

# The distinct roles of Ras and Rac in PI 3-kinase-dependent protrusion during EGF-stimulated cell migration

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## Summary

Cell migration involves the localized extension of actin-rich protrusions, a process that requires Class I phosphoinositide 3-kinases (PI 3-kinases). Both Rac and Ras have been shown to regulate actin polymerization and activate PI 3-kinase. However, the coordination of Rac, Ras and PI 3-kinase activation during epidermal growth factor (EGF)-stimulated protrusion has not been analyzed. We examined PI 3-kinase-dependent protrusion in MTLn3 rat adenocarcinoma cells. EGF-stimulated phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P<sub>3</sub>] levels showed a rapid and persistent response, as PI 3-kinase activity remained elevated up to 3 minutes. The activation kinetics of Ras, but not Rac, coincided with those of leading-edge PtdIns(3,4,5)P<sub>3</sub> production. Small interfering RNA (siRNA) knockdown of K-Ras but not Rac1 abolished PtdIns(3,4,5)P<sub>3</sub> production at the leading edge and

inhibited EGF-stimulated protrusion. However, Rac1 knockdown did inhibit cell migration, because of the inhibition of focal adhesion formation in Rac1 siRNA-treated cells. Our data show that in EGF-stimulated MTLn3 carcinoma cells, Ras is required for both PtdIns(3,4,5)P<sub>3</sub> production and lamellipod extension, whereas Rac1 is required for formation of adhesive structures. These data suggest an unappreciated role for Ras during protrusion, and a crucial role for Rac in the stabilization of protrusions required for cell motility.

Supplementary material available online at  
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Key words: Ras, Rac, Cdc42, Rho, Lamellipodia

## Introduction

Cell migration is an essential property of cells engaged in embryogenesis, inflammation, wound healing and tumor metastasis. Motility toward a source of chemoattractant depends on localized extension of actin-rich protrusions, such as lamellipodia. Studies with metastatic rat carcinoma line MTLn3 have shown that actin polymerization at the leading edge of the lamellipod relies upon the synergistic interaction of Arp2/3 complex with actin-severing protein cofilin, which generates new actin barbed ends at the leading edge (Bailly et al., 2001; Chan et al., 2000; DesMarais et al., 2004; Ichetovkin et al., 2002). Arp2/3-mediated actin polymerization in MTLn3 is likely to be activated by Wiskott-Aldrich syndrome protein (WASP) family proteins, which are in turn regulated by upstream Rho GTPases (Bompard and Caron, 2004; Machesky et al., 1999; Miki and Takenawa, 2003; Weaver et al., 2003).

The kinetics of actin polymerization in globally epidermal growth factor (EGF)-stimulated MTLn3 cells, measured either by lamellipod extension or free barbed end formation, is highly reproducible. Lamellipod extension begins after 30-60 seconds of EGF stimulation, and is maximal within 180-300 seconds

(Chan et al., 1998; Segall et al., 1996). Barbed end formation, visualized by the incorporation of fluorescently labeled biotin-actin, exhibits early and late transients, at 1 and 3 minutes (Chan et al., 1998; Mouneimne et al., 2004). Early barbed end formation at 1 minute is linked to directional sensing, whereas late barbed end formation at 3 minutes seems to be involved in the production of mechanical protrusive force (Ghosh et al., 2004; Mouneimne et al., 2004). Whereas phosphoinositide 3-kinase (PI 3-kinase) is required for protrusion in MTLn3 cells (Hill et al., 2000), inhibition of PI 3-kinase primarily suppresses the late, more than the early, barbed end transient (Mouneimne et al., 2004). A similar diminution of late actin polymerization was observed in *Dictyostelium pi3k1/2*-null cells (Chen et al., 2003; Funamoto et al., 2002). These data are consistent with recent data from a variety of systems, suggesting that PI 3-kinase activation at the leading edge of moving cells is required for maximal speed of migration, but is not involved in directional sensing (Andrew and Insall, 2007; Ferguson et al., 2007; Hoeller and Kay, 2007; Nishio et al., 2007).

How is PI 3-kinase activity regulated at the leading edge of

the lamellipod? Class I PI 3-kinases can be directly activated by interactions with receptor tyrosine kinases and their substrates, with small GTPases, and with  $\beta\gamma$ -subunits from activated trimeric G-proteins (Vanhaesebroeck et al., 1997). In polarized neutrophils, the asymmetrical accumulation of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5) $P_3$ ] at the leading edge has been proposed to involve a PI 3-kinase-dependent positive feedback loop between PI 3-kinase and Rac (Bourne and Weiner, 2002; Srinivasan et al., 2003; Weiner et al., 2002), consistent with the fact that Rac activates PI 3-kinase by binding to the breakpoint cluster region (BCR) of p85 regulatory subunits (Bokoch et al., 1996; Zheng et al., 1994) and PtdIns(3,4,5) $P_3$  production enhances the activity of guanine nucleotide exchange factors for Rac activation (Hawkins et al., 1995; Welch et al., 2003). However, membrane localization of Akt-PH-green fluorescent protein (GFP), a probe for phosphatidylinositol (4,5)-biphosphate [PtdIns(3,4) $P_2$ ] and PtdIns(3,4,5) $P_3$ , is blocked by the Rho GTPase inhibitor toxin B in formyl-methionyl-leucyl-proline (fMLP)- but not in insulin-stimulated cells (Servant et al., 2000; Srinivasan et al., 2003; Weiner et al., 2002), suggesting that PI 3-kinase localization is differentially regulated by G-protein-coupled receptors as opposed to receptor tyrosine kinases. Although RacB and PI 3-kinase have similarly been proposed to constitute a positive feedback loop in *Dictyostelium* (Park et al., 2004; Sasaki et al., 2004), the mechanism is less clear because there is no homolog of the PI 3-kinase p85 regulatory subunit and therefore no obvious way for activated Rac to regulate PI 3-kinase activity.

