics of the interaction without overexpression of fascin, these experiments were carried out with SW480 inducible knock-down (IKD)-F11 cells knocked-down for human fascin 1 (IKD-F11 Fas– cells), in which GFP-tagged *Xenopus tropicalis* fascin 1 (GFP-*Xt*fascin), which is not susceptible to the short hairpin RNA (shRNA) to human fascin 1 (Hashimoto et al., 2007), was transiently expressed. The expression level of transfected GFP-*Xt*fascin in individual cells was similar to that of endogenous fascin in SW480 cells (Fig. 3A). FRET/FLIM measurements of cells migrating on LN for 30 minutes demonstrated transient decreases in donor fluorescent lifetime in cells co-expressing PKCγ-mRFP, compared with control cells expressing GFP-*Xt*fascin alone (Fig. 3B; mean FRET efficiency of 8.3%, n=5 cells). The quantitative measurement represents the mean FRET efficiency from entire cells, yet it was also striking that within individual cells the largest

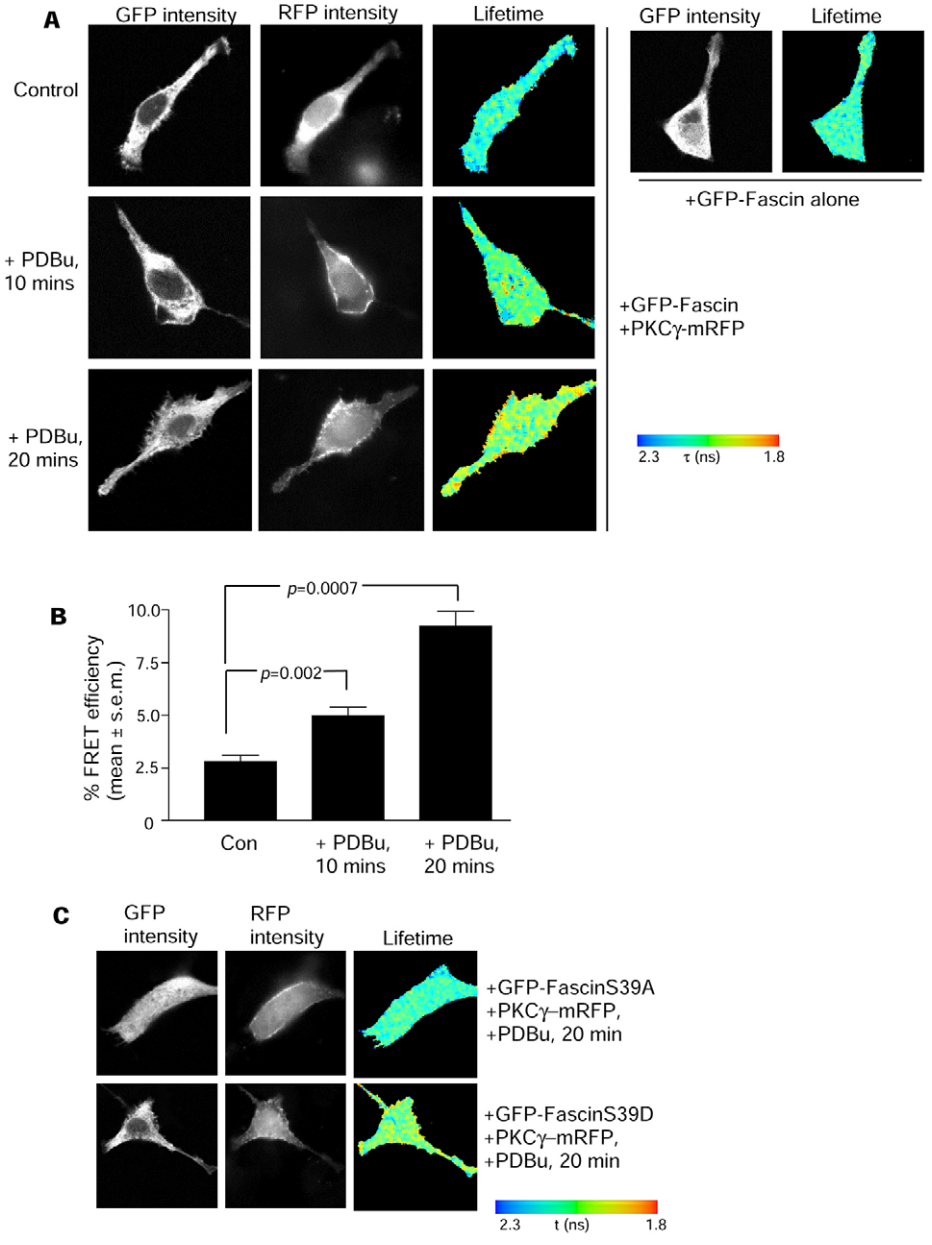

Fig. 2. PKC activity drives the interaction of fascin with PKCγ. (A) SW480-Pa cells transiently expressing GFP-fascin, alone or plus mRFP-PKCγ, were treated with solvent or PDBu for the indicated times, and then fixed and imaged using FLIM to measure FRET. PDBu and TPA activate conventional PKC equivalently at early timepoints. (B) Quantification of data from nine cells from three independent experiments as in A. (C) SW480-Pa cells transiently co-expressing either GFP-fascin-S39A or GFP-fascin-S39D with PKCγ-mRFP were treated with PDBu for 20 minutes, then fixed and imaged using FLIM to measure FRET. Only GFP-fascin-S39D interacted with active PKCγ. (A,C) Intensity multiphoton GFP images (donor) and, where relevant, epifluorescence images for RFP (acceptor). Lifetime images are depicted using a pseudocolour scale where red is a low lifetime (1.8 nseconds) and blue is high (2.3 nseconds).

