<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>pRCP-driven ±5°1 recycling suppresses Rac and promotes RhoA activity via the RacGAP1-IQGAP1 complex</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Authors(s)</strong></td>
<td>Jacquemet, G.; Green, D. M.; Bridgewater, R. E.; et al.</td>
</tr>
<tr>
<td><strong>Publication date</strong></td>
<td>2013-09-09</td>
</tr>
<tr>
<td><strong>Publication information</strong></td>
<td>The Journal of Cell Biology, 202 (6): 917-935</td>
</tr>
<tr>
<td><strong>Publisher</strong></td>
<td>The Rockefeller University Press</td>
</tr>
<tr>
<td><strong>Item record/more information</strong></td>
<td><a href="http://hdl.handle.net/10197/5566">http://hdl.handle.net/10197/5566</a></td>
</tr>
<tr>
<td><strong>Publisher's version (DOI)</strong></td>
<td>10.1083/jcb.201302041</td>
</tr>
</tbody>
</table>
RCP-driven α5β1 recycling suppresses Rac and promotes RhoA activity via the RacGAP1-IQGAP1 complex

Guillaume Jacquemet,¹* David M. Green,¹* Rebecca E. Bridgewater¹, Alexander von Kriegsheim³, Martin J. Humphries,¹ Jim C. Norman,² and Patrick T. Caswell¹

¹Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, M13 9PT, UK
²Beatson Institute for Cancer Research, Glasgow. G61 1BD, UK
³Systems Biology Ireland, Conway Institute, University College Dublin, Belfield, Dublin, Ireland

* These authors contributed equally to this work

Corresponding author:
Patrick T. Caswell, Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK
Tel.: +44 (0) 161 3060505
Fax: +44 (0) 161 2755082
E-mail: patrick.caswell@manchester.ac.uk

Running head: Local Rac suppression promotes pseudopod extension

eTOC summary statement: “RCP-driven α5β1 recycling suppresses Rac activity through the RacGAP1-IQGAP1 complex to permit local activation of RhoA and drive invasive migration.”

Character count: 41700

Manuscript #201302041; revision submitted 31/07/13
Abstract

Inhibition of αvβ3 or expression of mutant p53 promotes invasion into fibronectin (FN)-containing extracellular matrix (ECM) by enhancing Rab-coupling protein (RCP)-dependent recycling of α5β1 integrin. RCP and α5β1 cooperatively recruit receptor tyrosine kinases (RTKs), including EGFR1, to regulate their trafficking and downstream signalling via PKB/Akt which, in turn, promotes invasive migration. Here, we identify a novel PKB/Akt substrate, RacGAP1, that is phosphorylated as a consequence of RCP-dependent α5β1 trafficking. Phosphorylation of RacGAP1 promotes its recruitment to IQGAP1 at the tips of invasive pseudopods, and RacGAP1 then locally suppresses the activity of the cytoskeletal regulator Rac, and promotes the activity of RhoA in this subcellular region. This Rac to RhoA switch promotes the extension of pseudopodial processes and invasive migration into FN-containing matrices, in a RhoA-dependent manner. Thus, the localised endocytic trafficking of α5β1 within the tips of invasive pseudopods elicits signals that promote the reorganisation of the actin cytoskeleton, protrusion and invasion into FN-rich ECM.
Introduction

Tumour cells invade 3-dimensional ECM as individual cells, or as collective sheets and strands (Friedl and Alexander, 2011). Both individual and collective cell migration have been documented in vivo, and together these strategies play an important role in escape from the primary tumour and seeding of metastases (Friedl and Alexander, 2011; Sahai, 2007).

Cell migration is well-studied within the context of 2D, planar substrates, with clear roles described for RhoGTPases such as Rac in establishing and maintaining a broad ruffling lamellipodium at the cell front and for RhoA in controlling acto-myosin contractility and retraction of the cell rear (Ridley et al., 2003). Individual cell invasive migration has been broadly categorised as ‘mesenchymal’ (protease-dependent with protrusion driven by Rac and/or Cdc42) or ‘amoeboid’ (exhibiting little protease-dependence with protrusion driven by RhoA-mediated acto-myosin contractility and blebbing; Friedl and Alexander, 2011). The cycling of RhoGTPases between active and inactive states is controlled by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which can determine the reciprocal relationship between RhoA and Rac activities (Guilluy et al., 2011). In invasive melanoma cells, specific GAPs and GEFs balance the activities of Rac and RhoA to control switching between modes of migration in 3D (Sanz-Moreno et al., 2008). The use of Förster resonance energy transfer (FRET)-based activity probes has revealed the spatiotemporal activities of RhoGTPases to be highly complex, with active RhoA seen at the cell front on 2D substrates (Machacek et al., 2009; Pertz et al., 2006). Furthermore, in 3D matrix and in vivo, highly invasive mutant p53-expressing pancreatic cancer cells with a clear elongated morphology have high levels of RhoA activity at the cell front (Timpson et al., 2011), suggesting that mesenchymal invasion dependent on mutant p53 could be driven by RhoA.

The interaction between invading cells and the surrounding ECM is governed by integrins, which act as receptors for ECM proteins (Humphries et al., 2006). Integrins are α/β heterodimers
that function to link the ECM to the cytoskeleton, recruiting a range of signalling molecules to regulate cellular function such as cell migration, and RhoGTPases are key effectors of integrin signalling (Huveneers and Danen, 2009; Hynes, 2002; Legate et al., 2009). Integrin function is regulated by the binding of intracellular factors, such as talin and kindlins, which control the integrin activation (Shattil et al., 2010; Moser et al., 2009). In addition, integrins are internalized from the plasma membrane (PM), and endosomal sorting determines the degradation or recycling of the receptor (Wickström and Fässler, 2011; Caswell et al., 2009; Bridgewater et al., 2012). Integrin recycling can be targeted to specific regions of the cell, and can therefore control propagation of intracellular signals in a localised manner (Caswell et al., 2008, 2007; Dozynkiewicz et al., 2012; Rainero et al., 2012). The pathways that regulate integrin trafficking have been implicated in many aspects of cell migration in 2D, and accumulating evidence indicates that the trafficking of integrins, particularly the fibronectin (FN) receptor α5β1, can dictate the migratory properties of invasive cancer cells (Caswell & Norman, 2008).

In fibroblasts and tumour cells, inhibition of αvβ3 (or αvβ3 recycling) promotes the recycling of α5β1, and rapid, random migration in 2D (Caswell et al., 2008; Christoforides et al., 2012; White et al., 2007). Similarly, in carcinoma cells, expression of gain-of-function mutant p53 can switch on a rapid α5β1 recycling pathway (Muller et al., 2009). Rab-coupling protein (RCP, also known as Rab11-FIP1) is central to the control of α5β1 recycling in these contexts, and drives pseudopod extension and invasion into FN-rich 3D matrix (Muller et al., 2009; Caswell et al., 2008). RCP-mediated α5β1 recycling does not influence adhesion; rather RCP and α5β1 act by recruiting RTKs, e.g. EGFR1 and c-Met, and coordinating the recycling of associated RTKs to potentiate their signalling via PKB/Akt (Muller et al., 2009; Caswell et al., 2008; Muller et al., 2012). Production of phosphatidic acid (PA) by DGKα localizes RCP, and as a consequence α5β1/RTK trafficking, toward the front of invading cells, providing a spatial cue for protrusion (Rainero et al., 2012). Whilst it is clear that the RhoA-ROCK-cofilin pathway controls the rapid, random migration of fibroblasts (White et al., 2007), and that
RCP-dependent integrin trafficking influences invasive migration via effects on RTK trafficking and signalling (Caswell et al., 2008; Muller et al., 2012, 2009), the mechanism through which RCP/\(\alpha 5\beta 1\)-mediated RTK trafficking and signalling impacts upon the cytoskeleton to promote pseudopodial extension at the cell front and migration in 3D-matrix are not known.

Here we describe RacGAP1 (also known as Mgcracgap, or hCYK-4), a component of the centralspindlin complex, as a novel PKB/Akt substrate that is phosphorylated on threonine (T) 249 as a consequence of RCP-driven \(\alpha 5\beta 1\)/EGFR trafficking and signalling. Phosphorylation of RacGAP1 on T249 does not influence formation of the centralspindlin complex, but promotes recruitment of RacGAP1 to IQGAP1-containing complexes within the tips of invasive pseudopods. RCP-driven \(\alpha 5\beta 1\) recycling suppresses Rac activity through the RacGAP1-IQGAP1 complex, and permits the concomitant activation of RhoA. Surprisingly, suppression of Rac is necessary and sufficient to promote pseudopod extension and invasive migration in 3D, both of which require RhoA activity, identifying a novel pathway that regulates the acquisition of an elongated mode of individual cell migration into FN-rich ECM.

Results

RacGAP1 is a novel PKB/Akt substrate phosphorylated downstream of RCP/\(\alpha 5\beta 1\)-mediated RTK signalling

We have previously shown that inhibition of \(\alpha \nu \beta 3\) integrin (using cRGDFV, a selective cyclic peptide inhibitor of \(\alpha \nu \beta 3\)) promotes RCP-dependent trafficking and signalling via PKB/Akt to induce extension of pseudopodial protrusions in 3D-matrix (Caswell et al., 2008). In order to localise PKB/Akt activity, we employed the FRET probe Akind (Yoshizaki et al., 2007). Only low levels of PKB/Akt activity were detectable in A2780 cells migrating on cell-derived matrix (CDM) under basal conditions (Fig. 1A; compared to inactive Akind-3A, Fig. S1A). Inhibition of \(\alpha \nu \beta 3\) promoted activation of PKB/Akt at the front of cells migrating with RCP/\(\alpha 5\beta 1\)-driven pseudopodia (Fig. 1A). Furthermore,
suppression of PKBβ/Akt2 greatly reduced RCP/α5β1-driven invasion (Fig. S1B, C), in line with previous findings (Irie et al., 2005; Dillon et al., 2009).

We next sought to identify the PKB/Akt targets required for invasion downstream of RCP/α5β1. Immunoprecipitation (IP) using an antibody recognising the phosphorylated PKB/Akt consensus sequence (RxRxxS*/T*) revealed putative PKB/Akt substrates of 75-85kDa enriched upon addition of cRGDfV (unpublished data). Mass spectrometric (MS) analysis of proteins within this molecular weight range, combined with hierarchical clustering of spectral count data, identified a subset of proteins that were hyperphosphorylated upon cRGDfV stimulation, with RacGAP1 (also known as MgcRacGAP, hCYK-4) showing the highest increase following cRGDfV addition (Fig. 1B).

