

ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility

Emmanuel Vial, Erik Sahai, and Christopher J Marshall*

Cancer Research UK Centre for Cell and Molecular Biology, Institute of Cancer Research, 237 Fulham Road, London, SW3 6JB, United Kingdom

*Correspondence: chris.marshall@icr.ac.uk

Summary

We describe two signaling events downstream of ERK-MAP kinase contributing to cell motility in colon carcinoma cells. The Fos family member Fra-1 is expressed in an ERK-dependent manner. Silencing of Fra-1 expression with short interfering RNAs leads to losses of cell polarization, motility, and invasiveness in vitro. These effects of ablating Fra-1 are a consequence of activation of a RhoA-ROCK pathway by β 1-integrin, leading to an increase in the amount of stress fibers and stabilization of focal adhesions. We propose that Fra-1 promotes cell motility by inactivating β 1-integrin and keeping RhoA activity low. This depression of RhoA activity is necessary to permit a second ERK-dependent signaling event via uPAR, the receptor for urokinase-type plasminogen activator, to activate Rac and to drive motility through polarized lamellipodia extension.

Introduction

Unraveling the signaling pathways responsible for the establishment of a metastatic phenotype in carcinoma cells is of crucial importance for the understanding of the pathology of cancer. The process of metastasis has several components including the ability to invade through acquisition of cell motility, degradation of extracellular matrix and basement membranes, survival signaling, and cell proliferation. At present, it is not clear whether there are distinct metastatic genes activated during tumor progression or whether oncogenes and tumor suppressor genes, activated at earlier stages of oncogenesis, are the major genetic changes contributing to the metastatic phenotype (Bernards and Weinberg, 2002).

Much evidence points to the involvement of the Rho family of GTPases in cell motility and invasive phenotypes (Jaffe and Hall, 2002). Rho family members Rac, Cdc42, and RhoA are key regulators of the actin cytoskeleton (Hall, 1998). In the process of cell migration, Rac is required for the formation of lamellipodia at the leading edge of the migrating cells and is thought to be the driving force for cell movement (Jaffe and Hall, 2002). Cdc42, while not directly required for cell movement, has been suggested to be involved in the regulation of cell polarity, controlling the direction of cell movement (Allen et al., 1998; Nobes and Hall, 1999). Rho regulates the assembly of actin stress fibers and associated focal adhesions through activation of its downstream effectors mDia and the ROCKI and ROCKII kinases

(Amano et al., 1997; Watanabe et al., 1999). The role of Rho in cell movement is less clear; its activity is required to induce actomyosin contractility, driving the translocation of the cell body retraction at the rear (Allen et al., 1997). Nevertheless, Rho may also negatively influence cell migration by increasing stress fiber-dependent adhesions to the substrate (Cox et al., 2001). A tight control of the activity of Rho seems to be required to balance the opposing effects of cell body contraction and adhesion.

As well as contributions of the Rho family GTPases to the metastatic phenotype, several lines of evidence point to involvement of Ras oncoproteins (Varghese et al., 2002; Webb et al., 1998). Three classes of Ras effectors are now well established: the Raf family of protein kinases, phosphatidylinositol-3 kinases (PI3Ks), and the Ral guanine nucleotide exchange factors (Ral-GEFs) (Downward, 1998; Marshall, 1996; Wolthuis and Bos, 1999). The Raf kinases phosphorylate the dual specific kinase Mek, which in turn phosphorylates and activates the MAP kinases ERK1 and ERK2. The role of Raf-ERK MAP kinase signaling downstream of Ras has been highlighted in a number of studies of invasive growth (Janda et al., 2002; Webb et al., 1998). The importance of signaling through Raf in oncogenesis is emphasized by the discovery of oncogenic mutations of BRAF (Davies et al., 2002). The ERK-MAPK signaling pathway activated by Raf appears to be of critical importance since its inhibition abolishes growth in mice of the HT29 human colon tumor

SIGNIFICANCE

The acquisition of cell motility is a key property of tumor cells with an invasive phenotype. We show that the ERK-MAPK pathway downstream of oncogenic Ras can regulate the activity of the small GTPases Rac and RhoA and thereby contribute to tumor cell motility and invasion. ERK-MAPK signaling to RhoA is via transcriptional upregulation of the Fra-1 transcription factor and to Rac via transcriptional upregulation of the urokinase receptor (uPAR). Those two molecules are responsible for the coordinated regulation of GTPase activity permitting the formation of cellular protrusions. Thus, inhibition of ERK-MAPKs may provide a route to blocking tumor cell motility and invasion as well as proliferation.

cell line that contains an activating BRAF mutation (Sebolt-Leopold et al., 1999).

Activated ERKs have many substrates including cytosolic, cytoskeletal components, and transcription factors. The AP-1 family of transcription factors are known to be targets of ERK signaling. AP-1 consists of dimers composed of transcription factors of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families (Angel and Karin, 1991). AP-1 has been shown to be a major mediator of transformation by Ras (Mechta et al., 1997). The signaling pathway from Ras to AP-1 appears to be through the Raf-ERK MAP kinase cascade since signaling through Raf and Mek1 (Treinies et al., 1999) modifies AP-1 activity and composition. The importance of AP-1 activity for Ras-dependent transformation has been demonstrated in a number of studies in which c-Jun inhibition has been shown to revert the transformed phenotype induced by Ras in fibroblasts (Johnson et al., 1996; Lloyd et al., 1991; Suzuki et al., 1994). In contrast to c-Jun, a requirement for c-Fos in Ras-mediated transformation is less clear. Fra-1 is the predominant Fos family protein contributing to AP-1 activity in Ras- and Mek1-transformed fibroblasts (Mechta et al., 1997; Treinies et al., 1999). The ERK-MAPK pathway controls Fra-1 expression transcriptionally and posttranscriptionally in colon carcinoma cells containing KRAS oncogenes (E.V. and C.J.M., unpublished results). An important role for Fra-1 in Ras-induced tumorigenesis is suggested by the fact that antisense-mediated inhibition of Fra-1 antagonizes the Ras-induced colony formation in soft agar of thyroid cell lines (Vallone et al., 1997).

We demonstrate that ERK-MAPK-dependent expression of Fra-1 is required for motility and invasiveness of colon carcinoma cells. We show that Fra-1 suppresses β 1-integrin signaling, resulting in the downregulation of RhoA activity. Depression of Rho-GTP levels prevents ROCK from inducing excessive contractile and adhesive forces, which would lead to increased focal adhesions, inhibition of protrusions, and loss of cell motility. We propose that Fra-1 promotes motility in cancer cells by keeping RhoA activity low. In addition, we show that the activation of Rac required for cell motility can also be mediated by ERK-MAPK signaling. We provide evidence for a Fra-1-independent role of ERK signaling, via expression of uPAR, to activate Rac and regulate lamella/ruffling activity. Our data suggest that in colon carcinoma cells, there are two signaling pathways downstream of ERK regulating motility and invasiveness: (1) a Fra-1-dependent pathway leading to the downregulation of RhoA necessary for the extension of the ruffles into active extending protrusions; (2) a Fra-1-independent uPAR-dependent pathway leading to the activation of Rac1 and the initial formation of ruffles.

Results

Fra-1 RNAi decreases tumor cell motility and invasion

Fra-1 is the Fos family member predominantly expressed in Hct-116 and BE colon carcinoma cells and is regulated by the ERK-MAPK pathway via both transcriptional and posttranscriptional mechanisms (E.V. and C.J.M., unpublished results). To investigate the role of Fra-1 in colon cancer cells, short interfering RNAs (siRNAs) specific for the *fosL1* gene, which encodes Fra-1, were transfected into BE cells. BE cells contain an oncogenic KrasG13D mutation as well as a BRAF oncogenic mutation G463V (Davies et al., 2002). As shown by immunoblotting, Fra-1

protein expression was dramatically reduced (90%–95%), as soon as 2 days after transfection, and the silencing persisted for more than 6 days (ten generations) (Figure 1A). After transfection of the siRNAs, most of the immunofluorescence staining for nuclear Fra-1 disappeared, confirming its functional inhibition (Figure 1B). Furthermore, the overall AP-1 DNA binding activity in BE cells, essentially composed of c-Jun and Fra-1, was reduced by 90% upon Fra-1 silencing (Supplemental Figure S1 online at <http://www.cancer-cell.org/cgi/content/full/4/1/67/DC1>).

BE cells display strong invasive properties in vitro. These tumor cells efficiently migrate through a three-dimensional Matrigel matrix, but upon inhibition of Fra-1 expression, the proportion of invading cells was reduced by more than 80% (Figure 1D). Most of the cells remained at the bottom of the filter (and in the filter), where initially seeded, and were unable to enter and migrate through the Matrigel (Figure 1C). This decrease was not due to a defect in cell growth since Fra-1 silencing did not affect cell proliferation (data not shown); instead, we observed that random motility on a tissue culture dish was severely impaired. The average migration speed was reduced from approximately 18 to 1.8 μ m/hour (Figure 1E). This loss of motility is likely to be responsible, at least in part, for the loss of invasiveness. These observations show that in BE colon carcinoma cells, Fra-1 (AP-1) activity is required for motility and invasiveness. A similar effect on cell motility was observed when Hct-116, a colon carcinoma cell line, which contains a KrasG13D oncogene, was transfected with Fra-1-specific siRNAs (see Supplemental Movies S1A and S1B on *Cancer Cell* website).

