PAK1-mediated activation of ERK1/2 regulates lamellipodial dynamics

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Summary

PAK1 is a member of the p21-activated kinase (PAK) family of serine/threonine kinases that are activated by the Rho GTPases Rac and Cdc42, and are implicated in regulating morphological polarity, cell migration and adhesion. Here we investigate the function of PAK1 in cell motility using macrophages derived from PAK1-null mice. We show that CSF1, a macrophage chemoattractant, transiently stimulates PAK1 and MAPK activation, and that MAPK activation is reduced in PAK1$^{-/-}$ macrophages. PAK1 regulates the dynamics of lamellipodium extension as cells spread in response to adhesion but is not essential for macrophage migration or chemotaxis towards CSF1. Following adhesion, PAK1$^{-/-}$ macrophages spread more rapidly and have more lamellipodia than wild-type cells; however, these lamellipodia were less stable than those in wild-type macrophages. ERK1/2 activity was reduced in PAK1$^{-/-}$ macrophages during adhesion, and inhibition of ERK1/2 activation in wild-type macrophages was sufficient to increase the spread area and mimic the lamellipodial dynamics of PAK1$^{-/-}$ macrophages. Together, these data indicate that PAK1 signals via ERK1/2 to regulate lamellipodial stability.

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Key words: PAK1, ERK1/2, Cell adhesion, Cell migration, Macrophages, Rho GTPases

Introduction

Cells alter their morphology dynamically in response to stimuli that induce cell migration and during cell division. Extracellular matrix composition affects cell shape by activating integrins, inducing intracellular signalling pathways that regulate cell morphology, including activation of the Rho GTPase family members RhoA, Rac1 and Cdc42 (Rose et al., 2007). Rac1 and Cdc42 promote cell spreading by inducing actin polymerisation and lamellipodium extension (Choma et al., 2004; Price et al., 1998; Vidali et al., 2006; Wells et al., 2004).

Downstream effectors of Rac and Cdc42 include the PAK (p21-activated kinase) family of serine/threonine kinases. Mammals have six PAKs, which are divided into two groups based upon sequence homology: group A consists of PAK1-PAK3 and group B of PAK4-PAK6. The group A PAKs are highly homologous, sharing 88% sequence homology within the p21-binding domain (PBD), which binds to Rac1 and Cdc42, and 93% homology within the kinase domain (Jaffer and Chernoff, 2002). Despite this high level of homology, unique functions for each of the three PAKs are indicated from studies of knockout mice. Whereas PAK1- and PAK3-null mice initially appear normal and healthy, knockout of PAK2 is embryonic lethal (Hofmann et al., 2004). Closer analysis of PAK3-null mice indicated mental retardation as a result of defects in synaptic plasticity (Meng et al., 2005), whereas PAK1-null mice exhibited undefined immune defects (Hofmann et al., 2004).

Many potential targets have been identified for PAK1-PAK3. These include various members of the mitogen-activated protein kinase (MAPK) pathways (Beeser et al., 2005; Frost et al., 1996; Frost et al., 1997; King et al., 1998), the cytoskeletal regulators myosin II (Zeng et al., 2000), myosin light chain kinase (MLCK) (Sanders et al., 1999) and stathmin/Op18 (Daub et al., 2001; Wittmann et al., 2004), and the apoptosis regulator BAD (Schurmann et al., 2000). PAK1 affects both the actin cytoskeleton (Edwards et al., 1999; Sanders et al., 1999) and the microtubule network (Daub et al., 2001; Wittmann et al., 2004), and is thereby implicated in cell migration (Adam et al., 1998; Ching et al., 2007; Sells et al., 1999; Zhou et al., 2003), phagocytosis (Dharmawardhane et al., 1999; Diakonova et al., 2002), and cell spreading in platelets (Suzuki-Inoue et al., 2001) and fibroblasts (ten Klooster et al., 2006). In fibroblasts, PAK1 appears to inhibit spreading by competing with Rac1 for binding to β-PIX (ten Klooster et al., 2006). PAK1 could also affect cell shape via regulation of the MAPKs ERK1 and ERK2. PAK1 can phosphorylate and activate both MEK1 (MAPK/ERK kinase 1) and Raf, which are upstream activators of ERKs (Frost et al., 1997; King et al., 1998). Although the MAPKs are typically associated with regulation of transcription, they also affect cell migration and adhesion. For example, ERK1/2 is required for integrin-induced cell scattering (Honma et al., 2006) and for neutrophil migration downstream of Cdc42 (Szczur et al., 2006). ERK1/2 can localise to the plasma membrane (Glading et al., 2001; Harding et al., 2005), to endosomes (Kermorgant et al., 2004) and to focal adhesions (Fincham et al., 2000), and is activated upon adhesion in a PAK-dependent manner (Eblen et al., 2002; Sundberg-Smith et al., 2005). ERK1/2 has also been reported to phosphorylate paxillin, promoting lamellipodium formation and spreading in a FAK- and Rac-dependent manner (Ishibe et al., 2004).