RacGAP1 is a Rac and Cdc42-specific GAP, and plays a critical role in the regulation of RhoGTPases during cytokinesis (Bastos et al., 2012; Canman et al., 2008). This led us to speculate that RacGAP1 could constitute a central link between RCP/α5β1-mediated trafficking and signalling and RhoGTPase cytoskeletal regulators. In vitro phosphorylation, using purified mannose-binding protein (MBP)-RacGAP1 and recombinant active PKB/Akt, established RacGAP1 as a direct substrate for PKB/Akt (Fig. 1C). Moreover, immunoblotting of PKB substrates captured using the RxRxxS*/T* antibody demonstrated that RacGAP1 phosphorylation was promoted by cRGDfV addition, and was significantly reduced by the broad spectrum kinase inhibitor staurosporine (Fig. 1D).

**RacGAP1 is required for RCP/α5β1 driven pseudopod extension and invasive migration**

We next tested the functional importance of RacGAP1 in 3D cell migration. RCP/α5β1-driven pseudopodial migration leads to increased invasive capability within dense plugs of high concentration collagen I rich in the α5β1 ligand FN (Fig. 2A-C; Caswell et al., 2008). RacGAP1 knockdown (Fig. S1D) had no significant effect on the speed or persistence of cells migrating on CDM, or on the formation of protrusions under basal conditions (Fig. 2A, B, Fig. S1F, G). Upon
cRGDfV stimulation, however, RacGAP1-depleted cells were unable to extend invasive pseudopods (Fig. 2A, B, Fig. S1H). Moreover, RacGAP1 knockdown specifically reduced cRGDfV-driven invasion into FN-rich ECM (Fig. 2C, Fig. S1I), and this was rescued by expression of siRNA-resistant RacGAP1 (Fig. 2D, Fig. S1L). Expression of mutant forms of p53, either endogenously (MDA-MB-231 cells), or exogenously in H1299 cells, promotes invasion via RCP/α5β1-dependent trafficking (Muller et al., 2009), and depletion of RacGAP1 levels in these cell lines significantly reduced invasive migration (Fig. 2E, F; Fig. S1M-O). These data demonstrate that RacGAP1 is required for RCP/α5β1-dependent invasive migration in several cell lines.

**Phosphorylation of RacGAP1 on T249 promotes recruitment to IQGAP1**

MS analysis of *in vitro* phosphorylated MBP-RacGAP1 and FLAG-RacGAP1 IPd from 293T cells identified a single predominant PKB/Akt phosphorylation site in RacGAP1, but could not distinguish between T249 (within the sole PKB/Akt consensus within RacGAP1; previously reported in MS analysis (Moritz et al., 2010)) and T251 (unpublished data). *In vitro* phosphorylation using recombinant active PKB/Akt, with either putative phosphorylation site mutated to alanine, revealed T249 to be the predominant PKB/Akt phosphorylation site within RacGAP1 (Fig. 3A). We generated stable FLAG-RacGAP1-expressing A2780 cells using lentiviral transfection, and noted that cells expressing wild type (wt) or mutant RacGAP1 lost endogenous RacGAP1 expression (Fig. S1J). Furthermore, these cell lines showed proliferation rates equivalent to control, suggesting that they are fully functional during cytokinesis when expressed at this level (Fig. S1K). IP with PKB/Akt substrate-specific antibodies revealed that phosphorylation of FLAG-RacGAP1 was increased by addition of cRGDfV, and this was opposed by mutation of RacGAP1’s T249 to alanine (Fig. 3B, Fig. S2K). Taken together, these data suggest that T249 is the major PKB/Akt phosphorylation site within RacGAP1.
T249 is situated within a region of RacGAP1 that is predicted to be unstructured (Fig. S1E). Nevertheless, we determined the consequences of mutating T249 on the recruitment of known interactors (Yüce et al., 2005; Mishima et al., 2002). IP of FLAG-RacGAP1^{WT} from A2780 cells revealed robust associations with MKLP1 and Ect2, which were unaltered by treatment with cRGDFV, or by mutation of T249 (Fig. 3C). Hence, PKB/Akt-mediated phosphorylation of RacGAP1 at T249 has no influence on formation of the centralspindlin complex or its recruitment of Ect2.

We recently showed that the cytoskeletal adaptor protein IQGAP1 recruits RacGAP1 to sites of integrin activation in fibroblasts, in order to restrict the activity of Rac as cells spread on FN (Jacquemet et al., 2013). In A2780 cells, little association was seen between endogenous IQGAP1 and RacGAP1, however stimulation with cRGDFV increased IQGAP1-RacGAP1 complex formation (Fig. 3D). cRGDFV treatment also stimulated the recruitment of FLAG-RacGAP1^{WT} to IQGAP1, but not RacGAP1^{249A} (Fig. 3E, F), and RacGAP1^{249D} showed an increased level of association with IQGAP1 even in the absence of cRGDFV (Fig. 3E, F). In addition, a proximity ligation assay (PLA) revealed close association between IQGAP1 and RacGAP1 in cRGDFV-treated cells expressing RacGAP1^{WT}; however, very little signal was detectable in cells expressing GFP or FLAG-RacGAP1^{249A} (Fig. 3G). Together, these data indicate that PKB/Akt-phosphorylation of RacGAP1 on T249 promotes recruitment of RacGAP1 to IQGAP1-containing complexes.

**IQGAP1 recruits phosphorylated RacGAP1 to the tips of protrusions as cells migrate in 3D**

RacGAP1 plays a well-documented role in cytokinesis, and localises to the central spindle and midbody in mammalian cells (Lekomtsev et al., 2012). RacGAP1 is also expressed in interphase cells, and plays roles in nuclear transport of a Rac/STAT module and maintaining RhoA signalling at cell-cell junctions (Kawashima et al., 2009, 2006; Ratheesh et al., 2012). Endogenous RacGAP1 is localised to the nucleus, but also appears in a granular distribution in the cytoplasm (Fig. 4A), and in cells migrating on CDM, RacGAP1 accumulates at the cell rear but appears to be excluded from the
cell front (Fig. 4A). Following treatment with cRGDFV the levels of RacGAP1 were increased within pseudopods towards the cell front (Fig. 4A). Knockdown of IQGAP1 (Fig. S2A) had relatively little influence on the localisation of RacGAP1 under basal conditions (Fig. 4B); however in cRGDFV treated IQGAP1 knockdown cells, RacGAP1 was excluded from the cell front (Fig. 4B).

Stable overexpression of RacGAP1 resulted in an increase in the nuclear pool of RacGAP1 (Fig. 4C). FLAG-RacGAP1<sup>wt</sup> predominantly localised to the nucleus in unstimulated cells, but was recruited to the tips of invasive pseudopods upon treatment with cRGDFV. Here, RacGAP1 colocalised with IQGAP1 within structures that resemble filopodia (Fig. 4C). RacGAP1<sup>249A</sup> did not localise to the cell front in cRGDFV-stimulated cells, and was found to be predominantly in the nucleus (Fig. 4C). Conversely, FLAG-RacGAP1<sup>249D</sup> was found in the nucleus and cytoplasm, but was recruited to pseudopod tips even under basal conditions (Fig. 4C). Together, these data indicate that phosphorylation of RacGAP1 on T249 promotes its recruitment to the front of invasive cells through association with IQGAP1.

The RacGAP1-IQGAP1 complex drives pseudopod extension and invasive migration

As phosphorylation of RacGAP1 downstream of RCP-α5β1 trafficking determines its association with IQGAP1 and subcellular localisation, we hypothesised that formation of the RacGAP1-IQGAP1 complex was required for RCP/α5β1 driven invasion. Knockdown of IQGAP1 had a profound influence on cell migration: cells plated on CDM displayed multiple protrusions, and the speed and persistence of migration were reduced in both the presence and absence of cRGDFV (Fig. 5A, Fig. S2A, B, C). IQGAP1 knockdown cells were unable to extend and maintain long invasive pseudopods (Fig. 5A), and invasive migration of cells into collagen I plugs was also suppressed, with this effect greatest in the presence of both FN and cRGDFV (Fig. 5B, Fig. S2D). Furthermore, IQGAP1 depletion inhibited the invasive migration of MDA-MB-231 and H1299-p53<sup>273H</sup> cells (which express
mutant p53), but not in H1299 cells null for p53 (Fig. S2E-H), indicating that IQGAP1 is required for RCP/α5β1-driven pseudopod extension and invasion in FN-rich matrix.

Stable expression of RacGAP1 had no discernible effect on the ability of A2780 cells to extend invasive pseudopods or invade plugs of collagen I/FN in response to cRGDFV (Fig. 5C, D). Expression of FLAG-RacGAP249A had little effect on basal migration, but prevented cRGDFV or mutant p53-driven pseudopod extension and invasion into collagen/FN (Fig. 5C, D, Fig S2I). Conversely, stable expression of FLAG-RacGAP1249D promoted pseudopod extension, and significantly increased invasion into FN-rich collagen even in the absence of cRGDFV (Fig. 5C, D), and this was dependent on expression of IQGAP1 (Fig. 5E, Fig. S2J). Taken together, these data indicate that phosphorylation of RacGAP1 on T249 by PKB/Akt is an essential step in the acquisition of a migratory phenotype in cells invading FN-rich ECM, and highlight the fundamental role of IQGAP1 as a scaffold for RacGAP1.

**α5β1 recycling suppresses Rac activity and promotes activation of RhoA**

Inhibition of αvβ3 promotes α5β1 recycling and downstream signalling via the RhoA effectors ROCK and coflin to promote rapid, random migration on 2D-substrates (White et al., 2007). Together with the identification of RacGAP1 as a Rac inactivator required for α5β1-driven invasive migration, this led us to hypothesise that α5β1 may not utilise the canonical Rac-driven cytoskeletal machinery to promote elongated invasive migration.

Using Raichu-Rac and -RhoA FRET probes (Itoh et al., 2002; Yoshizaki et al., 2003), we analysed the dynamic activity of Rac and RhoA in live cells migrating in 3D-matrix by FRET-FLIM. For both probes, the dynamic range was ascertained using dominant negative or constitutively active probe mutants, and these membrane-targeted probes were distributed around the cell periphery without concentrating at the front, similar to the intact GTPases (Fig. S1A, S3A). FLIM measurements were made by creating regions of interest around the plasma membrane at the front, middle and
rear of the cell (Fig. S3B). Under basal conditions, high FRET efficiency indicated that Rac was activated towards the cell front, and this high Rac activity was maintained as cells migrated (Fig. 6A, C, D). However, upon treatment with cRGDFIV, FRET efficiency was significantly reduced at the cell front, but not in other regions (Fig. 6B, C, D), indicating that Rac activity was suppressed at the tips of extending pseudopods. Low FRET efficiency revealed a low level of RhoA activity towards the cell front as cells migrated under basal conditions (Fig. 6E, G, H); stimulation of α5β1 recycling, however, promoted RhoA activity within the tips of pseudopods (Fig. 6F, G, H). Again, the switch in RhoGTPase activity was more pronounced within peripheral regions at the cell front, and this level of RhoA activity was maintained as cells migrated (Fig. 6G, H). Expression of mutant p53 also suppressed Rac activity, and promoted activation of RhoA, at the front of cells migrating on CDM (Fig. 6I), indicating that this RhoGTPase switch is a general feature of cells that utilise the RCP/α5β1 machinery for motility in 3D.