Fra-1 downregulates Rho-GTP but does not alter Rac or Cdc42 levels

We next sought to determine the mechanism by which Fra-1 regulates motility. Small GTPases of the Rho family play key roles in regulating the actin cytoskeleton and the cellular responses required for cell migration (Ridley, 2001). A link between AP-1 and Rho-GTPase function in invasion has been suggested by a study showing that c-Jun activity is necessary, upstream of Rho-like GTPases, for EGF-induced cell motility and invasiveness (Malliri et al., 1998). RhoA, Rac, and Cdc42 activities were assessed in pulldown assays by measurement of the levels of active, GTP bound proteins. Upon Fra-1 inhibition, the levels of active RhoA were increased with no change in RhoA expression (Figure 2A), demonstrating that Fra-1 inhibits RhoA activity in BE cells. In contrast, the levels of active Rac and Cdc42 were not altered.

We next analyzed the effect of Fra-1 inhibition on cell morphology and actin organization. BE cells are strongly dedifferentiated carcinoma cells. Phase contrast analysis showed that BE cells display very elongated, mainly bipolar, fibroblastoid shape (Figure 2B). They migrated as single cells, forming extended protrusions followed by translocation of the cell body (Supplemental Movie S2A on *Cancer Cell* website). Upon Fra-1 inhibition, the cells became rounder and flatter and did not form protrusions. This was accompanied by a dramatic increase in the number of stress fibers and in the size and number of focal adhesions (Figure 2B), consistent with an increase in RhoA-GTP. In fibroblasts, the formation of protrusions, which are the driving force of cell movement, is dependent upon the creation of Rac-dependent (Ridley et al., 1992), actin-rich structures at the leading edge of the cell, forming membrane ruffles. Consistent with the absence of an effect on Rac-GTP, Fra-1-silenced

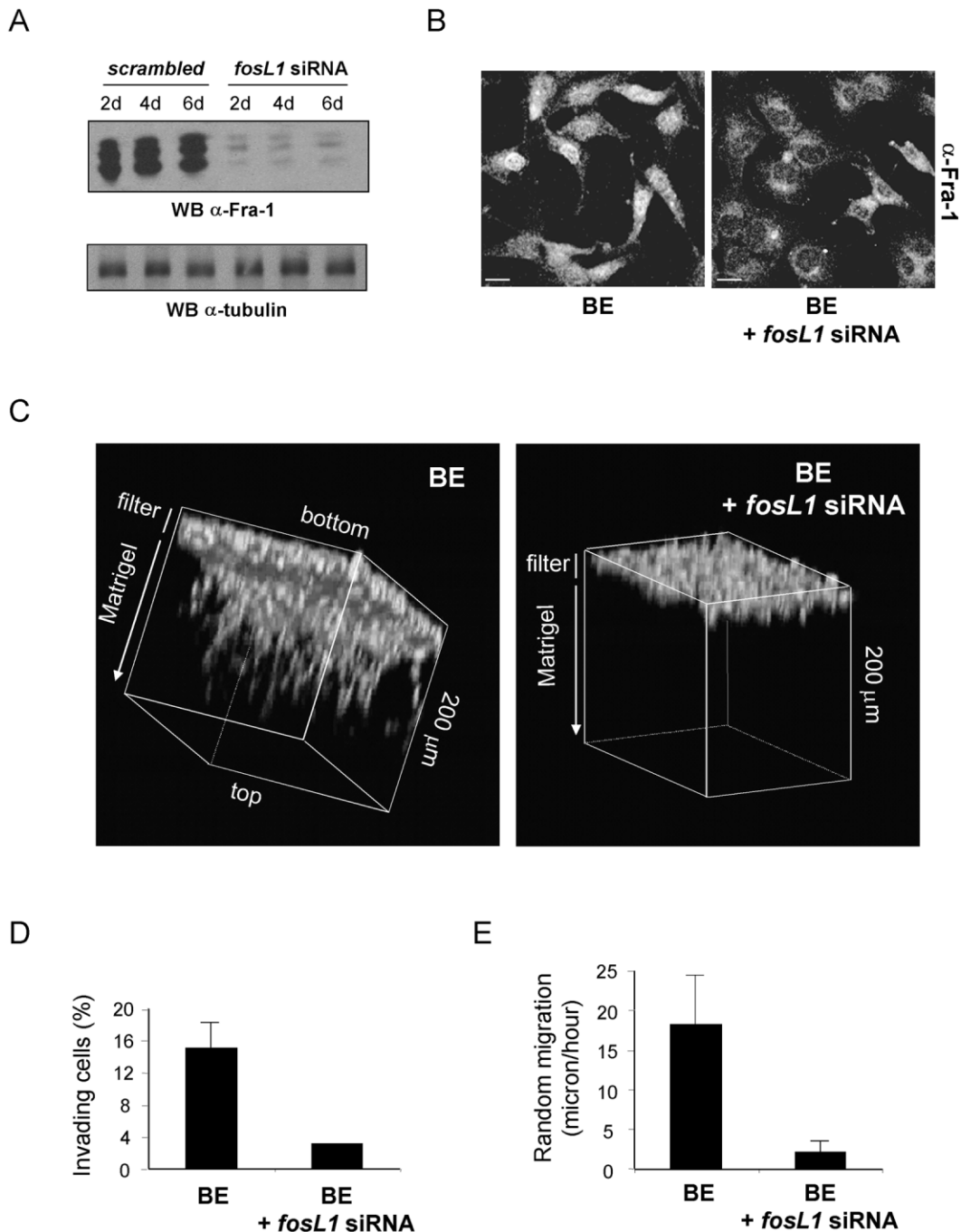


Figure 1. Fra-1 silencing inhibits cell motility and invasiveness

A: Immunoblot analysis of Fra-1 expression in BE cells after transfection of *fosL1*-specific siRNAs to silence Fra-1 expression or scrambled siRNAs.

B: Immunofluorescence analysis of Fra-1 expression in BE cells 48 hr after transfection of *fosL1* or scrambled siRNAs. Bars, 15 μ m.

C: Three-dimensional representations of fluorescence of GFP-expressing BE cells (BE-GFP) in Matrigel invasion assays. Forty-eight hours after transfection of siRNAs, cells were seeded at the bottom of the filter and allowed to migrate into Matrigel toward the chemoattractant (top) for 4 days.

D: Quantitative analysis of invasion of silenced and control BE-GFP cells. Values represent mean \pm SD of two independent experiments.

E: Random migration of Fra-1-silenced or control BE cells was recorded by timelapse microscopy. A minimum of 20 cells was monitored in three independent experiments. Values represent mean cell speeds \pm SD from one representative experiment.

BE cells still displayed ruffling activity, but unlike control cells ruffles were all around the cell and not localized to a leading edge. Furthermore, these ruffles did not extend into protrusions (Supplemental Movie S2B on *Cancer Cell* website). These results show that upon Fra-1 silencing, loss of motility results

from the absence of extending protrusions associated with an increase in RhoA activity, stress fibers, and focal adhesions rather than loss of membrane ruffles.

The ERK-MAPK pathway controls Fra-1 expression in these colon carcinoma cells (E.V. and C.J.M., unpublished results).

