RhoB regulates uPAR signalling

Daniela Alfano[1,2,*], Pia Ragno[2], M. Patrizia Stoppelli[3] and Anne J. Ridley[1,‡]

[1]Randall Division of Cell and Molecular Biophysics, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, UK
[2]Department of Chemistry and Biology, University of Salerno, Via Ponte don Melillo 1, 84084 Fisciano, Salerno, Italy
[3]Institute of Genetics and Biophysics Adriano Buzzati-Traverso, Consiglio Nazionale delle Ricerche (CNR), Via P. Castellino 111, 80131 Naples, Italy
*Present address: Institute of Genetics and Biophysics Adriano Buzzati-Traverso, Consiglio Nazionale delle Ricerche (CNR), Via P. Castellino 111, 80131 Naples, Italy
‡Author for correspondence (anne.ridley@kcl.ac.uk)

Accepted 12 January 2012
Journal of Cell Science 125, 2369–2380

doi: 10.1242/jcs.091579

Summary

Urokinase-type plasminogen activator (uPA) and its receptor, uPAR, play important roles in promoting cancer cell adhesion, migration and invasion. Rho GTPases are key coordinators of these processes; the Rho GTPase Rac1 has previously been implicated in uPA- and/or uPAR-induced migratory or morphological cell responses. We used RNAi to deplete 12 different Rho GTPases to screen for effects on uPA-stimulated migration, and found that depletion of RhoB significantly reduces uPA-induced migration and invasion of prostate carcinoma cells. RhoB depletion did not affect the expression or surface levels of uPAR but reduced the uPAR-induced increase in levels of several integrins and inhibited uPAR signalling to the actin regulator cofilin, the cell-adhesion signal-transduction adaptor molecule paxillin and the serine/threonine kinase Akt. uPAR rapidly activated RhoB and increased RhoB expression. RhoB depletion also reduced cell adhesion to and spreading on vitronectin, which is a uPAR ligand. This correlated with decreased association between integrins and uPAR and reduced integrin β1 activity. Our results indicate that RhoB is a key regulator of uPAR signalling in cell adhesion, migration and invasion.

Key words: Rho GTPases, Cell migration, Signal transduction, uPA, uPAR

Introduction

Urokinase-type plasminogen activator (uPA) stimulates changes to cells and their environments both through its proteolytic activity and through binding to its cell surface receptor, the uPA receptor (uPAR). uPA is a protease that converts the pro-enzyme plasminogen into plasmin, a wide-spectrum serine protease able to degrade most of the extracellular matrix (ECM) components and activate latent collagenases. uPA and plasmin are involved in thrombolysis, inflammation, cell migration, tissue remodelling, cancer invasion and vascularization (Irigoyen et al., 1999). uPA is secreted in the pro-enzyme form (pro-uPA), which can be activated in the extracellular milieu by a single proteolytic cleavage occurring between Lys158 and Ile159, thus generating a two-chain enzyme. uPA is a multi-domain protein that includes an amino-terminal growth factor-like domain (GFD; residues 1–49) followed by a 'kringle' region (residues 50–131) linked by the 'connecting peptide' (residues 135–158) to the catalytic protease domain (residues 158–411). High affinity binding to uPAR occurs through the GFD of uPA and does not involve the catalytic domain or alter its catalytic activity (Stoppelli et al., 1985; Vassalli et al., 1985; Vincenza Carriero and Stoppelli, 2011). uPAR is a three-domain (D1, D2 and D3) glycosylphosphatidylinositol-anchored protein that binds uPA or the uPA amino-terminal fragment (ATF; residues 1–135), mainly through its N-terminal D1 domain (Llinas et al., 2005; Vincenza Carriero et al., 2009). Independently of its catalytic domain, uPA acts through uPAR to induce dynamic reorganization of the actin cytoskeleton, cell adhesion to the extracellular matrix (ECM) and cell migration. uPAR also binds the ECM protein vitronectin (VN), thus mediating cell adhesion (Montuori et al., 2005). uPAR activates a number of signalling molecules, including Rho family GTPases, Src family kinases and ERK1/2 Ser/Thr kinases (Blasi and Sidenius, 2010; Smith and Marshall, 2010). Because uPAR is not a transmembrane receptor, uPA- and VN-dependent signalling require physical and/or functional interaction of uPAR with a variety of transmembrane receptors, such as integrins, the EGF receptor and fMLP receptors (Alfano et al., 2005; Franco et al., 2006; Montuori et al., 2011).

In human cancer, increased expression of uPA and uPAR is associated with a high risk of metastases and an unfavourable clinical outcome (Harbeck et al., 2002; Danø et al., 2005). Prostate cancer continues to be one of the deadliest cancers and currently ranks as the second most frequent cause of cancer-related death in males in the United States. The uPA–uPAR system could serve as a useful molecular marker to predict a poor prognosis for prostate cancer and might be a therapeutic target for prostate cancer intervention (Sheng, 2001; Sehgal et al., 2006; Almasi et al., 2011; Rabbani et al., 2010). uPAR and uPA overexpression seems to be associated with a more aggressive phenotype of prostate cancer cells, because they stimulate cell invasion, survival and tumorigenicity (Pulukuri et al., 2005).

Rho family GTPases are crucial regulators of cytoskeletal and adhesion dynamics, thereby stimulating cell migration and invasion (Heasman and Ridley, 2008). Although there are at least 20 human Rho GTPases, only a few have been rigorously tested for their involvement in migratory responses (Vega and Ridley, 2008). The uPA–uPAR system has been reported to act through Rac, Cdc42 and RhoA (Smith et al., 2008; Smith and Marshall, 2010). uPAR overexpression by itself induces an increase in Rac-dependent lamellipodia and cell migration, and increases the level of activated Rac (Kjoller and Hall, 2001; Aguirre-Ghiso et al., 2003). Conversely, downregulation of uPAR by RNA interference (RNAi) results in the loss of actin-rich membrane ruffles, and reduces Rac activity (Smith et al., 2008). RhoA, however, stimulates uPAR transcription whereas Rac1 does

Journal of Cell Science

not (Muller et al., 2000). To date, only RhoA and Rac1 have been tested in uPA-induced migration (Kiian et al., 2003), whereas the involvement of other Rho GTPases has not been investigated.

The aim of this work was to identify which Rho GTPase(s) mediate uPA–uPAR-induced migration and invasion. Using RNAi-mediated knockdown of 12 different Rho GTPases, we identified RhoB as a key regulator of uPAR-dependent responses in prostate cancer cells, including cell migration, invasion and adhesion to vitronectin. RhoB affects uPAR-induced integrin expression, association of uPAR with integrins and integrin activity. In addition, uPAR activates RhoB and stimulates RhoB expression. These results indicate a previously unknown role for RhoB in uPAR responses.

Results

uPA and uPAR expression regulates cell motility and invasiveness of PC3 prostate cancer cells

One of the key functions of the uPA–uPAR system is to promote invasion, a crucial process for tumour metastasis (Blasi and Carmeliet, 2002). We investigated the contribution of uPA and uPAR to the migration and invasion of the highly invasive human prostate cancer cell line, PC3 (Dedhar et al., 1993). We first assessed uPA and uPAR expression in PC3 cells. uPA and uPAR were both expressed in PC3 cells and their expression was reduced by specific short interfering RNAs (siRNAs) (Fig. 1A,B). We then investigated the effects of uPA and uPAR depletion in invasion assays. PC3 cells efficiently invaded through Matrigel towards serum acting as a chemoattractant; this invasion was reduced by 50% following uPA depletion and 40% following uPAR depletion, compared with control cells (Fig. 1C). To determine whether uPA also affected cell migration in two dimensions we performed a scratch-wound-healing assay with PC3 cells and monitored them by time-lapse microscopy. uPA depletion substantially reduced PC3 cell migration into the wound area, both in 10% FCS (Fig. 1D) and 0.5% FCS (data not shown). uPA and uPAR are therefore required for efficient PC3 cell migration and invasion.

uPAR-induced migration and invasion requires a specific subset of Rho GTPases

The uPA–uPAR system is known to act through Rho GTPases to affect cell migration and invasion (Smith and Marshall, 2010), but a comprehensive analysis of Rho GTPase involvement downstream