RCP is the Rab11 effector that controls the recycling of α5β1 and associated RTKs in invasive cancer cells (Caswell et al., 2008; Muller et al., 2009). Whilst knockdown of RCP had little effect on the balance between Rac and RhoA activity under basal conditions, RCP knockdown cells were unable to respond to cRGDFIV and switch RhoGTPase activity (Fig. 6J, Fig. S3C). This is consistent with the inability of RCP knockdown cells to extend pseudopodial extensions and invade FN-rich 3D-matrix (Rainero et al., 2012; Caswell et al., 2008), and indicated a requirement for RCP-dependent trafficking in the RhoGTPase switch.

Because the activities of Rac and RhoA are reciprocally related (Guilluy et al., 2011), we tested whether suppression of Rac activity alone influenced RhoA. Treatment with a small molecule inhibitor of Rac, NSC-23766, promoted an increase in activity of RhoA at the front of cells moving on CDM (Fig. 6K), indicating that suppressing Rac activity is sufficient to permit RhoA activation at the cell front.
The RacGAP1-IQGAP1 complex suppresses Rac activity and promotes activation of RhoA

Biochemical experiments revealed that FLAG-RacGAP1 immunoprecipitated from A2780 cells could directly promote GTPase activity of recombinant Rac, but not Ras (Fig. S3D). Furthermore, whilst RacGAP1 knockdown had little influence on the activities of Rac and RhoA in cells migrating on CDM under basal conditions, RacGAP1 knockdown cells were unable to switch RhoGTPase activity when treated with cRGDFV (Fig. 7A). Similarly, IQGAP1 knockdown had relatively little influence on RhoGTPase activity under basal conditions, but prevented inactivation of Rac and activation of RhoA at the cell front when cells were stimulated with cRGDFV (Fig. 7B).

As RacGAP1-IQGAP1 association is regulated by phosphorylation on T249, we sought to determine the requirement for RacGAP1 phosphorylation in the RhoGTPase switch. FLAG-RacGAP1<sup>249A</sup> was unable to support the suppression of Rac activity and activation of RhoA at the cell front in cells stimulated with cRGDFV (Fig. 7C, D). Conversely, expression of FLAG-RacGAP1<sup>249D</sup>, which is recruited to IQGAP1 and the cell front under basal conditions, was sufficient to suppress Rac and promote RhoA activity in cells migrating on CDM (Fig. 7C, D). Taken together, these data suggest that the formation of the RacGAP1-IQGAP1 complex drives the localised suppression of Rac activity, and concomitant activation of RhoA, as cells migrate within FN-rich matrices.

Suppression of Rac drives invasion into FN-rich ECM

Given that the RacGAP1-IQGAP1 complex suppressed Rac and activated RhoA at the front of invasive cells, we determined the requirement for these RhoGTPases in 3D-migration within FN-rich ECM. Rac1 knockdown had little influence on the extension of invasive pseudopods, or speed and persistence of migration on CDM, in the presence of cRGDFV (Fig. 8A, Fig. S4A, E-G), indicating that RCP/α5β1-driven pseudopodial migration is Rac-independent. However, the migration of Rac1
knockdown cells under basal conditions was characterised by extension of long invasive pseudopods in the direction of migration, and this effect was reversed by expression of an siRNA-resistant GFP-Rac1 (Fig. 8A, B, Fig. S4G). Similar results were obtained using a chemical inhibitor of Rac activation (Fig. S3I, J, K). Furthermore, whilst invasion of cRGDfV-treated or mutant p53-expressing cells into FN-rich collagen plugs was largely unaffected by knockdown of Rac1, Rac1 depletion promoted a striking increase in invasion in the absence of these RCP/α5β1-promoting factors (Fig. 8E, F, G). These data indicate that Rac1 activation is not required for α5β1-driven invasive migration, and that suppression of Rac1 levels, or Rac1 inactivation, are sufficient to drive pseudopodial invasion into FN-rich ECM.

**RhoA is required for α5β1-driven invasion**

RhoA-depleted cells were unable to extend and maintain long pseudopodial projections in the presence of cRGDfV (Fig. 8A), and showed reduced speed and persistence migration in CDMs regardless of stimulation, and this was rescued by expression of siRNA-resistant GFP-RhoA (Fig. 8C, D, Fig. S3E, F). Furthermore, RhoA knockdown abrogated cRGDfV- or mutant p53-stimulated invasion into FN-rich collagen (Fig. 8E-G). These data demonstrate that RhoA is required for RCP/α5β1-driven invasion.

**RCP-dependent α5β1 trafficking promotes formation of F-actin rich spikes and cell elongation in FN-rich collagen gels**

The migration of cells with an elongated morphology in 3D has been categorised as ‘mesenchymal’, dependent on Rac/Cdc42, and distinct from ‘amoeboid’ (RhoA-driven) migration (Friedl and Alexander, 2011; Sahai, 2007). However, here we have described movement of elongated cells within 3D matrix that is independent of Rac, but dependent on RhoA. We therefore characterised the morphology and actin dynamics of cells migrating with RCP/α5β1-driven pseudopodia.
Mesenchymally migrating HT1080 cells (Wolf et al., 2003), display a morphology on CDM that is consistent with high Rac activity, with numerous wave-like dynamic protrusions, resembling small lamellipodia (Fig. 9A). A2780 cells migrating in 3D under basal conditions also display wave-like protrusions at the leading edge, consistent with high Rac activity, but these protrusions appear less dynamic and smaller than those observed in HT1080 cells (Fig. 9A). Strikingly, cRGDFV treatment drastically changed the morphology of cells, triggering the formation of numerous short, dense and linear filamentous actin spikes that appear at the cell front and precede forward movement (Fig. 9A, B).

In CDM, RCP-dependent α5β1 trafficking promotes elongation and extension of pseudopodial processes in the direction of migration (Fig. 1A, Caswell et al., 2008; Rainero et al., 2012). In FN-rich collagen gels, under basal conditions, the morphology of A2780 cells was restricted by the dense fibrillar network and cells remained relatively rounded and displayed short projections (Fig. 9C). In the presence of cRGDFV, the morphology of cells changed drastically, and cells became less spherical and more elongated, with long, thick actin-rich protrusions formed, reminiscent of pseudopods observed in CDM (Fig. 9C-E).

Taken together, these data suggest that the RCP/α5β1 pathway promotes a mode of elongated migration that is distinct from previously classified ‘mesenchymal’ and ‘amoeboid’ migration in 3D matrix, and is characterised by the extension of long pseudopodial processes tipped by linear arrays of actin spikes.
Discussion

Here we determine the mechanism through which RCP-driven α5β1 recycling promotes invasive migration into FN-rich ECM. PKB/Akt is locally activated within the pseudopod tip as a consequence of RCP/α5β1 trafficking, and phosphorylates RacGAP1, a Rac- and Cdc42-specific GAP, on T249. This promotes recruitment of RacGAP1 to IQGAP1 within the tips of invasive pseudopods as cells migrate in 3D, and provides the platform for local suppression of Rac1 activity, and activation of RhoA, to drive protrusion and invasion in FN-rich 3D-matrix (Fig. 10).

Whilst IQGAP1 can both positively and negatively regulate small GTPase activity, it possesses no intrinsic GAP or GEF activity. IQGAP1 can bind directly to active RhoGTPases, e.g. Cdc42, prolonging their activity (Ho et al., 1999, Brown and Sacks, 2006). However, in spreading fibroblasts IQGAP1 is recruited to sites of integrin activation, and recruits RacGAP1 in order to suppress Rac1 (Jacquemet et al. 2013). We now show that the phosphorylation-dependent recruitment of RacGAP1 to IQGAP1 at the front of invading cells suppresses Rac1 activity (Fig. 6 and 7), and is central to the mechanism through which RCP/α5β1 controls cancer cell invasion. A large body of evidence has suggested that IQGAP1 plays a role in tumourigenesis and invasive migration (Jameson et al., 2013; Johnson et al., 2009; Mataraza et al., 2003). Consistent with this, we found that IQGAP1 knockdown inhibits invasive migration (Fig. 5A, B, Fig. S2D-F). RacGAP1 expression is also associated with tumourigenesis (Lu et al., 2004; Wang et al., 2011), and we provide evidence here that RacGAP1 requires IQGAP1 to facilitate invasive migration (Fig. 5C-E). Further studies will reveal whether cooperation and simultaneous upregulation of RacGAP1 and IQGAP1 is a feature of human cancers, in particular those that express mutant p53 or soluble αvβ3 ligands such as osteopontin.

We have shown a novel function of RacGAP1 in invasive migration. The PKB/Akt phosphorylation site within RacGAP1, T249, is not within any recognisable structural region (Fig. S1E), and is therefore unlikely to directly influence GAP activity as reported for Aurora kinase.
(Minoshima et al., 2003). Furthermore, T249 is outside of the regions known to interact with MKLP1, Ect2 or Rab11-FIP3 (Simon et al., 2008; Yüce et al., 2005; Mishima et al., 2002), and phosphorylation at T249 did not influence binding of MKLP1 or Ect2 (Fig. 3C) or the rate of proliferation of cancer cells (Fig. S1K). These observations suggest that PKB/Akt-phosphorylation does not regulate cytokinesis, and instead plays a role in the recruitment of RacGAP1 to IQGAP1 at the cell periphery during invasive migration.

Rac and RhoA control different modes of migration, and plasticity exists within migratory systems to allow switching between modes (Friedl and Alexander, 2011; Deakin and Turner, 2011; Sanz-Moreno et al., 2008; Sahai, 2007). Mesenchymal migration is thought to be adhesion-dependent, and amoeboid migration less so (Friedl and Alexander, 2011), although amoeboid tumour cells can form adhesions (Deakin and Turner, 2011; Poincloux et al., 2011). More recently, ‘lobopodial’ migration has been described in matrices exhibiting linear elasticity, including CDMs and dermal explants, and this requires integrin adhesion formation and RhoA-ROCK (Petrie et al., 2012). Our evidence indicates that RCP/α5β1-driven invasive migration is distinct from previously described modes of migration, firstly in the requirement for the ECM component FN, which is important in metastatic progression (Ghajar et al., 2013; Psaila and Lyden, 2009; Reticker-Flynn et al., 2012), but not present in many in vitro invasion assays. We demonstrate that RCP/α5β1-driven invasive migration is further distinguished by morphology and RhoGTPase requirement: it is characterised by extension of long pseudopodial protrusions driven by RhoA (Fig. 8A-C), and antagonised by Rac1 (Fig. 8A-C). Furthermore, RCP/α5β1 induces the formation of short linear arrays of actin spikes at the leading edge, which appear to drive protrusion in 3D-matrix (Fig. 9A), rather than wave-like lammellipodia. We have noted that ROCK activity is dispensable for protrusion, and is only required for retraction of the cell rear (unpublished data), leading us to speculate that cytoskeletal reorganisation could be driven by other classes of RhoA effectors. Formin homologous domain (FHD) proteins are capable of generating spike-like F-actin structures by polymerising actin from the
barbed end, and members of this family play important roles in cancer cell invasion (Brandt et al., 2009; Kitzing et al., 2010; Vega et al., 2011).