We have investigated the role of PAK1 in cell adhesion and migration by comparing macrophages derived from wild-type

(WT) and PAK1-null (PAK1$^{-/-}$) mice. We report that deletion of PAK1 in macrophages results in enhanced cell spreading but reduces lamellipodial stability. PAK1 is required for optimal ERK1/2 activation during adhesion and CSF1 stimulation whilst inhibition of ERK1/2 in WT macrophages mimicked the lamellipodial dynamics and enhanced spreading observed in PAK1$^{-/-}$ macrophages. These results indicate that PAK1 affects lamellipodial dynamics by regulating ERK1/2 activity.

Results

PAK1 is not required for macrophage differentiation or migration but regulates MAPK activity

To determine which of the six PAK isoforms are expressed in mouse bone-marrow-derived macrophages (BMM), isoform-specific antibodies and/or RT-PCR was used. This analysis indicated that BMMs express PAK1, PAK2 and PAK3 but not PAK4, PAK5 or PAK6 (data not shown). Western blotting using an antibody (C19) against PAK1, PAK2 and PAK3, or a PAK1-specific antibody, confirmed that PAK1 protein was expressed in WT BMMs but was not detectable in PAK1$^{-/-}$ BMMs (Fig. 1A). The C19 antibody also showed that there was no change in PAK2 protein expression in PAK1$^{-/-}$ BMMs. PAK3 migrates at the same molecular mass as PAK1 upon SDS-PAGE (Gujdar et al., 2003), but no band was detectable with the C19 antibody in this region in PAK1$^{-/-}$ BMMs, implying that PAK3 protein levels are very low compared with PAK1 levels. Rac1 levels were not altered in PAK1$^{-/-}$ BMMs (not shown).

Macrophage colony-stimulating factor 1 (CSF1) acts through the tyrosine kinase receptor Fms/CSF1R to stimulate macrophage survival and differentiation, and also acts as a macrophage chemoattractant (Pixley and Stanley, 2004). Similar surface levels of the macrophage differentiation marker F4/80 (Hirsch et al., 1981) were observed in WT and PAK1$^{-/-}$ BMMs, indicating that loss of PAK1 did not prevent CSF1-mediated macrophage differentiation (Fig. 1B). CSF1 stimulation of WT macrophages rapidly induced PAK1 Thr423 phosphorylation (Fig. 1C), a site associated with PAK1 activation (Zenke et al., 1999). PAK1 phosphorylation was increased within 2 minutes, and returned to basal levels by 10 minutes. Deletion of PAK1 did not affect CSF1-mediated phosphorylation of Akt (Fig. 1D), thus it is not essential for CSF1 signalling. However, the phosphorylation of ERK1/2 and p38 MAPK was reduced in PAK1$^{-/-}$ BMMs after CSF1 stimulation (Fig. 1D), consistent with a role for PAKs in regulating the activity of kinases upstream of MAPK, including Raf and MEK1 (Beeser et al., 2005; Frost et al., 1997; King et al., 1998; Zhang et al., 1995). However, phosphorylation of MEK1 on S298 (Fig. 1D) or c-Raf on S338, both described as PAK substrates, was not altered in PAK1$^{-/-}$ BMMs, indicating that deletion of PAK1 alone is not sufficient to affect these sites (data not shown).

PAK1 is required for chemotaxis but not translocation of leukocytes and HEK293 cells stimulated with ligands for G-protein-coupled receptors (Li et al., 2003; Wang et al., 2002). PAK1 deletion did not affect the migration speed of macrophages in CSF1-containing growth medium: both WT and PAK1$^{-/-}$ BMMs migrated at 0.4 μm/minute. Chemotaxis towards CSF1 was also unaffected in PAK1$^{-/-}$ BMMs (Fig. 1E). PAK1 is therefore not required for long term CSF1 responses such as differentiation and chemotaxis, but does contribute to early CSF1 signalling to MAPKs.