Ras is also an upstream activator of PI 3-kinase, and has been implicated in growth factor-induced membrane ruffling (Bar-Sagi et al., 1987; Kundra et al., 1995; Price et al., 1999; Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1997). In mammalian cells, EGF has been shown to activate Ras at the cell periphery in a study using a fluorescence resonance energy transfer (FRET)-based biosensor (Mochizuki et al., 2001). Overexpression of constitutively active Ras<sup>V12</sup> induced strong membrane ruffling, whereas inhibition of endogenous Ras prevented scatter factor/hepatocyte growth factor (SF/HGF)-stimulated actin reorganization and spreading (Bar-Sagi and Feramisco, 1986; Ridley, 1995; Ridley et al., 1992; Rodriguez-Viciana et al., 1997). Similarly, cAMP-induced motility was defective in *Dictyostelium* cells expressing either Ras-null or RasG<sup>N17</sup> mutants (Insall et al., 1996; Kae et al., 2004; Sasaki et al., 2004; Tuxworth et al., 1997), and platelet-derived growth factor (PDGF)-stimulated motility was dependant on Ras activity in 3T3 cells (Kundra et al., 1995).

p85/p110 $\alpha$  PI 3-kinase activity is required for EGF-stimulated lamellipod extension in MTLn3 cells (Condeelis, 2001; Hill et al., 2000; Mouneimne et al., 2004). However, the relative contribution of Ras and Rac to PI 3-kinase-mediated protrusion has not been examined, and the hypothesis that Rac and PI 3-kinase form a positive feedback loop at the leading edge has not been tested in EGF-responsive cells. In this study we have examined the mechanism of PI 3-kinase activation and PI 3-kinase-dependent lamellipod extension in EGF-stimulated cells. We find a crucial role of Ras but not Rac in EGF-stimulated PI 3-kinase activation at the leading edge of cells and lamellipod extension. Rac is required for cell motility, but this is because of its effects on formation of adhesions behind

the edge of cell protrusions. Thus, Ras and Rac play distinct, but coordinated, roles during EGF-stimulated protrusion in carcinoma cells.

## Results

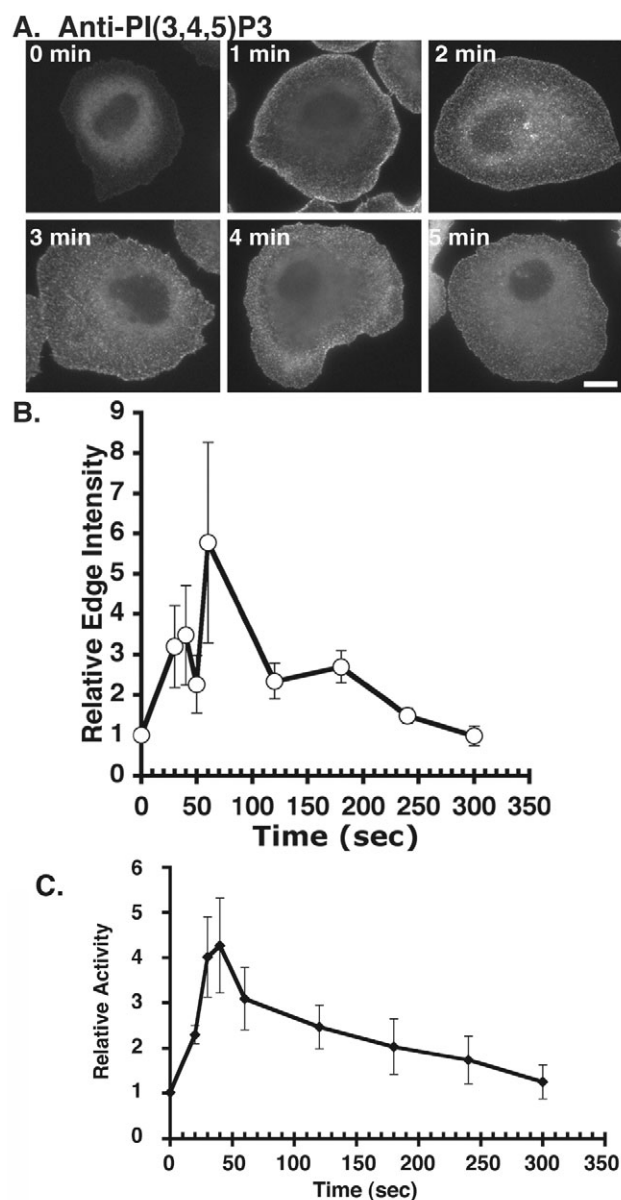
### EGF-induced PtdIns(3,4,5) $P_3$ production at the leading edge of carcinoma cells

We measured the production of PtdIns(3,4,5) $P_3$  at the leading edge of EGF-stimulated MTLn3 cells using a monoclonal anti-PtdIns(3,4,5) $P_3$  antibody (Echelon Bioscience, Salt Lake City, UT). In quiescent MTLn3 cells, minimal membrane-associated PtdIns(3,4,5) $P_3$  was observed (Fig. 1A, 0 minutes). Anti-PtdIns(3,4,5) $P_3$  staining increased rapidly after EGF stimulation, appearing as punctate structures in a narrow band at the cell edge. PtdIns(3,4,5) $P_3$  staining was sixfold above basal at 1 minute (Fig. 1B). Staining of EGF-stimulated cells was specifically blocked by pre-absorption of the antibody with PtdIns(3,4,5) $P_3$ , but not by other phosphoinositides (S.-C.Y. et al., unpublished). After the peak at 1 minute, leading-edge PtdIns(3,4,5) $P_3$  remained 2.5-fold above basal until 3 minutes, when the protrusion of the lamellipod in MTLn3 cells is maximal (Chan et al., 1998; Segall et al., 1996). PtdIns(3,4,5) $P_3$  staining returned to basal levels by 4-5 minutes of stimulation. These data show that EGF-induced PtdIns(3,4,5) $P_3$  production is rapid, persists throughout the first 3 minutes of EGF stimulation, and is evident at the leading edge of the lamellipod. Given that the half-life of PtdIns(3,4,5) $P_3$  in MTLn3 cells is under 10 seconds (S.-C.Y. et al., unpublished), the sustained elevation of PtdIns(3,4,5) $P_3$  at the leading edge of the lamellipod requires the presence of sustained local activation of PI 3-kinase.

We next examined the kinetics of phosphotyrosine-associated PI 3-kinase activity. Phosphotyrosine-associated PI 3-kinase activity peaked sharply at ~4.3-fold above basal at 40 seconds, followed by a slower decline that returned to basal activity at 5 minutes (Fig. 1C). The correlation between phosphotyrosine-associated PI 3-kinase activity and the kinetics of leading-edge PtdIns(3,4,5) $P_3$  suggests that PI 3-kinase activation at the leading edge of the lamellipod is regulated at least in part by direct binding to tyrosine-phosphorylated proteins.