RhoGTPases are major downstream effectors of integrins, and are required for many of the myriad functions of this family of adhesion receptors, in particular those relating to cell migration (Ridley et al., 2003). In fibroblasts, αvβ3 (and αvβ3 recycling) promotes persistent lamellipodial migration in 2D through Rac, whilst α5β1 (and α5β1 recycling) signals via RhoA/ROCK to favour rapid, random migration (White et al., 2007; Morgan et al., 2013; Danen et al., 2005). The reciprocity of these phenotypes is reflected in the plasticity of carcinoma cells as they invade: αvβ3 recycling promotes invasion into 3D-matrix in the absence of FN, whilst α5β1 recycling promotes invasion into FN-rich ECM (Christoforides et al., 2012; Jacquemet et al., 2013; Caswell et al., 2008; Muller et al., 2009; Rainero et al., 2012). DGKα-dependent production of PA recruits RCP to the tips of invasive pseudopods via a C2 domain, and restricts the localisation of α5β1 recycling vesicles (Rainero et al., 2012). Thus the RCP/α5β1 pathway encodes spatial information, which is transduced via PKB/Akt to the RacGAP1-IQGAP1 complex, to locally suppress Rac1 activity and activate RhoA at the cell front. Whilst RacGAP1 can directly inactivate Rac1, the mechanism through which RhoA is activated remains unclear. Chemical inhibition of Rac is sufficient to promote RhoA activity at the cell front (Fig. 6K), consistent with the findings of others (Guilluy et al., 2011), and also to promote pseudopod extension (Fig. S4I). This suggests that suppression of Rac is a key event that promotes RhoA activity and initiates pseudopod extension. Although RacGAP1 phosphorylation does not influence binding of RacGAP1 to the RhoA GEF Ect2 (Fig. 3C), it is possible that RacGAP1 localises the activity of this RhoA GEF in migrating cells as is the case at adherens junctions in epithelial cells (Ratheesh et al., 2012).

Our findings indicate that RCP regulates α5β1 trafficking to sustain localised signalling to RhoGTPases. Phosphorylation of RacGAP1 by PKB/Akt, as a consequence of integrin-mediated EGFR1 trafficking and signalling, promotes recruitment to the front of invading cells via IQGAP1. This
leads to suppression of Rac activity, which is sufficient to promote pseudopod extension and invasion by permitting activation of RhoA. FN is key to RCP/α5β1-driven invasion, indicating that the extracellular environment is a critical determinant of the mode of migration and RhoGTPase requirement of cells migrating in 3D, highlighting the importance of both intrinsic and extrinsic factors in determining the metastatic dissemination of tumour cells.
Materials and Methods

Cell culture and transient transfection

A2780 cells were cultured in RPMI and H1299, MDA-MB-231 and HT1080 cells in DMEM (Sigma) supplemented with 10% FCS, and grown at 37°C and 5%CO₂. Transient transfections and knockdowns were performed using the Amaza nucleofector (A2780s, Solution T, programme A-23; 3µg plasmid DNA or 1µM siRNA), Lipofectamine 2000 for siRNA transfection (MDA-MB-231, H1299, IQGAP1 siRNA; 100nM siRNA, 2 rounds of transfection) or Lipofectamine LTX (for A2780 and HT1080 meGFP-Lifeact transfections) according to manufacturer’s instructions. CDMs were prepared as described previously (Caswell et al., 2008; Cukierman et al., 2001). In brief, tissue culture plates were gelatin-coated, crosslinked with glutaraldehyde, quenched, and equilibrated in DMEM containing 10% FCS. Human dermal fibroblasts were seeded at near confluence and grown for 8-10 days in DMEM containing 10% FCS and 50 µg/ml ascorbic acid. Matrices were denuded of living cells by incubation with PBS containing 20mM NH₄OH and 0.5% Triton X-100, and DNA residue was removed by incubation with DNase I.

Plasmids and reagents

RNAi oligonucleotides were purchased from Dharmacon as follows: ON-TARGETplus non-targeting siRNA (single oligo or pool as appropriate); IQGAP1#1 (GAACGUGGCUUAUGAGUAC); IQGAP1#2 (J-004694-08); RacGAP1 (SMARTpool, oligo 6: GCGAAGUGCUCUGGAUGUU, oligo 8: GAAGUCACAUCUGCCUGUU); Rac1 (SMARTPool or Rac1#1: CGGCACCACUGUCCAACA); RhoA (SMARTpool or RhoA#1 AUGGAAAGCAGGUAGAGUU); RCP (J-015968-10). shRNA vectors for PKB/Akt isoforms were prepared using mU6Pro and the following sequences: Akt1 #1 (GCTACTTCTCCTCAAGAA), Akt1#2 (CGAGTTTGAGTACCTGAAG), Akt2#1 (CGTGGTGAATACATCAAGA), Akt2#2 (TCTGTCATCAAAGAAGGCT), Mammalian expression vectors encoding siRNA resistant GFP-Rac1 (pEGFP-C2, full length, resistant to Rac1#1) and GFP-RhoA (pcDNA-DEST53, full length, resistant
to RhoA#1) were kind gifts from Anne Ridley (Vega et al., 2011; Reymond et al., 2012). Akind, Raichu-Rac (Raichu-1011X) and Raichu-RhoA (Raichu-1237X) in the pCAGGS backbone were kind gifts from M. Matsuda (Yoshizaki et al., 2003, 2007; Itoh et al., 2002). Bacterial expression vector pMAL encoding MBP-RacGAP1 was a kind gift from T. Kitamura. A codon optimised RacGAP1-FLAG was synthesised by Genscript, and cloned into the lentiviral vector pWPLX using BamHI and EcoRI (Addgene). Point mutations were introduced using Quickchange Lightning site-directed mutagenesis (Agilent Technologies); for MBP-RacGAP1 the following primers were used: T249A 5’-gaccaggagcgaaggaacaggtctttacaac-3’, T251A 5’-gccaaggaacaggtctttacaac-3’. For codon-optimized RacGAP1-FLAG the following primers were used: T249A 5’-agccggcggaagggaaagcactctgc-3’, T249D 5’-cgagccggcggaagggaaagcactctgcagcc-3’. Rac inhibitor NSC23766 was purchased from Millipore and used at a concentration of 25μM. cRGDfV was purchased from BACHEM and added directly to the culture medium at a concentration of 2.5μM. The broad specificity kinase inhibitor staurosporine was purchased from Calbiochem and used at a concentration of 10μM.

Rabbit anti-IQGAP1 (H-109), rabbit anti-RacGAP1 (B-7), rabbit anti-MKLP1 (N-19), rabbit anti-RhoA (119) and mouse anti-PKBβ/Akt2 (F-7) were purchased from Santa Cruz Biotechnology. Mouse anti-RacGAP1 (1G6) and mouse anti-FLAG (M2), mouse anti-α-tubulin and mouse anti-β-actin were purchased from Sigma-Aldrich. Mouse anti-Rac1 (23A8) was purchased from Millipore. Rabbit anti-RxRxxS*/T* (110B7E) and rabbit anti-PKBα/Akt1 (C73H10) were purchased from Cell Signaling Technology. Rabbit anti-Ect2 (raised against Ect2 aa 1-421) was a kind gift from M. Petronczki (Su et al., 2011).

**in vitro kinase assay**

MBP-RacGAP1 and mutants were produced in BL-21 strain E.coli and purified using Amylose resin (NEB). MBP-RacGAP1 (18μg) was incubated with recombinant active PKBβ/Akt2 (δPH/S474D; 0.5μg 0.155 units; Millipore) and 10mM ATP (including 10μCi γ^32-P ATP) in reaction buffer (100mM
Tris pH7.5, 10mM MgCl₂, 30mM β-mercaptoethanol, 20% glycerol). Reactions were terminated by addition of reducing sample buffer.

**Immunoprecipitation**

For IPs, A2780 cells were serum starved overnight, stimulated with cRGDfV for 30mins and EGF (30ng/ml) for 5mins before lysis in the appropriate buffer.

For PKB/Akt substrate IPs, A2780 cells were lysed in lysis buffer (200mM NaCl, 75mM Tris-HCl pH 7, 15mM NaF, 1.5mM Na₃VO₄, 7.5mM EDTA, 7.5mM EGTA, 1.5% (v/v) Triton X-100, 0.75% (v/v) NP-40, 50μg/ml leupeptin, 50μg/ml aprotinin, and 1mM AEBSF. Lysates were passed three times through a 27-gauge needle and clarified by centrifugation at 10,000xg for 10 min at 4 °C. Magnetic beads conjugated to sheep anti-rabbit IgG (Invitrogen) were bound to anti-RXRXXS*/T*. Antibody-coated beads were incubated with lysates for 2hrs at 4°C with constant rotation. Unbound proteins were removed by extensive washing in lysis buffer, and specifically associated proteins were eluted from the beads by boiling for 10 min in Laemmli sample buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blotting or LC-MS/MS.

For co-IPs, A2780 cells were lysed in CSK buffer (10mM Pipes pH 6.8, 150mM NaCl, 150mM sucrose, 3mM MgCl₂, 0.5% (v/v) Triton X-100, 10μg/ml leupeptin, 10μg/ml aprotinin, 0.5mM AEBSF, 2mM Na₃VO₄). Lysates were clarified (centrifugation at 10,000xg for 10 min at 4 °C) and incubated with rabbit anti-IQGAP1 antibody-coated sheep anti-rabbit magnetic beads (Invitrogen) or mouse anti-FLAG antibody-coated sheep anti-mouse magnetic beads (Invitrogen) for 1-2hrs at 4°C. Complexes bound to the beads were isolated using magnets, washed three times with ice-cold lysis buffer and eluted in Laemmli reducing sample buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blotting.
**Lentiviral production and transduction**

Lentiviruses were produced by transfecting 293T cells with three plasmids (psPAX2, pMD2.G and pWPLX). Conditioned medium containing viruses was collected after 5 days, then use immediately to infect cells or stored at -80°C. Infection rate in A2780 and MDA-MB-231 cells was close to 100%, and stable transfectants retained expression of RacGAP1 and mutants over several months.

**SDS-PAGE and quantitative western blotting**

Protein extracts were separated under denaturing conditions by SDS-PAGE (4–12% Bis-Tris gels; Invitrogen) and transferred to nitrocellulose membrane. Membranes were blocked and incubated overnight at 4°C with the appropriate primary antibody, and then at room temperature for 1 hours with the appropriate fluorophore-conjugated secondary antibody. Membranes were scanned using an Odyssey infrared imaging system (LI-COR Biosciences). Band intensity was determined by digital densitometric analysis using Odyssey software (version 2.1; LI-COR Biosciences). Blots shown are representative of at least 3 independent experiments.