Deletion of PAK1 increases BMM spreading upon adhesion

WT and PAK1$^{-/-}$ BMMs were indistinguishable morphologically (Fig. 2A; supplementary material Movies 1 and 2): the majority of cells

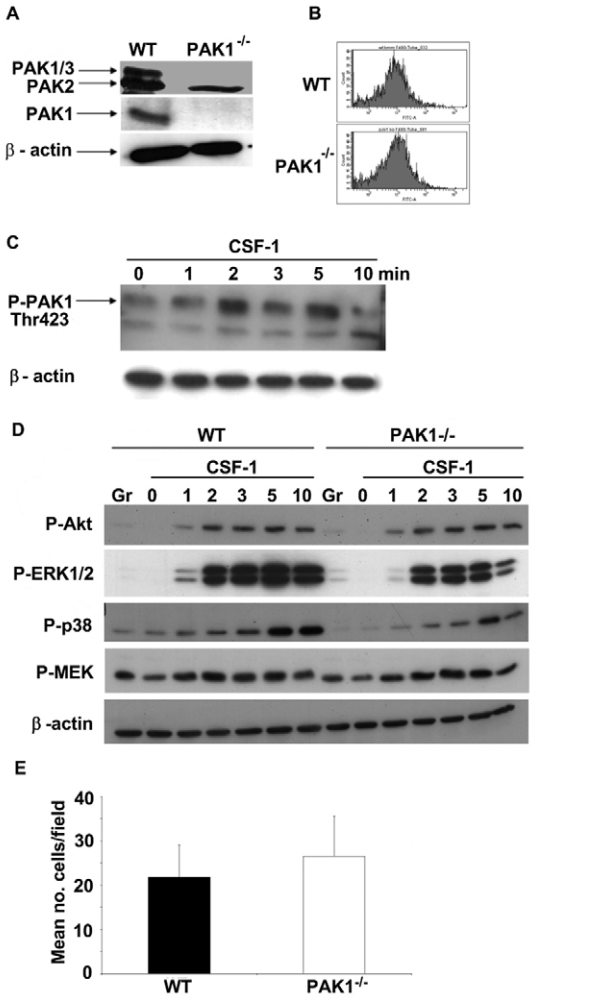

Fig. 1. PAK1 regulates CSF1-induced MAPK activation but not macrophage differentiation or chemotaxis. (A) Lysates from WT and PAK1$^{-/-}$ BMMs were immunoblotted for PAK1 and PAK2 using a group-1-specific polyclonal antibody (C19) or a PAK1-specific polyclonal antibody. β-actin was detected as a loading control. Gr, cells in growth medium. (B) Flow cytometry analysis of WT and PAK1$^{-/-}$ BMM surface F4/80 expression levels, detected using a FITC-F4/80 antibody. Background fluorescence levels were established with a FITC-IgG2b negative control antibody. (C) WT BMMs were stimulated with 33 ng/ml CSF1, and lysates were immunoblotted for Thr423-*P*-PAK1 (P-PAK1) and β-actin as a loading control. (D) WT and PAK1$^{-/-}$ BMMs were stimulated with 33 ng/ml CSF1 and lysates were immunoblotted for Ser473-*P*-Akt, Thr202/Tyr204-*P*-ERK1/2, Thr180/Tyr182-*P*-p38 and Ser298-*P*-MEK1/2 levels. β-actin was detected as a loading control. Western blots are representative of three separate experiments. (E) To investigate chemotaxis, 1×10^5 WT or PAK1$^{-/-}$ BMMs were placed into the upper chamber of a Transwell with 33 ng/ml CSF1 in the lower chamber. After 24 hours, cell migration was evaluated by determining the cell number in ten randomly selected fields. Results are the mean ± s.e.m. of three experiments performed in triplicate.

were elongated and often assumed a polarised migratory morphology with a lamellipodium at the front and tail at the back, whereas some cells were round and flat, extending lamellipodia in several directions. Following adhesion, the migration speed of WT and PAK1$^{-/-}$ BMMs was similar. They migrated most rapidly in the first 30 minutes and then the speed decreased to reach a steady-state migration speed of approximately 0.4 μm/minute from around 60 minutes (Fig. 2A; supplementary material Movies 1 and 2). Since PAK1 is a target for Cdc42 and Rac, both of which contribute to cell spreading during