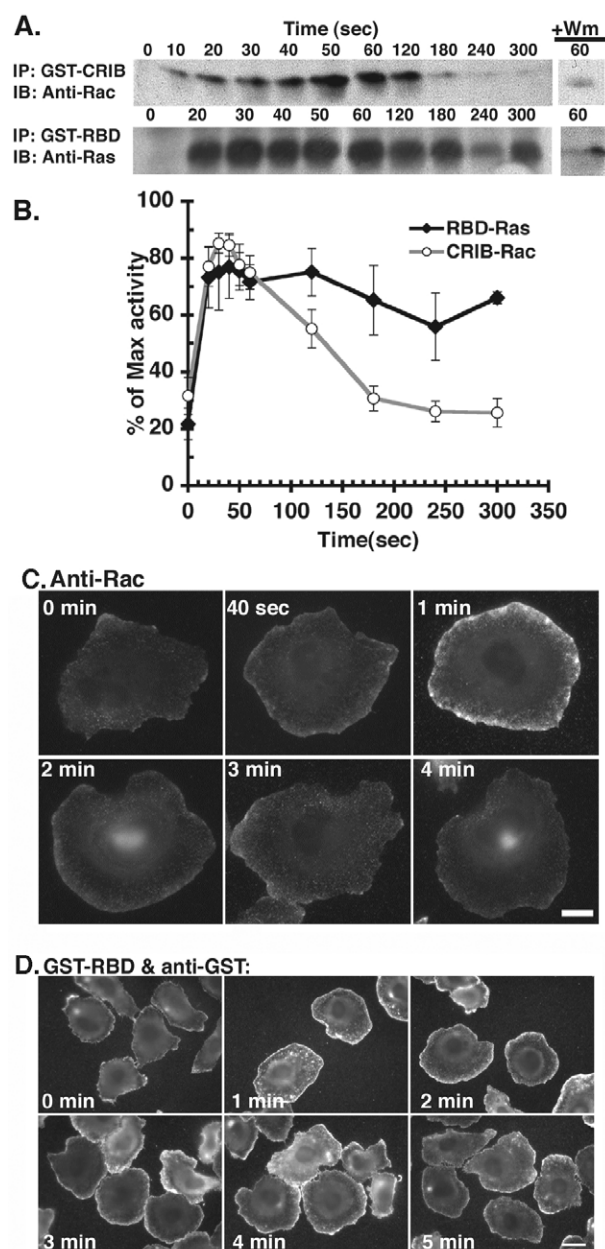
### Kinetics of Ras and Rac activation in EGF-stimulated carcinoma cells

We compared the time-course of PtdIns(3,4,5) $P_3$  production to that of Rac and Ras activation, because both GTPases have been implicated in PI 3-kinase activation (Bokoch et al., 1996; Pacold et al., 2000; Rodriguez-Viciana et al., 1994). Rac and Ras activation in MTLn3 cells was measured in GST-pull-down assays using the CRIB domain from Pak1 and the Ras-binding domain (RBD) from Raf, respectively (Benard et al., 1999; de Rooij and Bos, 1997). Total levels of Ras and Rac in cells lysates did not change during 5 minutes of EGF stimulation (data not shown). In resting MTLn3 cells, the levels of activated Rac and Ras were low (Fig. 2A). Both GTPases were activated rapidly after EGF stimulation. Rac activity was maximal at 50-60 seconds, but the activation was transient and returned almost to basal levels by 3 minutes (Fig. 2A,B, open circles). Consistent with previous findings (Benard et al., 1999), Rac GTPase activity was inhibited by 100 nM wortmannin (Fig. 2A, top panel). Although EGF-induced Ras

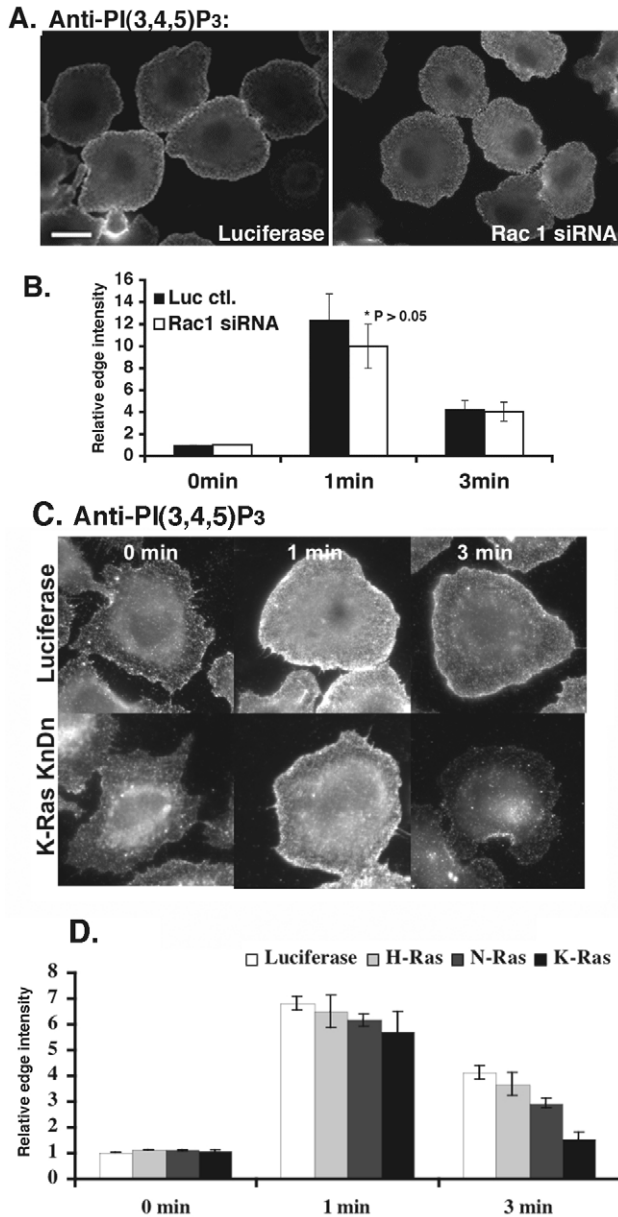


**Fig. 1.** EGF-stimulated PtdIns(3,4,5) $P_3$  production at the leading edge of lamellipod is rapid and sustained. Quiescent MTLn3 cells were stimulated with 5 nM EGF and fixed at various times. (A) Selected images of EGF-stimulated cells stained with anti-PtdIns(3,4,5) $P_3$  antibody. Bar, 10  $\mu$ m for all panels. (B) Quantification of leading-edge PtdIns(3,4,5) $P_3$  production following EGF stimulation. The average edge fluorescence (0–0.66  $\mu$ m from the perimeter) was measured as described in the Materials and Methods, normalized for the edge fluorescence of unstimulated cells and plotted as a function of time. The data are the mean  $\pm$  s.e.m. from four experiments. (C) Anti-phosphotyrosine immunoprecipitates from EGF-stimulated cells were assayed for PI 3-kinase activity. The data are the mean  $\pm$  s.e.m. from four experiments.

activation was also maximal at 40–60 seconds, Ras activation persisted throughout 5 minutes of stimulation (Fig. 2A,B, closed diamonds). Interestingly, Ras activation is also inhibited by wortmannin treatment (Fig. 2A), suggesting that Ras activity is regulated in part by PI 3-kinase. These data show