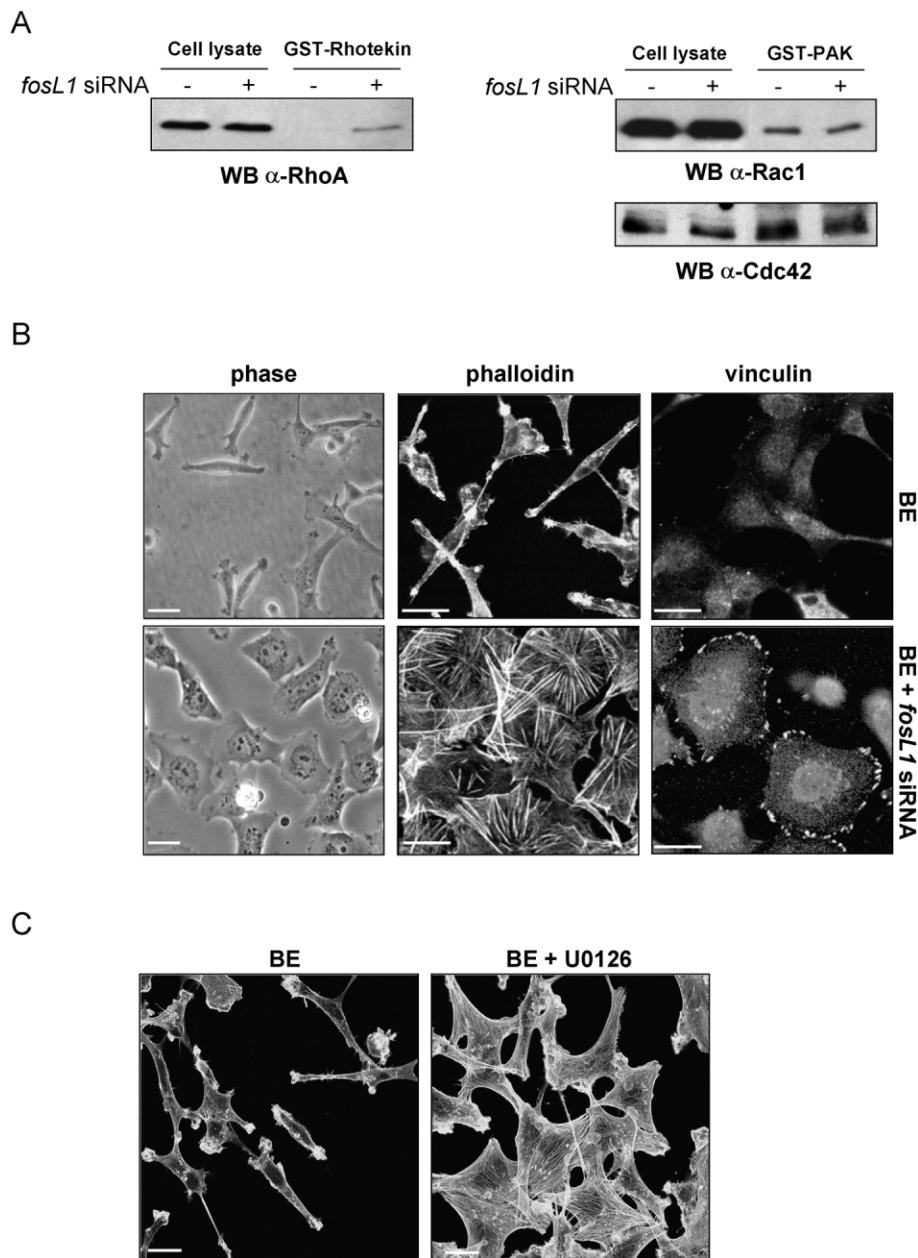


Figure 2. Fra-1 silencing increases Rho-GTP levels, stress fibers, and focal adhesions

A: Forty hours after transfection of siRNAs, pull-down assays were performed for GTP bound RhoA, Cdc42, or Rac.

B: Control and Fra-1-silenced cells were visualized by phase contrast microscopy or staining with anti-vinculin antibody or Texas red-coupled phalloidin for F-actin. Bars, 15 μ m.

C: Control BE cells and cells treated for 18 hr with the Mek inhibitor U0126 were stained for F-actin.

We therefore tested whether inhibition of the ERK-MAPK pathway with the Mek inhibitor U0126 could recapitulate the effects induced by Fra-1 inhibition. We found that upon Mek inhibition, as for Fra-1 inhibition, BE cell motility was dramatically reduced (Supplemental Movie S3 on *Cancer Cell* website), and there was also an increase in stress fibers and focal adhesions (Figure 2C and data not shown). These effects on cytoskeleton and cell motility only started after 10–12 hr of U0126 treatment, when Fra-1 protein levels were efficiently suppressed (>80% reduction) (data not shown). In contrast to Fra-1 inhibition, Mek inhibition resulted in the inability to form ruffles, suggesting that in these cells, ruffling activity is dependent on the ERK-MAPK pathway, but is independent of Fra-1 (Supplemental Movie S3 online).

Elevated Rho-GTP suppresses motility

The above experiments suggest that the increased RhoA activity resulting from Fra-1 inhibition might be the cause of increased stress fiber formation and focal adhesions, which in turn inhibit protrusions and cell motility. To test this hypothesis, we microinjected BE cells with a constitutively active RhoA mutant (RhoA-V14). The microinjected cells were much rounder than the control cells and were completely devoid of protrusions (Figures 3A and 3D), and, like cells in which Fra-1 had been silenced, RhoA-V14-expressing cells displayed membrane ruffles all around the periphery of the cell (data not shown). The motility of the RhoA-V14-expressing cells was dramatically reduced (Figure 3E), consistent with the hypothesis that high RhoA activity suppresses motility by preventing Rac-induced protrusions. Contrasting with the inhibition of Fra-1 expression, however,

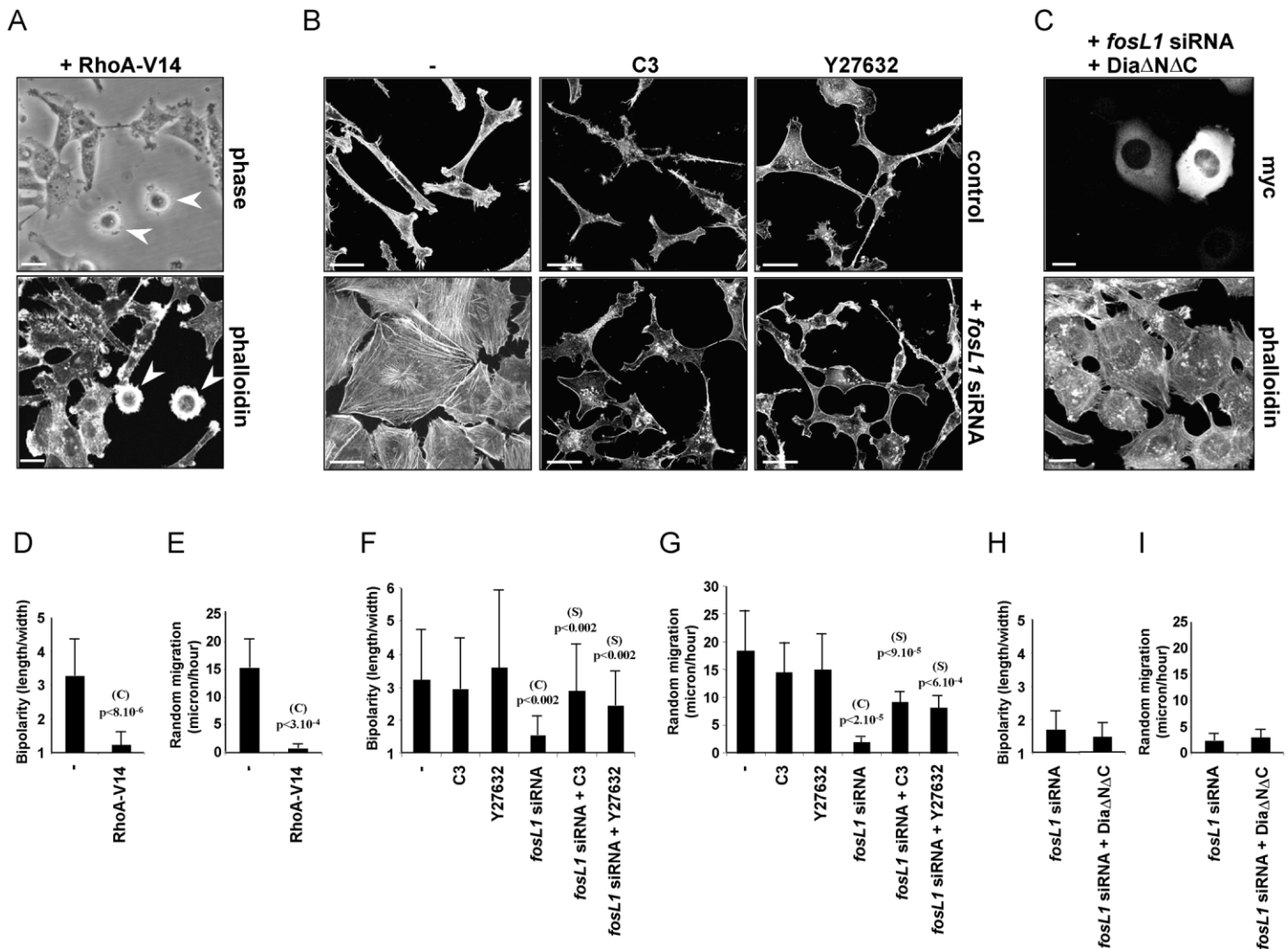


Figure 3. Rho activity antagonizes cell motility

A: BE cells were microinjected with EF-RhoA-V14 and EGFP-C1 and incubated for 6 hr before fixation and staining for F-actin. Microinjected cells were identified using GFP fluorescence.

B: BE cells were transfected with *fosL1* or scrambled siRNAs, incubated for 24 hr, then Y27632 (10 μ M) or Tat-C3 (0.5 μ M) was added and the cells were incubated for a further 24 hr before staining for F-actin.