**Mass spectrometric data acquisition and analysis**

MBP-RacGAP1 and RxRxxS/T pull-downs samples were analysed by liquid chromatography–tandem mass spectrometry using a nanoACQUITY UltraPerformance liquid chromatography system (Waters) coupled online to an LTQ Velos (Thermo Fisher Scientific). Peptides were separated on a bridged ethyl hybrid C18 analytical column (75mm × 250μm, 1.7μm particle size; Waters) using a 45-min linear gradient from 1% to 25% (vol/vol) acetonitrile in 0.1% (vol/vol) formic acid at a flow rate of 200nl/min. Peptides were selected for fragmentation automatically by data-dependent analysis. Tandem mass spectra were extracted using extract_msn (Thermo Fisher Scientific) executed in Mascot Daemon (version 2.2.2; Matrix Science). Peak list files were searched against the IPI Human database (version 3.70, release date 4th March 2010) modified to contain ten additional
contaminants and reagent sequences of non-human origin. Searches were submitted to an in-house Mascot server (version 2.2.03; Matrix Science; Perkins et al., 1999). Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine and phosphorylation of serines, threonines and tyrosines were set as variable modifications. Only tryptic peptides were considered, with up to one missed cleavage permitted. Monoisotopic precursor mass values were used, and only doubly and triply charged precursor ions were considered. Mass tolerances for precursor and fragment ions were 5 ppm and 0.5 Da respectively. Data were further analysed using the search engine X! Tandem (version 2007.01.01.1; Craig and Beavis, 2003) implemented from within Scaffold (version 3.00.03; Proteome Software). Data were validated in Scaffold using a threshold of identification of at least 90% probability at the peptide level, at least 99% probability at the protein level and assignment of at least two unique, validated peptides. Identified proteins were hierarchically clustered on the basis of uncentred Pearson’s correlation using Cluster 3.0 (C Clustering Library, version 1.50; De Hoon et al., 2004) and visualised using Java TreeView (version 1.1.6r2; Saldanha, 2004).

Microscopy

For immunofluorescence imaging, cells were plated onto CDM for >4 hours, treated with or without cRGDfV, and fixed in 4% paraformaldehyde. After permeabilisation and blocking, cells were stained using primary (rabbit anti-RacGAP1, rabbit anti-iIQGAP1 or mouse anti-FLAG) and Cy2 or Cy3 conjugated secondary antibodies as indicated. Images were captured on a spinning disk confocal inverted microscope (Marianis, 3i) using a 63x objective (Plan-Apochromat NA 1.46), and Slidebook 5.0 software (3i, Denver). Linear adjustments to brightness and contrast made using ImageJ (adjustments were equivalent for all channels between comparable images).

For long-term timelapse, A2780 cells were plated onto CDM in normal culture medium for >4 hours and imaged in the presence or absence of cRGDFV after >4 hours in a 37°C 5% CO₂
atmosphere. Phase contrast images were captured on an inverted AS-MDW microscope system (Leica) using a 20x objective (HC Plan Fluotar Ph2 NA 0.50) every 10mins using a Coolsnap HQ CCD camera and Image Pro 6.3 software (Media Cybernetics). Images and movies were analysed using ImageJ (manual tracking plugin to determine speed/persistence).

For live-cell imaging of actin dynamics during invasive migration, A2780 and HT1080 cells transiently transfected with Life-act-GFP were plated on CDM coated glass-bottom dishes for 4 h. For optimal image resolution, normal culture medium was replaced by Ham’s F-12 (Gibco) containing 25 mM HEPES in presence of 10% (vol/vol) FCS, prior to addition of cRGDfV (>1 hour) where appropriate image and subsequent acquisition. Images were collected every 3 min at 37°C on a spinning disk confocal inverted microscope (Marianis, 3i) using a 100x objective (Plan-Apochromat NA 1.4) and Slidebook 5.0 software (3i, Denver). For each protrusive event, the average integrated density of life-act GFP at protrusion sites was normalized to the average integrated density of life-act GFP in the whole cell.

For imaging of cells within FN-rich collagen gels, A2780 cells were embedded in the collagen gel and plated on glass-bottom dishes for 30 min. Prior to fixation, cells were allowed to invade in the presence or absence of cRGDfV for 24h. Cells were stained using TRITC-phalloidin (Invitrogen), imaged using a Leica TCS SP5 AOBS inverted confocal and a 40x objective (HCX PL Apo NA 1.25) with 1.7x confocal zoom using LCS software (Leica). Z sections were acquired every 0.4µm. Maximum projections were produced using imageJ and 3D reconstructions using the Imaris software (Bitplane Scientific Software). 2D shape analyses were performed using the imageJ particle analyses plugin using the maximum projection images, and the 3D shape analyses were performed using the imageJ plugin 3D Shape.
**FLIM**

Fluorescence lifetime imaging (FLIM) is a well-established method for calculation of FRET efficiency, and because it involves measurement of the fluorescence lifetime of the donor molecule in FRET, it avoids many of the caveats associated with intensity-based FRET measurements including artefacts introduced by concentration of probes and donor bleed-through (Becker, 2012). We used frequency domain FLIM to calculate FRET efficiency, based on the polar plot method (Redford and Clegg, 2005). This method allows calculation of the nearest single lifetime for multi-exponential donors such as CFP. For analysis of PKB/Akt, Rac and RhoA activities, A2780 cells were transiently transfected with Raichu probes (Akind-1711, Akind 1714 (Akind-3A), Raichu-1011X (Rac1), Raichu 1012X (Rac1 G12V), Raichu 1013X (Rac1 T17N), Raichu-1237X, Raichu 1238X (RhoAQ63L), Raichu-1239X (RhoA S19N)), and seeded onto CDM 20 hours later. For optimal image resolution, normal culture medium was replaced by Ham’s F-12 (Gibco) containing 25 mM HEPES in presence of 10% FCS. After >4 hours on CDM, cells were treated with cRGDFV for a further 1-2 hours where necessary, and fluorescence lifetime imaging microscopy (FLIM) performed using a Marianis system equipped with Lambert instruments FLIM module (3i, Denver) and a 63x objective (Plan-Apochromat NA 1.46). Image capture and analysis was performed using Slidebook (3i, Denver). FRET efficiency ($E$) was calculated using the formula $E=100x\left(1-\frac{\tau_{DA}}{\tau_0}\right)$; where $\tau_0$ represents the lifetime of the donor (CFP), and $\tau_{DA}$ represents the lifetime of the donor in the presence of the acceptor (CFP/YFP probe).

**Inverted invasion assays**

Inverted invasion assays were modified from those described previously (Hennigan et al., 1994). In brief, Collagen I (BD, final concentration approx. 5μg/ml) supplemented with 25μg/ml FN as indicated was allowed to polymerize in transwell inserts (Corning) for 1 h at 37 °C. Inserts were then inverted and cells were seeded directly onto the opposite face of the filter. Transwell inserts were finally placed in 0.1% serum medium, and medium supplemented with 10% FCS and 30ng/ml EGF was placed on top of the matrix, providing a chemotactic gradient. Where appropriate, 2.5μM
cRGDFV was added to the matrix before plug polymerization and also to the medium throughout the system. 48-72h after seeding, migrating cells were visualised with Calcein-AM and visualized by confocal microscopy with serial optical sections being captured at 15μm intervals using a Leica SP2 confocal microscope and a 20x objective. Individual confocal images are presented in sequence with increasing penetrance from left to right. Invasion was quantified using the area calculator plugin in ImageJ, measuring the fluorescence intensity of cells invading 45μm or more, and expressing this as a percentage of the fluorescence intensity of all cells within the plug.

**Proximity ligation assay**

Proximity ligation assays were performed according to the manufacturer's instructions (Duolink) using the Duolink In Situ PLA Probes Anti-Rabbit PLUS and Anti-Mouse MINUS, and the Duolink In Situ Detection Reagents Red (Sigma). Cells were counterstained using FITC-phalloidin (Invitrogen), imaged using a spinning disk confocal microscope (Marianis, 3i) using a 100x objective, and images analysed using ImageJ.

**GAP Assay**

GAPs assays were performed using a RhoGAP assay kit (Cytoskeleton) in a similar manner to Bastos et al. (2012). Briefly, two 15cm plates (A2780-GFP or A2780-FLAG-RacGAP1WT) were lysed per GAP assay condition, and immunoprecipitated with anti-FLAG antibodies. Immunoprecipitates were washed copiously, and with final washes in TBS. GTP was added to mixtures of immunoprecipitate and GTPase for 30 mins at 37C, before incubation with Cytophos reagent and measurement of optical density at 650nm.

**Proliferation Assay**

Cells were seeded at 5000 cells per well in 96-well plates. Cell number was assayed at varying timepoints over 7 days using CellTitre 96 kit (Promega). Briefly, cells were seeded into 96 well plates
and allowed to proliferate for up to 7 days. Dye solution was added to each well and the plate returned to 37°C for 4h before addition of stop solution. The plate was incubated at room temperature overnight, and absorbance read at 570nm. Cell number was normalised to the number of cells (absorbance at 570nm) 16 hours post-seeding (day 0).

**Statistical analysis**

Statistical analyses were performed as appropriate, and p values are indicated by an asterisk in the figure legends. Z-tests were performed where n>30, in all other cases Student’s t-test was used (unpaired, two-tailed, unequal variance).