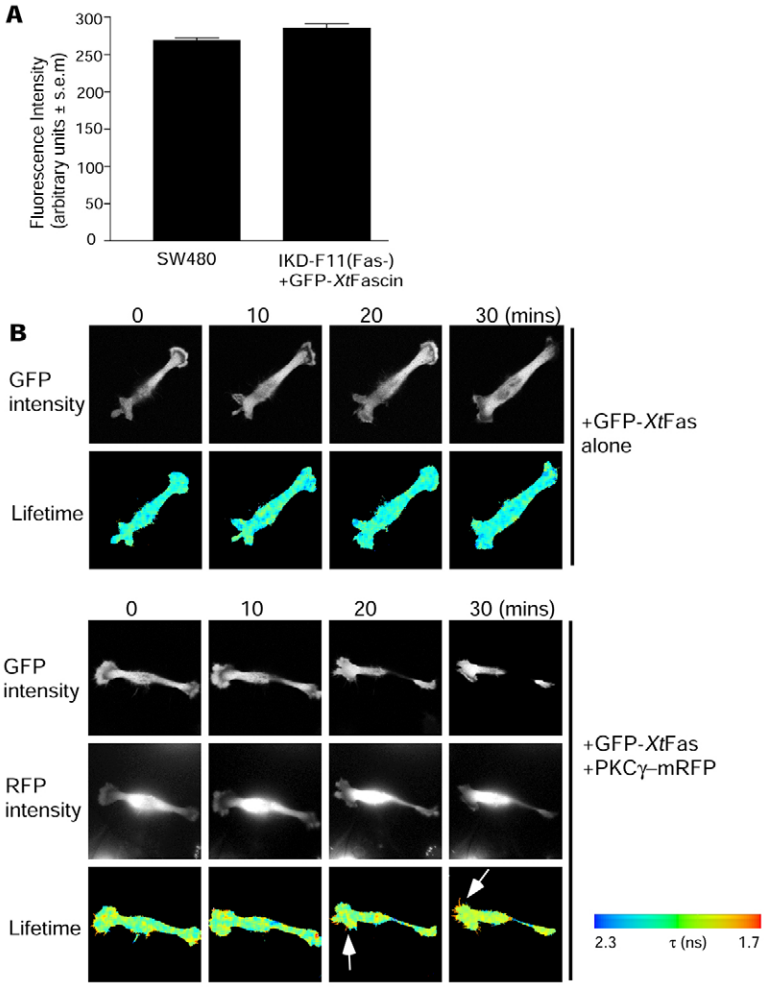

Fig. 3. Fascin and PKCγ interact in living carcinoma cells migrating on LN. (A) Level of expression of GFP-*Xt*fascin in SW480 IKD-F11 Fas– cells compared with the endogenous fascin of SW480 cells. Cells were stained with antibody reactive with human and *Xenopus* fascin 1 and the mean fluorescence intensity calculated from 10 cells in each population and from three independent experiments. (B) IKD-F11 Fas– cells transiently expressing GFP-*Xt*fascin alone (top panels) or GFP-*Xt*fascin and PKCγ-mRFP (bottom panels) were plated on 15 nM LN for 2 hours. Transfected migrating cells were imaged over time, using FLIM to measure FRET. Panels show intensity multiphoton GFP images from each cell (donor) and, where relevant, an epifluorescence image for mRFP (acceptor) at selected timepoints. Lifetime images are depicted by a pseudocolour scale with red as low lifetime (1.7 nseconds) and blue as high (2.3 nseconds). Arrows indicate examples of strong interaction in filopodia. Representative of a total of nine experiments for control and test cells.

decreases in GFP fluorescent lifetime, indicative of strong protein interaction, were spatially localised at leading edge protrusions and their associated filopodia (Fig. 3B, arrows).

The fascin/PKCγ interaction in migrating cells depends on PKCγ kinase activity and Rac activity

To further examine the dependence of the fascin/ PKCγ interaction on PKC kinase activity and Rac in migrating cells, we tested the effects of the pharmacological inhibitors of PKC and Rac on the fascin/PKCγ interaction in migrating IKD-F11 Fas– cells reconstituted with GFP-*Xt*fascin. Whereas control migrating cells co-expressing GFP-*Xt*fascin and PKCγ-mRFP had strong FRET efficiency, BIM-treated cells showed near-complete inhibition of FRET, indicating that the interaction with fascin depends on active PKCγ (Fig. 4A,B). FRET was also almost totally inhibited in NSC23766-treated cells (Fig. 4A,B). No changes in donor fluorescence lifetime were detected in migrating cells expressing GFP-*Xt*fascin alone (Fig. 4A). To confirm the effect of pharmacological inhibition of PKC by an independent method, we also examined the ability of a kinase-dead form of PKCγ, PKCγ-K380A-mRFP (Rosenberg and Ravid, 2006), to interact with GFP-*Xt*fascin in migrating cells. The FRET efficiency was significantly decreased from that obtained with wild-type PKCγ-mRFP, and was essentially at background level (Fig. 4C). Thus, the fascin/PKCγ interaction depends strictly on active PKCγ.

Rac specifically regulates the fascin/PKCγ interaction

The results obtained with NSC23766 compound had implicated Rac as an upstream regulator of the interaction of fascin and PKCγ at cell edges (Fig. 1, Fig. 4B). To corroborate these results by an independent method and to examine the role of Rac in more depth, we examined the effect of dominant-negative N17Rac1 on the fascin/PKCγ interaction in IKD-F11 Fas– cells rescued with GFP-*Xt*fascin and migrating on laminin. To test whether Rac is a specific regulator of the interaction, the effect of dominant-negative N17Cdc42 was also examined. Cdc42 is another small GTPase regulator of actin nucleation that promotes filopodial assembly and cell migration in normal and cancer cells (Nobes and Hall, 1995; Rohatgi et al., 1999; reviewed by Ridley, 2006). Cdc42 and Rac both regulate the organisation of fascin and F-actin in protrusions of skeletal myoblasts and fibroblasts (Adams and Schwartz, 2000). In the migrating colon carcinoma cells, N17Rac1-myc caused significant inhibition of the fascin/PKCγ interaction, whereas N17Cdc42-myc had no significant effect (Fig. 5). These data demonstrate a specific role of Rac1 as an upstream regulator.

To gain further insight into the mechanism of Rac1 regulation, the impact of either constitutively active V12Rac1 or V12Cdc42 was examined in serum-starved IKD-F11 Fas– cells rescued with GFP-*Xt*fascin. After serum starvation, the GFP-*Xt*fascin/PKCγ–mRFP interaction was at a low level with the GFP fluorescent lifetime similar to that in cells expressing GFP-*Xt*fascin alone (Fig. 6A,B). Expression of the active GTPases induced the expected characteristic changes in cell shape, with multiple protrusions and short filopodia in the cells expressing V12Rac1-HA (influenza haemagglutinin tag) and a hyper-filopodial morphology in cells expressing V12Cdc42-HA (Fig. 6B). Nevertheless, only V12Rac1-HA significantly activated the fascin/PKCγ interaction, which again was strongest in filopodia (Fig. 6B, and quantified from three independent experiments in Fig. 6C). Together with the analysis of the effects of the dominant-negative forms of the GTPases in migrating cells (Fig. 5), these results definitively establish that Rac1, but not Cdc42, is a positive regulator of the fascin/PKCγ interaction.

Rac regulation can be bypassed by constitutive activation of PKCγ

Conventional PKCs are activated by a complex process involving priming phosphorylation events and subsequent membrane

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translocation, membrane tethering, and release of autoinhibition in the presence of elevated diacylglycerol and Ca^{2+} (Griner and Kazanietz, 2007). To establish whether signal-dependent plasma membrane localisation of active PKCγ is essential for its interaction with fascin, we tested if constitutively active (A25E) PKCγ would interact with fascin in serum-starved cells. The FRET efficiency of PKCγA25E-mRFP with fascin was significantly increased over that of wild-type PKCγ–mRFP, and was similar to the FRET efficiency in the presence of V12Rac1 (Fig. 6B,C). Furthermore, the fascin/PKCγA25E interaction was not significantly inhibited by NSC treatment of cells (Fig. 6C), demonstrating that any basal Rac activity present in this experiment can be bypassed by expression of a constitutively active PKCγ.

Pak participates downstream of Rac to regulate the fascin/PKCγ interaction in migrating cells

Our initial results indicated that Rac activity regulates PKCγ kinase activity by an indirect mechanism (Fig. 1D). To gain insight into the mechanism downstream of Rac, we examined whether the Rac effector p21-activated kinase (Pak) has a role in regulation of the fascin/PKCγ interaction in migrating cells. The Pak family member Pak1 is expressed in colonic epithelial cells and colorectal adenocarcinomas (Carter et al., 2004). Pak1 is a well-established downstream effector of Rac1 that contributes to cell migration and to other motility processes (reviewed by Jaffer and Chernoff, 2002). Overexpression of wild-type Pak1 did not significantly alter the efficiency of FRET interaction between GFP-*Xt*fascin and PKCγ-mRFP in IKD-F11 cells migrating on laminin (Fig. 7A,B). By contrast, overexpression of the autoinhibitory domain (AID; amino acids 83-149) of Pak1, which inhibits activation of Pak1, 2 and 3 without interfering with other Rac effectors (Zhao et al., 1998), significantly reduced the efficiency of interaction by about 50% (Fig. 7A,B). The remaining interacting population of fascin and PKCγ was predominantly localised to punctate regions at both the leading edge and the rear of the cell (Fig. 7A, lifetime image for Pak1-AID-expressing cell). These data indicate that Pak1 participates as an upstream regulator of the fascin/PKCγ interaction. To confirm that this activity of Pak1 is mediated downstream of Rac, we examined endogenous active Pak1 in SW480 cells on laminin in the absence or presence of NSC23766. NSC23766 treatment decreased active Pak1 in laminin-adherent cells, as determined by the amount of T423-phosphorylated Pak1 detected on immunoblots of cell extracts, whereas BIM treatment did not alter Pak1 activity status (Fig. 7C). Thus, Pak1 activity is regulated downstream of Rac and is not dependent on PKC activity.