C: BE cells were transfected with *fosL1* siRNAs, incubated 42 hr, microinjected with EF-Dia Δ N Δ C, and incubated for 6 hr before staining for F-actin. Microinjected cells were identified by staining with anti-myc antibody.

The average length/width ratios (polarized phenotype) of the EF-RhoA-V14 microinjected cells (**D**), Y27632- and Tat-C3-treated cells (**F**), and EF-Dia Δ N Δ C microinjected cells (**H**) and the corresponding control cells were calculated and the motility of the same cells recorded (**E**, **G**, **I**, respectively). A minimum of 20 cells was monitored in each experiment. Results are representative of 2–3 independent experiments. Statistical significance was evaluated by calculating p values: (C) compared to control cells, (S) compared to Fra-1-silenced cells. Bars, 15 μ m.

the RhoA-V14-expressing cells did not display large stress fibers but instead a very large actin-rich network at the cell border (Figure 3A). These results argue that elevated levels of RhoA-GTP in BE cells inhibit the formation of protrusions, polarized phenotype, and motility, probably via the reorganization of the actin cytoskeleton.

To confirm that Rho signaling led to the loss of protrusions and motility, treatment with cell-permeable C3 toxin disrupted stress fibers and focal adhesions and partially restored membrane protrusions and motility in Fra-1-silenced cells (Figures 3B, 3F, and 3G). Inhibition of the Rho effector ROCK/Rho-kinase using the synthetic inhibitor Y27632 also restored polarized phenotype and motility in those cells (Figures 3B, 3F, and 3G). In contrast, microinjection of a dominant-negative mutant of the

Rho effector mDia (Dia Δ N Δ C) did not lead to the disruption of stress fibers and restoration of protrusions or motility (Figures 3C, 3H, and 3I). These data demonstrate that RhoA and ROCK but not mDia functions are required to inhibit protrusions and motility upon Fra-1 inhibition.

These results argue that upon Fra-1 inhibition, the increase in Rho-GTP levels is responsible, via ROCK and the reorganization of the actin cytoskeleton, for the lack of protrusions and inhibition of motility.

Fra-1 suppresses β 1-integrin signaling to Rho-GTP

We next sought to determine the relationship between Fra-1 and RhoA-GTP levels. The composition of the extracellular ma-

trix and specific integrin signals can influence the adhesive and migratory response of cells via the regulation of the Rho family of small GTPases. Integrin engagement has been shown to regulate Rho activity in a complex fashion. Initial adhesion of suspended cells to fibronectin can transiently depress RhoA activity (Arthur and Burridge, 2001), but more sustained interaction leads to Rho activation whose magnitude is dependent on fibronectin concentration (Cox et al., 2001). Integrins also regulate Rac and Cdc42, leading to cell spreading and adhesion (del Pozo et al., 2000; Price et al., 1998).

To determine whether, upon Fra-1 inhibition, integrin signaling was responsible for RhoA activation, we analyzed expression of a wide range of integrin subunits. Using a series of conformation-dependent monoclonal antibodies (9EG7, HUTS-4, HUTS-21, 12G10, 15/7) which detect the activated state of $\beta 1$ -containing integrins (Lenter et al., 1993; Luque et al., 1996; Mould et al., 1995; Picker et al., 1993), we found that staining for activated $\beta 1$ -integrin was seen upon Fra-1 silencing (Figure 4A and data not shown). In contrast, both control and Fra-1-silenced cells were stained using pan- $\beta 1$ -integrin antibodies such as P5D2 (Figure 4A) or K20 (data not shown). Similar results were found when Mek was inhibited in BE cells (data not shown). Each of these antiactive $\beta 1$ antibodies demonstrated a fibrillar staining pattern on Fra-1-silenced cells, but did not stain control BE cells (9EG7, HUTS-21, 15/7) or showed a low diffuse staining (12G10, HUTS-4). While 12G10 has been shown to detect activated integrin irrespective of ligand occupancy (Mould et al., 2002), it has been suggested that antibodies, such as 9EG7, detect activation-specific epitopes only if ligand is present (Bazzoni et al., 1995). Figures 4B and 4C show that silencing Fra-1 leads to increased expression of fibronectin, and therefore it is possible that the increased staining with activation-specific antibodies is a consequence of increased ligand production. To test this possibility, we added exogenous fibronectin to control BE cells and to Fra-1-silenced cells. Staining with activation-specific antibodies, 9EG7 (Figure 4D), and HUTS-21 (data not shown) was seen in the silenced cells and it was increased in the presence of fibronectin, but no active $\beta 1$ -integrin was detected in the control cells with or without exogenous fibronectin. Thus, the increased detection of active $\beta 1$ in Fra-1-silenced cells is not merely a consequence of increased ligand production but reflects a change in the competence to bind the ligand. As a functional measure of $\beta 1$ -integrin activity, we measured the adhesion of control and Fra-1-silenced cells to extracellular matrix components. Figure 4E shows that silencing Fra-1 increased $\beta 1$ -dependent cell adhesion to the $\beta 1$ ligands fibronectin, laminin, and collagen but not to vitronectin, which is not a $\beta 1$ ligand. As expected, adhesion to fibronectin, laminin, and collagen was abrogated in the presence of the $\beta 1$ -integrin blocking antibody A1B2. Moreover, treatment of control BE cells with the $\beta 1$ -activating antibody TS2/16 mimicked the effect of silencing Fra-1. These results demonstrate that Fra-1 functions to keep $\beta 1$ -integrins in an inactive state. In addition, the observation that in Fra-1-silenced cells, activated $\beta 1$ was present in fibrillar structures (Figure 4A) suggests that the activated $\beta 1$ -integrins cluster after silencing Fra-1. Clustering of integrins increases avidity for ligands (Humphries, 2000) and can be modulated by intracellular signaling pathways (Stewart et al., 1998). Interestingly co-staining with antibodies against fibronectin and activated $\beta 1$ showed that the fibrillar structures consisted of strands of fibronectin connecting acti-

vated $\beta 1$ -integrins on adjacent cells (Figure 4F). These structures may increase intercellular adhesion and thereby contribute to decreased cell motility. We also observed that these structures were connected to stress fibers inside the cells.

To determine whether $\beta 1$ -integrin signaling was involved in the activation of RhoA, we silenced its expression using RNAi (Figure 5A) or inhibited its function with blocking antibodies. Silencing of $\beta 1$ -integrin abrogated the increase in Rho-GTP seen in Fra-1-silenced cells (Figure 5B, upper panel). A similar inhibition of Rho activation was observed when we used $\beta 1$ -integrin-blocking antibody (A1B2) in Fra-1-silenced cells (Figure 5B, lower panel). These results indicate that activation of $\beta 1$ -integrin signaling is required for the activation of Rho when Fra-1 is silenced. To confirm these results, we investigated whether activation of $\beta 1$ -integrin signaling in BE cells could lead to the activation of RhoA. Incubation of BE cells with a $\beta 1$ -integrin-activating antibody (TS2/16) led to an increase in Rho-GTP, to levels similar to those seen after inhibition of Fra-1 (Figure 5C). In Fra-1-silenced cells, TS2/16 did not produce a further increase in Rho activity, indicating that physiological maximum levels were reached. Altogether, these results demonstrate that upon Fra-1 inhibition, activation of $\beta 1$ -integrin signaling is responsible for the activation of RhoA. Consistent with this conclusion, rather than Rho signaling activating $\beta 1$ -integrin signaling, we found that microinjection of BE cells with constructs expressing RhoA-V14 or an active version of ROCK1 (ROCK1 Δ C) did not lead to the formation of active $\beta 1$ complexes although they induced formation of focal adhesions (Figure 5D).

An ERK-uPAR-Rac pathway is required for ruffling activity

As described earlier, Fra-1-silenced BE cells still formed ruffles. In fibroblasts, the small GTPase Rac has been shown to be the driving force of cell movement by regulating the formation of membrane ruffles at the leading edge of cells (Ridley et al., 1992). In BE cells, ruffling activity and motility are dependent on Rac, as shown by microinjection of dominant negative Rac-N17 (Figure 6A and Supplemental Figure S2 and Movie S4 on *Cancer Cell* website).