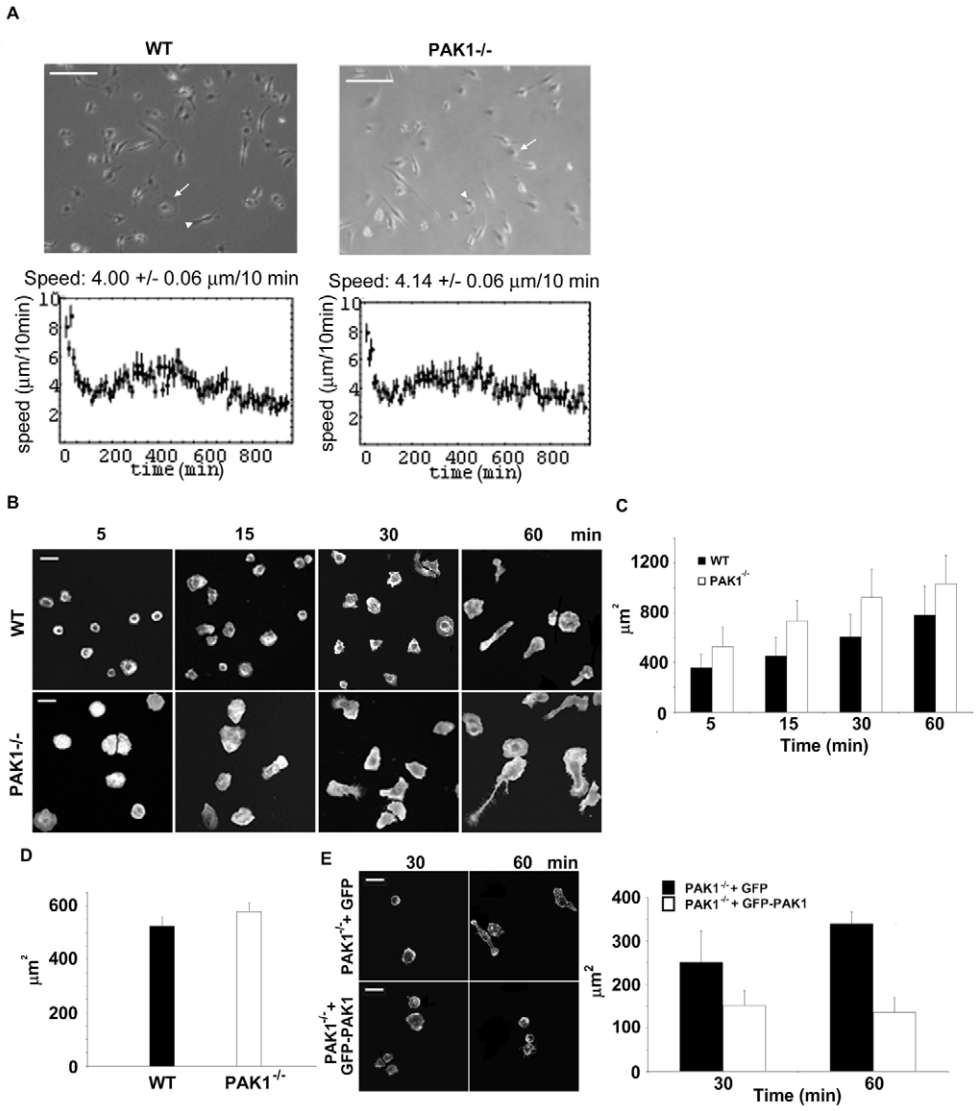

Fig. 2. PAK1 regulates macrophage spreading but not migration speed. (A) WT and PAK1$^{-/-}$ BMMs were seeded onto tissue culture plastic in growth medium. Upper panels are representative micrographs of migrating BMMs 8 hours after seeding. Scale bars: 100 µm; arrows indicate round, flat cells; arrowheads indicate lamellipodia on migrating cells. Cell migration was observed by time-lapse microscopy for 8 hours. Mean migration speed at each time point was determined from cell tracks, *n*=70 and *n*=60 for WT and PAK1$^{-/-}$ BMMs, respectively. Results are representative of three separate experiments (lower panels). (B) WT and PAK1$^{-/-}$ BMMs were allowed to adhere to uncoated glass coverslips in macrophage starvation medium supplemented with 33 ng/ml CSF1 for the indicated times. Cells were stained for F-actin. Scale bars: 10 µm. (C) The images in B were analysed to quantify the spread area of WT and PAK1$^{-/-}$ BMMs. Data are the mean ± s.e.m. of three separate experiments; *n*=~50 cells per time point per experiment. (D) Quantification of WT and PAK1$^{-/-}$ BMM spread area as determined in C, using images of cells 24 hours after adhesion. Data are the mean ± s.e.m. of three separate experiments; *n*=~50 cells per experiment. (E) PAK1$^{-/-}$ BMMs nucleofected with GFP or GFP-PAK1 were allowed to adhere to uncoated glass coverslips in growth medium for the indicated times. Cells were stained for F-actin; Scale bar: 10 µm. The spread area of GFP- and GFP-PAK1-expressing cells was determined. Data are the mean ± s.d. of two separate experiments; *n*=~20 cells per experiment.

adhesion (Price et al., 1998; Wells et al., 2004), we investigated the contribution of PAK1 to spreading. The spread area of PAK1$^{-/-}$ BMMs was already significantly higher than WT BMMs by 5 minutes after adhesion, and this was maintained up to 60 minutes (Fig. 2B,C). This was not due to an intrinsic difference in the volume of WT and PAK1$^{-/-}$ BMMs, which was determined using a Casy cell counter (data not shown). Interestingly, the increase in spread area was transient: by 24 hours after adhesion, the spread area was not significantly different between WT and PAK1$^{-/-}$ BMMs (Fig. 2D). The transient increased spreading of PAK1$^{-/-}$ BMMs was reduced by expression of GFP-PAK1 compared with GFP alone (Fig. 2E).