**Fig. 2.** The kinetics of EGF-stimulated Ras and Rac activation and translocation. (A,B) GST-CRIB (from hPAK) or GST-RBD (from Raf 1) were immobilized on glutathione-Sepharose beads and incubated with EGF-stimulated cell lysates as described. Glutathione-Sepharose pull-downs were separated by 14% SDS-PAGE gel and blotted with anti-Rac or anti-Ras antibody. (A, upper panel) A representative anti-Rac immunoblot of a GST-CRIB pull-down from EGF-stimulated cells. (Lower panel) A representative anti-Ras immunoblot of a GST-RBD pull-down from EGF-stimulated cells. Where indicated, cells were treated with 100 nM wortmannin prior to EGF stimulation. Total Ras and Rac levels did not change during 5 minutes of EGF stimulation (data not shown). (B) Ras (closed diamonds) and Rac (open circles) pull-down assays were quantified by densitometry. Data were expressed as percentage of maximum activity, and show the mean  $\pm$  s.e.m. from nine experiments. (C) Representative fluorescent images of MTLn3 cells stimulated with EGF for various times and stained with anti-Rac antibodies. Bar, 10  $\mu$ m. (D) Representative images of EGF-stimulated MTLn3 cells stained with GST-RBD followed by anti-GST antibody, to visualize endogenous activated Ras. Bar, 10  $\mu$ m.



**Fig. 3.** Inhibition of Ras but not Rac reduces PtdIns(3,4,5)P<sub>3</sub> production at the leading edge of lamellipodia. (A,B) MTLn3 cells were transfected with control or Rac1 siRNA. (A) Representative images of control siRNA or Rac1 siRNA-treated cells stimulated with EGF for 3 minutes and stained with anti-PtdIns(3,4,5)P<sub>3</sub> antibody are shown. (B) Quantification of leading-edge PtdIns(3,4,5)P<sub>3</sub> in siRNA-treated cells. The data are mean  $\pm$  s.e.m. from four experiments. Bar, 10  $\mu$ m in all panels. (C,D) MTLn3 cells were transfected with control siRNA or pools of four siRNA duplexes specific for H-Ras, K-Ras or N-Ras. (C) Representative images of control siRNA or K-Ras siRNA-treated cells stimulated with EGF for 0, 1 or 3 minutes and stained with anti-PtdIns(3,4,5)P<sub>3</sub> antibody are shown. (D) Quantification of leading-edge PtdIns(3,4,5)P<sub>3</sub> in siRNA-treated cells. The data are mean  $\pm$  s.e.m. from 40 cells per condition, and are representative of two independent experiments.

that both Ras activation and leading-edge PtdIns(3,4,5)P<sub>3</sub> production exhibit a persistent activation, whereas Rac activation is largely gone by 3 minutes.

We also measured the localization of Rac and Ras. Staining of EGF-stimulated cells with anti-Rac antibody after various periods of EGF stimulation showed that Rac was rapidly but transiently recruited to the cell edge in EGF-stimulated cells (Fig. 2C). Anti-Rac staining was specific, as it was abolished by pre-absorption with GST-Rac or by small interfering RNA (siRNA) knockdown of Rac expression (supplementary material Fig. S1A,B). We also measured the intracellular distribution of activated Ras in EGF-stimulated cells by staining with GST-RBD followed by anti-GST antibodies. In resting cells, there was a minimal level of activated Ras detected in the plasma membrane. After EGF stimulation, an increase in fluorescent intensity was observed at both plasma membrane and perinuclear regions as previously reported (Chiu et al., 2002; Hancock, 2003; Mochizuki et al., 2001) (Fig. 2D). GST-RBD immunostaining at the leading edge persisted for at least 3 minutes, and did not return to basal levels until 4–5 minutes of EGF stimulation (Fig. 2D). The Ras staining was specific, as it was abolished by pretreatment of cells with the farnesylation inhibitor manumycin A (data not shown), by incubating with recombinant GST prior to incubation with anti-GST antibody, and by combined knockdown of N-Ras and K-Ras (supplementary material Fig. S1C,D). Taken together, data in Figs 1 and 2 show that the kinetics of Ras activation and membrane localization is similar to the sustained kinetics of PI(3,4,5)P<sub>3</sub> production in EGF-stimulated cells, whereas Rac activation and membrane localization is transient.

#### Inhibition of Ras but not Rac reduces leading-edge PtdIns(3,4,5)P<sub>3</sub> production

To examine the requirement for Ras versus Rac in the regulation of PtdIns(3,4,5)P<sub>3</sub> production at the leading edge, we knocked down each GTPase using siRNA. Rac1 is the predominant Rac isoform in MTLn3 cells, as levels of Rac2 and Rac3 were barely detectable by quantitative RT-PCR (Q-RT-PCR) (M.S., unpublished). To inhibit Rac1, we transiently transfected MTLn3 cells with siRNA duplexes directed against luciferase (negative control) and Rac1. Optimal suppression of Rac1 occurred at 48 hours post-transfection, when Rac1 knockdown efficiency was approximately 80–85% as detected by anti-Rac immunoblots (see supplementary material Fig. S2A, upper panel). We measured the effect of siRNA-mediated Rac1 knockdown on EGF-stimulated production of PtdIns(3,4,5)P<sub>3</sub> at the leading edge. Control or Rac1 siRNA-treated cells were stimulated with EGF for 1 or 3 minutes, and then stained with anti-PtdIns(3,4,5)P<sub>3</sub> antibodies. Knockdown of Rac1 expression had no effect on PtdIns(3,4,5)P<sub>3</sub> production at the leading edge (Fig. 3A): in both control and Rac1 siRNA-treated cells, EGF-stimulated production of PtdIns(3,4,5)P<sub>3</sub> at the leading edge of both was maximal at 1 minute, and remained 3–4-fold above basal levels at 3 minutes (Fig. 3B). These results show that inhibition of Rac expression does not affect PtdIns(3,4,5)P<sub>3</sub> production at the leading edge of the lamellipod.