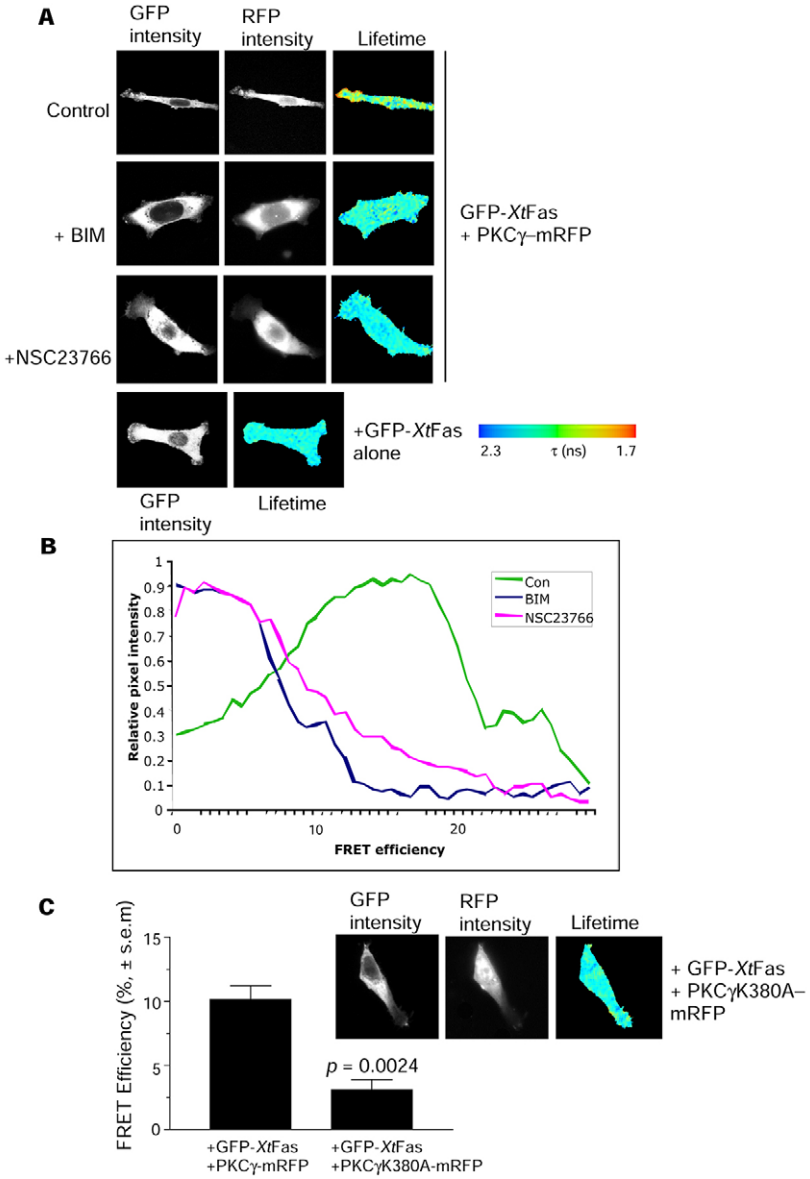

Fig. 4. PKCγ and Rac activities regulate the interaction of fascin and PKCγ in migrating cells. (A,B) SW480 IKD-F11 Fas– cells transiently transfected with GFP-*Xt*fascin alone (bottom line in A) or GFP-*Xt*fascin and mRFP-PKCγ (all other panels and in B) were plated on 15 nM LN for 2 hours, with or without pretreatment with 1 μM BIM for 30 minutes or 100 μM NSC23766 for 4 hours, then fixed, mounted and imaged using FLIM to measure FRET. (A) Intensity multiphoton GFP images from each cell (donor) and, where relevant, an epifluorescence image for mRFP (acceptor). Lifetime images are depicted by a pseudocolour scale with red as low lifetime (1.7 nseconds) and blue as high (2.3 nseconds). (B) Histogram demonstrating mean cumulative FRET efficiency data from 16 cells per condition and three independent experiments. The graph represents the range of FRET efficiencies seen per cell in each condition, normalised to pixel intensity, and demonstrates a clear leftward shift in BIM- and NSC-treated samples where FRET is inhibited. (C) A kinase-dead form of PKCγ, PKCγK380A, does not interact with fascin in cells migrating on laminin. The cumulative FRET efficiency graph shows the mean FRET efficiency for wild-type and kinase-dead PKCγ. Each column represents the mean from seven to nine cells per condition and three independent experiments. Insets show the GFP and RFP intensities, and lifetime plot from a representative cell expressing PKCγ–K380A. Pseudocolour scale as in A.

Discussion

To our knowledge, these data constitute the first report of regulation of a conventional PKC by Rac. Our data establish that in migrating human colon carcinoma cells PKCγ activation requires Rac activity. The activity status of PKCγ in turn controls the interaction of PKCγ with (phospho)fascin, such that the complex is concentrated spatially in the periphery and filopodia of the migrating cells. Rac regulation of PKCγ kinase activity is indirect and is mediated, at least in part, via p21-activated kinase (PAK). We also demonstrate that Cdc42 activity is not required for the fascin/PKCγ interaction.

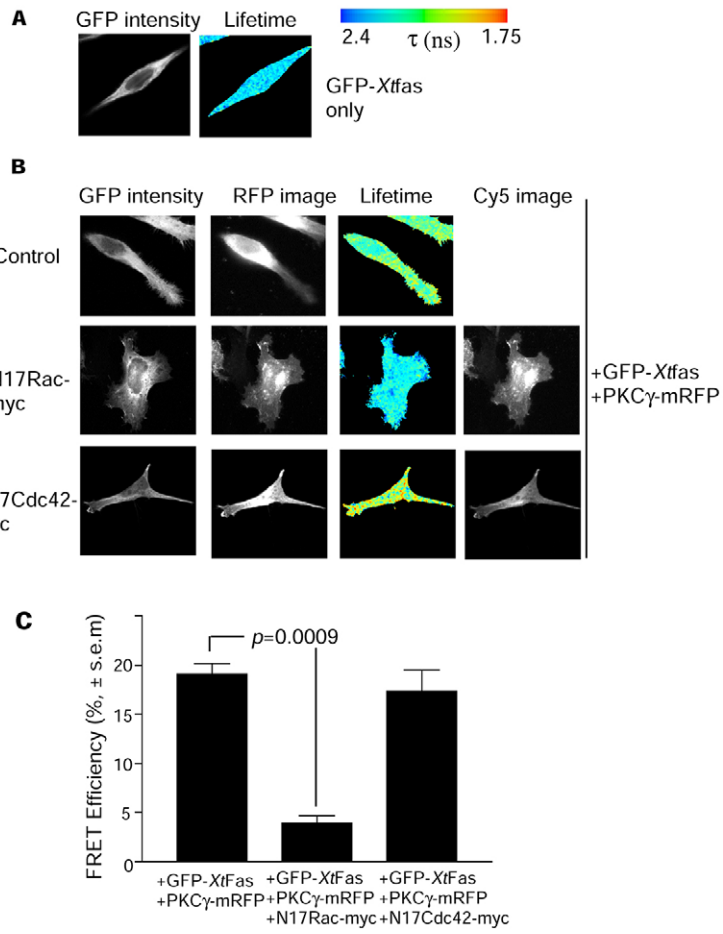

Fig. 5. The fascin–PKCγ interaction depends specifically on Rac1 activity in migrating cells. (A,B) IKD-F11 Fas– cells cultured in doxycycline were transiently transfected with GFP-*Xt*fascin and PKCγ-mRFP in conjunction with either N17Rac1-myc or N17Cdc42-myc, and allowed to migrate on 15 nM laminin for 6 hours. Cells were fixed and antibody stained to detect the GTPase and imaged using FLIM to measure FRET. Only N17Rac1 inhibited the FRET interaction. Lifetime images are depicted with a pseudocolour scale where red is a low lifetime (1.75 nseconds) and blue is high (2.4 nseconds). Representative of at least eight cells in each sample. (C) Cumulative FRET efficiency graph for cells migrating on laminin, under control conditions or in cells expressing N17Rac1-myc or N17Cdc42-myc. The mean FRET efficiency for each condition is from seven to nine cells per condition and three independent experiments.