While silencing Fra-1 did not block membrane ruffling, inhibition of ERK1/2 MAPK activation by treatment with the Mek inhibitors U0126 or PD184352 abrogated membrane ruffling suggesting that membrane ruffling was dependent on the ERK-MAPK pathway. Recently, overexpression of urokinase-type plasminogen activator receptor (uPAR) has been shown to result in the migration of fibroblasts through the activation of Rac (Kjoller and Hall, 2001). Both the Ras-ERK-MAPK pathway and AP-1 have been suggested to regulate uPAR expression (Aguirre Ghiso et al., 1999; Dang et al., 1999; Muller et al., 2000). In BE cells, we found that uPAR expression was dependent on the ERK-MAPK pathway, since accumulation of the uPAR mRNA was inhibited after addition of the Mek1/2 inhibitor PD184352 (Figure 6B). Silencing of Fra-1 by RNAi reduced uPAR mRNA levels by at most 20% demonstrating that uPAR expression is mainly Fra-1 independent (Figure 6B). To test whether uPAR was required for Rac activity in BE cells, we treated cells with a blocking antibody against uPAR or siRNAs. The monoclonal antibody R3, which recognizes the D1 domain, essential for the binding of uPA and vitronectin has been shown to block Rac activation in cells transfected with uPAR (Kjoller and Hall, 2001). Treatment of BE cells with antibody R3 inhibited Rac activity

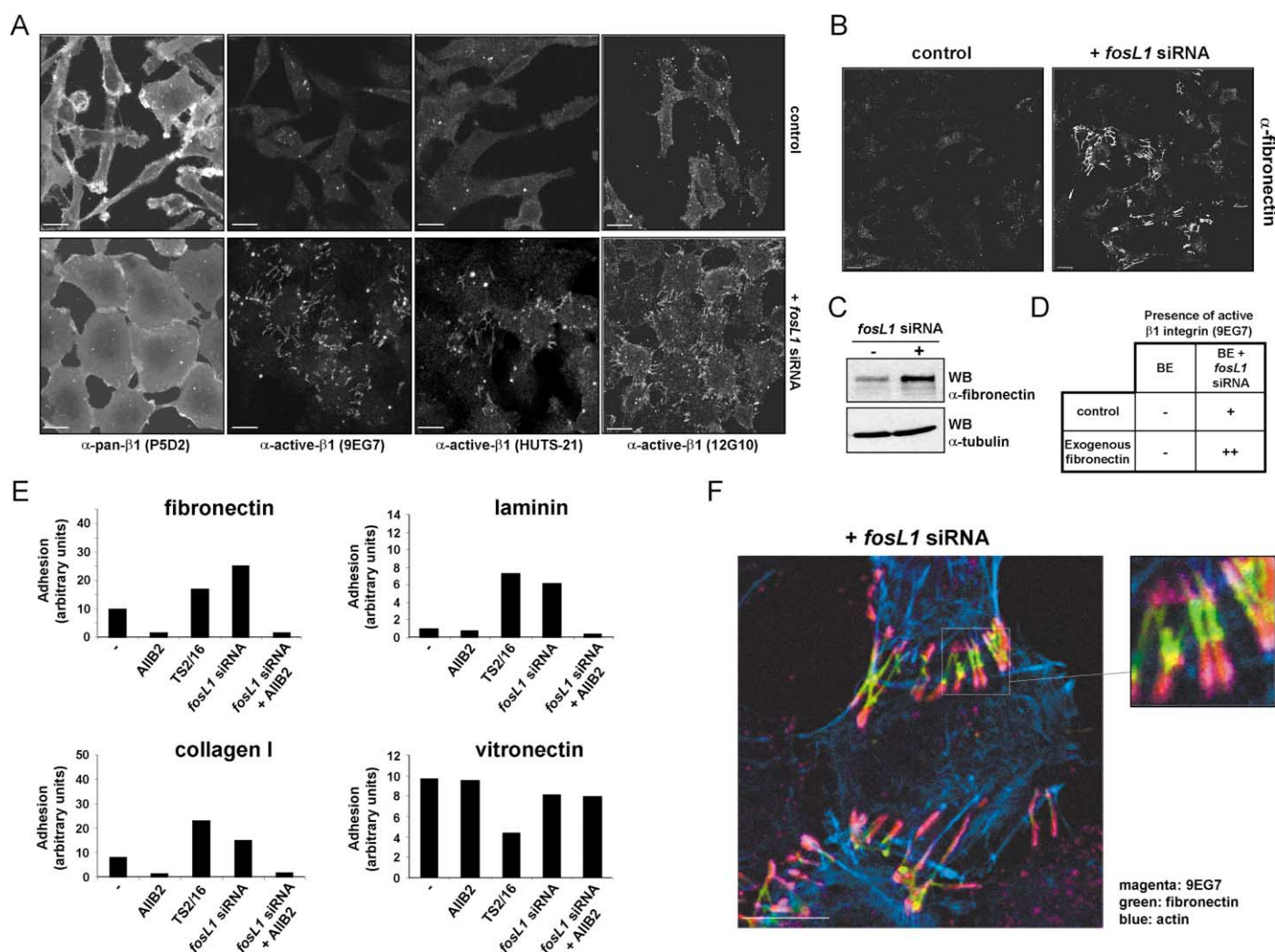


Figure 4. Fra-1 silencing leads to β 1-integrin activation

A: BE cells were transfected with siRNAs, incubated 48 hr, fixed but not permeabilized, then stained with anti-pan β 1-integrin antibody (P5D2) or antiactive β 1-integrin antibodies (9EG7, HUTS-21, 12G10). Bars, 15 μ m.

B: Fixed but not permeabilized control and Fra-1-silenced cells were stained with anti-extracellular fibronectin antibody (FN-15). Bars, 15 μ m.

C: Immunoblot analysis of fibronectin protein accumulation in control or Fra-1-silenced cells.

D: BE cells were transfected with scrambled or *fosL1* siRNAs, incubated 48 hr, detached from the plates, then human fibronectin (150 μ g/ml) was added and the cells replated. After a further 24 hr incubation, the cells were fixed and stained for active β 1-integrin (9EG7).

E: BE cells were transfected with siRNAs, incubated 48 hr, then detached from the plates using a nonenzymatic cell dissociation buffer (Gibco 13151-014). Activating (TS2/16) or blocking (AlIB2) β 1-integrin antibodies were added where appropriate, cells incubated 15 min in suspension at 37°C, then plated on different extracellular matrix protein coated wells for 15 min (fibronectin, collagen I) or 30 min (laminin, vitronectin). Quantitative adhesion assays were performed using the cytotoxicity screening kit (Chemicon international).

F: Fra-1-silenced BE cells were costained for active β 1-integrin (9EG7), fibronectin, and actin.

by approximately 50% (Figure 6C, upper panel). In confirmation of these results, silencing uPAR expression resulted in loss of actin-rich membrane ruffles (Figure 6D) and reduced Rac-GTP levels (Figure 6C, lower panel). These results argue that in BE tumor cells, uPAR drives Rac activation.

The results presented above indicate that a high Rho activity inhibits the formation of uPAR-Rac-driven protrusions in BE cells. We further confirmed this by showing that, in BE cells, the polarized phenotype can be increased by more than 2-fold by overexpressing uPAR, and this increase is abolished in pres-

ence of active RhoA (Figure 6E). Furthermore, Rac-dependent cell spreading and motility resulting from overexpression of uPAR in 293T cells was blocked by expression of RhoAV14 (Supplemental Figure S3 on website).

Discussion

We have shown that ERK-MAPK signaling acts to regulate Rho and Rac activity in colon carcinoma cells and thereby plays a major role in cell motility and invasion. ERK-dependent tran-