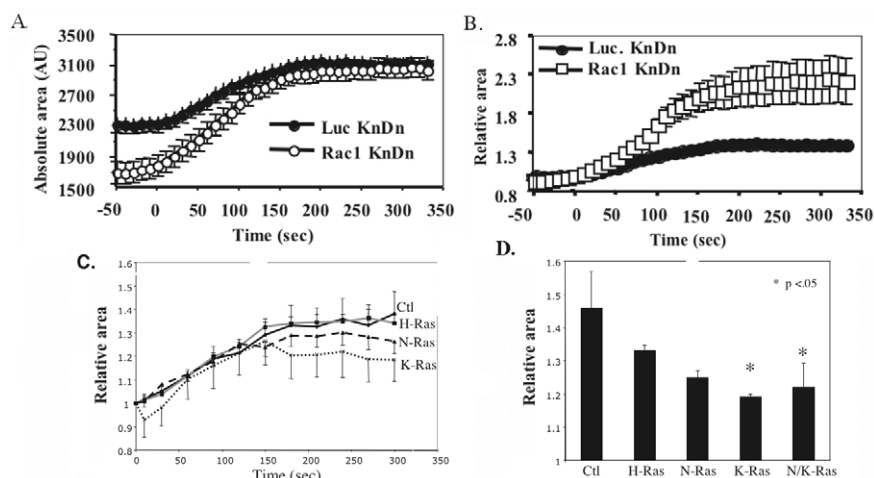
Q-RT-PCR analysis of MTLn3 cells showed that N-Ras and K-Ras were expressed at similar levels, and that we were able to achieve 70–80% knockdown of N-Ras and K-Ras using a pool of four distinct siRNA oligonucleotides for each protein (see supplementary material Fig. S2B). Although knockdown of H-Ras was only 40% efficient, control MTLn3 cells express

20-fold less H-Ras than N-Ras or K-Ras (supplementary material Fig. S2B). Anti-PtdIns(3,4,5) $P_3$  staining of EGF-stimulated cells showed that knockdown of K-Ras had little effect on PtdIns(3,4,5) $P_3$  levels at 1 minute, but markedly inhibited production at 3 minutes (Fig. 3C,D). By contrast, transfection of cells with control siRNA (luciferase) or H-Ras siRNA had no effect on PtdIns(3,4,5) $P_3$  production at either time point (Fig. 3D). siRNA knockdown of N-Ras has no effect at 1 minute and a modest effect at 3 minutes (Fig. 3D). Similarly, treatment of cells with the farnesyltransferase inhibitor manumycin A also reduced PI(3,4,5) $P_3$  production at 3 minutes (data not shown). Thus, EGF-stimulated PtdIns(3,4,5) $P_3$  production is inhibited by knockdown of K-Ras and, to a lesser extent, N-Ras, but not by knockdown of Rac1.

### Specific inhibition of Ras but not Rac blocks EGF-stimulated protrusion

Both Rac and Ras have been implicated in membrane protrusion and PI 3-kinase activation (Bokoch et al., 1996; Rodriguez-Viciana et al., 1997; Welch et al., 2003). Moreover, overexpression of constitutively active Rac in MTLn3 cells is sufficient to induce lamellipod extension (El-Sibai et al., 2007). To determine whether Rac is necessary for EGF-stimulated protrusion in MTLn3 cells, we used time-lapse video microscopy to measure the surface area of control or Rac1 siRNA-treated cells during EGF stimulation. Although Rac1 siRNA-treated cells showed a mixed morphology, with both rounded and normally spread cells, all of these cells were capable of extending lamellipodia in response to EGF (supplementary material Movie 1). Although the mean area of quiescent Rac1 siRNA-treated cells was less than that of control cells ( $P<0.01$ ), Rac1 siRNA-treated cells achieved a final area that was not significantly different than that of the control cells after 3 minutes of EGF stimulation (Fig. 4A). Thus, protrusion relative to initial area was greater in the Rac siRNA-treated cells than in control cells ( $P<0.001$  at 1 and  $P<0.01$  at 3 minutes; Fig. 4B), but this reflected the smaller initial size rather than a true hyperprotrusion. Similar results were obtained after knockdown of Rac1 with a pool of four distinct siRNA oligonucleotides (supplementary material Fig. S2A, upper panel; supplementary material Fig. S3) and in cells treated with the Rac-specific inhibitor NSC23766, whose efficacy against Rac was confirmed in a CRIB domain pull-down assay (see supplementary material Fig. S4A,B). These results show that Rac1 is not required for the protrusion step in EGF-stimulated cell motility in carcinoma cells.

To determine the effects of specific inhibition of Ras on EGF-stimulated lamellipod extension, we measured EGF-stimulated protrusion in cells transfected with pools of four siRNA oligonucleotides for each Ras isoform. Protrusion was measured in control or Ras siRNA-treated cells stimulated with EGF; data from a single experiment (Fig. 4B) and pooled data



**Fig. 4.** Specific inhibition of Ras, but not Rac, inhibits EGF-stimulated protrusion.

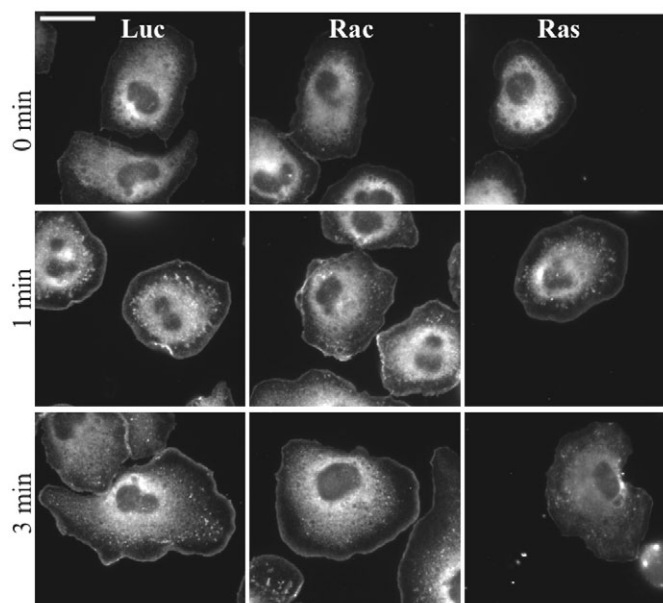
(A) MTLn3 cells were transfected with control or Rac1 siRNA. Time-lapse images of EGF-stimulated cells were recorded (20 seconds per frame) with a  $20\times 0.4$  NA objective, and surface areas were measured using NIH Image. The data show the mean  $\pm$  s.e.m. from 41 cells per time point. (B) Data from A were normalized to the initial area of each cell. (C) MTLn3 cells were transfected with control siRNA or isoform-specific siRNA targeting H-Ras, K-Ras, N-Ras or both N-Ras and K-Ras. The surface area of control siRNA or Ras siRNA-treated cells was measured after various times of EGF stimulation. Cell areas were normalized to the initial area of each cell. Data show the mean  $\pm$  s.e.m. from 9–14 cells per time point. (D) Pooled data from two experiments showing maximal EGF-stimulated protrusion in cells treated with control or anti-Ras siRNA. Data are the mean  $\pm$  s.d.

from two experiments (Fig. 4C) are shown. Whereas a slight decrease in cell protrusion is seen with H-Ras siRNA, protrusion was inhibited by almost 45% in cells treated with N-Ras siRNA, and nearly 60% in cells treated with K-Ras siRNA (Fig. 4C). Whereas inhibition by N-Ras siRNA did not reach statistical significance, inhibition by K-RAS siRNA was significant ( $P<0.05$ ). Knockdown of both N-Ras and K-Ras did not exacerbate the phenotype seen with K-Ras siRNA alone. We also saw an inhibition of EGF-stimulated protrusion in MTLn3 cells microinjected with the Y13-259 inhibitory anti-Ras antibody (data not shown). These data clearly demonstrate a requirement for K-Ras, and to a lesser extent N-Ras, in EGF-stimulated protrusion.