Conventional PKCs are activated by a complex process that involves priming phosphorylation events in the cytoplasm, and release of autoinhibition and membrane translocation in response to increased concentrations of intracellular Ca^{2+} and diacylglycerol (Griner and Kazanietz, 2007). We have established by pharmacological inhibition and with kinase-dead and constitutively active mutant forms of PKCγ that formation of the fascin/PKCγ complex depends crucially on PKCγ kinase activity. Fascin is a substrate of PKC (Ono et al., 1997) and we have identified that a phosphomimetic form of fascin interacts preferentially with active PKCγ. Previously, we mapped the phosphofascin binding site to the regulatory domain of PKCα (Anilkumar et al., 2003). Thus, phosphorylation of fascin by a conventional PKC is needed for the formation of the fascin–active PKC complex. The strong interaction of fascin with constitutively active PKCγ, which is active independent of a plasma membrane localisation, provides the first demonstration that membrane localisation per se is not crucial for formation of the fascin/PKCγ complex. However, under physiological conditions of cell adhesion and migration on laminin, the interaction of active PKCγ and fascin is concentrated at cell edges and in filopodia. Integrin ligation at sites of adhesion is a strong stimulus for activation of conventional PKCs (reviewed by Berrier and Yamada, 2007). Extracellular matrix ligation of integrins also regulates Rac and PAK activities (del Pozo et al., 2000). Thus, extracellular matrix-activated integrin complexes could provide a mechanism to bring active PKCγ and fascin into close proximity with active Rac and PAK, which then act locally to promote PKCγ binding to fascin.

PKC activity is implicated in tumour migration and invasion but the mechanisms remain poorly understood (Griner and Kazanietz, 2007). Our data establish that PKCγ activity in colon carcinoma cells is indirectly regulated by Rac and identify a PAK, probably Pak1, as a component of the mechanism. Interestingly, Pak1 expression is increased in correlation with malignant progression in several forms of human carcinoma, including colorectal carcinoma (Carter et al., 2004; Holm et al., 2006; Ching et al., 2007; O'Sullivan et al., 2007). In breast carcinomas, Pak1 overexpression correlates with tamoxifen insensitivity of oestrogen receptor-positive tumours (Holm et al., 2006; Bostner et al., 2007). In colorectal cancers, Pak1 expression was noted to be significantly higher in lymph node metastases than in the normal colon, adenomas or primary adenocarcinomas. From a composite scoring of primary tumours and metastases, highest Pak1 expression was found to be correlated with reduced patient survival (Carter et al., 2004). In several carcinoma xenograft models, Pak1 expression and kinase activity have been functionally linked to cell motility and tumour development (Ching et al., 2007; O'Sullivan et al., 2007). Thus, Pak1, like fascin (see Introduction), contributes to an aggressive, invasive tumour phenotype. Our data place Pak1 as a component of one of the pathways by which fascin impacts on colon carcinoma cell migration. Because Pak1–AID also inhibits Pak2 and Pak3, our data with this reagent do not rule out the possibility that these PAKs are also involved. A Pak1 kinase-independent cell cycle arrest has also been attributed to Pak1–AID, indicating multiple possible mechanisms for the activity of this inhibitory reagent (Thullberg et al., 2007).

How Pak1 regulates PKCγ activation and whether this mechanism is direct or indirect remains to be elucidated. A possible candidate effector of Pak1 could be the F-actin severing protein cofilin. LIM kinase and cofilin are important downstream mediators of Pak1 in the control of F-actin dynamics during formation of dendritic spines (Zhao et al., 2006), axon specification (Jacobs et al., 2007) and lamellipodial protrusion (Delorme et al., 2007). We speculate that localised control of newly forming protrusions by PAK and cofilin could indirectly drive the interaction of fascin and active PKCγ. Alternatively or additionally, the regulation of myosin IIB phosphorylation by PAK that impacts on myosin filament assembly and cell contractility status could regulate integrin signalling to PKCγ (Even-Faitelson and Ravid, 2006).

Whereas PAK is clearly an important mediator downstream of Rac for the fascin/active PKCγ complex, our FRET efficiency data demonstrate that inhibition of PAK reduces fascin/PKCγ interaction by around 50%. This contrasts with the complete blockade of the complex that results from pharmacological or dominant-negative

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inhibition of Rac activity. The residual Rac-dependent, but PAK-independent, fascin- and PKCγ-interacting population appears to have a restricted localisation in punctate structures at the front and rear of migrating cells. Several PAK-independent effector mechanisms of Rac have been identified, including PAK-independent actin polymerisation through formation of a complex containing WAVE and Abi1 (Abou-Kheir et al., 2008), and also mechanisms dependent on microtubules (Wittman et al., 2004). It will be of future interest to identify which Rac-dependent mechanism parallel to PAK contributes to regulation of the fascin–PKCγ complex. This could potentially explain the observed Rac specificity of regulation.

The demonstration of strong, Rac-dependent fascin/PKCγ interaction in filopodia suggests the existence of an actin-bundling-independent mechanism to localise phosphorylated fascin in filopodia. Although phosphorylated fascin does not bundle F-actin and overexpression of phosphomimetic fascin-S39D or –S39E reduces the assembly of cell protrusions (Ono et al., 1997; Adams et al., 1999; Shonukan et al., 2003; Vignjevic et al., 2006; Hashimoto et al., 2007), the report that fascinS39E concentrates transiently at protrusive tips in melanoma cells (Vignjevic et al., 2006) is consistent with our direct observations of localised fascin–PKCγ interaction in live migrating colon carcinoma cells. The generality of this localisation in cells of other tissue origins remains to be established.

In conclusion, we have put forward the novel model that fascin impacts on filopodial dynamics by two pathways: (1) the previously described crosslinking of F-actin by non-phosphorylated fascin that leads to the structural organisation of unipolar actin bundles in filopodia and lamellipodial protrusions and is dependent on both Cdc42 and Rac (Adams and Schwartz, 2000; Vignjevic et al., 2003; 2006, Aratyn et al., 2007); and (2) the Rac-dependent, spatially-localised interaction of phosphofascin with active PKC that we report here and that is independent of Cdc42. Localised high levels of interacting fascin–PKCγ at the plasma membrane of migrating carcinoma cells may provide for a rapid cycling of fascin between the S39-phosphorylated and -unphosphorylated states that facilitates the effective local positioning and remodelling of filopodia and protrusions for directional migration. In view of the fact that Rac regulates both of these biochemical activities of fascin, and that high levels of phosphomimetic fascin inhibit cell migration and tumour development and metastasis (Hashimoto et al., 2007), we propose that modulation of the Rac-to-fascin pathway and the phosphofascin–PKC complex could provide a novel, broadly applicable, biologically based strategy to limit carcinoma cell migration and metastasis.