To observe membrane dynamics during spreading in WT and PAK1$^{-/-}$ BMMs, cells were visualised by time-lapse microscopy. Upon adhesion, WT BMMs generally produced stable lamellipodia that gradually extended and did not collapse or retract back into the cell body. Membrane ruffling was frequently observed on the dorsal surface of the lamellipodia. By contrast, PAK1$^{-/-}$ BMMs extended unstable lamellipodia upon adhesion and these frequently collapsed back into the cell body (Fig. 3A; supplementary material Movies 3 and 4). Quantification indicated that although lamellipodia of PAK1$^{-/-}$ BMMs extended a similar distance from the cell body to those of WT BMMs, the lamellipodial perimeter in PAK1$^{-/-}$ BMMs was significantly shorter (Fig. 3B). This is consistent with the time-lapse data indicating that PAK1$^{-/-}$ BMM lamellipodia are less stable and collapse before they can encompass large lengths of the cell perimeter. The membrane dynamics of lamellipodia in WT and PAK1$^{-/-}$ BMMs was further analysed in kymographs. In WT BMMs, lamellipodia extended smoothly at a constant speed before they reached a plateau, and then ruffled with slight fluctuations at the membrane edge. However, in PAK1$^{-/-}$ BMMs, lamellipodia extended less regularly, with frequent membrane retractions (Fig. 3C).

The reduction in lamellipodial stability appeared inconsistent with the observation that PAK1$^{-/-}$ BMMs spread to a greater area than WT BMMs. Further analysis of the time-lapse movie data revealed that PAK1$^{-/-}$ BMMs consistently had more lamellipodia at a given time point than WT BMMs (Fig. 3D). This suggests that PAK1$^{-/-}$ BMMs spread to a greater area through the extension of smaller, less stable, but more numerous lamellipodia rather than production of fewer, broader lamellipodia.