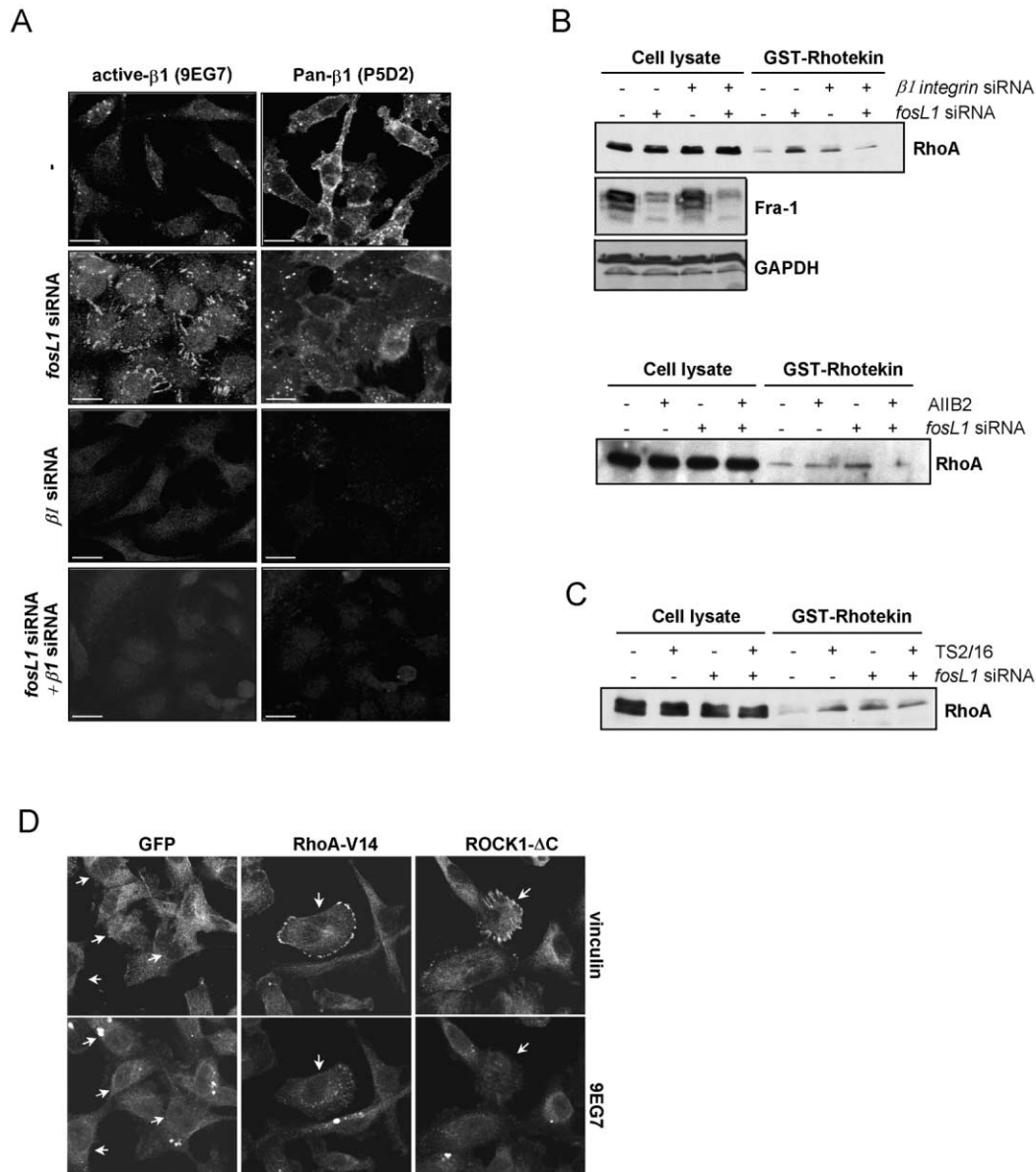


Figure 5. Rho is activated by a $\beta 1$ -integrin-dependent signaling

A: BE cells were transfected with scrambled or *fosL1* siRNAs together with scrambled or $\beta 1$ -integrin-specific siRNAs, incubated for 48 hr then fixed and stained with anti-pan $\beta 1$ -integrin antibody (P5D2) or antiactive $\beta 1$ -integrin antibody (9EG7). Bars, 15 μ m.

B: Upper panel: Fra-1- and $\beta 1$ -integrin-silenced BE cells were subjected to pulldown assays for Rho-GTP. Total cell lysates were immunoblotted for RhoA, Fra-1, and GAPDH. Lower panel: BE cells were transfected with scrambled or *fosL1* siRNAs, incubated 48 hr, detached from the plates, the $\beta 1$ -integrin-blocking antibody AlIB2 (10 μ g/ml) added, and the cells replated. After 24 hr incubation, RhoA pulldown assays were performed.

C: BE cells were transfected with the *fosL1* or scrambled siRNAs, incubated 24 hr, TS2/16 $\beta 1$ -integrin-activating antibody added, and cells incubated for 24 hr before performing a RhoA pulldown assay.

D: BE cells were microinjected with EF-RhoA-V14 or Pcagg-ROCK $\Delta 3$ and EGFP-C1, incubated for 6 hr, and fixed. Cells were stained with antiactive $\beta 1$ -integrin antibody (9EG7) or anti-vinculin antibody. Microinjected cells were identified using GFP fluorescence.

scriptional activation of the *fosL1* gene leads to its product, Fra-1, repressing Rho-GTP levels through inactivation of $\beta 1$ -integrin signaling. This depression of Rho-GTP levels is necessary to prevent signaling by the Rho effector ROCK, which would lead to the formation of excessive stress fibers and focal adhesions. Activation of this pathway prevents membrane ruffles, mediated by the small GTPase Rac, from being extended

into membrane protrusions. Thus inhibition of this pathway by Fra-1 is necessary to allow the extension of the ruffles into active protrusions required for cell motility. In addition to the regulation of Rho-GTP levels, the activation of Rac is also regulated through ERK signaling via expression of the receptor for urokinase type plasminogen activator, uPAR. This receptor has been shown to mediate Rac activation via binding to integrins

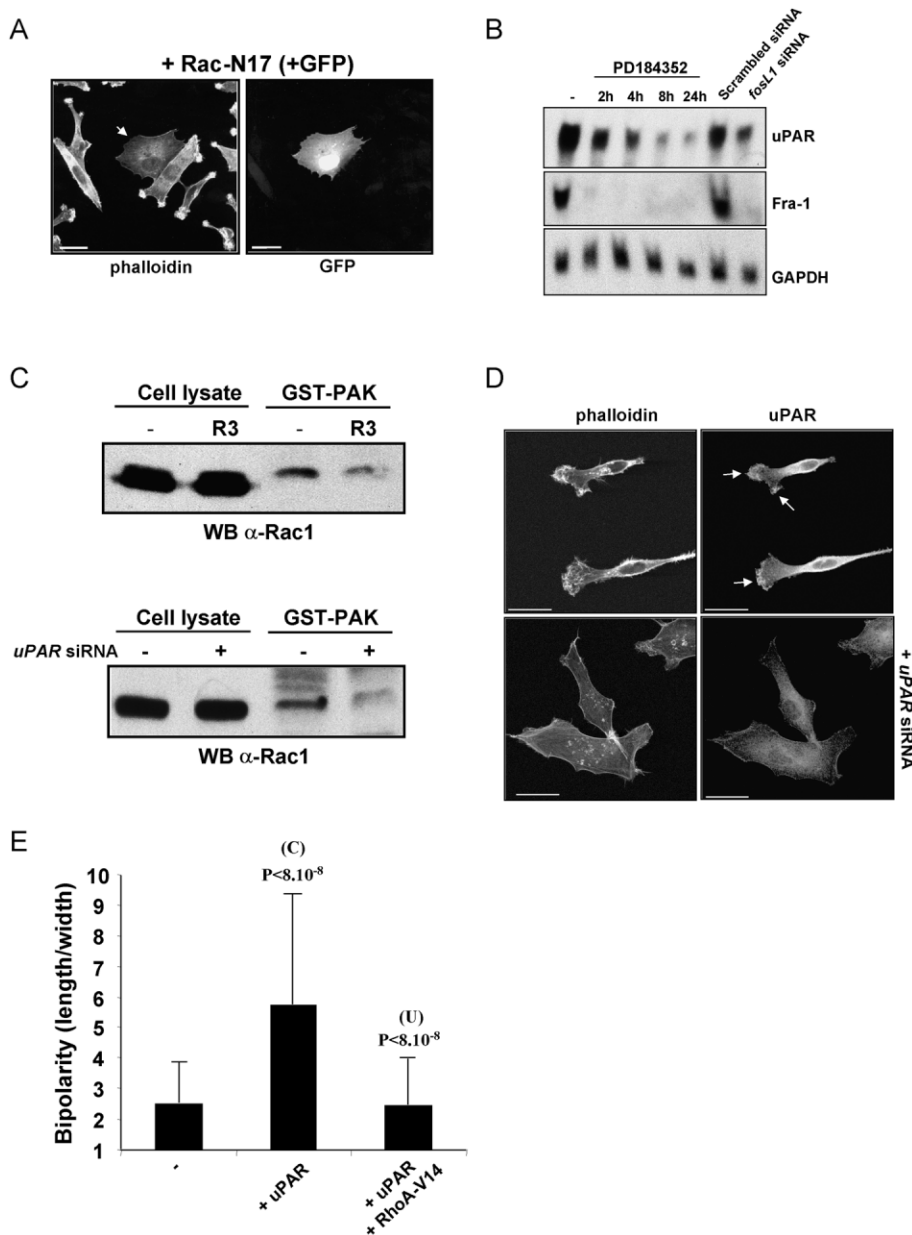


Figure 6. Ruffling is controlled by an ERK-uPAR-Rac pathway

A: BE cells were microinjected with EF-Rac-N17 and EGFP-C1 and incubated for 6 hr. Microinjected cells were identified using GFP fluorescence. Texas red-coupled phalloidin was used to visualize F-actin.

B: Northern blot analysis of uPAR and Fra-1 expression in BE cells treated with PD184352 (1 μ M) or transfected with the *fosL1* or scrambled siRNAs for 48 hr. Levels of uPAR mRNA normalized to GAPDH levels in PD184352-treated cells: t0(100%), t2(43%), t4(22%), t8(13%), t24(11%); in siRNA transfected cells: control(100%), *fosL1* (83%).

C: Upper panel: BE cells were incubated 18 hr with R3 uPAR antibody (30 μ g/ml) before a Rac pull-down assay was performed. Lower panel: BE cells were transfected with uPAR or scrambled siRNAs and lysed 72 hr later and a Rac pull-down assay performed.