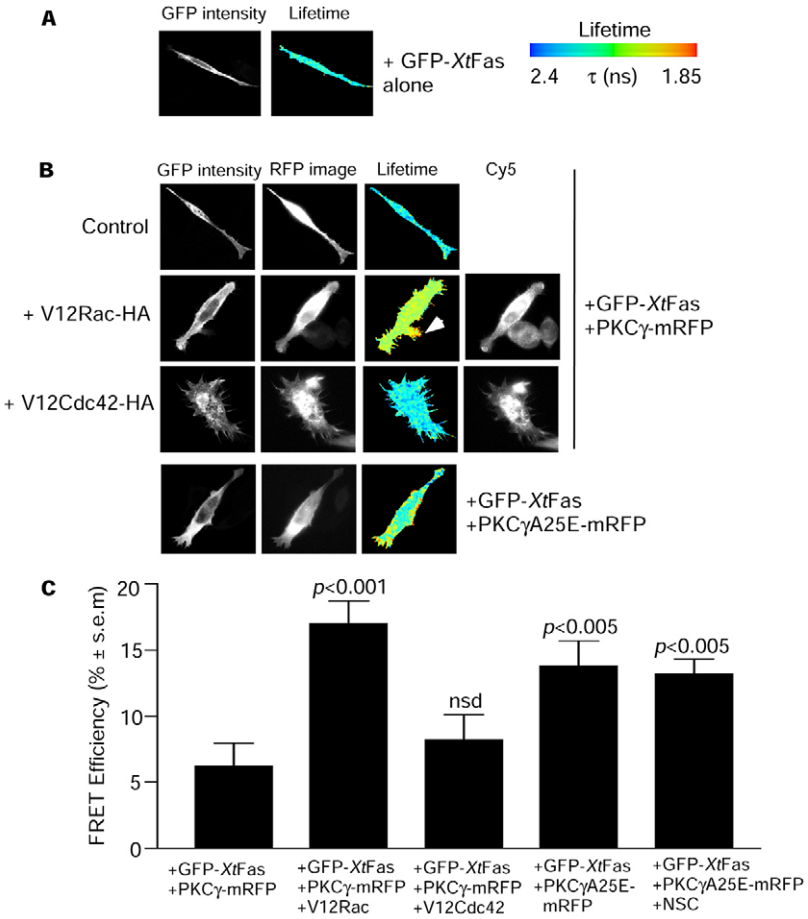

Fig. 6. Constitutively active Rac1 or PKCγ, but not Cdc42, activates the interaction of fascin and PKCγ in quiescent cells. (A) Lifetime of cells expressing GFP-*Xt*fascin alone. (B) IKD-F11 Fas– cells were transiently transfected with GFP-*Xt*fascin and the indicated mRFP acceptor constructs in the absence or presence of constitutively active Rac1 or Cdc42. Quiescent, serum-starved cells were fixed and antibody-stained to detect the GTPase and imaged using FLIM to detect FRET. Lifetime images are depicted with a pseudocolour scale where red is a low lifetime (1.85 nseconds) and blue is high (2.4 nseconds). FRET was increased in the presence of V12Rac1-HA (arrow indicates examples of strong interaction in filopodia) or PKCγA25E-mRFP, but not in the presence of V12Cdc42-HA. (C) Cumulative FRET efficiency graph for control cells or cells expressing the indicated constructs. The mean FRET efficiency for each condition is from seven to nine cells per condition and three independent experiments.

Materials and Methods

Cells and materials

SW480, SW1222 and DLD-1 cell lines from human colonic adenocarcinomas were cultured in DMEM containing 10% FCS. SW480-Pa cells expressing Tet repressor and SW480 IKD-F11 cells, in which fascin 1 is inducibly knocked down by culture in 0.5 μg/ml doxycycline (Sigma) for 48 hours, were as described (Hashimoto et al., 2007). The PKCγ–mRFP expression plasmid was prepared by subcloning cDNA for human PKCγ (GenBank clone BM926293, from Research Genetics) into pcDNA3.mRFPC, using the PCR primers 5′GTACAAGCTTGTTTCTGGGGCCATGGCTGGT and 5′GTACGGATCCCATGACGGGCACAGGCACTGG. A constitutively active A25E mutant (Baier-Bitterlich et al., 1996) and the kinase-dead K380A mutant (Rosenberg and Ravid, 2006) were generated in this plasmid by PCR-based site-directed mutagenesis using the Quickchange II XL kit (Stratagene) according to manufacturer's instructions, with the PCR primer pairs 5′CAGAAAGGGGGAGCTGAGGCAGAAGGTG and 5′CACCTTCTGCCTCAGCTCCCCCTTTCTG (for A25E), and 5′GAGCTCTACGCCATCGCGATCTTGAAAAAGGAC and 5′GTCCTTTTTCAAGATCGCGATGGCGTAGAGCTC (for K380A). Expression plasmids for V12Rac1-HA, V12Cdc42-HA, N17Rac1-myc, N17Cdc42-myc, wild-type PAK1 and PAK1-AID were as described (Adams and Schwartz, 2000; Parsons et al., 2005). pEGFP plasmids for expression of fascin 1 from *H. sapiens* or *X. tropicalis* were as described (Hashimoto et al., 2007). BIM and NSC23766 were obtained from Calbiochem and PDBu from Sigma. Mouse monoclonal antibody to Rac1 was from Upstate. Antibodies to Pak1 and Pak1-T423-PO_4 were from Cell Signaling. Rac-GTP levels were measured by the G-LISA method (Cytoskeleton) according to manufacturer's procedures, using SW480 cells plated on 15 nM laminin for 2 hours in the absence or presence of 100 μM NSC23766. C2C12 cells plated for 15 minutes on 50 nM fibronectin were used as a positive control for the Rac-GTP assay. All measurements were carried out in triplicate.