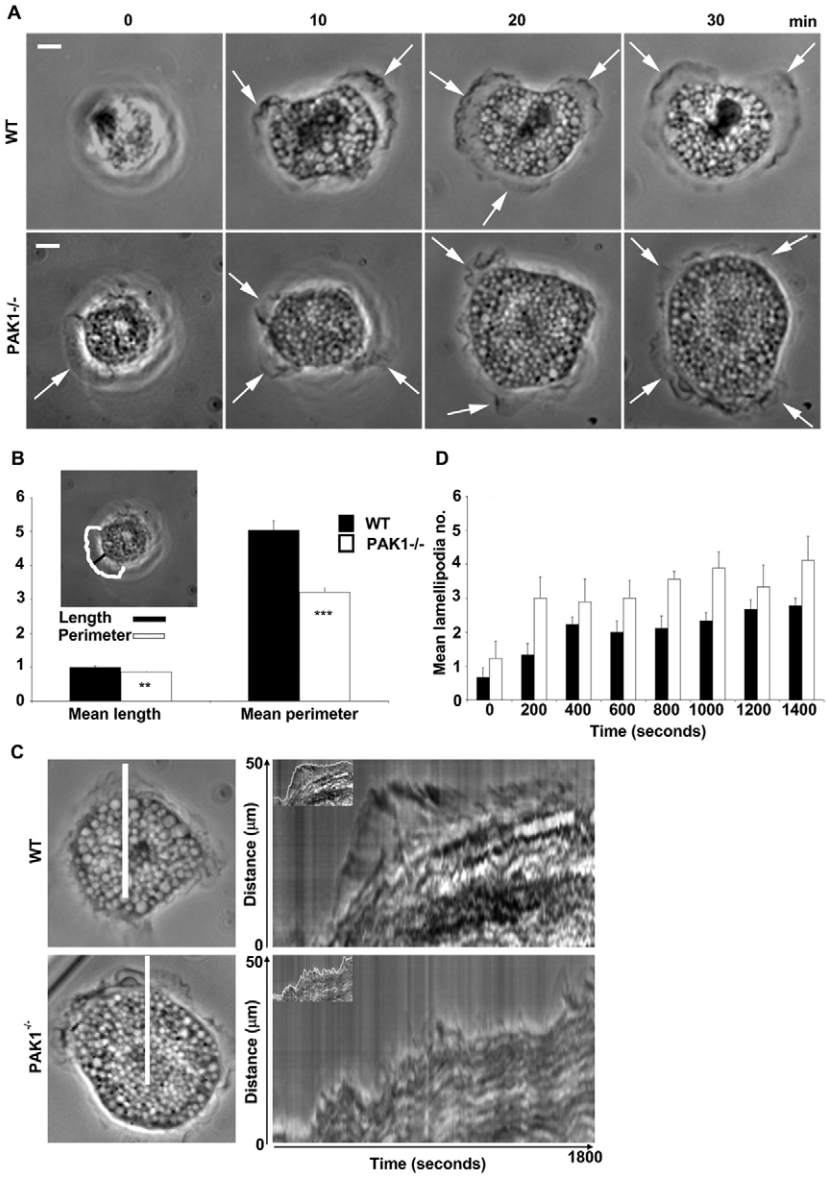

Fig. 3. PAK1 is required for lamellipodial stability during spreading. (A) WT and PAK1$^{-/-}$ BMMs were plated onto glass-bottomed culture dishes in growth medium and cell spreading was visualised by time-lapse microscopy. Images at the indicated time points of the movies are shown. Scale bars: 1 μm. (B) The length and the perimeter of WT and PAK1$^{-/-}$ lamellipodia was determined using movies as in A. Data shown are the mean ± s.e.m; n=145 and n=225 lamellipodia from WT and PAK1$^{-/-}$ BMMs, respectively, from six separate experiments. $^{**}P<0.01$ and $^{***}P<0.001$, Student's t-test. (C) Kymographs of extending lamellipodia. The final frame from the movie used (left panels) indicates the region of kymograph production. Right panels show kymographs; the inset shows the kymograph with the membrane edge highlighted (white line). (D) Quantification of the number of lamellipodia per cell observed at the specified times in movies. Mean ± s.e.m.; n=9 cells from six separate experiments for both WT and PAK1$^{-/-}$ BMMs.

PAK1 promotes ERK1/2 activation at the cell periphery

As described above, PAK1 is required for optimal CSF1-induced ERK1/2 activation. ERK1/2 localise at the cell membrane (Glading et al., 2001) and in focal adhesions (Fincham et al., 2000) and promote lamellipodium formation and spreading in epithelial cells (Ishibe et al., 2004). ERK1/2 activation during adhesion can be dependent on PAKs (Eblen et al., 2002; Sundberg-Smith et al., 2005).

ERK1/2 activity was reduced in PAK1$^{-/-}$ BMMs compared with WT BMMs 10 minutes after adhesion (Fig. 4A). This suggests that PAK1 is required for optimal activation of ERK1/2 during spreading. By contrast, p38MAPK activity was unchanged in PAK1$^{-/-}$ BMMs at this time point (Fig. 4A). In addition, MEK1 phosphorylation on Ser298 was not affected by lack of PAK1 (Fig. 4A), indicating that PAK1 is not essential for phosphorylation of this PAK-regulated site.

To address further the regulation of ERK1/2 activation during macrophage adhesion, we analysed the intracellular distribution of ERK1/2. In WT BMMs, ERK1/2 was enriched in regions of the cell periphery, as well as localising in the cytoplasm and occasionally the nucleus (Fig. 4B). ERK1/2 distribution was unchanged in PAK1$^{-/-}$ BMMs (Fig. 4B). However, visualisation of activated ERK1/2 with an antibody against Thr202-*P*/Tyr204-*P* revealed that PAK1$^{-/-}$ BMMs have reduced levels of activated ERK1/2 at the cell periphery compared with WT BMMs (Fig. 4C). PAK1, therefore, affects ERK1/2 activation but not its localisation. This suggests that PAK1-mediated activation of ERK1/2 at the cell periphery is required for the production of stable lamellipodia during spreading and that loss of this regulation promotes unstable lamellipodia.

ERK1/2 are activated upstream by MEKs. MEK1/2 activity, as measured by phosphorylation of S217/221 (Alessi et al., 1994) was not altered in PAK1$^{-/-}$ BMMs before or 10 minutes after adhesion (Fig. 4D), implying that the reduced ERK1/2 activity in spreading cells lacking PAK1 is not due to decreased MEK activity.

Inhibition of ERK1/2 activation promotes a PAK1$^{-/-}$ phenotype

To establish whether ERK1/2 activation affected membrane extension during macrophage adhesion and spreading, WT BMMs were incubated with U0126, a specific inhibitor of ERK1/2 upstream kinase MEK (Ge et al., 2002). Western blotting indicated that 1 μg/ml U0126 produced a partial inhibition of ERK1/2 in WT BMMs (results not shown), mimicking the decrease in ERK1/2 activity observed in PAK1$^{-/-}$ BMMs during spreading. U0126-treated WT BMMs spread to a greater area than untreated WT BMMs (Fig. 5A), similarly to the response of PAK1$^{-/-}$ BMMs (Fig. 2A,B). Quantification confirmed that MEK-inhibited WT BMMs spread to a greater area than untreated WT BMMs (Fig. 5B), suggesting that the increased spreading of PAK1$^{-/-}$ BMMs is a consequence of reduced ERK1/2 activation. Similarly, a different MEK inhibitor, PD98059, increased spreading of WT BMMs at 60 minutes (data not shown).