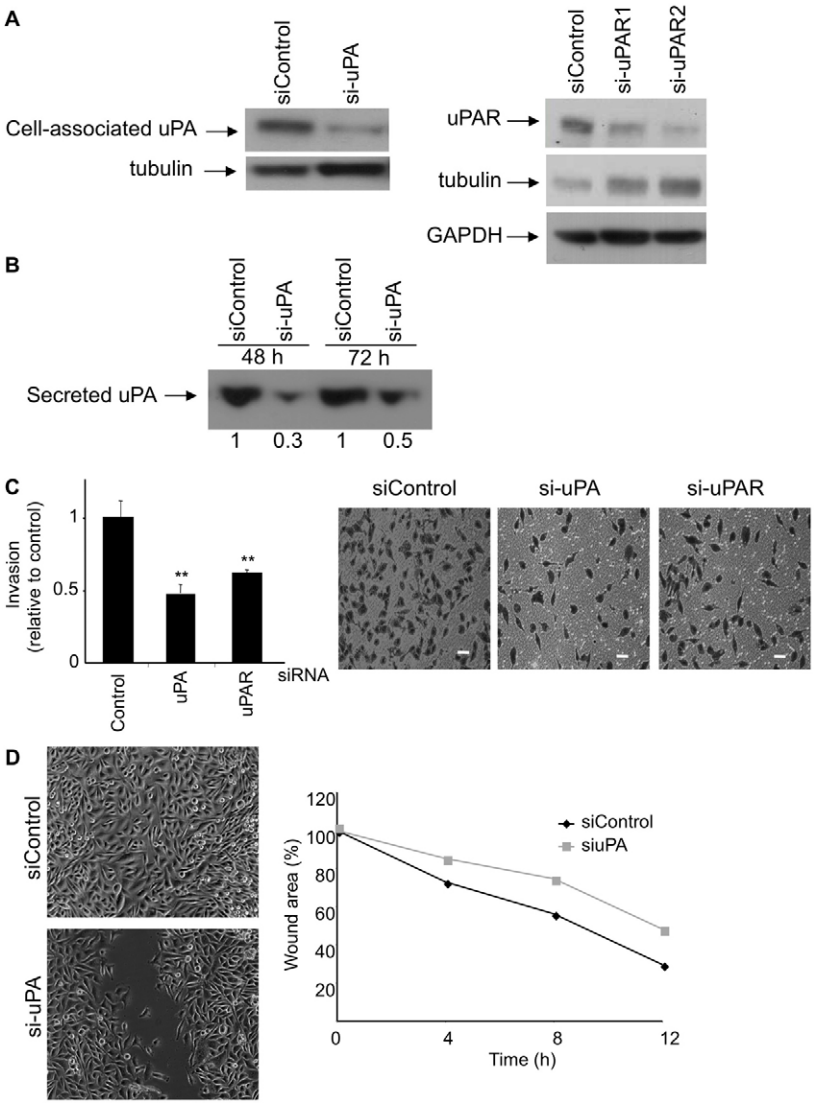

Fig. 1. Expression of uPA and uPAR regulates migration and invasion of PC3 cells. (**A**) To knockdown endogenous uPA and uPAR, PC3 cells were transfected with a control siRNA (siControl), a siRNA targeting uPA (si-uPA) or two different siRNAs targeting uPAR (si-uPAR1 and 2). After 72 hours, cell lysates were immunoblotted with anti-uPA, monoclonal R4 anti-uPAR or anti-tubulin and anti-GAPDH antibodies as loading controls. (**B**) To determine the level of secreted uPA following uPA knockdown, medium was collected from cells 48 and 72 hours after siRNA transfection, concentrated and analysed by immunoblotting. Relative uPA levels were quantified by densitometric scanning (numbers below the blot, normalized at each time point to siControl). (**C**) PC3 cells transfected with control, uPA or uPAR siRNAs were plated on Matrigel-coated Transwell filters containing 10% FCS in the bottom well. (Left) The invasion values are the means ± s.e.m. of three experiments performed in triplicate, normalized to the number of invading cells in the absence of FCS, shown as fold change relative to control. $^{**}P \leq 0.01$ compared with siControl-transfected cells. (Right) Representative images of cells on the Transwell filters. Scale bars: 50 μm. (**D**) A confluent monolayer of PC3 cells was wounded using a pipette tip and cells were monitored by time-lapse microscopy, acquiring an image every 3 minutes for 15 hours. (Left) Representative images 15 hours after scratch wounding. (Right) Scratch wound areas were determined from phase-contrast images taken at the indicated time points from movies, and are shown as a percentage of the initial wound area.

of uPAR has not been carried out. PC3 cells were therefore transiently transfected with siRNAs targeting 12 of the 20 known Rho GTPase family members, in order to assess the involvement of specific Rho GTPases in uPA-stimulated migration. These 12 Rho GTPases were chosen on the basis of phenotypes observed in other experiments knocking down Rho GTPases in PC3 cells in our laboratory (Vega and Ridley, 2011; Bai et al., 2011) (unpublished data). The efficiency of Rho GTPase knockdown was determined by western blotting of all Rho GTPases except for RhoD, for which no reliable antibody was available (Fig. 2A). Specific siRNAs strongly reduced the expression of the Rho GTPases analysed. To determine which Rho GTPases affect uPAR-dependent cell migration, PC3 cells were plated on collagen IV-coated Transwells and allowed to migrate toward the N-terminal region of uPA, known as the amino-terminal fragment (ATF, residues 1–135). ATF binds uPAR (Stoppelli et al., 1985; Vassalli et al., 1985) but lacks the catalytic uPA domain, thus its pro-migratory effect is only due to its interaction with uPAR and not to its proteolytic activity. ATF-stimulated migration of PC3 cells; Rac1, Cdc42, RhoB, RhoC or RhoF depletion inhibited ATF-induced migration. By contrast, depletion of RhoA, RhoD, RhoE, RhoG, RhoJ, RhoQ or RhoU did not significantly ($P>0.05$) affect migration towards ATF (Fig. 2B; siRNAs are listed in supplementary material Table S1). Rac1 and Cdc42 are known to affect uPAR-dependent migration (Kjoller and Hall, 2001; Aguirre-Ghiso et al., 2003; Chandrasekar et al., 2003; Kiian et al., 2003), thus we further analysed the functions of RhoB, RhoC and RhoF. We found that RhoB, RhoC and RhoF depletion also reduced invasion through Matrigel in an ATF gradient, whereas RhoA did not (Fig. 2C). uPAR-stimulated migration and invasion therefore requires a subset of specific Rho GTPases; we chose to focus our study on the involvement of RhoB in uPA responses, because RhoB depletion seems to impair uPAR-dependent invasion more strongly than RhoC and RhoF, suggesting a specific role in uPAR signalling. Expression of GFP–RhoB rescued the chemotactic response of RhoB-depleted cells to ATF, and thus knockdown of RhoB and not an off-target gene is responsible for the observed effect (Fig. 2D). Western blot analysis showed GFP–RhoB expression in control and RhoB-knockdown cells (Fig. 2D, right panel).

RhoB contributes to uPAR signalling

To investigate how RhoB affected uPAR-induced migration and invasion, we tested the effects of knocking down RhoB on uPAR

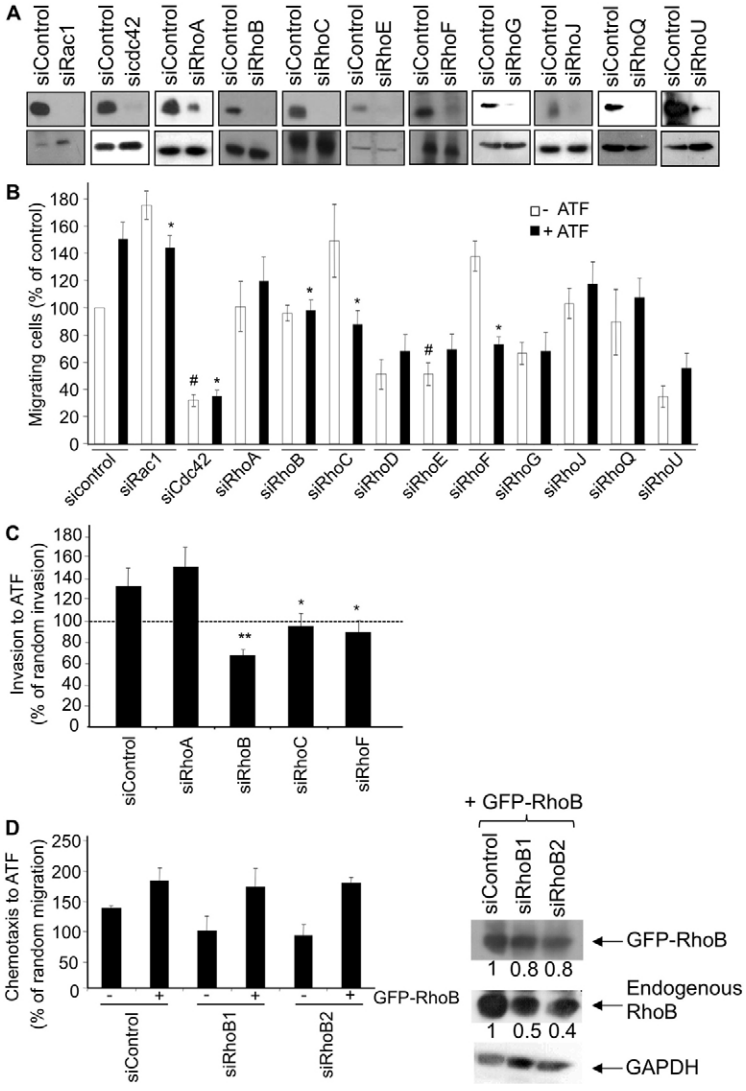

Fig. 2. uPAR-induced migration and invasion requires a specific subset of Rho GTPases. PC3 cells (3×10^5) were seeded in six-well plates and transfected with siRNAs targeting the indicated Rho GTPases or with a control siRNA (siControl). (A) Transfected cells were lysed and analysed 72 hours after transfection by immunoblotting for the indicated Rho GTPases, or tubulin as a loading control. Results shown are for siRNA-1 for each Rho GTPase (supplementary material Table S1). Similar results were obtained with other siRNAs to each gene. (**B**) Migration assay. Transfected cells were plated on collagen-coated Transwell filters with and without ATF in the bottom well. The values are the means ± s.e.m. of three experiments performed in triplicate. Results with the different siRNAs for each Rho GTPase listed in supplementary material Table S1 were pooled (see supplementary material Fig. S1 for results with individual siRNAs) and are shown as a percentage of siControl without ATF; $^{\#}P\leq0.05$ compared with siControl −ATF; $^{*}P\leq0.05$ compared with siControl +ATF. (**C**) Transfected cells were plated on Matrigel-coated Transwell filters to assess their invasion ability in an ATF gradient. Both migration and invasion results are pooled from experiments using all of the siRNAs to each gene listed in supplementary material Table S1. (**D**) PC3 cells were transfected with RhoB-targeting siRNAs (siRhoB1 or siRhoB2) or control siRNA (siControl) and after 24 hours cells were transfected with a construct encoding GFP–RhoB or GFP alone; 24 hours later, cells were allowed to migrate towards ATF to examine their chemotaxis (left). The levels of endogenous RhoB and ectopic GFP–RhoB protein were determined by immunoblotting (right). In C and D 100% values represent random cell migration or invasion for each siRNA treatment in the absence of ATF. The values are the means ± s.e.m. of three experiments performed in triplicate; $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$ compared with siControl.

levels and uPAR-induced signalling. RhoB depletion did not affect uPAR protein expression as shown by western blot (Fig. 3A) or affect surface levels of uPAR (Fig. 3B). It also did not alter mRNA levels as determined by real-time quantitative PCR (qPCR) analysis (Fig. 3C).

uPAR signalling is known to involve integrins (Smith and Marshall, 2010). Because RhoB depletion impaired uPAR-mediated responses but did not affect uPAR levels, we investigated the possibility that RhoB could regulate integrin levels. ATF treatment increased the cell surface levels of β1, β5 and αv integrins as detected by flow cytometry (Fig. 3D). This in part reflected changes in total levels of these integrins (Fig. 3E). RhoB depletion reduced the ATF-induced increase in the surface levels of β1, β5 and αv integrins (Fig. 3D). This reduction in integrin levels could explain how RhoB specifically affects uPAR-dependent migration and invasion but not basal migration.

We then investigated the involvement of two regulators of cell migration and adhesion, cofilin and paxillin, in uPAR-dependent signalling. Cofilin severs actin filaments to increase sites for actin polymerization in lamellipodia, and it is inhibited by phosphorylation on Ser3 (Bamburg, 1999). Paxillin localizes to integrin-mediated adhesion sites and is a multi-domain adaptor and scaffolding molecule that is regulated by tyrosine phosphorylation (Schaller and Parsons, 1995). Western blot analysis of ATF-stimulated PC3 cells showed changes in the phosphorylation of both these proteins. ATF transiently stimulated cofilin Ser3 phosphorylation, returning to basal levels by 60 minutes, and induced an increase in paxillin phosphorylation by 1 minute, which was still maintained at 60 minutes (Fig. 4A).

uPA has previously been shown to activate the phosphoinositide 3-kinase (PI3K)–Akt pathway in other cell types (Alfano et al., 2006). Accordingly, we found that ATF stimulated Akt phosphorylation in PC3 cells (Fig. 4A). RhoB depletion prevented ATF-induced cofilin, paxillin and Akt phosphorylation (Fig. 4B,C). These results indicate that RhoB contributes to three different aspects of uPAR signalling: actin dynamics (cofilin), cell adhesion (paxillin) and cell survival (Akt).