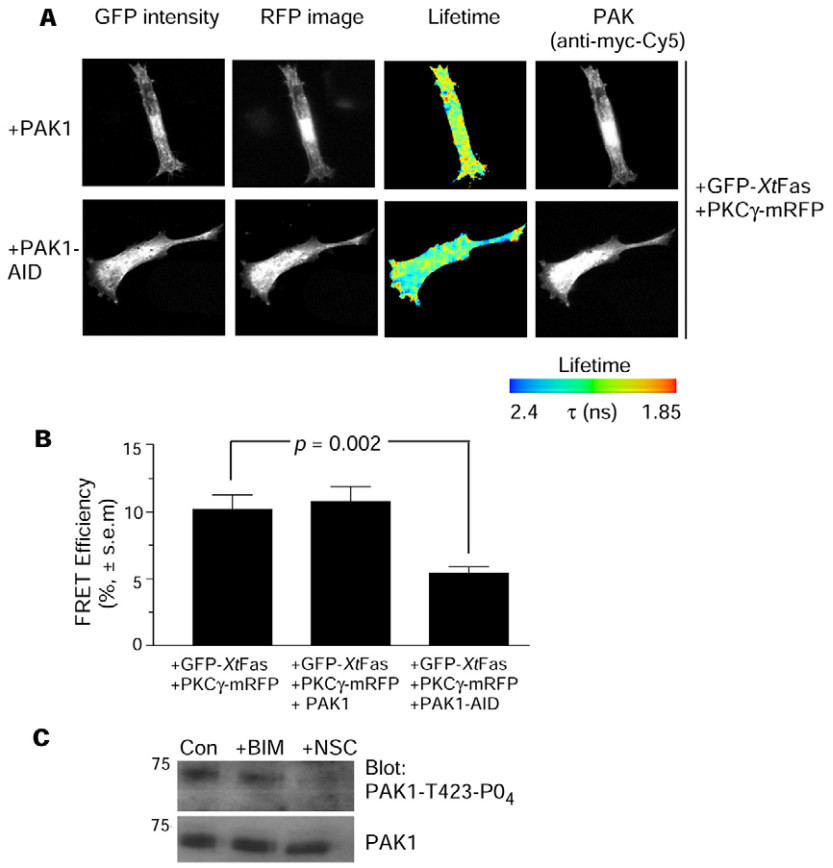

Fig. 7. Role of Pak1 in regulating the fascin–PKCγ interaction in migrating cells. (A) IKD-F11 Fas– cells cultured in doxycycline were transiently transfected with GFP-*Xt*fascin, PKCγ-mRFP and either wild-type Pak1-myc or Pak1AID-myc and allowed to migrate on 15 nM laminin for 6 hours. Cells were fixed and antibody-stained to detect Pak1 and imaged using FLIM to measure FRET. Pak1-AID inhibited the FRET interaction. Lifetime images are depicted with a pseudocolour scale where red is a low lifetime (1.85 nseconds) and blue is high (2.4 nseconds). Representative of at least eight cells in each sample. (B) Cumulative FRET efficiency graph for control cells or cells co-expressing the indicated Pak1 constructs. The mean FRET efficiency for each condition is from seven to nine cells per condition and three independent experiments. (C) Pak1 activity is Rac dependent. SW480-Pa cells were pretreated with BIM, NSC23766 or solvent (Con), as described in the Materials and Methods, plated on 15 nM laminin for 2 hours in the continued presence of inhibitors, then lysed and analysed by SDS-PAGE and immunoblotting for total Pak1 or Pak1–T423PO$_4$ as a reporter of Pak1 activation. Representative of three independent experiments.

Fluorescence lifetime measurements by time-correlated single-photon counting FLIM

SW480 cells were transfected with expression constructs as stated using Fugene 6 transfection reagent and manufacturer's procedures (Roche). For fixed samples, cells were fixed in 4% paraformaldehyde and permeabilised in 0.2% (w/v) Triton X-100 in PBS. After quenching with 1 mg/ml sodium borohydride in PBS for 10 minutes at room temperature, cells were washed in PBS and mounted in Mowiol containing 2.5% DABCO (Sigma). For live time-domain fluorescence lifetime imaging microscopy (FLIM) experiments with randomly migrating cells, cells were plated following transfection onto 3 cm glass-bottom dishes coated with 15 nM laminin, allowed to adhere and migrate for 2 hours, and the media replaced with phenol red-free Optimem (Gibco) prior to imaging. In some experiments, cells were treated with inhibitors or activators using previously optimised dose and time conditions (Hashimoto et al., 2007). Serum-starvation was started 24 hours after transfection and was carried out for 16 hours before fixation for FLIM. FLIM was performed with a multi-photon microscope system as described (for details, see Parsons et al., 2005; Peter et al., 2005). Fluorescence lifetime imaging capability was provided by time-correlated single-photon counting electronics (Becker & Hickl, SPC 700). A 40× objective was used throughout (Nikon, CFI60 Plan Fluor N.A. 1.3) and data were collected at 500±20 nm through a bandpass filter (Coherent Inc. 35-5040). Acquisition times of the order of 300 seconds at low 890 nm excitation power were used to achieve sufficient photon statistics for fitting, while avoiding either pulse pile-up or significant photobleaching.

Analysis of data for FRET experiments

Data were analysed as previously described (Parsons et al., 2005; Prag et al., 2007). Briefly, bulk measurements of Forster resonance energy transfer (FRET) efficiency (i.e. intensity-based methods) cannot distinguish between an increase in FRET efficiency (i.e. the coupling efficiency) and an increase in FRET population (i.e. the concentration of FRET species) because the two parameters are not resolved. Measurements of FRET based on analysis of the fluorescence lifetime of the donor resolve this issue when analysed using multi-exponential decay models. For measurements of bulk interactions (i.e. where only single exponential decays are fit to the data), measured efficiencies will appear significantly lower owing to the assumption that all donors are associated with one or more acceptors. The assumption that non-interacting and interacting fractions are present allows the determination of the efficiency of interaction. The FRET efficiency is related to the molecular separation of donor and acceptor, and the fluorescence lifetime of the interacting fraction by:

$$\eta_{fret}=(R_0^6/(R_0^6+r^6))=1-\tau_{fret}/\tau_d,$$

where R_0 is the Förster radius, R the molecular separation, η_{fret} is the lifetime of the interacting fraction and τ_d the lifetime of the donor in the absence of acceptor. The donor-only control is used as the reference against which all other lifetimes are calculated in each experiment. η_{fret} and τ_d can also be taken to be the lifetime of the interacting fraction and non-interacting fraction, respectively. Quantification was made from all pixels within each cell analysed. All data were analysed using TRI2 software (developed by Paul Barber, Gray Cancer Institute, London, UK). Histogram data presented here are plotted as mean FRET efficiency from the stated number of cells over three experiments ± s.e.m. ANOVA was used to test statistical significance between different populations of data. Fig. 4B demonstrates the range of lifetime efficiencies per cell normalised for pixel intensity for each experimental condition. In the figures, lifetime images of representative cells are presented using a pseudocolour scale, whereby blue depicts normal GFP lifetime (no FRET) and red depicts lower GFP lifetime (areas of FRET).

Confocal microscopy

Confocal images were collected on a model LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss) using a 63×/1.4Plan-APOCHROMAT oil immersion objective. Fascin immunostaining was carried out with mouse monoclonal 55k2 (Dako). Expression of GTPases was detected with 12CA5 mouse monoclonal antibody to influenza haemagglutinin-tag (HA) (obtained from Cancer Research UK) or 9E10 mouse monoclonal to c-myc (Evan et al., 1985). Species-specific FITC-, Cy3- or Cy5-tagged secondary antibodies were from ImmunoResearch Laboratories.

Analysis of PKCγ activation and PKCγ immunoprecipitation

The PKC isoforms expressed by colon carcinoma cells were identified by immunoblotting whole cell extracts with a panel of mouse monoclonal antibodies to the PKC family (BD Biosciences). For analysis of PKCγ activation, SW480 cells were treated with solvent, 1 μM BIM, 50 nM TPA or 100 μM NSC23766, under previously optimised conditions (Hashimoto et al., 2007), lysed in RIPA buffer containing 1 μM Calyculin A (Calbiochem) and protease inhibitor cocktail (Boehringer Mannheim), and resolved by SDS-PAGE and western blotting. Blots were probed with anti-PKCγ–T655PO$_4$ (Upstate) or anti-PKCγ (Santa Cruz Biotechnology) antibodies and developed with anti-rabbit HRP-conjugated secondary antibody (Dako) and ECL (Pierce) on Kodak X-ray film. For PKCγ immunoprecipitation, protein A/G beads (Santa Cruz Biotechnology) were incubated with anti-PKCγ antibodies, washed and then incubated with RIPA cell lysates for 4 hours in the presence or absence of NSC23766 or BIM. After washing thoroughly, beads were boiled in sample buffer and analysed by SDS-PAGE and western blotting.

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References

Abou-Kheir, W., Isaac, B., Yamaguchi, H. and Cox, D. (2008). Membrane targeting of WAVE2 is not sufficient for WAVE2-dependent actin polymerization: a role for IRSp53 in mediating the interaction between Rac and WAVE2. *J. Cell Sci.* **121**, 379-390.

Adams, J. C. (2004). Roles of fascin in cell adhesion and motility. *Curr. Opin. Cell Biol.* **16**, 590-596.

Adams, J. C. and Schwartz, M. A. (2000). Stimulation of fascin spikes by thrombospondin-1 is mediated by the GTPases Rac and Cdc42. *J. Cell Biol.* **150**, 807-822.

Adams, J. C., Clelland, J. D., Collett, G. D., Matsumura, F., Yamashiro, S. and Zhang, L. (1999). Cell-matrix adhesions differentially regulate fascin phosphorylation. *Mol. Biol. Cell* **10**, 4177-4190.