**Online supplementary material**

Fig. S1 shows FRET efficiencies of active/inactive versions of probes used in this study, extent of knockdown of PKB/Akt isoforms and effect on invasion, extent of knockdown of RacGAP1 in cell lines used and effects on migration/pseudopod extension on CDM and invasion, stable expression of FLAG-RacGAP1 and mutants, a schematic of RacGAP1, and that stable RacGAP1/mutant cell lines proliferate normally. Fig. S2 shows extent of knockdown of IQGAP1 in cell lines used and effects on migration on CDM and invasion, effect of RacGAP1^{249A} on invasion in MDA-MB-231 cells and the phosphorylation of RacGAP WT vs 249A. Fig. S3 shows the localisations of Raichu probes and endogenous Rac1 or GFP-RhoA, regions of interest used to analyse fluorescence lifetime, the extent of knockdown of RCP, and in vitro GAP assays. Fig. S4 shows the extent of knockdown of Rac1 and RhoA in the cell lines used and effects on migration/pseudopod extension on CDM, rescue of Rac1 and RhoA knockdown using siRNA-resistant GFP-Rac1 and –RhoA and the effect of a Rac inhibitor on pseudopod length. Video 1 shows FLAG-RacGAP1^{WT} expressing cells migrating in the presence and absence of cRGDFV, the inhibition of pseudopod extension by expression of FLAG-RacGAP1^{249A}, and the adoption of pseudopodial migration by cells expressing FLAG-RacGAP1^{249O}. Videos 2-5 show the activities of Raichu-Rac and -RhoA in cells +/- cRGDFV. Video 6 shows the effect of Rac and RhoA
knockdown on migration on CDM. Video 7 shows the dynamics of mEGFP-Lifect in HT1080 cells and A2780 cells +/-cRGDFV migrating through CDM.
Acknowledgements

We thank Prof. Michiyuki Matsuda (Kyoto University, Japan), Prof. Toshio Kitamura (Tokyo University), Dr. Mark Petronczki (LRI, U.K.) and Prof. Anne Ridley (Kings College, U.K.) for their kind gifts of reagents. Bioimaging and mass spectrometry was performed using facilities within the Faculty of Life Sciences, University of Manchester and Beatson Institute for Cancer Research; we thank Kurt Anderson, Juliana Schwarz and Peter March for help with the microscopy, Ben Atkinson and Glenn Redford (3i, Denver, CO) for help with FLIM, and Stacey Warwood and David Knight for help with mass spectrometry. The work of our laboratories is supported by the Wellcome Trust (P.T.C, grant 090453; M.J.H, grant 092015) and Cancer Research UK (J.C.N). GJ was supported by a Wellcome Trust studentship.
**Abbreviations List**

CDM: Cell-derived matrix

cRGDFV: Cyclo(-Arg-Gly-Asp-D-Phe-Val)

EGFR: EGF receptor

FHD-protein: Formin homologous domain protein

FMNL: Formin like

FN: Fibronectin

GAP: GTPase activating protein

GEF: Guanine nucleotide exchange factor

IQGAP1: IQ motif-containing GTPase activating protein 1

MKLP1: Mitotic kinesin-like protein 1

PKB: Protein kinase B

Rab11-FIP: Rab11-family interacting protein

RCP: Rab-coupling protein

RTK: Receptor tyrosine kinase
References


Shattil, S.J., C. Kim, and M.H. Ginsberg. 2010. The final steps of integrin activation: the end game. *Nature reviews. Molecular cell biology*. 11:288–300.


Figure Legends

**Figure 1: RacGAP1 is a PKB/Akt substrate required for pseudopod extension and invasion**

(A) A2780 cells expressing Akind on CDM were stimulated with 2.5μM cRGDFV as indicated. Fluorescence lifetime images were captured, and representative lifetime maps are shown. FRET efficiency was calculated for regions of interest (dotted ROIs). Scale bar=10μm, (n>18/condition).

(B) PKB/Akt substrates were immunoprecipitated (IP) using anti-RxRxS*/T* from lysates of EGF-stimulated cells treated with cRGDFV as indicated. IPs were separated by SDS-PAGE and analysed by MS/MS. Hierarchical clustering of identified proteins is shown, with increasing abundance indicated by intensity. RacGAP1 is indicated with an asterisk.

(C) Purified MBP-RacGAP1 was incubated in vitro phosphorylation reactions as indicated. Proteins were separated by SDS-PAGE, protein loading confirmed by Coomassie staining and incorporation of radioactive ATP was measured by phosphorimager.

(D) Lysates of EGF-stimulated A2780 cells treated with cRGDFV and staurosporine as indicated were subjected to IP using anti-RxRxS*/T* as in (B) or an isotype-matched control. IPs were analysed by SDS-PAGE and western blotting for RacGAP1, and quantified using the Odyssey system.

Data represent mean ± SEM from ≥3 independent experiments, * p<0.05, **p<0.001

**Figure 2: RacGAP1 is required for pseudopod extension and invasion**

(A) A2780 cells were transfected with control or RacGAP1-specific SMARTpool oligonucleotides, seeded onto CDMs, and stimulated with cRGDFV as indicated. Images were captured every 10mins using a 20x objective. Representative images are shown, scale bar=50μm. Pseudopod length (B, n>400/condition) was measured for all moving cells within the 20th frame.

(C) A2780 cells were transfected as in (A), and seeded into inverted invasion assays after 16 hours in the presence or absence of FN and cRGDFV as indicated.
(D) A2780 cells stably expressing GFP or FLAG-RacGAP\textsuperscript{WT} were transfected with control or RacGAP RNAi oligo\#6, treated as in (C), seeded into inverted invasion assays in the presence of cRGDfV and FN.

(E) MDA-MB-231 cells were transfected as in (A) and seeded into inverted invasion assays in the presence of FN.

(F) H1299 cells stably expressing mutant p53 (273H) or control vector (VEC) were transfected as in (A) and seeded into inverted invasion assays in the presence of FN.

Data represents mean ± SEM from ≥3 independent experiments, * p<0.05, **p<0.01, *** p<0.001.

Figure 3: RacGAP1 phosphorylation on T249 promotes association with IQGAP1

(A) Purified MBP-RacGAP1 and mutants were subjected to in vitro phosphorylation and incorporation of \textsuperscript{32}P measured as in 1C.

(B) PKB/Akt substrates were IPd from lysates of A2780 cells stably expressing GFP, FLAG-RacGAP1 or FLAG-RacGAP1\textsuperscript{249A} as in 1D.

(C) Lysates of A2780 cells stably expressing GFP(-), FLAG-RacGAP1, FLAG-RacGAP1\textsuperscript{249A} or FLAG-RacGAP1\textsuperscript{249D} were subjected to IP using FLAG antibodies. IPs were analysed by SDS-PAGE and western blotting for FLAG, Ect2 and MKLP1.

(D) Lysates of A2780 cells were subjected to IP using rabbit IQGAP1 antibodies or an isotype-matched control. IPs were analysed by SDS-PAGE and western blotting for RacGAP1 and IQGAP1.

(E) Cells as in (C) were lysed and IP performed using rabbit IQGAP1 antibodies. IPs were analysed by SDS-PAGE and western blotting for FLAG and IQGAP1.

(F) Western blots from IPs as in (E) were quantified using the Odyssey system.

(G) Cells stably expressing GFP, FLAG-RacGAP1\textsuperscript{WT} or FLAG-RacGAP1\textsuperscript{249A} on CDM were treated with cRGDfV for 1 hour, and fixed and stained with antibodies against FLAG and IQGAP1 before
performing PLA. PLA signal was quantified by measuring the integrated density within the whole cell using ImageJ (n>60/condition), scale bar=20μm.

Data represent mean ± SEM from ≥3 independent experiments, * p<0.05, ** p<0.01, ***p<0.001.

**Figure 4: IQGAP1 recruits RacGAP1 to the tips of invasive pseudopods as cells migrate in 3D**

(A, B) A2780 cells were subjected to control or IQGAP1 #1 RNAi and seeded onto CDMs. Cells were stimulated with cRGDFV as indicated for 2 hours before fixing and staining with rabbit anti-RacGAP1/anti-rabbit Cy2 antibodies and Phalloidin-Texas Red.

(C) A2780 cells stably expressing GFP, FLAGRacGAP1<sup>wt</sup>, <sup>-249A</sup> or <sup>-249D</sup> on CDMs were stimulated with cRGDFV as indicated and fixed and stained with rabbit anti-IQGAP1/anti-rabbit Cy2 and mouse anti-FLAG/anti-mouse Cy3 antibodies. Images were captured using a spinning disk confocal microscope, representative pseudocoloured images are shown. Scale bar=10μm, yellow arrow indicates direction of migration.

**Figure 5: The RacGAP1-IQGAP1 complex promotes integrin-dependent invasive migration**

(A) A2780 cells were subjected to control or IQGAP1 #1 RNAi and seeded onto CDMs. Images were captured and pseudopod length determined as in (2A, B, n>100/condition).

(B) A2780 cells were treated as in (A) and seeded into inverted invasion assays in the presence or absence of FN and cRGGfV as indicated.

(C) A2780 cells stably expressing GFP, RacGAP1<sup>wt</sup>, RacGAP1<sup>-249A</sup> or RacGAP1<sup>-249D</sup> were seeded onto CDMs, stimulated with cRGDFV as indicated, and images captured and pseudopod length measured as in (2A, B, n>40/condition).

(D) A2780 cells as in (C) were seeded into inverted invasion assays in the presence of FN and stimulated with cRGDFV as indicated.

(E) A2780 cells stably expressing GFP, RacGAP1<sup>wt</sup>, or RacGAP1<sup>-249D</sup> were transfected as in (A), and seeded into inverted invasion assays in the presence of FN and cRGDFV.
Figure 6: Integrin trafficking suppresses Rac activity and activates RhoA

(A, B) A2780 cells expressing Raichu-Rac were seeded onto CDMs and stimulated with cRGDFV as indicated. Fluorescence lifetime images were captured at 1 minute intervals, and representative lifetime maps are shown.

(C) FRET efficiency was calculated for regions of interest at the cell periphery at the front, middle (average of the two sides) or back, from lifetime maps generated as in (A, B; single images, or averages of all frames from timelapse movies, n>30/condition).

(D) FRET efficiency at the cell front was calculated as in (C) (for each frame of timelapse movies, n>9/condition).

(E, F) A2780 cells expressing Raichu-RhoA were analysed as in (A, B).

(G) FRET efficiency was calculated as in (C, n>35/condition).

(H) FRET efficiency at the cell front was calculated as in (D, n>15/condition).

(I) H1299 cells stably expressing mutant p53 (273H) or control vector (VEC) were transfected with Raichu-Rac or Raichu-RhoA, FLIM performed as in (A, B) and FRET efficiency at the cell front calculated as in (C, n>8/condition).

(J) A2780 cells were transfected with control or RCP-specific siRNA and allowed to recover for 24 hours. Cells were then transfected with Raichu-Rac or Raichu-RhoA, FLIM performed as in (A, B) and FRET efficiency at the cell front was calculated as in (C, n>13/condition).

(K) A2780 cells expressing Raichu-RhoA were seeded onto CDMs, treated with vehicle or the Rac inhibitor NSC-23766 for 2 hours, FLIM performed as in (A,B) and FRET efficiency at the cell front calculated as in (C, Control: n=8; NSC-23766: n=10).

Data represents mean ± SEM from ≥3 independent experiments, * p<0.05, ** p<0.01, *** p<0.001.

Scale bar=10μm.
Figure 7: Integrin trafficking suppresses Rac activity and activates RhoA through the RacGAP1-IQGAP1 complex

(A) A2780 cells were subjected to control or RacGAP1 oligo#6 RNAi and allowed to recover for 24 hours. Cells were then transfected with Raichu-Rac or Raichu-RhoA as indicated, seeded onto CDM, FLIM performed and FRET efficiency at the cell front calculated as in (6A-C, n≥15/condition).

(B) A2780 cells were subjected to control or IQGAP1 oligo#1 RNAi and allowed to recover for 24 hours. Cells were then transfected with Raichu-Rac or Raichu-RhoA as indicated, seeded onto CDM and FLIM performed and FRET efficiency at the cell front calculated as in (6A-C, n≥8/condition).

(C) A2780 cells stably expressing RacGAP1WT, RacGAP1249A or RacGAP1249D were transfected with Raichu-Rac, seeded onto CDMs and FLIM performed and FRET efficiency at the cell front calculated as in (6A-C). Representative images are shown (n≥8/condition).