ATF increases RhoB activation and expression

Because RhoB is required for uPAR responses, we assessed RhoB activation following ATF stimulation. RhoB activation was observed in PC3 cells between 1 minute (data not shown) and 3 minutes (Fig. 5A) after ATF stimulation with the same concentration (10 nM) used to stimulate cell migration; 1 nM ATF did not stimulate RhoB (data not shown). RhoB has previously been shown to be upregulated at the mRNA and protein levels in response to several growth factors (Wheeler and Ridley, 2004), thus we investigated whether ATF also affected RhoB expression. qPCR analysis showed that ATF increased *RHOB* mRNA levels in PC3 cells as early as 1 hour (threefold), with maximal levels at 6 hours (sevenfold; Fig. 5B); a parallel increase of RhoB protein, peaking at 6 hours after stimulation was also observed (Fig. 5C). The early induction of *RHOB* mRNA at 1 hour is consistent with it being an immediate early

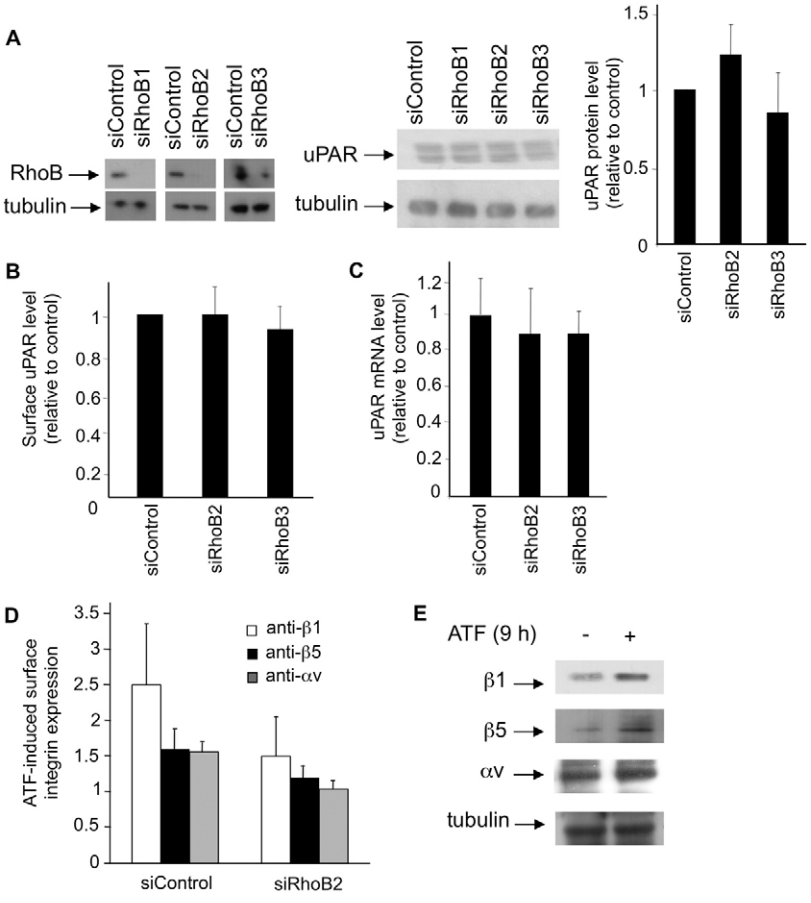

Fig. 3. RhoB knockdown alters integrin levels but not uPAR expression. PC3 cells were transfected with control siRNA (siControl) or three RhoB-targeting siRNAs (siRhoB1, siRhoB2 or siRhoB3) and analysed after 48 hours. (**A**) Transfected cell lysates were analysed by immunoblotting with polyclonal anti-RhoB antibody, monoclonal R4 anti-uPAR antibodies, or anti-tubulin antibodies as a loading control (left panels). Relative uPAR and tubulin levels were quantified by densitometric scanning and uPAR values were normalized to tubulin (right panel). Values are the ratio of uPAR levels in si*RHOB*-treated cells to those in siControl cells (means ± s.e.m. of three separate experiments). (**B**) Cell surface levels of uPAR were determined by flow cytometry. Values are the mean fluorescence of the population ± s.d. from two separate experiments each performed in duplicate. Results were normalised to siControl values. (**C**) uPAR mRNA levels were quantified by qPCR and normalized to the corresponding *GAPDH* mRNA levels. Values are the ratio of uPAR mRNA levels in si*RHOB*-treated cells to siControl cells (means ± s.e.m. of three separate experiments). (**D**) PC3 cells were serum-starved for 24 hours, then stimulated with 10 nM ATF or left unstimulated for 9 hours. Cells were stained for surface levels of β1, β5 and αv integrins. Values are the mean fluorescence of the population ± s.d. of two separate experiments each performed in duplicate, relative to control cells not treated with ATF. (**E**) Cell lysates from control and ATF-stimulated cells were analysed by immunoblotting with antibodies to the indicated integrins. Tubulin was used as a loading control.

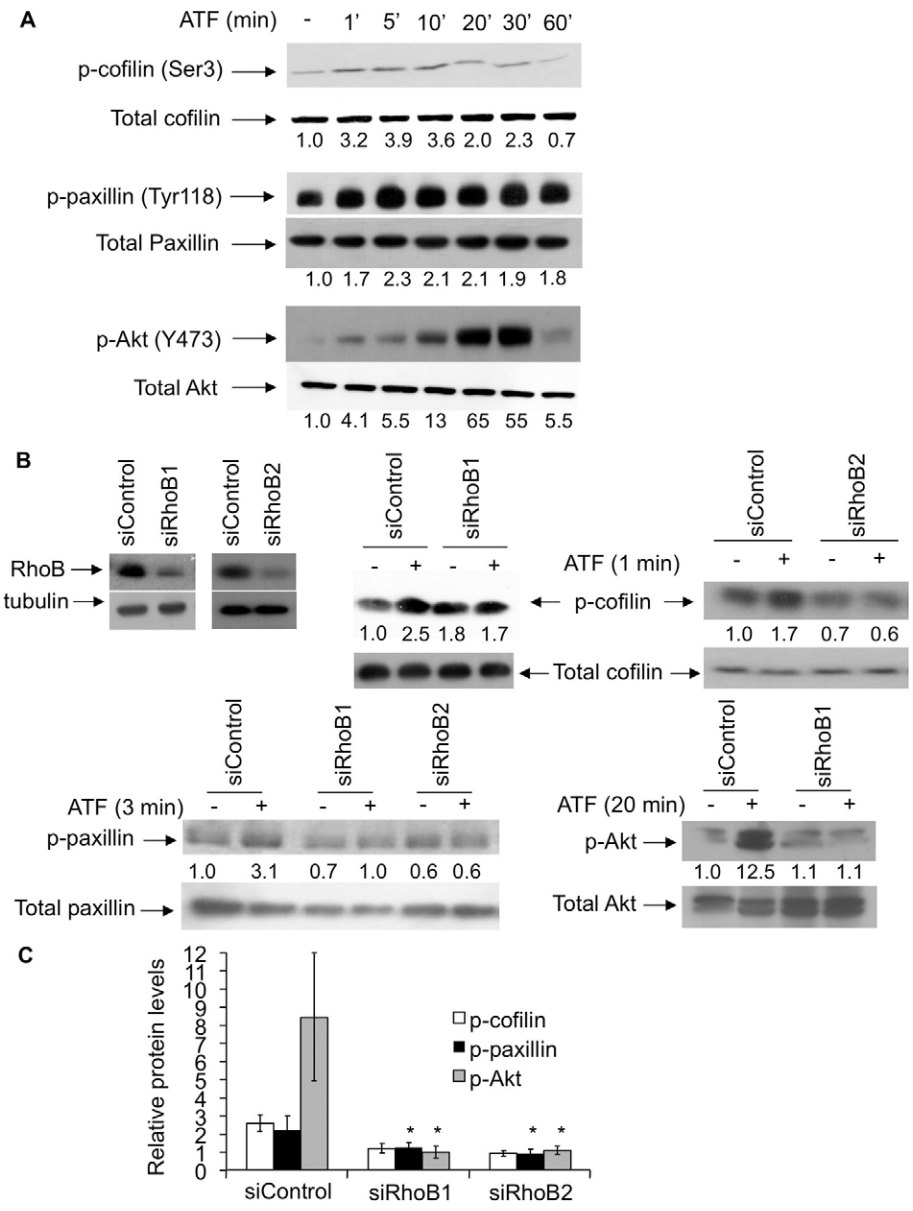

Fig. 4. RhoB depletion inhibits uPAR-induced paxillin, Akt and cofilin phosphorylation. (**A**) ATF stimulates cofilin, paxillin and Akt phosphorylation in PC3 cells. Cells were serum-starved for 24 hours, stimulated with 10 nM ATF and lysed at the indicated time points. Cell lysates were analysed by immunoblotting with antibodies to the indicated phosphorylated proteins. GAPDH was used as a loading control. Relative protein levels were quantified by densitometric scanning. Phosphorylated cofilin (p-cofilin) levels were normalized to cofilin levels, phosphorylated paxillin levels to paxillin levels and phosphorylated Akt levels to Akt levels. Values are the ratio of level of the phosphorylated proteins in ATF-stimulated to unstimulated cells. (**B**) Cells were transfected with control siRNA (siControl) or the indicated siRNAs targeting RhoB. (Left) Transfected cell lysates were analysed by immunoblotting with polyclonal anti-RhoB antibody. (Right and below) After 72 hours, cells were serum-starved, stimulated with 10 nM ATF and lysed at 1, 3 or 20 minutes, as indicated. Cell lysates were analysed by immunoblotting with antibodies to the indicated phosphorylated proteins. Levels of total paxillin or cofilin were used as loading controls. Relative protein levels were quantified by densitometric scanning. Phosphorylated cofilin, paxillin and Akt levels were normalised respectively to total cofilin, paxillin and Akt levels. Values are levels of the phosphorylated proteins relative to those in unstimulated siControl cells. Blots shown are representative of at least three independent experiments. (**C**) Graph showing densitometric analysis of immunoblots. Values are the fold increase over basal levels (−ATF) for phosphorylated cofilin, paxillin and Akt, normalized to total protein levels of the respective protein (n=3 for each protein; means ± s.d. *P<0.05 versus control).

gene (Huang and Prendergast, 2006; Vasilaki et al., 2010). The later upregulation at 6 hours could reflect another layer of transcriptional regulation. uPA has previously been reported to stimulate RhoA activation in MCF-7 breast cancer cells (Jo et al., 2002), and Cdc42 and Rac activation in MDA-MB-231 breast cancer cells (Sturge et al., 2002). We demonstrate here that ATF both stimulates RhoB activation and increases RhoB expression.

uPAR expression regulates PC3 cell adhesion to vitronectin

uPAR mediates adhesion of a variety of cell types to VN, because it is a non-integrin VN receptor (Deng et al., 1996; Wei et al., 1996; Madsen and Sidenius, 2008; Blasi and Sidenius, 2010). Consistent with this, uPAR overexpression in PC3 cells increased cell elongation and spread area (Fig. 6A), and increased their adhesion to VN (Fig. 6B). uPAR expression also increased cell migration speed (Fig. 6C) and induced the extension of numerous microspikes on the plasma membrane of PC3 cells plated on VN (Fig. 6D). Endogenous uPAR contributes to PC3 cell adhesion to VN, because the function-blocking 399R anti-uPAR antibody inhibited adhesion of both PC3 and uPAR-transfected PC3 cells to VN (Fig. 6E).