Anilkumar, N., Parsons, M., Monk, R., Ng, T. and Adams, J. C. (2003). Interaction of fascin and protein kinase Calpha: a novel intersection in cell adhesion and motility. *EMBO J.* **22**, 5390-5402.

Aratyn, Y. S., Schaus, T. E., Taylor, E. W. and Borisy, G. G. (2007). Intrinsic dynamic behavior of fascin in filopodia. *Mol. Biol. Cell* **8**, 3928-3940.

Baier-Bitterlich, G., Uberall, F., Bauer, B., Fresser, F., Wachter, H., Grunicke, H., Utermann, G., Altman, A. and Baier, G. (1996). Protein kinase C-theta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Mol. Cell. Biol.* **16**, 1842-1850.

Berrier, A. L. and Yamada, K. M. (2007). Cell-matrix adhesion. *J. Cell Physiol.* **213**, 565-573.

Bostner, J., Ahnström Waltersson, M., Fornander, T., Skoog, L., Nordenskjöld, B. and Stål, O. (2007). Amplification of CCND1 and PAK1 as predictors of recurrence and tamoxifen resistance in postmenopausal breast cancer. *Oncogene* **26**, 6997-7005.

Carter, J. H., Douglass, L. E., Deddens, J. A., Colligan, B. M., Bhatt, T. R., Pemberton, J. O., Konicek, S., Hom, J., Marshall, M. and Graff, J. R. (2004). Pak-1 expression increases with progression of colorectal carcinomas to metastasis. *Clin. Cancer Res.* **10**, 3448-3456.

Ching, Y. P., Leong, V. Y., Lee, M. F., Xu, H. T., Jin, D. Y. and Ng, I. O. (2007). P21-activated protein kinase is overexpressed in hepatocellular carcinoma and enhances cancer metastasis involving c-Jun NH2-terminal kinase activation and paxillin phosphorylation. *Cancer Res.* **67**, 3601-3608.

Delorme, V., Machacek, M., DerMardirossian, C., Anderson, K. L., Wittmann, T., Hanein, D., Waterman-Storer, C., Danuser, G. and Bokoch, G. M. (2007). Cofilin activity downstream of Pak1 regulates cell protrusion efficiency by organizing lamellipodium and lamella actin networks. *Dev. Cell* **13**, 646-662.

del Pozo, M. A., Price, L. S., Alderson, N. B., Ren, X. D. and Schwartz, M. A. (2000). Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. *EMBO J.* **19**, 2008-2014.

Edwards, R. A. and Bryan, J. (1995). Fascins, a family of actin bundling proteins. *Cell Motil. Cytoskeleton* **32**, 1-9.

Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610-3616.

Even-Faitelson, L. and Ravid, S. (2006). PAK1 and aPKCzeta regulate myosin II-B phosphorylation: a novel signaling pathway regulating filament assembly. *Mol. Biol. Cell* **17**, 2869-2881.

Gao, Y., Dickerson, J. B., Guo, F., Zheng, J. and Zheng, Y. (2004). Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc. Natl. Acad. Sci. USA* **101**, 7618-7623.

Griner, E. M. and Kazanietz, M. G. (2007). Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer* **7**, 281-294.

Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, A. L., Toker, A. and Stossel, T. P. (1995). Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilised human platelets. *Cell* **82**, 643-653.

Hashimoto, Y., Shimada, Y., Kawamura, J., Yamasaki, S. and Imamura, M. (2004). The prognostic relevance of fascin expression in human gastric carcinoma. *Oncology* **67**, 262-270.

Hashimoto, Y., Skacel, M., Lavery, I. C., Mukherjee, A. L., Casey, G. and Adams, J. C. (2006). Prognostic significance of fascin expression in advanced colorectal cancer: an immunohistochemical study of colorectal adenomas and adenocarcinomas. *BMC Cancer* **6**, 241.

Hashimoto, Y., Parsons, M. and Adams, J. C. (2007). Dual actin-bundling and protein kinase C-binding activities of fascin regulate carcinoma cell migration downstream of Rac, and contribute to metastasis. *Mol. Biol. Cell* **18**, 4591-4602.

Holm, C., Rayala, S., Jirström, K., Stål, O., Kumar, R. and Landberg, G. (2006). Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients. *J. Natl. Cancer Inst.* **98**, 671-680.

Jacobs, T., Causeret, F., Nishimura, Y. V., Terao, M., Norman, A., Hoshino, M. and Nikolić, M. (2007). Localized activation of p21-activated kinase controls neuronal polarity and morphology. *J. Neurosci.* **27**, 8604-8615.

Jaffer, Z. M. and Chernoff, J. (2002). p21-activated kinases: three more join the Pak. *Int. J. Biochem. Cell Biol.* **34**, 713-717.

Kureishy, N., Sapountzi, V., Prag, S., Anilkumar, N. and Adams, J. C. (2002). Fascins, and their roles in cell structure and function. *BioEssays* **24**, 350-361.

Machesky, L. M. and Hall, A. (1997). Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. *J. Cell Biol.* **138**, 913-926.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Ono, S., Yamakita, Y., Yamashiro, S., Matsudaira, P. T., Gnarra, J. R., Obinata, T. and Matsumura, F. (1997). Identification of an actin binding region and a protein kinase C phosphorylation site on human fascin. *J. Biol. Chem.* **272**, 2527-2533.

O'Sullivan, G. C., Tangney, M., Casey, G., Ambrose, M., Houston, A. and Barry, O. P. (2007). Modulation of p21-activated kinase 1 alters the behavior of renal cell carcinoma. *Int. J. Cancer* **121**, 1930-1940.

Parsons, M., Vojnovic, B. and Ameer-Beg, S. (2004). Imaging protein-protein interactions in cell motility using fluorescence resonance energy transfer (FRET). *Biochem. Soc. Trans.* **32**, 431-433.

Parsons, M., Monypenny, J., Ameer-Beg, S. M., Millard, T. H., Machesky, L. M., Peter, M., Keppler, M. D., Schiavo, G., Watson, R., Chernoff, J. et al. (2005). Spatially distinct binding of Cdc42 to PAK1 and N-WASP in breast carcinoma cells. *Mol. Cell. Biol.* **25**, 1680-1695.

Pelosi, G., Pastorino, U., Pasini, F., Maissoneuve, P., Fraggetta, F., Iannucci, A., Sonzogni, A., De Manzoni, G., Terzi, A., Durante, E. et al. (2003). Independent prognostic value of fascin immunoreactivity in stage I nonsmall cell lung cancer. *Br. J. Cancer* **88**, 537-547.

Peter, M., Ameer-Beg, S. M., Hughes, M. K., Keppler, M. D., Prag, S., Marsh, M., Vojnovic, B. and Ng, T. (2005). Multiphoton-FLIM quantification of the EGFP-mRFP1 FRET pair for localization of membrane receptor-kinase interactions. *Biophys. J.* **88**, 1224-1237.

Prag, S., Parsons, M., Keppler, M. D., Ameer-Beg, S. M., Barber, P., Hunt, J., Beavil, A. J., Calvert, R., Arpin, M., Vojnovic, B. et al. (2007). Activated ezrin promotes cell migration through recruitment of the GEF Dbl to lipid rafts and preferential downstream activation of Cdc42. *Mol. Biol. Cell* **18**, 2935-2948.

Puppa, G., Maisonneuve, P., Sonzogni, A., Masullo, M., Chiappa, A., Valerio, M., Zampino, M. G., Franceschetti, I., Capelli, P., Chilosi, M. et al. (2007). Independent prognostic value of fascin immunoreactivity in stage III-IV colonic adenocarcinoma. *Br. J. Cancer* **96**, 1118-1126.

Ridley, A. J. (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol.* **16**, 522-529.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M. W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97**, 221-231.