To further explore the formation of lamellipodia in Rac and Ras knockdown cells, we measured the EGF-stimulated recruitment of Arp2/3 to the leading edge, which corresponds to the zone of new barbed end formation (Bailly et al., 1999). We found that Arp2/3 recruitment in Rac1 siRNA-treated cells was indistinguishable from that in control cells (Fig. 5). By contrast, in K-Ras knockdown cells, Arp2/3 recruitment to the cell edge was normal at 1 minute but was markedly suppressed at 3 minutes of EGF stimulation.

### Rac is required for cell locomotion

As our data show that Ras, but not Rac, is required for EGF-stimulated PtdIns(3,4,5) $P_3$  production and protrusion, we tested whether Rac might be required for other steps in the motility cycle. We performed a motion analysis of time-lapse movies of control and Rac1 siRNA-treated cells, recorded over 1 hour in the presence of 5% serum (supplementary material Movie 2). The Rac1 siRNA-treated cells showed an



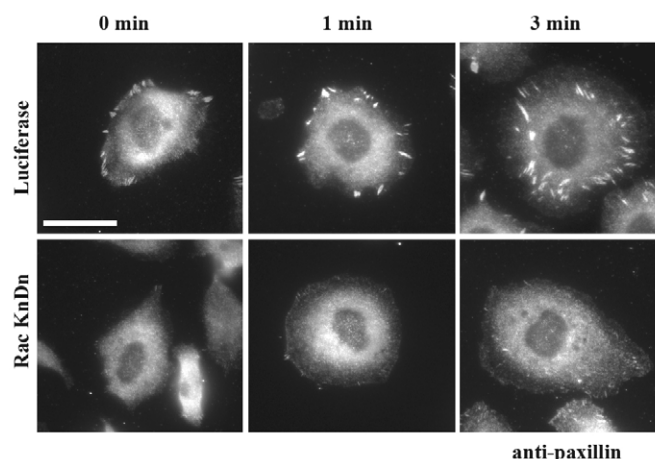
**Fig. 5.** Specific inhibition of Ras, but not Rac, inhibits EGF-stimulated Arp2/3 recruitment. MTLn3 cells were transfected with control (left panels), Rac1 (middle panels) or K-Ras (right panels) siRNA. Cells were stimulated with EGF for 0, 1 or 3 minutes, fixed and stained with anti-Arp2/3 antibodies.

approximately fourfold reduction in net path; data are provided for a single siRNA oligonucleotide and a pool of four distinct siRNA oligonucleotides (Table 1). Similar results were obtained using the Rac-specific inhibitor NSC23766 (Table 1), which also caused a fourfold decrease in net path.

We note that unlike control MTLn3 cells, in which EGF stimulation suppresses ruffling (Segall et al., 1996), we see significant membrane ruffling in Rac1 siRNA-treated cells (supplementary material Movie 1). Ruffling can result from the dissipation of a lamellipod, when membrane protrusions fail to adhere at the leading edge (Burrage and Wennerberg, 2004). In addition, the surface area of unstimulated Rac knockdown cells is approximately 30% less than in control cells (Fig. 4A), which could result from a defect in cell adhesion. To test whether Rac1 knockdown affects formation of adhesive structures, we stained control and Rac siRNA-treated cells with anti-paxillin antibodies. In control cells, EGF stimulates an increase in cell area, with a proliferation of focal adhesions behind the edge of the protruding cell (Fig. 6, upper panels). However, knockdown of Rac1 leads to a significant reduction in focal adhesion in both unstimulated and EGF-stimulated cells (Fig. 6, lower panels). These data suggest that the reduced motility of Rac1 knockdown cells is because of a reduced capacity to form adhesions after protrusion.

## Discussion

PtdIns(3,4,5) $P_3$  production at the leading edge of acutely EGF-stimulated carcinoma cells is rapid and persistent, as PtdIns(3,4,5) $P_3$  levels remain elevated for up to 3 minutes. Given that the half-life of PtdIns(3,4,5) $P_3$  in MTLn3 cells is less than 10 seconds. (S.-C.Y. et al., unpublished), the kinetics



**Fig. 6.** Rac1 knockdown inhibits formation of focal adhesions. MTLn3 cells were transfected with control (luciferase, upper panels) or Rac1 (lower panels) siRNA. Cells were stimulated with EGF for 0, 1 or 3 minutes, fixed and stained with anti-paxillin antibodies.

of PtdIns(3,4,5) $P_3$  accumulation reflect the kinetics of PI 3-kinase activation. Although the mechanism of PI 3-kinase activation at the cell edge at 1 minute is not yet clear, phosphotyrosine-associated PI 3-kinase activity is also maximal at 1 minute, and PtdIns(3,4,5) $P_3$  accumulation at 1 minute is not significantly inhibited by suppression of either Ras or Rac. Thus, leading-edge PtdIns(3,4,5) $P_3$  at 1 minute is probably due in part to direct binding of Class IA PI 3-kinase to tyrosine phosphorylated EGF receptor substrates such as Gab1 (Holgado-Madruga et al., 1996). Recent data from our laboratory suggest that Cdc42 may also be involved in regulation of the early PtdIns(3,4,5) $P_3$  peak (El-Sibai et al., 2007).

By contrast, siRNA knockdown of Ras, but not Rac, inhibits PtdIns(3,4,5) $P_3$  production at the leading edge at 3 minutes. Consistent with this, EGF-stimulated PtdIns(3,4,5) $P_3$  production is kinetically correlated with the activation of Ras at the leading edge, whereas Rac is only transiently activated and its activity is markedly reduced by 3 minutes. The K-Ras isoform plays the major role in this process, although a modest reduction in PtdIns(3,4,5) $P_3$  levels was seen after N-Ras knockdown.