RhoB specifically regulates PC3 cell adhesion to vitronectin

RhoB depletion has previously been reported to reduce the adhesion of macrophages to ICAM-1- and serum-coated surfaces (Wheeler and Ridley, 2007). Depletion of RhoB inhibited adhesion of both PC3 and uPAR-overexpressing PC3 cells to VN (Fig. 7A), suggesting that RhoB affects uPAR-mediated adhesion. The function-blocking 399R anti-uPAR antibody did not further reduce the adhesion of RhoB-depleted cells, suggesting that the effect of RhoB on adhesion is predominantly through uPAR (Fig. 7B). Consistent with this,

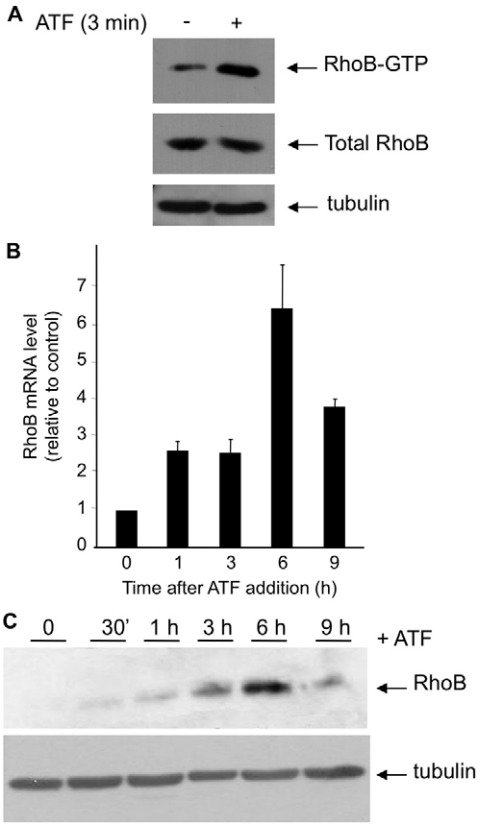

Fig. 5. uPAR increases RhoB activation and expression. PC3 cells were serum-starved for 24 hours, then stimulated with 10 nM ATF and harvested at the indicated time points. (**A**) RhoB activity in ATF-stimulated or untreated cells was evaluated by GST-RBD pulldown assay. The levels of total RhoB or active RhoB pulled down by GST-RBD were analysed by immunoblotting with anti-RhoB antibodies. Tubulin was used as a loading control. (**B**) *RHOB* mRNA levels in ATF-treated cells were analysed by qPCR. *RHOB* mRNA levels were normalized to the corresponding *GAPDH* mRNA levels. Values are shown as fold increase relative to *RHOB* mRNA levels in untreated cells. (**C**) RhoB protein levels in ATF-treated PC3 cells were analysed by immunoblotting with RhoB-specific antibodies. Tubulin was used as a loading control. Data are representative of three independent experiments.

RhoB knockdown specifically decreased PC3 cell adhesion to VN but not to collagen IV or uncoated plastic (Fig. 7C). RhoB-depleted PC3 cells had a smaller spread area and were rounder than control cells on VN (Fig. 7D). GFP–RhoB expression rescued the spread area of RhoB-depleted cells (Fig. 7E), showing that the effects of siRNA targeting of RhoB were specific and not due to off-target effects. RhoB-depleted PC3 cells migrated more slowly than control cells (Fig. 7F), and again this was rescued by GFP–RhoB. The smaller spread area of RhoB-depleted cells was not due to a smaller cell volume or diameter, as measured in suspension (Fig. 7G). However, RhoB overexpression was unable to rescue the spread area of uPAR-depleted cells (Fig. 7H), confirming that RhoB-mediated adhesion of PC3 cells on VN is uPAR dependent. Hence, RhoB depletion impaired PC3 cell adhesion specifically to VN, which is a high affinity ligand of uPAR (Blasi and Sidenius, 2010). We propose that RhoB depletion reduces adhesion to VN, thereby reducing cell migration speed.

RhoB depletion reduces the association of uPAR with integrins

In order to elucidate the mechanism through which RhoB affects uPAR-dependent adhesion and spreading, we investigated the possibility that RhoB could affect the association of uPAR with integrins, because integrins generally mediate uPAR signalling (Smith and Marshall, 2010). The level of uPAR in β1 integrin or αv integrin immunoprecipitates was reduced in RhoB-depleted cells compared with control cells (Fig. 8A). Interestingly, RhoB depletion also reduced the association of talin with β1 and αv integrins (Fig. 8A). Given that talin is a crucial integrin-activating protein (Calderwood, 2004) we investigated the activation status of surface β1 integrin by flow cytometry. RhoB depletion reduced the level of active β1 integrin (Fig. 8B). Taken together these results suggest that RhoB affects uPAR-dependent adhesion and spreading by reducing the interaction between uPAR and active integrins.

Discussion

uPA and uPAR are well known to stimulate the migration and invasion of cancer cells thereby contributing to cancer progression and metastasis, and to signal through integrins (Blasi and Carmeliet, 2002). Rac1 is required for uPAR-mediated migratory responses (Smith and Marshall, 2010), but the involvement of Rho GTPases other than Rac1, Cdc42 and RhoA in uPA–uPAR signalling has not been tested. We investigated the role of 12 different Rho GTPases in uPA–uPAR responses in prostate carcinoma cells and found that silencing RhoB impaired uPAR-dependent signalling in cell migration and invasion. uPAR stimulation induced RhoB activation and also increased RhoB levels. We demonstrate that RhoB mediates uPAR-induced upregulation of surface integrin levels and signalling to paxillin, cofilin and Akt. It also controls the association of uPAR and the integrin-activating protein talin with integrins and modulates β1 integrin activation. These results demonstrate that RhoB is a crucial mediator of uPAR signalling and that it acts through integrins.

uPAR can bind independently or simultaneously to its two ligands, uPA and VN (Madsen et al., 2007; Smith and Marshall, 2010). We demonstrate that RhoB contributes to both uPA-driven uPAR responses and adhesion to VN. First, by using the amino-terminal fragment (ATF) of uPA, which binds to uPAR and activates uPAR signalling but is devoid of proteolytic activity, we could dissociate proteolytic effects of uPA from its effects on uPAR (Vincenza Carriero et al., 2009). This allowed us to demonstrate that uPAR-induced migration and invasion of PC3 cells required RhoB. Depletion of uPAR also inhibited uPA-induced migration and invasion, demonstrating the importance of uPAR signalling in PC3 cell migratory responses.

We show that RhoB is required for adhesion of PC3 cells to VN but not to other substrates, and for adhesion to VN of uPAR-overexpressing cells. However, RhoB overexpression does not rescue adhesion to VN after uPAR knockdown. Because uPAR is known to contribute to VN adhesion (Madsen et al., 2007) these results suggest that RhoB is specifically involved in mediating uPAR-dependent PC3 cell adhesion to VN. RhoB has previously been implicated in regulating cell adhesion in other cell types. For example, macrophages and fibroblasts from RhoB-null mice have defective adhesion and spreading (Liu et al., 2001; Wheeler and Ridley, 2007), and RhoB depletion inhibits adhesion of human lung epithelial cells (Bousquet et al., 2009). In

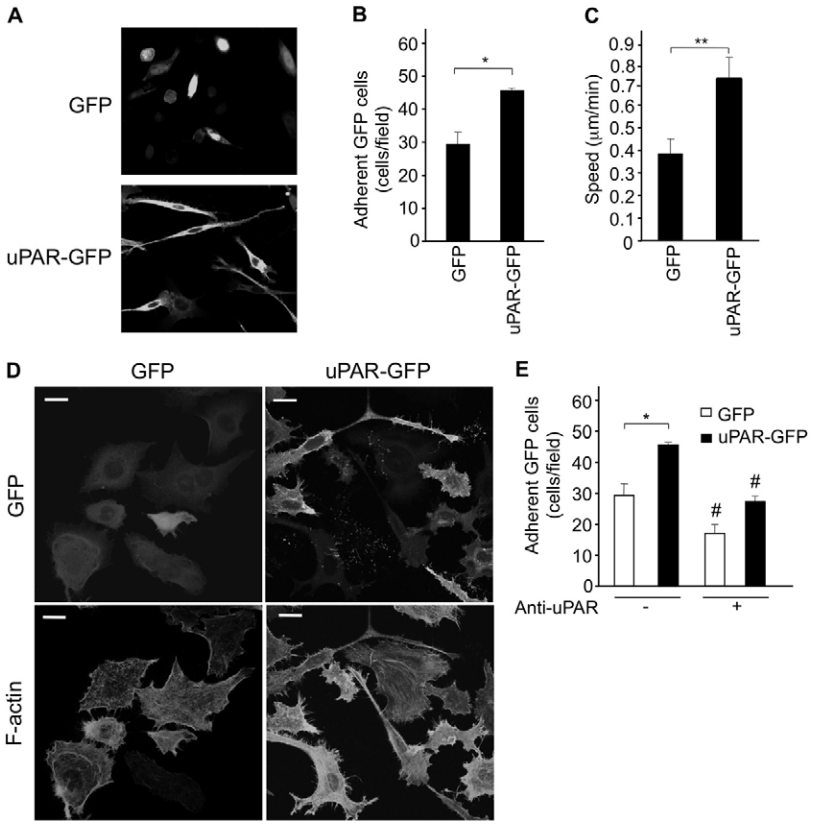

Fig. 6. uPAR expression regulates PC3 cell adhesion to vitronectin. (**A**) Representative fluorescence images of PC3 cells expressing uPAR–GFP or GFP alone as a control, plated on VN-coated plates. (**B**) PC3 cells expressing uPAR–GFP or GFP alone were plated onto VN-coated wells. After 1 hour, attached cells were fixed and GFP-expressing cells were counted. Data are from two different experiments, each carried out in triplicate, analysing 15 cells per field (~90 cells in total). Values are means ± s.d.; $*P<0.05$ compared with control. (**C**) Cell speed was determined by time-lapse microscopy, acquiring an image every 10 minutes over a period of 16 hours. Data are from 30 cells in two independent experiments. Values are means ± s.e.m., $**P<0.01$. (**D**) PC3 cells were transfected with a construct encoding uPAR–GFP or the GFP vector; 24 hours after transfection, cells were plated on VN-coated coverslips in the absence of serum. After a further 24 hours, cells were fixed and stained for F-actin. Scale bars: 20 μm. (**E**) PC3 cells expressing uPAR–GFP or GFP alone were pre-incubated for 30 minutes at 37℃ with a polyclonal 399 anti-uPAR antibody (25 μg/ml) or buffer as control, and then plated onto VN-coated wells. After 1 hour, attached cells were fixed and GFP-expressing cells were counted. Data are from two different experiments, each carried out in triplicate, analysing 15 cells per field (~90 cells in total). Values are means ± s.d.; $*P<0.05$ compared with control or $^{\#}P<0.05$ compared with the absence of anti-uPAR antibody.