D: Immunofluorescence analysis of uPAR and F-actin expression in BE cells, 72 hr after transfection of uPAR or scrambled siRNAs. Bars, 15 μ m.

E: BE cells were microinjected with EGFP-C1, PRC/CMV-uPAR, and EF-RhoA-V14, then incubated 6 hr. Microinjected cells were identified using GFP fluorescence or staining with anti-myc antibody. The polarized phenotype and the formation of protrusions were evaluated by measuring the length/width ratios. A minimum of 20 cells was measured in two independent experiments. Values represent mean \pm SD of the two experiments. P values were calculated, (C) compared to control cells, (U) compared to uPAR-expressing cells.

(Kjoller and Hall, 2001) and may be independent or dependent on uPA binding (Sturge et al., 2002). We show that uPAR expression is regulated by the ERK-MAPK pathway but is a transcriptional target largely independent of Fra-1 in these cells. These data establish a model in which motility and invasiveness of colon carcinoma cells are coordinated by two signaling pathways downstream of ERK. One component is a Fra-1-dependent pathway leading to the downregulation of RhoA necessary for the extension of the ruffles into active extending protrusions. The second component is a Fra-1-independent uPAR-dependent pathway leading to the activation of Rac1 and the initial formation of ruffles (see Figure 7).

Coordinated control of Rho family GTPase activity by ERK-MAP kinases contributes to motility of tumor cells

The results presented above indicate that efficient motility of colon carcinoma cells requires low Rho and high Rac activities,

which are both achieved through Ras-ERK-MAPK signaling. Previous studies have shown that overexpression of p190RhoGAP to downregulate Rho-GTP levels initiates the formation of protrusions and promotes migration (Arthur and Burridge, 2001; Nakahara et al., 1998). Interestingly, integrins such as β 1-integrin have been suggested to modulate p190RhoGAP activity and/or localization (Arthur et al., 2000; Nakahara et al., 1998; Sharma, 1998). It is possible that the β 1-integrin inactivation induced by Fra-1 might allow p190RhoGAP localization at the cell membrane that permits the Rac-dependent extension of protrusions by locally reducing Rho activity. A reduction in Rho activity may also promote cell motility by facilitating the turnover of focal adhesions necessary for cell movement (Ilic et al., 1995; Nobes and Hall, 1999; Parsons et al., 2000). Consistent with this, silencing Fra-1 led to the formation of very large Rho-dependent focal complexes (Figure 2B). Our results demonstrating the roles that

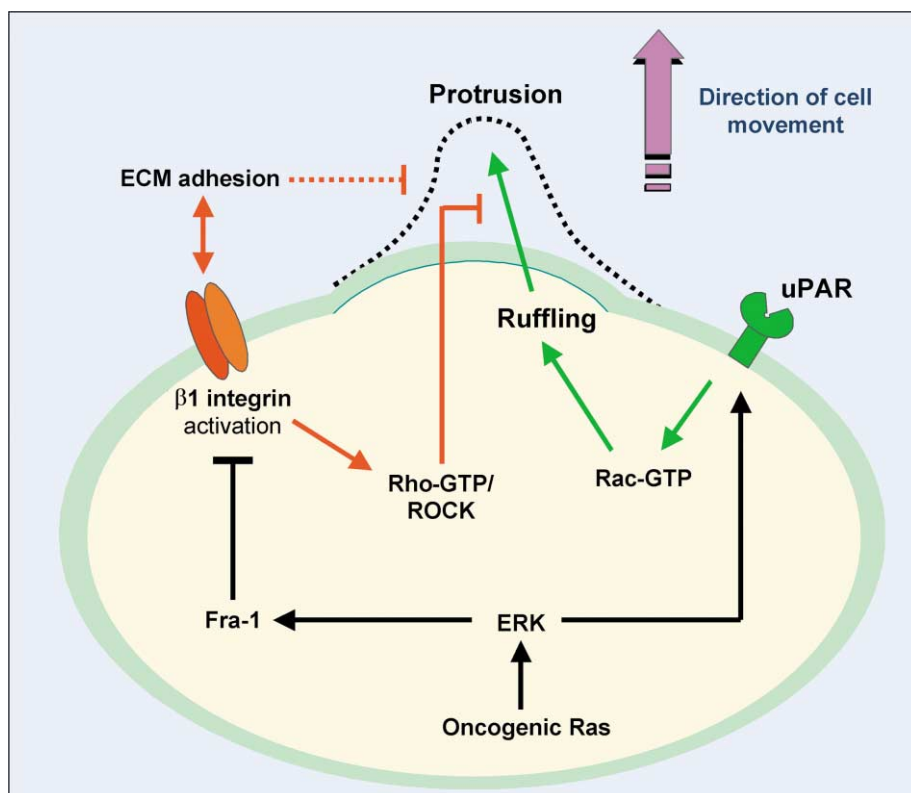


Figure 7. ERK-dependent signaling pathways regulating motility of colon carcinoma cells

ERK-MAP kinase signaling plays in regulating Rho and Rac activity are in disagreement with recent studies showing that overexpression of oncogenic Ras leads to the downregulation of Rac and the upregulation of Rho (Zondag et al., 2000; Sahai et al., 2001). However, in Ras-transformed fibroblasts with elevated Rho-GTP levels, signaling through ROCK is downregulated to permit cell motility (Sahai et al., 2001). In agreement with our conclusions, studies of Rho and Rac activation following PDGF stimulation show activation of Rac and the downregulation of Rho, inducing cell motility (Sander et al., 1999; Zondag et al., 2000). Thus a consistent theme of Rac-driven cell motility is that it requires signaling through ROCK to be low; this can be achieved by ERK-MAPK signaling downstream of Ras either through Fra-1-mediated suppression of Rho-GTP levels or uncoupling ROCK from Rho-GTP (Sahai et al., 2001).

We have previously shown that Rho activity is required in BE cells to prevent $p21^{Waf1}$ accumulating to growth inhibitory levels; therefore, conflicting changes in Rho activity may be required to promote the growth and motility of tumor cells. These apparently contradictory results may be reconciled if different Rho effector pathways are utilized to promote growth and motility. In Ras-transformed fibroblasts, downregulation of ROCK signaling is crucial for ERK-dependent motility but ROCK signaling is not involved in the regulation of $p21^{Waf1}$ levels (Sahai et al., 2001), and neither does ROCK regulate $p21^{Waf1}$ levels in BE cells (Sahai et al., 2001). Furthermore, although ERK signaling downregulates Rho-ROCK signaling by suppressing Rho activation, in BE cells it promotes the expression of another Rho effector, mDia (E.S. and E.V., unpublished observations); thus signaling through other Rho effectors involved in promoting cell proliferation may be maintained. An alternative possibility is that

different threshold levels of Rho activity regulate cell growth and motility. High levels of Rho activity may be required to drive ROCK-dependent cell contraction and antagonize cell motility whereas lower levels of activity may allow cell proliferation. It is worth noting that low levels of Rho activity in BE cells contribute positively to cell motility because total inhibition of Rho and ROCK slightly inhibited motility of the control cells by interfering with the cell body translation (Figure 3G and data not shown); this may explain why complete inhibition of Rho and ROCK only partially restored motility upon Fra-1 inhibition.

Fra-1 and integrin function

We have shown that in BE tumor cells in response to Fra-1 silencing, $\beta 1$ -integrins switch to an active, ligand binding state, which indicates that Fra-1 keeps $\beta 1$ -integrins in an inactive state without affecting the expression levels of $\beta 1$ -integrin. This effect of Fra-1 downregulates $\beta 1$ -dependent cell adhesion and activation of RhoA. These results suggest that either through transcriptional or nontranscriptional mechanisms, Fra-1 may interfere with the process of integrin "activation" or "inside-out signaling," which is the modulation of integrin affinity for the extracellular ligands in response to intracellular signals (Hughes and Pfaff, 1998). Suppression of integrin activation has been shown to be an important function of oncogenic Ras-Raf pathway (Hughes et al., 1997). While the mechanism through which Fra-1 mediates $\beta 1$ -integrin inactivation remains to be defined, it could regulate the expression of cytoplasmic or membrane proteins which are known to interact with integrins, and which may be implicated in the "activation" process, such as paxillin, α -actinin, talin, filamin, or FAK (Hughes and Pfaff, 1998). Alternately, Fra-1 might directly modify the integrin composition at the cell surface,

depriving $\beta 1$ -integrin from its appropriate partner. Fra-1 modulation of $\beta 1$ -integrin function may also occur through AP-1-regulated transcription, altering the extracellular matrix composition in a way which deprives the $\beta 1$ -integrin complexes expressed at the cell surface from their activating ligands, such as laminin or fibronectin. We found that expression of fibronectin was increased in Fra-1-silenced cells; however, addition of exogenous fibronectin was not sufficient to convert $\beta 1$ -integrin to its active state, arguing that Fra-1 affects integrin activation more directly than merely through affecting the composition of the extracellular matrix. Additionally, the presence in Fra-1-silenced cells of activated $\beta 1$ -integrin in fibronectin containing fibrillar structures connecting adjacent cells suggests that Fra-1 can also affect integrin clustering. Integrin clustering increases the avidity for binding ligands (Humphries, 2000; Stewart et al., 1998) and thereby enhances adhesion and signaling.