**Table 1. Rac is required for cell motility**

	Net path ( $\mu$ m)
Control siRNA	19.4 $\pm$ 2.0 (n=30)
Rac1 siRNA	5.4 $\pm$ 0.6 (n=37)
Control siRNA	12.0 $\pm$ 2.1 (n=15)
Rac1 siRNA (pooled oligonucleotides)	3.0 $\pm$ 0.6 (n=13)
Control cells (DMSO)	30.9 $\pm$ 1.7 (n=20)
NSC23766 (100 $\mu$ M)	7.1 $\pm$ 0.7 (n=20)

MTLn3 cells were transfected with control siRNA, a single Rac siRNA or a pool of four distinct Rac1 siRNAs. Alternatively, cells were treated with the Rac inhibitor NSC23766 or carrier (dimethyl sulfoxide, DMSO). Time-lapse phase images of cells in 5% serum were collected (1 frame/min). Net path was calculated using DIAS software (Solltech, Oakdale, CA).

Unlike fMLP-stimulated neutrophils, in which a positive feedback loop between Rac and PI 3-kinase has been postulated (Servant et al., 2000; Srinivasan et al., 2003; Weiner et al., 2002), EGF-stimulated PtdIns(3,4,5) $P_3$  production in MTLn3 cells is Rac independent, whereas wortmannin inhibits Rac activation. Thus, in carcinoma cells, EGF-stimulated Rac activation requires PI 3-kinase, but PI 3-kinase activation at the leading edge does not require Rac. However, our data do support the possibility of a positive feedback loop between Ras and PI 3-kinase, which may contribute to PI 3-kinase-dependent lamellipod extension. Treatment of cells with Ras siRNA reduces PtdIns(3,4,5) $P_3$  levels at the leading edge at 3 minutes, and EGF-stimulated Ras activation is reduced in wortmannin-treated cells. The possibility of a local positive feedback loop between Ras and PI 3-kinase is supported by previous studies showing that Ras-GTP activates PI 3-kinase by direct interaction with the p110 catalytic subunit (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994), and that PI 3-kinase contributes to Ras activation through the PtdIns(3,4,5) $P_3$ -mediated enhancement of Gab1 phosphorylation and recruitment of Grb2/Sos (Rodrigues et al., 2000; Yart et al., 2001).

We previously showed that PI 3-kinase is required for barbed end formation at 3 minutes, and is involved in the generation of protrusive force (Mouneimne et al., 2004). siRNA knockdown of K-Ras, and to a lesser extent N-Ras, inhibits EGF-stimulated PtdIns(3,4,5) $P_3$  production at 3 minutes but not 1 minute, and the effect of Ras knockdown on protrusion is most pronounced at later time points (3–5 minutes), whereas protrusion at early time points is relatively unaffected. These data support an important role for sustained Ras-mediated PtdIns(3,4,5) $P_3$  production in EGF-stimulated lamellipod extension. Ras has been previously implicated in growth factor-induced chemotaxis in both mammalian systems (Kundra et al., 1995; Price et al., 1999; Rodriguez-Viciana et al., 1997) and *Dictyostelium* (Insall et al., 1996; Kae et al., 2004; Sasaki et al., 2004; Tuxworth et al., 1997). A recent study in fibroblasts suggested that the K(B)-Ras isoform in particular was required for PDGF-stimulated migration (Liao et al., 2006).

The failure of Rac inhibition or siRNA knockdown to inhibit EGF-stimulated lamellipod formation is surprising, given that the formation of a Rac/IRSp53/WASP family verprolin-homologous protein 2 (WAVE2) complex and other Rac-dependent WAVE-activation complexes have been implicated in membrane ruffling and lamellipod extension (Gautreau et al., 2004; Miki et al., 1998; Miki et al., 2000; Weiner et al., 2006). It is unlikely that the residual 20% Rac activity after siRNA treatment accounts for the lack of effects on lamellipod extension at 3 minutes, given that we did see effects of Rac1 knockdown on motility. Interestingly, expression of constitutively active Rac does produce lamellipodia in MTLn3 cells (El-Sibai et al., 2007), suggesting that Rac can drive protrusion in these cells. However, in control MTLn3 cells, the kinetics of Rac activation after acute EGF stimulation are extremely transient, and other PI 3-kinase-dependent mechanisms appear to regulate protrusion. Whereas Rac is required for protrusion in many cell types, protrusion and chemotaxis in the absence of Rac activity have been previously described in colon carcinoma cells, macrophages and dendritic cells (O'Connor et al., 2000; West et al., 2000; Wheeler et al., 2006). Ruffling and/or protrusion induced by expression of

activated Rab5 or the Cdc42 effector MSE55, or by infection with *Salmonella typhimurium*, are also Rac1-independent (Burbelo et al., 1999; Jones et al., 1993; Spaargaren and Bos, 1999). We have not elucidated the mechanism of Rac-independent protrusion, but it could involve the recruitment of WAVE2 by PtdIns(3,4,5) $P_3$  production at the cell edge (Oikawa et al., 2004). Recent data from our laboratory have also implicated the PI 3-kinase-dependent activation of Cdc42 as playing an important role in protrusion in MTLn3 cells (El-Sibai et al., 2007).

If Rac siRNA-treated cells undergo EGF-stimulated protrusion, how can we explain their defect in motility? Time-lapse studies (supplementary material Movie 2) suggest that although Rac1 knockdown cells rapidly extend membrane protrusions, these structures are not stable and do not persist in any direction. Activated Rac has been reported to promote integrin-mediated adhesions in migrating cells, where new lamellipodia are stabilized by formation of adhesions (Kiosses et al., 2001), and we find that focal adhesions are greatly reduced in Rac1 knockdown cells. Thus, a likely explanation for the reduced motility of Rac1 siRNA-treated cells is the failure to establish stable adhesions after lamellipod extension. This is consistent with our previous report that overexpression of dominant-negative Rac greatly reduces both cell spreading and the number of focal adhesions during protrusion (Bouzahzah et al., 2001). Rac and PI 3-kinase activities have been shown to be required for recruitment of Arp2/3 complex to vinculin, an integrin-associated protein, at new adhesion sites at the leading edge of lamellipod in EGF-stimulated A431 cells (DeMali et al., 2002; DeMali and Burridge, 2003). This suggests that a coordination of membrane protrusion and Rac-mediated recruitment of adhesion complexes to the leading edge may be necessary for MTLn3 cells to migrate efficiently.

Although our data suggests that Ras-mediated activation of PI 3-kinase at 3 minutes is required for the generation of protrusive force, we do not yet understand the mechanistic role of EGF-stimulated leading-edge PtdIns(3,4,5) $P_3$  production at 1 minute. This event temporally coincides with the early transient of actin polymerization, which has been implicated in directional sensing in EGF-stimulated tumor cells (Ghosh et al., 2004; Mouneimne et al., 2004). EGF-induced PI 3-kinase activation at the leading edge at 1 minute may contribute to early barbed end formation, as residual barbed ends were seen after inhibition of cofilin with siRNA or blocking antibodies (approximately 20% of maximum) (Mouneimne et al., 2004). Alternatively, PI 3-kinase activation at 1 minute may initiate signaling events that contribute to the formation or stabilization of focal contacts at the leading edge at later times. The regulation and function of PI 3-kinase and Rac during the initial responses to EGF stimulation will be an important issue for future study.