macrophages, RhoB has been postulated to affect adhesion by affecting surface levels of β-integrins (Wheeler and Ridley, 2007). We demonstrate that RhoB mediates ATF-induced upregulation of surface integrin levels, explaining how RhoB specifically affects uPAR responses, and hence uPAR-dependent signalling. We also found that RhoB depletion reduces the level of activated β1 integrin; moreover the association of uPAR, as well as the integrin-activating protein talin, with integrins is strongly decreased in RhoB-depleted cells. RhoB could modulate uPAR–integrin and talin–integrin association through its effects on surface integrin levels and activity.

Integrins traffic dynamically between the plasma membrane and endosomes, and this trafficking is regulated by multiple stimuli (Caswell et al., 2009). RhoB might affect integrin surface levels and activity by regulating integrin endosomal trafficking. RhoB is known to regulate trafficking of tyrosine kinase receptors, including EGFR and PDGFRβ, to late endosomes and/or lysosomes and hence affect receptor signalling (Wherlock et al., 2004; Huang et al., 2007). The non-receptor tyrosine kinase Src is also dependent on RhoB for trafficking (Sandilands et al., 2004). RhoB has been postulated to affect protein trafficking by regulating actin polymerization on endosomes (Sandilands et al., 2004; Ridley, 2006).

We observed a striking ability of uPAR to activate RhoB: RhoB was rapidly activated by the addition of ATF. RhoB activation is also rapidly stimulated by exposure to ultra violet (UVB) and ionizing radiation, which, similar to ATF, subsequently increase RhoB protein levels (Canguilhem et al., 2005; Monferran et al., 2008). The activation of RhoB by uPAR could be mediated by integrins as co-receptors for uPAR. uPAR–integrin-β1 interaction has been reported to be required for uPA-induced activation of Rac1 and Cdc42 in MDA-MB-231 breast cancer cells (Sturge et al., 2002), and uPAR association with integrin β2 is involved in Rac1 and Cdc42 activation in microvascular endothelial cells (Margheri et al., 2006); moreover, uPAR-induced Rac activation is dependent on integrin β3 in a variety of cancer cell lines (Smith et al., 2008).

As well as activating RhoB, ATF induced RhoB mRNA and protein expression. RhoB gene expression is well known to be induced rapidly by a variety of different stimuli, including growth factors (Jähner and Hunter, 1991), stress stimuli such as UV irradiation and TGFβ (Jähner and Hunter, 1991; Fritz et al., 1995; Vasilaki et al., 2010). RhoB mRNA and protein have short half-lives (Fritz et al., 1995; Lebowitz et al., 1995) and thus it could be important to increase RhoB expression to maintain the levels required for sustained cell migration. Whether the uPAR-induced increase in RhoB levels is indeed required for uPAR-stimulated cell migration remains to be established, but interestingly RhoB expression is also induced by TGFβ, and RhoB contributes to TGFβ-induced migration of HaCaT keratinocytes and DU145 prostate cancer cells (Vasilaki et al., 2010).

RhoB is often considered a tumour suppressor because its expression is downregulated in some cancers, and this has been linked to its role in stimulating apoptosis (Huang and Prendergast, 2006; Vega and Ridley, 2008; Bousquet et al., 2009). By contrast, RhoB can either stimulate or inhibit cancer migration or invasion depending on the cellular context. For example, overexpression of RhoB suppresses invasion and/or migration of gastric cancer cell lines (Zhou et al., 2011) and hepatocellular carcinoma cell lines (Connolly et al., 2010). By

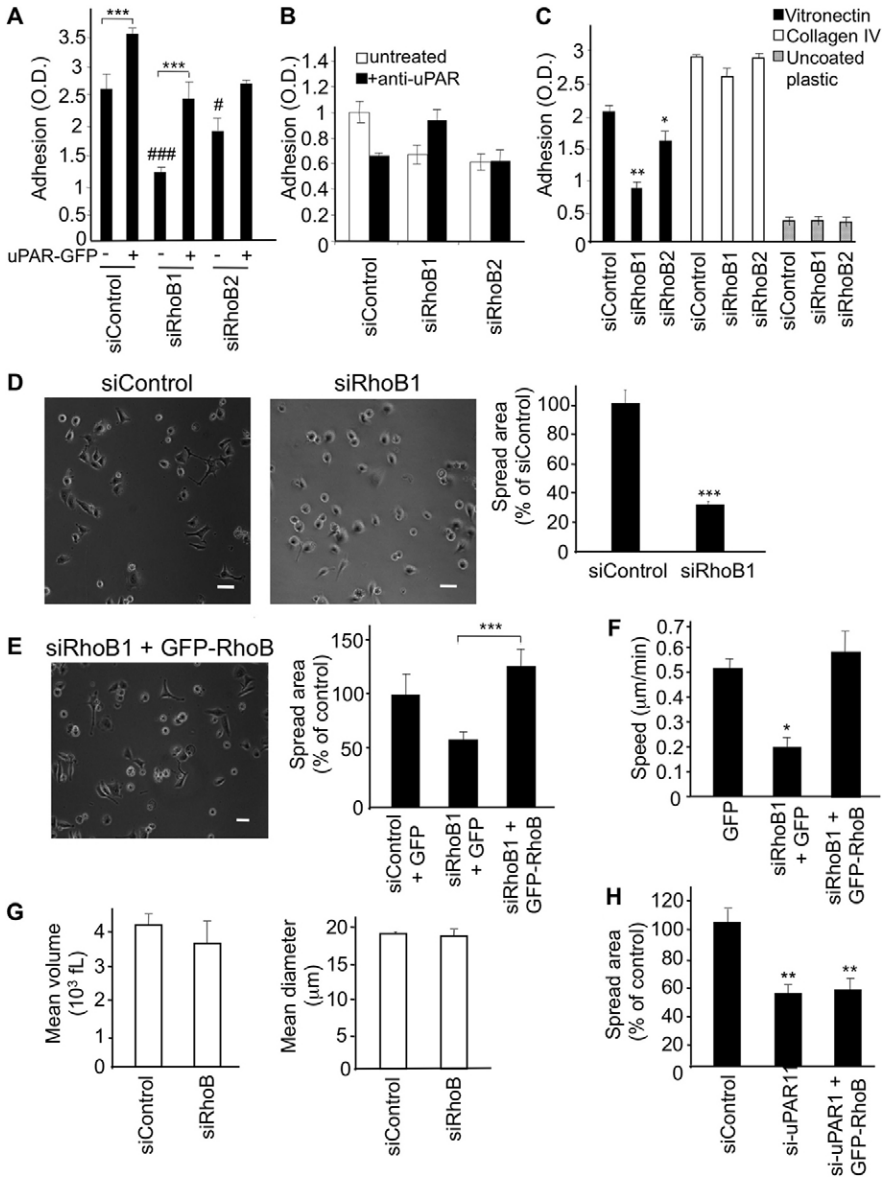

Fig. 7. RhoB specifically regulates PC3 cell adhesion to vitronectin. (**A**) PC3 cells were co-transfected with RhoB-targeting siRNA (siRhoB1 or siRhoB2) or control siRNA (siControl) and after 24 hours cells were transfected with a construct encoding uPAR–GFP or GFP alone; 24 hours later, cells were allowed to adhere to VN-coated wells for 30 minutes. The attached cells were fixed and stained with Crystal Violet. The stain was eluted, and the absorbance at 595 nm was measured by spectrophotometry. Values are the means ± s.d. of three experiments performed in triplicate, $*P<0.05$, $**P<0.01$, $***P<0.001$; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ compared with siControl. (**B**) PC3 cells were transfected with RhoB-targeting siRNA (siRhoB1 or siRhoB2) or control siRNA (siControl). After 48 hours they were incubated for 30 minutes at 37℃ with a polyclonal 399 anti-uPAR antibody (25 μg/ml) or buffer as control, and then cell were plated onto VN-coated wells and allowed to adhere for 1 hour. (**C**) Adhesion of PC3 cells transfected with control siRNA (siControl) or with RhoB-targeting siRNAs (siRhoB2 or siRhoB3) was determined 48 hours after transfection by allowing cells to adhere for 1 hour to plates coated with different substrates, as indicated. Values are the means ± s.d. of three independent experiments, each performed in triplicate, $*P<0.05$, $**P<0.01$ compared with control. (**D**) Phase-contrast images of PC3 cells transfected with control siRNA (siControl) or with RhoB-targeting siRNA (siRhoB1). 48 hours after transfection cells were seeded on VN-coated plates and cultured without serum, and 6 hours later they were imaged by time-lapse microscopy for 24 hours (left). Scale bars: 50 μm. Cell area was determined from time-lapse movie images using ImageJ software; $n\geq58$ cells from three independent experiments (right). Values are means ± s.d., $***P<0.001$ compared with control. (**E**) PC3 cells were transfected with RhoB-targeting siRNA (siRhoB1) or control siRNA (siControl). 24 hours later, cells were transfected with a construct encoding GFP–RhoB or GFP alone. After 48 hours, cells were added to VN-coated wells. (Left) Representative phase-contrast images; scale bars: 50 μm. (Right) Spread area of cells after seeding was determined using Image J. Values are means ± s.e.m., $***P<0.001$ compared with control. (**F**) Cell speed was determined by time-lapse microscopy, acquiring an image every 10 minutes over a period of 24 hours. Data are from 30 cells in two independent experiments. Values are means ± s.e.m., $*P<0.01$. (**G**) The volume and diameter of cells in suspension were measured. Graphs show data from three independent experiments, each carried out in triplicate. Results are normalized to control. Values are means ± s.d. (**H**) PC3 cells were transfected with uPAR-targeting siRNA (si-uPAR1) or control siRNA (siControl). 24 hours later, cells were transfected with a construct encoding GFP–RhoB or GFP alone. After a further 10 hours, cells were added to VN-coated wells and cultured in the absence of serum and imaged by time-lapse microscopy for 24 hours. After 24 hours, cell area was determined from time-lapse movie images; $n\geq100$ cells pooled from three independent experiments. Values are means ± s.d., $*P<0.05$ compared with siControl.