It has been suggested that cell migration requires a dynamic adhesion and de-adhesion process reflecting activation and inactivation of integrins and that maintaining integrins in their active state inhibits motility (Kuijpers et al., 1993). Our data suggest that in cells where there is constitutive activation of the ERK-MAPK pathway, the balance between integrin activation and inactivation is shifted toward inactivation to favor Rac-driven motility. However, a dynamic process of adhesion and de-adhesion predicts that some active $\beta 1$ -integrin will be required for cell motility. Indeed we observed that ablation of $\beta 1$ -integrin by siRNA reduced motility of BE cells (data not shown), consistent with this idea that some $\beta 1$ -integrin signaling is required for the motility of these cells.

Ras-Raf-ERK signaling and tumor cell invasion

Our studies show important roles of ERK-MAP kinase signaling in tumor cell invasion. This signaling pathway may regulate the activity of the Rho and Rac GTPases via the expression of Fra-1 and uPAR. Studies from Ozanne and collaborators have indicated an important role for AP-1 in mediating tumor cell invasion. Many of the genes that they have identified, such as CD44 and TSC-36, that are regulated through elevated AP-1 activity following v-Fos expression have been shown to play a role in invasion (Johnston et al., 2000; Lamb et al., 1997). Our studies provide a further link between AP-1 and invasion by showing how AP-1 activity regulates the activity of Rho. The presence of Fra-1 and uPAR in tumors, which are easily detectable, might give valuable information in the choice of the therapeutic strategy since Fra-1 and uPAR are likely to be associated with an high Ras-ERK-MAPK activity rather than a high Rho activity. Recently, Bernards and Weinberg (Bernards and Weinberg, 2002) have questioned whether there are separate genes for metastasis/invasion and suggested that these phenotypes may be the consequence of the action of oncogenes and tumor suppressor genes regulating cell proliferation or survival. Our results support this notion since they show that ERK-MAP kinase signaling downstream of the oncogene Ras, known to be involved in tumor cell proliferation and survival, is also involved in invasion and cell motility. However, since the outcome of ERK-MAP kinase signaling is dependent on cellular context (Marshall, 1995), it is possible that the requirement for ERK signaling for cell motility and invasion will be dependent on cell type and cooperating genetic changes.

Experimental procedures

Cell culture and reagents

BE and Hct-116 human colon carcinoma cell lines were grown routinely in DMEM supplemented with 10% fetal calf serum (FCS). Human fibronectin (BD Biosciences 356008) was added to the media at a concentration of 150 μ g/ml. The BE-GFP cell line is a pool of puromycin-resistant clones generated by cotransfection of BE cells with pBabepuro and EF-GFP. U0126 was dissolved in EtOH and used at 10 μ M (Promega #V1121), PD184352 (Sebolt-Leopold et al., 1999) (gift from P. Cohen) was used at 1 μ M, Y-27632 (Tocris) was used at 10 μ M, and TAT-C3 (Sahai et al., 2001) was used at 0.5 μ M in cell culture media.

Antibodies and DNA constructs

Antibodies to AP-1 components were from Santa Cruz: anti-Fra-1 (sc-605), anti-c-Jun (sc-7481), anti-JunB (sc-46), anti-Fra-2: (sc-171), anti-c-Fos (sc-052). Other antibodies: anti- β -tubulin: Sigma (T4026), anti-RhoA: Santa Cruz (sc-418), anti-Rac1: Upstate Biotechnology (05-389), anti-Cdc42: Santa Cruz (sc-8401), anti-vinculin: Sigma (V4505), anti-myc (9E10): ICR (Sutton, United Kingdom), anti-Pan $\beta 1$ -integrin (P5D2): Santa Cruz (sc-13590), anti-active $\beta 1$ -integrin: (9EG7) BD pharmingen (550531) and (HUTS-21) BD pharmingen (556048), anti- $\beta 1$ -integrin antibodies 12G10, 15/7, HUTS-4, and K20 were gifts from M. Humphries, blocking $\beta 1$ -integrin (A1B2) was a gift from F. Watt, anti-fibronectin: (EP5) Santa Cruz (sc-8422) for Western blotting and (FN-15) Sigma (F7387) for immunofluorescence, anti-GAPDH: RDI (TRK5G4-6C5), anti-uPAR: American Diagnostica (3931), R3 anti-uPAR: L. Kjoller, TS2/16: ATCC (HB-243), secondary antibodies for immunofluorescence from Jackson Stratech (Luton, United Kingdom).

EF-RhoA-V14 and EF-Rac-N17 plasmids were gift from R. Treisman, EF-Dia Δ N Δ C is described elsewhere (Sahai and Marshall, 2002); pCagg-ROCK Δ 3 expressing an active version of ROCK1 (ROCK1 Δ C) is a gift from S. Narumiya. EGFP-C1 was from Clontech; pRc/CMV-uPAR is a gift from L. Kjoller.

RNAi

Desalted and deprotected synthetic oligonucleotides were purchased from Dharmacon (Lafayette, Colorado) and were annealed following their standard protocol. The siRNA sequence targeting human Fra-1 is CACCAUGAGUGG CAGUCAG; human $\beta 1$ -integrin: UGAGCUUCUCUGCUGUUC; human uPAR: GGUGAAGAAGGCGUCCAA. Control oligonucleotides correspond to the inverse sequences. SiRNAs were transfected using the oligofectamine reagent (Invitrogen).

Biochemical methods

Northern blot analyses were performed using conventional methods. Radioactive probes were coding sequences of human uPAR, *fosL1*, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes. Accumulation of the mRNAs was quantified using a phosphorimager (Molecular Dynamics).

For Western blot analysis of tubulin, GAPDH, and fibronectin accumulation, total cell lysate was used. For Western blot analysis of Fra-1 and for DNA binding assays, cells were washed twice in ice-cold PBS, recovered by scraping, centrifuged and resuspended in 1 volume of 20 mM Hepes (pH 7.9), 600 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1 mM vanadate, 1 mM PMSF, 1% aprotinin. After a 30 min incubation at 0°C, the lysate was centrifuged and the supernatant added to 1 volume of 20 mM Hepes (pH 7.9), 0.2 mM EDTA, 1 mM DTT, 40% glycerol, 1 mM vanadate, 1 mM PMSF, 1% aprotinin.

Small G protein pulldown assays were performed using GST-PAK-CRIB or GST-rhotekin as previously described (Sahai et al., 2001).

Microinjections and immunofluorescence

For microinjections, all plasmids were injected in Phosphate-buffered saline (PBS) at a concentration of 100 ng/ μ l, except EGFP-C1, which was at 25 ng/ μ l.

For immunofluorescence, cells were washed with PBS, fixed with 4% formaldehyde in PBS and usually permeabilized with 0.2% Triton X-100 in PBS. After several PBS washes, cells were stained for the actin cytoskeleton with Texas red-phalloidin (Molecular Probes) or primary antibodies were added for 1 hr; after several PBS washes, appropriate secondary antibodies were added for 1 hr; cells were mounted after several further PBS washes and viewed using a Bio-Rad MRC1024 confocal microscope. Microinjected

cells were identified by GFP fluorescence or using 9E10 antibody for myc-tagged constructs.

Biological methods

The motility of cells was determined by recording phase contrast and GFP fluorescence images once every 2 min with a multichannel timelapse digital video-microscope for several hours (Digital Pixel). The recordings were played back and the movement of the cells over a 6 hr period was traced manually. The lengths of the tracings were measured with a ruler, scaled appropriately, and divided by six to give the distance moved per hour. In all videos, the format is 24 frames (48 min real time) per second.

To assess for the formation of polarized morphology and protrusions, the length/width ratio was calculated.

The inverse invasion assay was performed as previously described (Malliri et al., 1998). In brief, 40 hr after transfection of the siRNAs, BE-GFP cells were allowed to attach to the underside (bottom) of the growth factor-depleted Matrigel-coated polycarbonate chambers (Transwells 8 μ m pore size filters). The cells were then chemoattracted (10% FCS) across the filter and through the Matrigel above. Cells were fixed in 4% paraformaldehyde and GFP fluorescence was analyzed in z-sections (1 section every 4 μ m) from the bottom of the filter using a confocal microscope (Bio-Rad MRC1024). Three-dimensional reconstructions of the GFP-expressing cells into the Matrigel and then pixel quantification were done using the Volocity computer software (Improvision). Percentage of invading cells is the ratio of pixels above the filter (into Matrigel) to the total number of pixels above and below the filter.

Adhesion assays were performed using the cytomatrix screen kit (Chemicon International) according to their protocol.

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