## Materials and Methods

### Materials

Anti-PtdIns(3,4,5) $P_3$  antibody (IgG) was a generous gift from Echelon Biosciences (Salt Lake City, UT). Anti-phosphotyrosine antibody (PY20) was purchased from Signal Transduction Laboratories. Anti-EGF receptor antibody and anti-phospho-Erk antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Rac antibody (clone 23A8) and anti-phospho-Ser473 AKT antibodies were purchased from Upstate Biotechnology (Charlottesville, VA). Anti-Ras antibody (Y13-259) was provided by T. Michaeli (Albert Einstein College of Medicine, Bronx, NY). Manumycin A, *Clostridium Difficile* toxin B and wortmannin were obtained from CalBiochem (San Diego, CA). NSC23766 was a gift from Yi Zheng (Children's

Hospital Research Foundation, Cincinnati, OH). siRNA duplexes were purchased from Dharmacon.

### Cell culture and EGF stimulation

The rat mammary adenocarcinoma breast cancer cell line, MTLn3, has been previously described (Segall et al., 1996). In each experiment, MTLn3 cells were starved in L15 media (Gibco BRL) supplemented with 0.35% bovine serum albumin (BSA) in a 37°C, non-CO<sub>2</sub> incubator for 3–4 hours. Cells were then stimulated with 5 nM murine EGF (Upstate Biotechnology) for various times.

### Microscopy and immunofluorescence analysis

For immunofluorescence staining procedures, cells were plated on collagen-coated coverslips 24 hours prior to the experiment. After incubation without or with EGF for various times, cells were rapidly fixed and permeabilized by immersion in 3.7% paraformaldehyde, 0.1% glutaraldehyde and 0.15 mg/ml saponin in fix buffer (5 mM KCl, 137 mM NaCl, 4 mM NaHCO<sub>3</sub>, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 1.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, 5 mM PIPES, pH 7.2, 2 mM EGTA and 5.5 mM glucose) for 1 hour at 37°C. The fixed cells were incubated with primary antibodies for 1 hour, followed by secondary antibodies for 45 minutes, and mounted in 6 mg/ml of N-propyl gallate prepared in 50% glycerol-phosphate-buffered saline (PBS) (v/v).

All fluorescent images were obtained using a 60× 1.4 NA Olympus objective optic and a cooled CCD camera. For fluorescence quantification, all digital images were imported in NIH image software and analyzed using a previously described macro (DesMarais et al., 2002). This macro collects pixel intensities from the perimeter of the cell in a 0.22 µm stepwise manner. The pixel intensities in the leading-edge compartment, defined as 0.66 µm from the perimeter of the cells, were averaged and normalized to the edge intensity of non-stimulated cells.

### Immunoprecipitation and western blots

For immunoprecipitations, MTLn3 cells were plated on 100 mm tissue culture dishes. EGF-stimulated cells were rinsed with cold PBS containing 1 mM sodium vanadate and lysed in 20 mM Tris, 137 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1% NP-40, 10% glycerol, pH 7.5, supplemented with protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin]. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes. After blocking, membranes were incubated with primary antibodies at 4°C overnight followed by horseradish peroxidase (HRP)-conjugated secondary antibodies, and visualized using enhanced chemiluminescence (ECL) (Amersham Biosciences). For measurement of phosphotyrosine-associated PI 3-kinase activity, anti-phosphotyrosine (PY20; Signal Transduction Laboratory) immunoprecipitates were assayed for lipid kinase activity towards phosphatidylinositol, as described (Yu et al., 1998).

### GST-CRIB and GST-RBD protein purification and pull-down assays

GST fusion proteins containing the PAK1 GTPase-binding domain [hPAK(67–150), CRIB domain] or the Raf1 RBD (amino acids 51–131, RBD domain) were generous gifts from Klaus M. Hahn (Scripps Research Institute, La Jolla, CA) and Linda van Aelst (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), and were purified from *Escherichia coli*. To measure activation of Rac and Ras, EGF-stimulated cells were washed with cold PBS containing 1 mM sodium vanadate and lysed in magnesium lysis buffer (MLB) (25 mM HEPES, pH 7.5, 1% Igapal, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM EDTA, 1 mM vanadate) containing protease inhibitors (1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin). Immobilized GST-CRIB or RBD (8 µg per assay) was incubated with the cell lysates at 4°C for 30 minutes on a rotating wheel, washed three times with MLB and suspended in 50 µl Laemmli sample buffer. Proteins were separated by 14% SDS-PAGE, transferred to PVDF membranes and blotted with anti-Ras or anti-Rac antibodies.

### GST-CRIB and GST-RBD immunostaining

EGF-stimulated cells were fixed and incubated with purified GST-CRIB or GST-RBD for 1 hour, followed by anti-GST antibody and secondary antibody.

### siRNA and Q-RT-PCR analysis

Rac1 siRNA sequences were designed specifically against rat Rac1: 5'-AAAGAGAUCGGUGCUGUCAA-3'. Control siRNA duplexes were targeted against luciferase: 5'-CGTACGCGGAATACTTCGA-3'. Rac1 was also knocked down using a pool of four siRNA duplexes (Smartpools, Dharmacon). For knockdown of Ras isoforms, pools of four siRNA duplexes for each Ras isoform (Smartpools, Dharmacon) were used. Cells were transfected with 10 nM siRNA using Lipofectamine 2000 (Invitrogen) for 48 hours. For analysis of Rac1 suppression, SDS-sample buffer was added directly to non-stimulated cells, and anti-Rac expression was analyzed by western blotting. For analysis of Rac and Ras isoform suppression, total mRNA from MTLn3 cells was isolated using the RNeasy mini kit (Qiagen). mRNA levels were analyzed by Q-RT-PCR as previously described (Chan et al., 2005; Wang et al., 2002). For signaling and motility experiments, siRNA-treated cells were plated on collagen-coated coverslips or acid-

treated glass-bottom MatTek dishes (MatTek, Ashland, MA) at 24 hours after transfection.

### Area extension analysis in EGF live upshift assays

For time-lapse experiments, quiescent cells were EGF stimulated on the microscope using a heated stage and an objective heater. Images were collected at 10-second intervals using 20× 0.4 NA or 40× 0.55 NA objectives with a video camera. Cell surface area was analyzed using NIH Image. Statistical significance was calculated using a two-tailed Student's *t*-test.

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