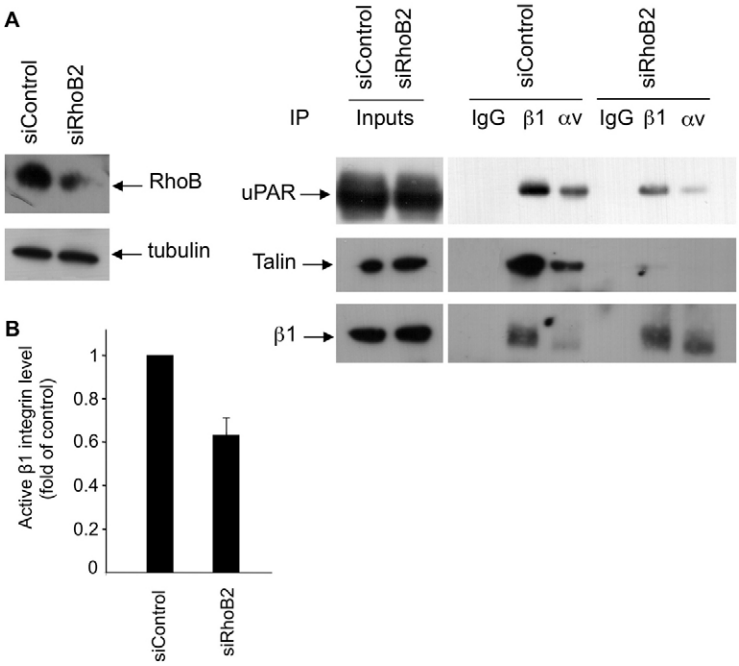

Fig. 8. RhoB depletion inhibits integrin association with uPAR. (**A**) PC3 cells were lysed 48 hours after transfection with siRhoB2 or siControl and analysed by immunoblotting with polyclonal anti-RhoB antibody, or anti-tubulin antibodies as a loading control. Total lysates were immunoprecipitated with anti-β1 or anti-αv antibodies. Both total lysates (inputs) and agarose bead eluates (IP) were subjected to electrophoresis and subsequently probed with the same antibodies used for immunoprecipitation, with R2 monoclonal anti-uPAR antibody and monoclonal anti-talin antibody. (**B**) PC3 cells were transfected with siRNAs, then serum-starved for 24 hours, and stained for surface levels of active β1 integrin (12G10 antibody), then analysed by flow cytometry. Values are the mean fluorescence of the population ± s.d. of two separate experiments each performed in duplicate, normalized to total β1 integrin levels.

contrast, in metastatic prostate cancer cells RhoB promotes migration (Yoneda et al., 2010), and RhoB depletion impairs TGF β-dependent migration (Vasilaki et al., 2010).

RhoB was required for uPAR-induced signal transduction to two proteins involved in cell migration, cofilin and paxillin. Cofilin induces actin filament severing in lamellipodia and is important for cell migration and invasion (Oser and Condeelis, 2009). We observed rapid cofilin phosphorylation in response to ATF, which is known to inhibit its activity. RhoA can stimulate cofilin phosphorylation through its downstream targets ROCK1 and ROCK2 (Oser and Condeelis, 2009). RhoB can also interact with ROCKs in vitro and can act through ROCK1 to stimulate NFκB activation (Rodriguez et al., 2007), but whether it regulates cofilin through ROCK is not known. uPA–uPAR was previously reported to increase tyrosine phosphorylation of paxillin as well as other focal adhesion proteins in endothelial cells (Tang et al., 1998). Because paxillin is activated by integrins and plays a central role in coordinating integrin-based adhesion turnover (Deakin and Turner, 2008), it is likely that RhoB regulates paxillin through its effects on integrins, and that paxillin then contributes to uPAR-induced migration.

uPA–uPAR play an important role in cancer dissemination, and thus downstream regulators of uPAR signalling could be targets for inhibiting cancer progression. Although RhoB is considered a tumour suppressor in some cancer cell types (Vega and Ridley, 2008), our data indicate that it could also play a role in cancer invasion as a downstream mediator of uPAR responses.

Materials and Methods

Reagents

Polyclonal anti-uPA antibody was a gift from P. A. Andreasen, Aarhus, Denmark. Monoclonal anti-uPAR R4 antibody was kindly provided by G. Hoyer-Hansen, Finsen Institute, Copenhagen, Denmark. Polyclonal anti-uPAR 399 antibody and ATF protein were purchased from American Diagnostica (Greenwich, CT). Polyclonal anti-RhoB and polyclonal anti-integrin β5 antibodies were from Santa Cruz Biotechnology (Insight Biotechnology, Wembley, UK). Antibodies to RhoA (clone 26C4), RhoB (119), RhoC (C-16) and Cdc42 (clone B-8) were from Santa Cruz Biotechnology, Rac1 (clone 23A) and anti-integrin αv were from Millipore (Watford, UK), mouse anti-RhoJ, rabbit anti-RhoQ, goat anti-RhoU, mouse anti-integrin β1 (clone P5D2) and mouse anti-active integrin β1 (clone 12G10) were from Abcam (Cambridge, UK). Monoclonal anti-RhoE antibody was described previously (Riento et al., 2003). Sheep anti-RhoF antibody was kindly provided by Harry Mellor, Bristol University, UK. Mouse anti-RhoG was a gift from Martin A. Schwartz (University of Virginia, USA). Polyclonal anti-phosphorylated Tyr118 paxillin was from Biosource, Invitrogen (Paisley, UK); monoclonal anti-paxillin (clone 349) was from BD Transduction Laboratories (Oxford, UK); polyclonal anti-phosphorylated Thr308 AKT was from Cell Signaling (New England Biolabs, Hitchin, UK). Collagen type IV, polyclonal anti-tubulin and monoclonal anti-talin antibodies were from Sigma-Aldrich (Dorset, UK). Vitronectin, Transwell filters and Matrigel-coated Transwell filters with a PET membrane with 8 μm pores were from BD Biosciences. ECL detection kit was from Amersham Biosciences. The complete mini EDTA-free protease inhibitor was from Roche (Welwyn Garden City, UK). Halt phosphatase inhibitor cocktail was from Pierce Biotechnology (Rockford, USA). All cell culture reagents, Lipofectamine 2000 and Oligofectamine were purchased from Invitrogen.

Cell culture and transfections

PC3 prostate cancer cells were cultured in Roswell Park Memorial Institute medium (RPMI) containing 10% FBS, L-glutamine (300 μg/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were seeded at 3×10^5 per well in six-well plates and transfected with siRNAs (final concentration 50 nM) in antibiotic-free medium using Oligofectamine according to the manufacturer's instructions. All Rho GTPase siRNAs were obtained from Dharmacon (Fisher Scientific UK, Loughborough, UK). A non-targeting siRNA was used as a control in all experiments (ON-TARGET control; Dharmacon). uPA and uPAR siRNAs were either from Qiagen or Sigma. All siRNA sequences are shown in supplementary material Table S1.

Immunoblotting

Cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.6, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) supplemented with 1 mM phenylmethylsulphonyl fluoride and 1× Complete mini EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor (Roche). Debris was removed by centrifugation at 10,000 ***g*** for 20 minutes at 4℃, and protein content was assessed by a Bradford protein assay. 50 μg proteins per sample were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore). Membranes were subsequently incubated for 2 hours at room temperature in TBST buffer [125 mM Tris-HCl (pH 8.0), 625 mM NaCl, 0.1% Tween 20] containing 5% BSA and further incubated at 4℃ for 16 hours with primary antibodies (used at a dilution of 1:500). Secondary HRP-conjugated mouse (GE Healthcare, Chalfont St Giles, UK) or rabbit (Dako, Ely, UK) antibodies were used at a dilution of 1:5000. Membranes were developed using the enhanced chemiluminescence system (GE Healthcare). X-ray films were scanned and analysed using a Bio-Rad GS-800 densitometer.

Co-immunoprecipitation

Immunoprecipitations were performed as previously described (Wei et al., 1996). PC3 cells (5×0^6/sample) were washed twice with microtubule stabilization buffer (0.1 M PIPES, pH 6.9, 2 M glycerol, 1 mM EGTA, 1 mM magnesium acetate) and then extracted in 0.2% Triton X-100 with additional protease inhibitors. The insoluble residue, enriched in cytoskeleton-associated proteins, was solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton-X-100, and protease inhibitors) and preincubated with non-immune serum and 10% protein-A–Sepharose for 2 hours at 4℃. After centrifugation, the supernatants were incubated with 2 µl of antisera to β1 integrin and αv integrin, or with 2 µl of non-immune serum for 2 hours at 4℃. After 30 minutes of incubation with 10% protein-A–Sepharose at room temperature, the immunoprecipitates were washed, subjected to 9% SDS-PAGE and analysed by western blotting using R2 monoclonal anti-uPAR antibody or monoclonal anti-talin antibody at a concentration of 1 µg/ml. Membranes were reprobed with monoclonal anti-β1 integrin or αv integrin antibody.

Measurement of uPA secretion

Cells were seeded in 24-well plates and, 24 hours later, were serum-starved for 16 hours. Equal amounts of protein from the conditioned medium (20 µg/sample) were concentrated by trichloroacetic acid precipitation, loaded under non-reducing conditions and resolved by SDS-PAGE, then subjected to immunoblotting analysis using a polyclonal anti-uPA antibody.

RhoB activity assay

The pull-down assay to measure RhoB activity was performed using a Rho activation assay kit, according to the manufacturer's protocol (Cytoskeleton, Denver, CO). Sub-confluent PC3 cells were serum-starved for 24 hours, then treated with acid buffer (50 mM glycine, 100 mM NaCl, pH 3) for 2 minutes to remove membrane-bound uPA. Cells were washed in serum-free medium. Cells were stimulated in serum-free medium with 10 nM ATF for 5 minutes at 37℃ before addition of lysis buffer (l50 mM Tris-HCl pH 7.5, 1 mM EDTA, 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 0.5% 2-mercaptoethanol) at 4℃. A protein assay was performed (Bio-Rad) and equal amounts of total protein were used for each pull-down assay. Lysates were incubated with GST–rhotekin–RBD on glutathione–agarose beads (Cytoskeleton) for 1 hour at 4℃, and then the beads were washed four times in lysis buffer. The agarose beads were boiled in SDS–PAGE sample buffer to release active Rho proteins, which were then processed for immunoblotting with an anti-RhoB antibody. Total cell lysate (30 µg) per sample was used to detect total RhoB.