To investigate whether the increased spreading observed in ERK1/2-inhibited WT BMMs was accompanied by similar defects in lamellipodial dynamics to PAK1$^{-/-}$ BMMs, cells were visualised during adhesion by time-lapse microscopy. U0126 treatment during spreading resulted in extension of less stable lamellipodia and an increased number of lamellipodia, particularly at early time points

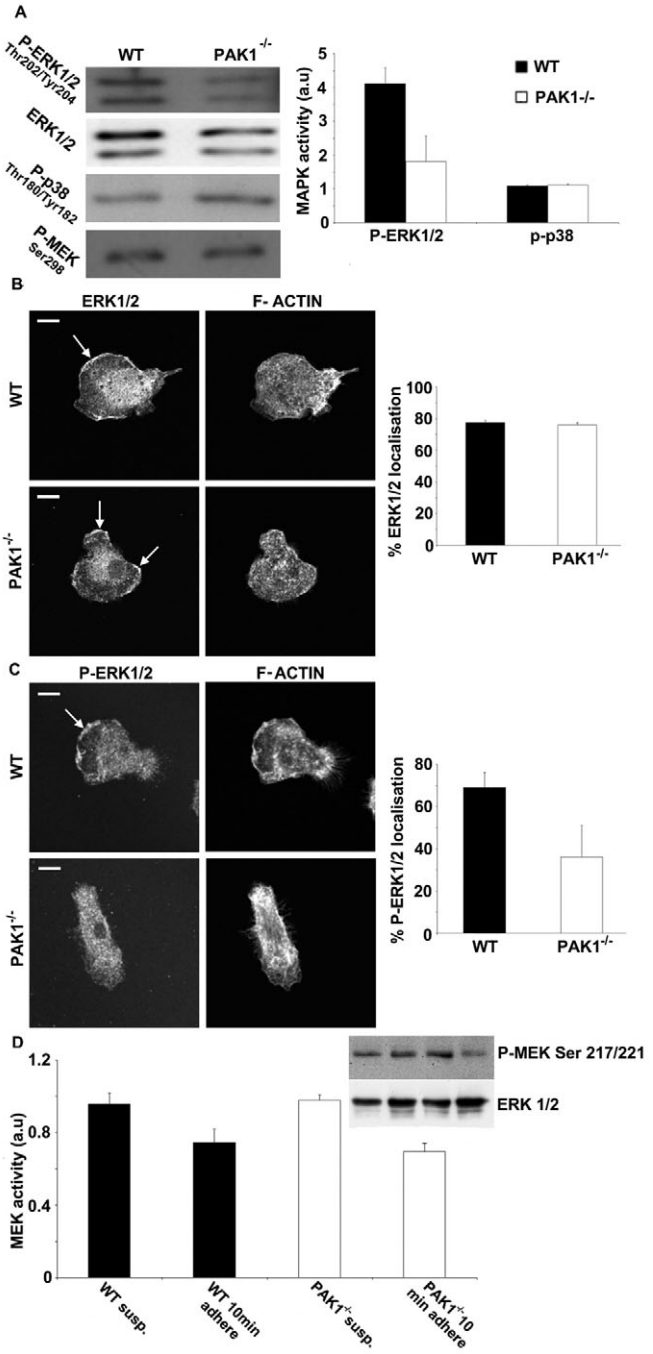

Fig. 4. PAK1 promotes ERK1/2 activation at the cell periphery. (A) WT and PAK1$^{-/-}$ BMMs were adhered onto tissue culture plastic for 10 minutes in growth medium. Lysates were immunoblotted for Thr202/Tyr204-*P*-ERK1/2, Thr180/Tyr182-*P*-p38, Ser298-*P*-MEK1/2 and total ERK1/2. Densitometry quantification of phosphorylated ERK1/2 and p38 levels equalised to total ERK1/2 protein levels are shown (a.u., arbitrary units). Data are mean ± s.d. of two separate experiments. (B) WT and PAK1$^{-/-}$ BMMs were plated onto glass coverslips for 10 minutes in growth medium and were stained using an ERK1/2 antibody and TRITC-phalloidin to visualise F-actin. Cells were imaged by confocal microscopy. ERK1/2 localisation was quantified by determining the number of cells with ERK1/2 staining at the periphery. The mean ± s.d. is shown for two separate experiments; n=60 (WT) and n=45 (PAK1$^{-/-}$). (C) BMMs were stained with a Thr202/Tyr204-*P*-ERK1/2 antibody (P-ERK1/2) and TRITC-phalloidin (F-actin). Localisation of ERK1/2-*P* was quantified by determining the number of cells with staining at the cell periphery. The mean ± s.d. is shown for two separate experiments; n=39 (WT) and n=62 (PAK1$^{-/-}$). (D) WT and PAK1$^{-/-}$ BMMs were kept in suspension or adhered onto tissue culture plastic for 10 minutes in growth medium. Lysates were immunoblotted for Ser217/221-*P*-MEK1/2, and total ERK1/2 as a loading control. Densitometry quantification of phosphorylated MEK1/2 equalised to total ERK1/2 protein levels is shown (a.u., arbitrary units). Data are mean ± s.d. of two separate experiments.