Rosenberg, M. and Ravid, S. (2006). Protein kinase Cgamma regulates myosin IIB phosphorylation, cellular localization, and filament assembly. *Mol. Biol. Cell* **17**, 1364-1374.

Shirsat, N. V., Pignolo, R. J., Kreider, B. L. and Rovera, A. (1990). A member of the ras gene superfamily is expressed specifically in T, B, and myeloid hemopoietic cells. *Oncogene* **5**, 769-772.

Shonukan, T., Bagayogo, I., McCrea, P. D., Chao, M. and Hempstead, B. (2003). Neurotrophin-induced melanoma cell migration is mediated through the actin-bundling protein fascin. *Oncogene* **22**, 3616-3623.

Thullberg, M., Gad, A., Beeser, A., Chernoff, J. and Strömblad, S. (2007). The kinase-inhibitory domain of p21-activated kinase 1 (PAK1) inhibits cell cycle progression independent of PAK1 kinase activity. *Oncogene* **26**, 1820-1828.

Vignjevic, D., Yarar, D., Welch, M. D., Peloquin, J., Svitkina, T. and Borisy, G. G. (2003). Formation of filopodia-like bundles in vitro from a dendritic network. *J. Cell Biol.* **160**, 951-962.

Vignjevic, D., Kojima, S., Aratyn, Y., Danciu, O., Svitkina, T. and Borisy, G. G. (2006). Role of fascin in filopodial protrusion. *J. Cell Biol.* **174**, 863-875.

Wittman, T., Bokoch, G. M. and Waterman-Storer, C. (2004). Regulation of microtubule destabilizing activity of Op18/stathmin downstream of Rac1. *J. Biol. Chem.* **279**, 6196-6203.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. and Mizuno, K. (1998). Cofilin phosphorylation and LIM-kinase 1 and its role in Rac-mediated actin reorganisation. *Nature* **393**, 809-812.

Yoder, B. J., Tso, E., Skacel, M., Pettay, J., Tarr, S., Budd, T., Tubbs, R. R., Adams, J. C. and Hicks, D. G. (2005). The expression of fascin, an actin-bundling motility protein, correlates with hormone receptor-negative breast cancer and a more aggressive clinical course. *Clin. Cancer Res.* **11**, 186-192.

Zhao, L., Ma, Q. L., Calon, F., Harris-White, M. E., Yang, F., Lim, G. P., Morihara, T., Ubeda, O. J., Ambegaokar, S., Hansen, J. E. et al. (2006). Role of p21-activated kinase pathway defects in the cognitive deficits of Alzheimer disease. *Nat. Neurosci.* **9**, 234-242.

Zhao, Z. S., Manser, E., Chen, X. Q., Chong, C., Leung, T. and Lim, L. (1998). A conserved negative regulatory region in PAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol. Cell. Biol.* **18**, 2153-2163.

Zigeuner, R., Droschl, N., Tauber, V., Rehak, P. and Langner, C. (2006). Biologic significance of fascin expression in clear cell renal cell carcinoma: systematic analysis of primary and metastatic tumor tissues using a tissue microarray technique. *Urology* **68**, 518-522.

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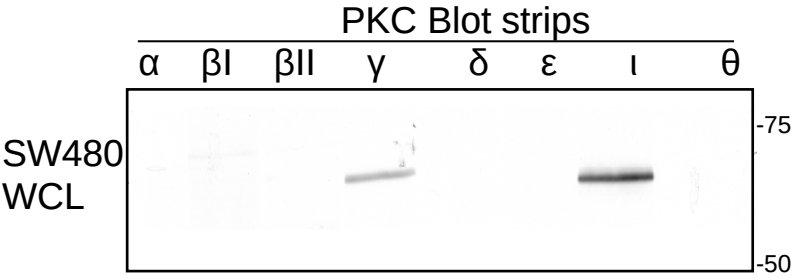
PKC Blot strips
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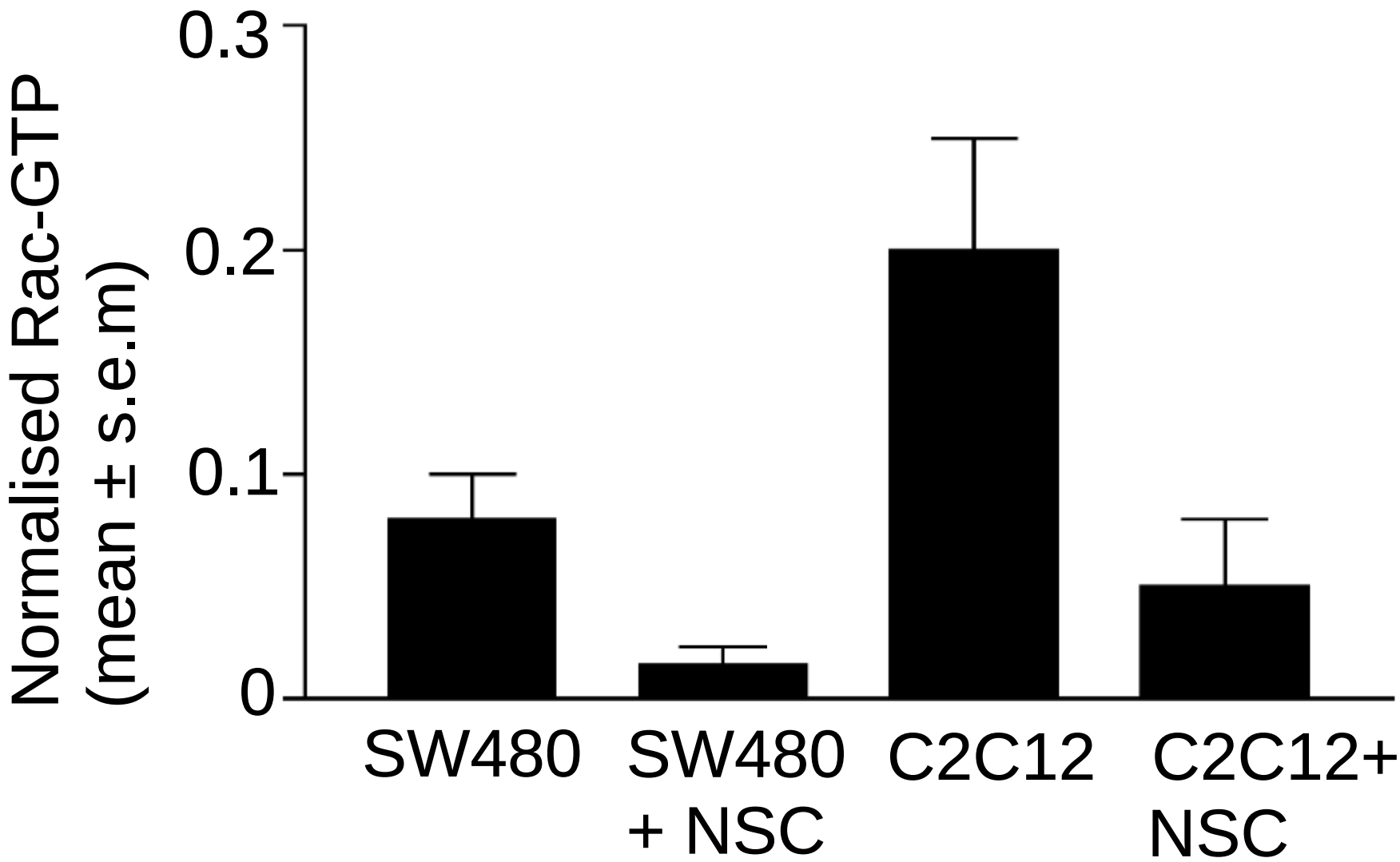
Normalised Rac-GTP
(mean ± s.e.m)
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0.2
0.1
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C2C12
C2C12+
NSC