Migration and invasion assays

Transwell filters coated with collagen IV (50 µg/ml, diluted in PBS) with a PET membrane with 8 µm pores (BD Biosciences) were rehydrated for 2 hours at 37℃ in medium without supplements. Transfected cells were washed once in PBS, and 10^5 cells were seeded into the upper chamber of the Transwells in serum-free medium containing 0.1% BSA. Medium containing 10 nM ATF and 0.1% BSA was added to the bottom chamber as a chemoattractant. Control wells without ATF were included to assess random migration. Cells were allowed to migrate for 4 hours at 37℃, in 5% CO_2. For invasion assays the Transwell filters were coated in Matrigel, and ATF or FCS was used as a chemoattractant, as indicated. The cells were allowed to invade for 22 hours at 37℃ and then the remaining cells and matrix were removed from the upper side of the membrane. The cells on the bottom part of the membrane were fixed in methanol containing 0.1% Crystal Violet. Ten separate bright-field images were randomly acquired of each Transwell filter using a Nikon TE2000-E microscope with a Plan Fluor 10× objective (Nikon, Kingston, UK). The cells in each image were counted and analysed in comparison with control-transfected cells. Migration or invasion in the absence of ATF (random migration) was taken as 100% for each siRNA treatment, to control for variations in final cell number.

For time-lapse microscopy, PC3 cells were seeded 24 hours after transfection in 24-well plates at sub-confluency (random migration assays) or confluency (scratch wound assays) in growth medium. For scratch wound assays, the confluent monolayer was wounded after 24 hours by scraping with a pipette tip and then imaged by time-lapse microscopy. During acquisition of movies, cells were incubated in a humidified chamber at 37℃, 5% CO_2 in serum-free medium. In some experiments CO_2 was not used and instead 25 mM HEPES was added. A phase-contrast image was acquired every 10 minutes for 24 hours on a fully motorized Nikon TE2000-E microscope with a Plan Fluor 10× objective using Metamorph 5.01 software (Molecular Devices, Wokingham, UK). For random migration assays, cell migration speed was quantified with ImageJ software using the plug-in 'manual tracking'. In each experiment, 30 randomly chosen cells were tracked and their mean migration speed was calculated. The area occupied by cells in scratch wounds was determined from time-lapse movie images taken at different time after wounding, using ImageJ analysis software (http://rsb.info.nih.gov/ij).

Adhesion assays

Flat-bottomed 96-well microtiter plates were coated with 10 µg/ml vitronectin, collagen or 1% heat-denatured BSA in PBS (uncoated plastic) as a negative control, and incubated overnight at 4℃. The plates were then blocked for 1 hour at room temperature with 1% heat-denatured BSA in PBS. Cells were harvested using trypsin and allowed to recover for 1 hour at 37℃, then washed three times in PBS. 10^5 cells were plated in each coated well and incubated for 1 hour at 37℃. Attached cells were fixed with 3% paraformaldehyde in PBS for 10 minutes and then incubated with 2% methanol for 10 minutes. The cells were finally stained with 0.5% Crystal Violet in 20% methanol. The stain was eluted using 0.1 M sodium citrate in 50% ethanol, pH 4.2, and the absorbance at 595 nm was measured in a spectrophotometer.

Flow cytometry analysis

PC3 cells were harvested in PBS containing 4 mM EDTA, washed in PBS containing Ca^{2+} and Mg^{2+}, and then 5×10^5 cells were incubated with 10 µg/ml of polyclonal anti-uPAR or polyclonal anti-integrin αv, monoclonal anti-integrin β1 or polyclonal anti-integrin β5 antibodies for 1 hour at 4℃. Purified immunoglobulin was used as a negative control. The cells were then washed and incubated with a fluorescein isothiocyanate-labelled goat anti-rabbit or anti-mouse IgG for 30 minutes at 4℃. For analysis of surface-active β1 integrin levels, cells were scraped into PBS, fixed in 4% PFA–PBS for 30 minutes on ice and blocked in 5% BSA–PBS for 30 minutes on ice. Cells were then incubated with 10 µg/ml anti-integrin β1 (12G10) overnight at 4℃. Finally, the cells were washed and analysed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA).

Quantitative PCR

PC3 cells were transfected with siRNAs. After 72 hours, cells were washed with PBS, then total RNA was isolated by acid-phenol extraction using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. 1 µg of total RNA was reverse-transcribed using QuantiTect Reverse Transcription (Qiagen, Hilden, Germany); 1 µl of a 1:10 dilution of the reverse transcription reaction was analysed by qPCR with a Bio-Rad IQ5 system, using IQ™SYBR Green Supermix for qPCR kit. The mRNAs measured were normalized to the internal glyceraldehyde-3-phosphate dehydrogenase mRNA. Primers, designed using Primer3 software and used at 0.25 µM, were as follows: for uPAR amplification, forward primer 5′-AAGGATACAGCTGGAGTCAG-3′ and reverse primer 5′-GAGTTCATTCACTACCTGTTC-3′; for RhoB amplification: forward primer 5′-CATTCTGACCACACTTGTACGC-3′ and reverse primer 5′- GGTTTCTTTTCC-CTCTCCTTGT-3′; for glyceraldehyde-3-phosphate dehydrogenase amplification, forward primer 5′-ACATGTTCCAATATGATTCCA-3′ and reverse primer 5′-TGGACTCCACGACGTACTCAG-3′. The relative level of expression was calculated with the formula 2–ΔΔCt.

Statistical analysis

Statistical analysis was carried out where indicated using data from three or more independent experiments each in triplicate, unless stated otherwise. Differences between data sets were determined by using an unpaired Student's *t*-test.

Acknowledgements

We are grateful to Ritu Garg for technical support, Dario Gallotta and Roberta Cotugno for assistance with flow cytometry, Francisco Vega, Ferran Valderrama, Sarah Heasman and Philippe Riou for comments and discussions, P. A. Andreasen, G. Hoyer-Hansen, H. Mellor and M. A. Schwartz for antibodies, and Nicolai Sidenius for providing the construct encoding uPAR–GFP.

Funding

This work was supported by grants from Cancer Research UK [grant number C6620/A8833] to A.J.R., Italian Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2007) [grant number 2007YJAP2M] to P.R.; Associazione Italiana per la Ricerca sul Cancro [grant number IG 4714] to P.R.; the European Molecular Biology Organization [grant number ASTF 270.00-2007 to D.A.]; and a short-term fellowship from the Federation of the Societies of Biochemistry and Molecular Biology to D.A.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.091579/-/DC1

References

Aguirre-Ghiso, J. A., Estrada, Y., Liu, D. and Ossowski, L. (2003). ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). *Cancer Res.* **63**, 1684-1695.

Alfano, D., Franco, P., Vocca, I., Gambi, N., Pisa, V., Mancini, A., Caputi, M., Vincenza Carriero, M., Iaccarino, I. and Stoppelli, M. P. (2005). The urokinase plasminogen activator and its receptor: role in cell growth and apoptosis. *Thromb. Haemost.* **93**, 205-211.

Alfano, D., Iaccarino, I. and Stoppelli, M. P. (2006). Urokinase signaling through its receptor protects against anoikis by increasing BCL-xL expression levels. *J. Biol. Chem.* **281**, 17758-17767.

Almasi, C. E., Brasso, K., Iversen, P., Pappot, H., Høyer-Hansen, G., Danø, K. and Christensen, I. J. (2011). Prognostic and predictive value of intact and cleaved forms of the urokinase plasminogen activator receptor in metastatic prostate cancer. *Prostate* **71**, 899-907.

Bai, S. W., Herrera-Abreu, M. T., Rohn, J. L., Racine, V., Tajadura, V., Survavanshi, N., Bechtel, S., Wiemann, S., Baum, B. and Ridley, A. J. (2011). Identification and characterization of a set of conserved and new regulators of cytoskeletal organization, cell morphology and migration. *BMC Biol.* **9**, 54.

Bamburg, J. R. (1999). Proteins of the ADF/cofilin family: essential regulators of actin dynamics. *Annu. Rev. Cell Dev. Biol.* **15**, 185-230.

Blasi, F. and Carmeliet, P. (2002). uPAR: a versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.* **3**, 932-943.

Blasi, F. and Sidenius, N. (2010). The urokinase receptor: focused cell surface proteolysis, cell adhesion and signaling. *FEBS Lett.* **584**, 1923-1930.

Calderwood, D. A. (2004). Integrin activation. *J. Cell Sci.* **117**, 657-666.

Canguilhem, B., Pradines, A., Baudoin, C., Boby, C., Lajoie-Mazenc, I., Charveron, M. and Favre, G. (2005). RhoB protects human keratinocytes from UVB-induced apoptosis through epidermal growth factor receptor signaling. *J. Biol. Chem.* **280**, 43257-43263.

Caswell, P. T., Vadrevu, S. and Norman, J. C. (2009). Integrins: masters and slaves of endocytic transport. *Nat. Rev. Mol. Cell Biol.* **10**, 843-853.

Chandrasekar, N., Mohanam, S., Gujrati, M., Olivero, W. C., Dinh, D. H. and Rao, J. S. (2003). Downregulation of uPA inhibits migration and PI3k/Akt signaling in glioblastoma cells. *Oncogene* **22**, 392-400.

Connolly, E. C., Van Doorslaer, K., Rogler, L. E. and Rogler, C. E. (2010). Overexpression of miR-21 promotes an in vitro metastatic phenotype by targeting the tumor suppressor RHOB. *Mol. Cancer Res.* **8**, 691-700.

Bousquet, E., Mazières, J., Privat, M., Rizzati, V., Casanova, A., Ledoux, A., Mery, E., Couderc, B., Favre, G. and Pradines, A. (2009). Loss of RhoB expression promotes migration and invasion of human bronchial cells via activation of AKT1. *Cancer Res.* **69**, 6092-6099.

Danø, K., Behrendt, N., Høyer-Hansen, G., Johnsen, M., Lund, L. R., Ploug, M. and Rømer, J. (2005). Plasminogen activation and cancer. *Thromb. Haemost.* **93**, 676-681.

Deakin, N. O. and Turner, C. E. (2008). Paxillin comes of age. *J. Cell Sci.* **121**, 2435-2444.

Dedhar, S., Saulnier, R., Nagle, R. and Overall, C. M. (1993). Specific alterations in the expression of α3β1 and α6β4 integrins in highly invasive and metastatic variants of human prostate carcinoma cells selected by in vitro invasion through reconstituted basement membrane. *Clin. Exp. Metastasis* **11**, 391-400.

Deng, G., Curriden, S. A., Wang, S., Rosenberg, S. and Loskutoff, D. J. (1996). Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? *J. Cell Biol.* **134**, 1563-1571.