(D) A2780 cells stably expressing RacGAP1WT, RacGAP1249A or RacGAP1249D were transfected with Raichu-RhoA, seeded onto CDMs and FLIM performed and FRET efficiency at the cell front calculated as in (6A-C). Representative images are shown (n≥4/condition).

Data represents mean ± SEM from ≥3 independent experiments, * indicates p<0.5, ** p<0.01.
Scale bar= 10μm

Figure 8: Integrin trafficking promotes invasive migration through the suppression of Rac activity and activation of RhoA

(A) A2780 cells were subjected to control, Rac1 or RhoA SMARTpool RNAi, and seeded onto CDMs after 24-36 hours. Cells were stimulated with cRGDFV as indicated, and images captured as in 2A.
Representative images are shown, scale bar=50μm.

(B-D) A2780 cells were co-transfected with GFP-Rac1 or GFP-RhoA (as indicated) alongside control, Rac1#1 or RhoA#1 RNAi oligonucleotides, seeded onto CDM as in (A), and images captured as in 2A.
Pseudopod length (B, >30 cells/condition) was measured as in 2A. Speed and persistence (C, D, ≥26 cells/condition) of migration was analysed using ImageJ.

(E) A2780 cells were subjected to control, Rac1 or RhoA SMARTpool RNAi, seeded into inverted invasion assays in the presence of FN and cRGDFV.

(F, G) MDA-MB-231 (F) or H1299-VEC/273H cells were subjected to control, Rac1 or RhoA SMARTpool RNAi and seeded into inverted invasion assays in the presence of FN.

Data represents mean ± SEM from ≥3 independent experiments, * indicates p<0.05, ** p<0.01, *** p<0.001; ns: not significant.

Figure 9: RCP/α5β1 promote formation of actin spikes at the cell front and elongated movement in 3D matrix

(A) HT1080 and A2780 cells expressing Lifeact-mE GFP were plated onto CDM for 4 hours prior to imaging. Actin dynamics were captured as cells move in 3D using a spinning disk confocal microscope. Arrows indicate dynamic protrusions. Scale bar=20 μm.

(B) Normalised actin density at protrusions was calculated by dividing the average integrated density at protrusions by the average integrated density within the whole cell (n>500/condition).

(C) A2780 cells were allowed to invade through a plug of collagen and FN for 24h prior to fixation. Cells were stained for actin and imaged top to bottom using a confocal microscope. The maximum projections were produced using ImageJ and the 3D reconstructions using Imaris. Scale bar=50 μm.

(D) The 2D shape descriptors were calculated from the maximum projections images, using the particle analysis plugin of ImageJ (n>46/condition).

(E) The 3D shape descriptors were calculated from the entire cell volume, using the 3D shape plugin of ImageJ. (n>46).

***p>0.01.
Figure 10: RCP-dependent α5β1 recycling regulates the localisation of RacGAP1 and downstream signalling to RhoGTPases.

(A) α5β1 trafficking is suppressed by αvβ3, or the transcriptional activity of p63, and Rac signalling predominates at the leading edge.

(B) Inhibition of αvβ3, or expression of gain-of-function mutant p53, promotes the association of RCP with α5β1, recruitment of EGFR1 and subsequent recycling. Production of PA by DGKα within the tips of pseudopods recruits RCP/α5β1/EGFR1 vesicles, and localises downstream signalling via PKB/Akt. Here PKB/Akt phosphorylates RacGAP1, allowing its recruitment to IQGAP1, providing a platform for the inactivation of Rac and activation of RhoA to promote pseudopod extension and invasion in FN-rich ECM.
Figure S1

(A) A2780 cells were transfected with Raichu-Rac G12V/T17N, Raichu-RhoA Q63L/T19N, or Akind-3A and seeded onto glass bottom plates after 16-24 hours. Fluorescence lifetime images were captured, and FRET efficiency was calculated for regions of interest around the entire cell periphery from lifetime maps. Data represent mean ± SEM from >3 independent experiments (Raichu-Rac G12V: n=34, Raichu-Rac T17N: n=45, Raichu-RhoA Q63L: n=19, Raichu-RhoA T19N: n=21, Akind-3A: n=17).

(B) A2780 cells were transfected with control, PKBα/Akt1 or PKBβ/Akt2 specific shRNA constructs. After 16 hours cells were seeded into inverted invasion assays in the presence of FN and stimulated with osteopontin (OPN) as indicated. Cells were visualised with calcein-AM 48 hours later, and serial confocal sections were captured at 15µm intervals using a 10x objective. Invasion was quantitated by measuring the fluorescence intensity of cells invading 45µm or more, and expressing this as a percentage of the fluorescence intensity of all cells within the plug. Data represent mean ± SEM from three independent experiments.

(C) A2780 cells transfected as in (A) were lysed after 48 hours. Lysates were subjected to SDS-PAGE/ western blot analysis to assess relative levels of PKBα/Akt1, PKBβ/Akt2 and β-actin.

(D) A2780 cells were transfected with Control, RacGAP1 SMARTPool, or individual RacGAP1 siRNA oligonucleotides, and lysed after 48 hours. Lysates were subjected to SDS-PAGE/ western blot analysis to assess and relative levels of RacGAP1 and α-Tubulin.

(E) Schematic depicting domain structure of RacGAP1, and phosphorylation and known interaction sites. CC- coiled coil domain, C1- C1 domain, GAP- GAP domain.

(F, G) A2780 cells were transfected with control or RacGAP1-specific siRNA oligonucleotides, and seeded onto CDMs after 24-36 hours. Cells were tracked using ImageJ, and speed (E, ≥18 cells) and persistence (F, ≥18 cells) of migration analysed using the manual tracking plugin. Data represent mean ±SEM from at > 3 independent experiments.
(H) A2780 cells were transfected with control or RacGAP1-specific siRNA oligonucleotides, and seeded onto CDMs after 24-36 hours. Cells were stimulated with 2.5μm cRGDFV, and images captured at 10 minute intervals using a 20x objective. Pseudopod length (>200 cells) was measured for all moving cells within the 20th frame using ImageJ. Graphs display mean ±SEM from >3 independent experiments. *** indicates p<0.001.

(I) A2780 cells were transfected with control or RacGAP1 specific siRNA oligonucleotides, and seeded into inverted invasion assays after 16 hours in the presence or absence of FN and cRGDFV. Cells were visualised and invasion quantified as in (B). Data represents mean ± SEM from three independent experiments. * indicates p<0.05.

(J) A2780 cells were stably transfected by lentivirus to express GFP (control), FLAG-RacGAP1wt, FALG-RacGAP1249A or FLAG-RacGAP1249D. Cells were lysed and lysates analysed by SDS-PAGE/western blot mouse anti-FLAG and mouse anti-RacGAP1 antibodies.

(K) A2780 cells stably transfected as in (J) were seeded into 96-well plates and cell viability assayed over 7 days using CellTitre 96 kit (Promega).

(L) A2780 cells stably expressing GFP or FLAG-RacGAP1wt were transfected with control oligo or RacGAP1 oligo#6 and lysed after 48 hours. Lysates were subjected to SDS-PAGE/ western blot analysis to assess relative levels of RacGAP1 and α-Tubulin.

(M) MDA-MB-231 cells were transfected with control or RacGAP1 SMARTpool oligos and lysed after 48 hours. Lysates were subjected to SDS-PAGE/ western blot analysis to assess relative levels of RacGAP1 and α-Tubulin.

(N, O) H1299 cells stably transfected with control plasmid (VEC; N) or mutant p53 (273H; O) cells were transfected with control or RacGAP1 SMARTpool oligos and lysed after 48 hours. Lysates were subjected to SDS-PAGE/ western blot analysis to assess relative levels of RacGAP1 and α-Tubulin.
Figure S2

(A) A2780 cells were subjected to two rounds of control or IQGAP1 RNAi and lysed 48 hours after the second transfection. Lysates were subjected to SDS-PAGE/western blot analysis to assess relative levels of IQGAP1 and α-Tubulin.

(B, C) A2780 cells were subjected to two rounds of control or IQGAP1 RNAi and seeded onto CDMs. Cells were stimulated with 2.5μm cRGDFV as indicated, and images captured at 10 minute intervals using a 20x objective. Displacement of cells was tracked using ImageJ, and speed and persistence of migration analysed using the manual tracking plugin. Graphs display mean ±SEM from >3 independent experiments. * indicates p<0.05, ** indicates p<0.001.

(D-F) A2780 (D), MDA-MB-231 (E) or H1299-VEC/273H (F) cells were transfected with control or IQGAP1-specific siRNA oligonucleotides, and seeded into inverted invasion assays after 16 hours in the presence or absence of FN and cRGDFV (A2780s) or FN (MDA-MB-231/H1299). Cells were visualised and invasion quantified as in (1B). Data represent mean ± SEM from three independent experiments** indicates p<0.01.

(G, H) MDA-MB-231 (G) or H1299-VEC/273H (H) cells were transfected and knockdown efficiency established as in (A).

(I) MDA-MB-231 cells stably transfected with control vector, RacGAP1\textsuperscript{wt}, RacGAP1\textsuperscript{249A} or RacGAP1\textsuperscript{249D} were seeded into inverted invasion assays in the presence of FN, and cells visualised and invasion quantified as in (1B). Data represent mean ± SEM from three independent experiments. *** indicates p<0.001.

(J) A2780 cells stably transfected with control vector, FLAG-RacGAP1\textsuperscript{wt} or FLAG-RacGAP1\textsuperscript{249D} were subjected to two rounds of control or IQGAP1 RNAi and lysed 48 hours after the second transfection. Lysates were subjected to SDS-PAGE/western blot analysis to assess relative levels of IQGAP1 and α-Tubulin.
(K) A2780 cells stably transfected with control vector, FLAG-RacGAP1\textsuperscript{wt} \text{FLAG-RacGAP1}\textsuperscript{249A} were treated as in (1B), and IP\text{d} using anti- RxRxxS*/T* or an iso-type-matched control. IPs were analysed by SDS-PAGE and western blotting for RacGAP1, and quantified using the Odyssey system. Data represent mean ± SEM from more than 3 independent experiments.

* indicates p<0.05.

**Figure S3**

(A) A2780 cells were transfected with Raichu-Rac1 and fixed and stained for endogenous Rac1, or transfected with Raichu-RhoA or GFP-RhoA and fixed. Images were captured using a spinning disk confocal microscope. Scale bar= 20\textmu m.

(B) A2780 cells were transfected with Raichu-Rac, seeded onto CDMs after 16-24 hours and stimulated with cRGDfV as indicated. Fluorescence lifetime images were captured at 1 minute intervals, and intensity images are shown. Dotted lines indicate regions of interest drawn for quantification of probe lifetime at the plasma membrane at the front, middle and rear of the cell. Scale bar=10\textmu m.

(C) A2780 cells were transfected with control or RCP-specific siRNA and allowed to recover for 24 hours. Cells were then retransfected with Raichu probes and lysed 24 hours later. Lysates were subjected to SDS-PAGE/ western blot analysis to assess relative levels of RCP and \(\alpha\)-Tubulin.