Franco, P., Vocca, I., Vincenza Carriero, M., Alfano, D., Cito, L., Longanesi-Cattani, I., Grieco, P., Ossowski, L. and Stoppelli, M. P. (2006). Activation of urokinase receptor by a novel interaction between the connecting peptide region of urokinase and αvβ5 integrin. *J. Cell Sci.* **119**, 3424-3434.

Fritz, G., Kaina, B. and Aktories, K. (1995). The ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments. *J. Biol. Chem.* **270**, 25172-25177.

Harbeck, N., Kates, R. E., Look, M. P., Meijer-Van Gelder, M. E., Klijn, J. G., Krüger, A., Kiechle, M., Jänicke, F., Schmitt, M. and Foekens, J. A. (2002). Enhanced benefit from adjuvant chemotherapy in breast cancer patients classified high-risk according to urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (n = 3424). *Cancer Res.* **62**, 4617-4622.

Heasman, S. J. and Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nat. Rev. Mol. Cell Biol.* **9**, 690-701.

Huang, M. and Prendergast, G. C. (2006). RhoB in cancer suppression. *Histol. Histopathol.* **21**, 213-218.

Huang, M., Duhadaway, J. B., Prendergast, G. C. and Laury-Kleintop, L. D. (2007). RhoB regulates PDGFR-beta trafficking and signaling in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **27**, 2597-2605.

Irigoyen, J. P., Muñoz-Cánoves, P., Montero, L., Koziczak, M. and Nagamine, Y. (1999). The plasminogen activator system: biology and regulation. *Cell. Mol. Life Sci.* **56**, 104-132.

Jähner, D. and Hunter, T. (1991). The ras-related gene rhoB is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. *Mol. Cell. Biol.* **11**, 3682-3690.

Jo, M., Thomas, K. S., Somlyo, A. V., Somlyo, A. P. and Gonias, S. L. (2002). Cooperativity between the Ras-ERK and Rho-Rho kinase pathways in urokinase-type plasminogen activator-stimulated cell migration. *J. Biol. Chem.* **277**, 12479-12485.

Kiian, I., Tkachuk, N., Haller, H. and Dumler, I. (2003). Urokinase-induced migration of human vascular smooth muscle cells requires coupling of the small GTPases RhoA and Rac1 to the Tyk2/PI3-K signalling pathway. *Thromb. Haemost.* **89**, 904-914.

Kjoller, L. and Hall, A. (2001). Rac mediates cytoskeletal rearrangements and increased cell motility induced by urokinase-type plasminogen activator receptor binding to vitronectin. *J. Cell Biol.* **152**, 1145-2257.

Lebowitz, P. F., Davide, J. P. and Prendergast, G. C. (1995). Evidence that farnesyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. *Mol. Cell. Biol.* **15**, 6613-6622.

Liu, A. X., Rane, N., Liu, J. P. and Prendergast, G. C. (2001). RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells. *Mol. Cell. Biol.* **21**, 6906-6912.

Llinas, P., Le Du, M. H., Gårdsvoll, H., Danø, K., Ploug, M., Gilquin, B., Stura, E. A. and Ménez, A. (2005). Crystal structure of the human urokinase plasminogen activator receptor bound to an antagonist peptide. *EMBO J.* **24**, 1655-1663.

Madsen, C. D. and Sidenius, N. (2008). The interaction between urokinase receptor and vitronectin in cell adhesion and signalling. *Eur. J. Cell Biol.* **87**, 617-629.

Madsen, C. D., Ferraris, G. M., Andolfo, A., Cunningham, O. and Sidenius, N. (2007). uPAR-induced cell adhesion and migration: vitronectin provides the key. *J. Cell Biol.* **177**, 927-939.

Margheri, F., Manetti, M., Serrati, S., Nosi, D., Pucci, M., Matucci-Cerinic, M., Kahaleh, B., Bazzichi, L., Fibbi, G., Ibba-Manneschi, L. et al. (2006). Domain 1 of the urokinase-type plasminogen activator receptor is required for its morphologic and functional, beta2 integrin-mediated connection with actin cytoskeleton in human microvascular endothelial cells: failure of association in systemic sclerosis endothelial cells. *Arthritis Rheum.* **54**, 3926-3938.

Monferran, S., Skuli, N., Delmas, C., Favre, G., Bonnet, J., Cohen-Jonathan-Moyal, E. and Toulas, C. (2008). αvβ3 and αvβ5 integrins control glioma cell response to ionising radiation through ILK and RhoB. *Int. J. Cancer* **123**, 357-364.

Montuori, N., Visconte, V., Rossi, G. and Ragno, P. (2005). Soluble and cleaved forms of the urokinase-receptor: degradation products or active molecules? *Thromb. Haemost.* **93**, 192-198.

Montuori, N., Bifulco, K., Vincenza Carriero, M., La Penna, C., Visconte, V., Alfano, D., Pesapane, A., Rossi, F. W., Salzano, S., Rossi, G. et al. (2011). The cross-talk between the urokinase receptor and fMLP receptors regulates the activity of the CXCR4 chemokine receptor. *Cell. Mol. Life Sci.* **68**, 2453-2467.

Muller, S. M., Okan, E. and Jones, P. (2000). Regulation of urokinase receptor transcription by Ras- and Rho-family GTPases. *Biochem. Biophys. Res. Commun.* **270**, 892-898.

Oser, M. and Condeelis, J. (2009). The cofilin activity cycle in lamellipodia and invadopodia. *J. Cell. Biochem.* **108**, 1252-1262.

Pulukuri, S. M., Gondi, C. S., Lakka, S. S., Jutla, A., Estes, N., Gujrati, M. and Rao, J. S. (2005). RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo. *J. Biol. Chem.* **280**, 36529-36540.

Rabbani, S. A., Ateeq, B., Arakelian, A., Valentino, M. L., Shaw, D. E., Dauffenbach, L. M., Kerfoot, C. A. and Mazar, A. P. (2010). An anti-urokinase plasminogen activator receptor antibody (ATN-658) blocks prostate cancer invasion, migration, growth, and experimental skeletal metastasis in vitro and in vivo. *Neoplasia* **12**, 778-788.

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

Riento, K., Guasch, R. M., Garg, R., Jin, B. and Ridley, A. J. (2003). RhoE binds to ROCK I and inhibits downstream signaling. *Mol. Cell. Biol.* **23**, 4219-4229.

Rodriguez, P. L., Sahay, S., Olabisi, O. O. and Whitehead, I. P. (2007). ROCK I-mediated activation of NF-kappaB by RhoB. *Cell. Signal.* **19**, 2361-2369.

Sandilands, E., Cans, C., Fincham, V. J., Brunton, V. G., Mellor, H., Prendergast, G. C., Norman, J. C., Superti-Furga, G. and Frame, M. C. (2004). RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. *Dev. Cell* **7**, 855-869.

Schaller, M. D. and Parsons, J. T. (1995). pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol. Cell. Biol.* **15**, 2635-2645.

Sehgal, I., Foster, T. P. and Francis, J. (2006). Prostate cancer cells show elevated urokinase receptor in a mouse model of metastasis. *Cancer Cell Int.* **6**, 21.

Sheng, S. (2001). The urokinase-type plasminogen activator system in prostate cancer metastasis. *Cancer Metastasis Rev.* **20**, 287-296.

Smith, H. W. and Marshall, C. J. (2010). Regulation of cell signalling by uPAR. *Nat. Rev. Mol. Cell Biol.* **11**, 23-36.

Smith, H. W., Marra, P. and Marshall, C. J. (2008). uPAR promotes formation of the p130Cas-Crk complex to activate Rac through DOCK180. *J. Cell Biol.* **182**, 777-790.

Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F. and Assoian, R. K. (1985). Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. *Proc. Natl. Acad. Sci. USA* **82**, 4939-4943.

Sturge, J., Hamelin, J. and Jones, G. E. (2002). N-WASP activation by a β1-integrin-dependent mechanism supports PI3K-independent chemotaxis stimulated by urokinase-type plasminogen activator. *J. Cell Sci.* **115**, 699-711.

Tang, H., Kerins, D. M., Hao, Q., Inagami, T. and Vaughan, D. E. (1998). The urokinase-type plasminogen activator receptor mediates tyrosine phosphorylation of focal adhesion proteins and activation of mitogen-activated protein kinase in cultured endothelial cells. *J. Biol. Chem.* **273**, 18268-18272.

Vasilaki, E., Papadimitriou, E., Tajadura, V., Ridley, A. J., Stournaras, C. and Kardassis, D. (2010). Transcriptional regulation of the small GTPase RhoB gene by TGFbeta-induced signaling pathways. *FASEB J.* **24**, 891-905.

Vassalli, J. D., Baccino, D. and Belin, D. (1985). A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. *J. Cell Biol.* **100**, 86-92.

Vega, F. M. and Ridley, A. J. (2008). Rho GTPases in cancer cell biology. *FEBS Lett.* **582**, 2093-2101.

Vega, F., Fruhwirth, G., Ng, T. and Ridley, A. J. (2011). RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. *J. Cell Biol.* **193**, 655-665.

Vincenza Carriero, M. and Stoppelli, M. P. (2011). The urokinase-type plasminogen activator and the generation of inhibitors of urokinase activity and signaling. *Curr. Pharm. Des.* **17**, 1944-1961.

Vincenza Carriero, M., Franco, P., Vocca, I., Alfano, D., Longanesi-Cattani, I., Bifulco, K., Mancini, A., Caputi, M. and Stoppelli, M. P. (2009). Structure, function and antagonists of urokinase-type plasminogen activator. *Front. Biosci.* **14**, 3782-3794.

Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V. and Chapman, H. A. (1996). Regulation of integrin function by the urokinase receptor. *Science* **273**, 1551-1555.

Wheeler, A. P. and Ridley, A. J. (2004). Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp. Cell Res.* **301**, 43-49.

Wheeler, A. P. and Ridley, A. J. (2007). RhoB affects macrophage adhesion, integrin expression and migration. *Exp. Cell Res.* **313**, 3505-3516.

Wherlock, M., Gampel, A., Futter, C. and Mellor, H. (2004). Farnesyltransferase inhibitors disrupt EGF receptor traffic through modulation of the RhoB GTPase. *J. Cell Sci.* **117**, 3221-3231.

Yoneda, M., Hirokawa, Y. S., Ohashi, A., Uchida, K., Kami, D., Watanabe, M., Yokoi, T., Shiraishi, T. and Wakusawa, S. (2010). RhoB enhances migration and MMP1 expression of prostate cancer DU145. *Exp. Mol. Pathol.* **88**, 90-95.

Zhou, J., Zhu, Y., Zhang, G., Liu, N., Sun, L., Liu, M., Qiu, M., Luo, D., Tang, Q., Liao, Z. et al. (2011). A distinct role of RhoB in gastric cancer suppression. *Int. J. Cancer* **128**, 1057-1068.