(D) A2780 cells stably expressing GFP or \text{FLAG-RacGAP1}\textsuperscript{WT} were lysed, and immunoprecipitated with anti-FLAG antibodies. Immunoprecipitates were washed copiously, and then applied to GAP assays against Rac1 or Ras. * indicates p<0.05.

(E) GAP assays performed using recombinant p50RhoGAP against Rac1 or Ras (positive and negative control). *** indicates p<0.001.

**Figure S4**
(A-D) A2780 (A), MDA-MB-231 (B) or H1299-VEC/273H (C/D) cells were transfected with control, Rac1- or RhoA-specific siRNA SMARTpool oligonucleotides and lysed after 48 hours. Lysates were subjected to SDS-PAGE/western blot analysis to assess relative levels of Rac1, RhoA and α-Tubulin.

(E-G) A2780 cells were transfected as in (A), seeded onto CDM and cells migration tracked in the presence or absence of cRGDFv using ImageJ. Speed and persistence (E, F, ≥19 cells) of migration were analysed using the manual tracking plugin. Pseudopod length (G, >250 cells) was measured for all moving cells within the 20th frame. Graphs display mean ±SEM from at least 3-independent experiments, *** indicates p<0.001.

(H) A2780 cells were co-transfected with GFP-Rac1 or GFP-RhoA alongside control, Rac1#1 or RhoA#1 RNAi oligonucleotides, and lysed after 36 hours. Lysates were subjected to SDS-PAGE/western blot analysis to assess relative levels of Rac1, RhoA and α-Tubulin. Levels of Rac1 or RhoA were normalised against tubulin, and relative expression is shown.

(I-K) A2780 cells were seeded onto CDM and allowed to adhere and begin migrating. Cells were treated with or without cRGDFV (2.5μM) and Rac inhibitor (25μM) as indicated. Images were captured at 10 minute intervals using a 20x objective. Pseudopod length (I, >250 cells) was measured for all moving cells within the 20th frame and speed (J, ≥15 cells) and persistence (K, ≥15 cells) of migration analysed using the manual tracking plugin in ImageJ. Graphs display mean ±SEM from at least 3-independent experiments, *** indicates p<0.001.

**Supplementary video 1**

A2780 cells stably expressing FLAG- RacGAP1wt, FLAG-RacGAP1249A or FLAG-RacGAP1249D were seeded onto CDMs, stimulated with or without 2.5μM cRGDFV, and phase contrast images were captured every 10 mins on an inverted AS-MDW microscope system (Leica) using a 20x objective.
Supplementary video 2

A2780 cells were transfected with Raichu-Rac, seeded onto CDMs after 16-24 hours and fluorescence lifetime images were captured at 1 minute intervals using Marianis inverted microscope system (3i, Denver) and a 63x objective. Movies of lifetime maps are presented, with colder clours representing low lifetime (and high activity) and warm colours representing high lifetime (and low activity).

Supplementary video 3

A2780 cells were transfected with Raichu-Rac, seeded onto CDMs after 16-24 hours and stimulated with 2.5μM cRGDFV. Fluorescence lifetime images were captured at 1 minute intervals using Marianis inverted microscope system (3i, Denver) and a 63x objective. Movies of lifetime maps are presented, with colder clours representing low lifetime (and high activity) and warm colours representing high lifetime (and low activity).

Supplementary video 4

A2780 cells were transfected with Raichu RhoA, seeded onto CDMs after 16-24 hours and fluorescence lifetime images were captured at 1 minute intervals using Marianis inverted microscope system (3i, Denver) and a 63x objective. Movies of lifetime maps are presented, with colder clours representing low lifetime (and high activity) and warm colours representing high lifetime (and low activity).

Supplementary video 5

A2780 cells were transfected with Raichu RhoA, seeded onto CDMs after 16-24 hours and stimulated with 2.5μM cRGDFV. Fluorescence lifetime images were captured at 1 minute intervals using Marianis inverted microscope system (3i, Denver) and a 63x objective. Movies of lifetime maps are
presented, with colder clours representing low lifetime (and high activity) and warm colours representing high lifetime (and low activity).

**Supplementary video 6**

A2780 cells were subjected to Rac or RhoA knockdown, seeded onto CDM and stimulated with (RhoA knockdown) or without (Rac1 knockdown) 2.5μM cRGDFV, and and phase contrast images were captured every 10mins on an inverted AS-MDW microscope system (Leica) using a 20x objective.

**Supplementary video 7**

HT1080 and A2780 cells were transfected with Life-Act-mEGFP, and after 16 hours plated onto CDM for 4 hours prior to imaging. Actin dynamics were captured as cells move in 3D using a spinning disk confocal inverted Marianis microscope system (3i, Denver) and a 63x objective.
Jacquemet et al. Figure 3

A

B

C

D

E

F

G

[Images and data from the figure are represented here.]

Relative Binding to IQGAP1

<table>
<thead>
<tr>
<th>cRGDF:</th>
<th>FLAG-RacGAP1&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>FLAG-RacGAP1&lt;sup&gt;249A&lt;/sup&gt;</th>
<th>FLAG-RacGAP1&lt;sup&gt;249D&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.00</td>
<td>1.10</td>
<td>1.20</td>
</tr>
<tr>
<td>+</td>
<td>1.30</td>
<td>1.40</td>
<td>1.50</td>
</tr>
<tr>
<td>**</td>
<td>1.60</td>
<td>1.70</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Relative RacGAP1 phosphorylation

<table>
<thead>
<tr>
<th>cRGDF:</th>
<th>FLAG-RacGAP1&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>FLAG-RacGAP1&lt;sup&gt;249A&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.00</td>
<td>0.10</td>
</tr>
<tr>
<td>+</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>**</td>
<td>0.40</td>
<td>0.50</td>
</tr>
</tbody>
</table>

PLA signal (Integrated density)

<table>
<thead>
<tr>
<th>cRGDF:</th>
<th>A2780-GFP</th>
<th>FLAG-RacGAP1&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>FLAG-RacGAP1&lt;sup&gt;249A&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.00</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>+</td>
<td>0.30</td>
<td>0.40</td>
<td>0.50</td>
</tr>
<tr>
<td>**</td>
<td>0.60</td>
<td>0.70</td>
<td>0.80</td>
</tr>
</tbody>
</table>

** [Statistical significance]
Jacquemet et al Figure 4

A

Control RNAi
+cRGDfV
Control RNAi
+cRGDfV
RacGAP1/F-actin
+/-cRGDfV
IQGAP1 RNAi oligo#1
+/-cRGDfV
IQGAP1

B

A2780-GFP
+/-cRGDfV
A2780-FLAG-RacGAP1WT
+/-cRGDfV
A2780-FLAG-RacGAP1249A
+/-cRGDfV
A2780-FLAG-RacGAP1249D
+/-cRGDfV

C

A2780-GFP
+/-cRGDfV
A2780-FLAG-RacGAP1WT
+/-cRGDfV
A2780-FLAG-RacGAP1249A
+/-cRGDfV
A2780-FLAG-RacGAP1249D
+/-cRGDfV
Jacquemot et al Figure 5

**Video 1**

A

Control RNAi -cRGDfV

IQGAP1 RNAi -cRGDfV

Control RNAi +cRGDfV

IQGAP1 RNAi oligo

0.2 0.4 0.6 0.8 1.0 Relative invasion (>45 µm)

cRGDfV: - + FN

Control RNAi IQGAP1 RNAi oligo

B

wt       249A    249D

wt        249A    249D

FLAG-RacGAP1

0 10 20 30 40 50 60 Pseudopod length (µm)

cRGDfV: - + - + - + + +

Control RNAi IQGAP1 RNAi oligo

C

D

E

wt       249A    249D

wt        249A    249D

FLAG-RacGAP1-249A -cRGDfV

FLAG-RacGAP1-249A +cRGDfV

FLAG-RacGAP1-249D -cRGDfV

FLAG-RacGAP1-249D +cRGDfV

Pseudopod length (µm)

cRGDfV: - - - - + + + +

wt       249A    249D

wt        249A    249D

FLAG-RacGAP1

0 10 20 30 40 50 60 Pseudopod length (µm)

cRGDfV: - - - - + + + +

Control RNAi IQGAP1 RNAi oligo

A2780- Control +cRGDfV

FLAG-RacGAP1 WT -cRGDfV

FLAG-RacGAP1 WT +cRGDfV

0 0.2 0.4 0.6 0.8 1.0 Relative invasion (>45 µm)

cRGDfV: - + + + +

Control RNAi IQGAP1 RNAi oligo

ns *** *** ns ***
Jacquemet et al. Figure 8

A

Control RNAi -cRGDfV  Control RNAi +cRGDfV
Rac1 RNAi -cRGDfV  Rac1 RNAi +cRGDfV
RhoA RNAi -cRGDfV  RhoA RNAi +cRGDfV

B

Pseudopod length (µm)

Control RNAi  Rac1 RNAi  RhoA RNAi +cRGDfV

C

Speed (µm/min)

Control RNAi  Rac1 RNAi  RhoA RNAi +cRGDfV

D

Persistence

Control RNAi  Rac1 RNAi  RhoA RNAi +cRGDfV

E

cRGDfV

Control RNAi  Rac1 RNAi  RhoA RNAi

F

Invansion (% cells >45 µm)

Control RNAi  Rac1 RNAi  RhoA RNAi

G

Relative invasion

Control RNAi  Rac1 RNAi  RhoA RNAi
Jacquemet et al. Figure 9

A

Lifeact-mEGFP

HT1080

Video 7

Lifeact-mEGFP

A2780 - cRGDfV

Video 7

Lifeact-mEGFP

A2780 + cRGDfV

Video 7

B

Normalised actin density

at protrusion sites

HT1080

A2780

cRGDfV: - +

A2780 + cRGDfV A2780 - cRGDfV

cRGDfV: - + cRGDfV: - +

Volume (µm^3 x 10^3)

Video 7

C

Maximum projection

3D reconstruction

D

2D shape descriptors

**

Aspect Ratio

 Circulariy

E

3D shape descriptors

**

Volume (µm^3 x 10^3)

Sphericity
Jacquemet et al. Supplementary Figure 2
Jacquemet et al. Supplementary Figure 3

**A**

Raichu-Rac1  Endogenous Rac1  Raichu-RhoA  GFP-RhoA

**B**

Raichu-Rac -cRGDfV  Raichu-RhoA -cRGDfV
Raichu-Rac +cRGDfV  Raichu-RhoA +cRGDfV

**C**

kDa  Control RNAi  RCP RNAi

Blot: RCP
Blot: α-Tubulin

**D**

Absorbance (650nm)

A2780-GFP  FLAG-RacGAP1  A2780-GFP  FLAG-RacGAP1

+Rac1  +Ras

**E**

Absorbance (650nm)

+Rac1  +Ras  p50RhoGAP