(Fig. 6A; supplementary material Movies 5 and 6). Kymograph analysis confirmed that lamellipodia in ERK1/2-inhibited BMMs were less stable and frequently switched from extension to retraction (Fig. 6B), similarly to those in PAK1$^{-/-}$ BMMs. This strongly suggests that PAK1-mediated activation of ERK1/2 is required for the controlled spreading of BMMs.

Discussion

We have characterised the role of PAK1 in macrophage spreading and migration and found that PAK1 acts via ERK1/2 to regulate lamellipodial dynamics. PAK1 can be activated by Rac and Cdc42 proteins, yet the morphology of PAK1$^{-/-}$ macrophages is similar to WT macrophages and does not resemble that of macrophages lacking Rac1 and Rac2 (Wheeler et al., 2006), which have elongated narrow protrusions. PAK1 deletion did not significantly affect migration or chemotaxis of macrophages, whereas under similar conditions, macrophages lacking Rac1 and Rac2 migrated faster than WT macrophages (Wheeler et al., 2006). Loss of Rac proteins, therefore, has a stronger morphological phenotype than loss of PAK1. It is possible that PAK2, which has been implicated in chemotaxis (Weiss-Haljiti et al., 2004), compensates for the absence of PAK1, or that other Rac targets are important for regulating cell shape. However, PAK1 did affect cell shape during macrophage spreading, because PAK1$^{-/-}$ BMMs exhibit a transient increase in spread area during adhesion-mediated spreading. Interestingly, a combination of increased lamellipodium formation and reduced lamellipodium stability were responsible for this phenotype, indicating the importance of regulating lamellipodial dynamics. Activation of the ERK1/2 pathway was attenuated in the PAK1$^{-/-}$ BMMs, and inhibition of ERK1/2 activation was sufficient to produce a similar phenotype to PAK1$^{-/-}$ BMMs during spreading. This suggests that PAK1-mediated regulation of lamellipodial stability and cell spreading requires activation of ERK1/2.

Cell spreading upon integrin engagement has previously been shown to induce the activation of the Rho, Rac and/or Cdc42 (del Pozo et al., 2003; Hamelers et al., 2005; Price et al., 1998). As a target of both Rac and Cdc42, PAK1 has been implicated in the regulation of spreading (Suzuki-Inoue et al., 2001; ten Klooster et al., 2006). Indeed, loss of PAK1 in fibroblasts resulted in enhanced cell spreading, supporting our observations in macrophages (ten Klooster et al., 2006). In fibroblasts, PAK1 is proposed to compete with Rac1 for β-PIX binding at the cell membrane, and thereby regulate Rac1 activity. Rac1 and β-PIX colocalised at the cell periphery in WT and PAK1$^{-/-}$ BMMs and no apparent change in Rac1 localisation was observed in PAK1$^{-/-}$ BMMs (data not shown) suggesting this mechanism does not account for the change in macrophage spreading.

Live cell imaging has provided new insight into how PAK1 regulates spread area through membrane dynamics. The increased spread area of PAK1$^{-/-}$ BMMs was not merely a result of increased lamellipodium extension but a consequence of increased lamellipodium formation and turnover. PAK1 could function in lamellipodial extension and stability through the regulation of cell adhesion. PAK1 can reportedly activate the integrin αMβ2 (Jones

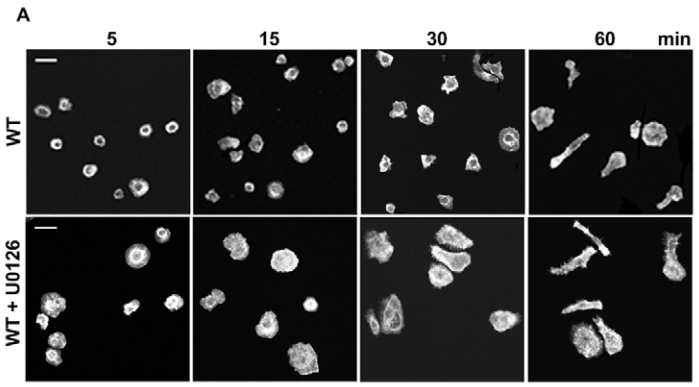

Fig. 5. Inhibition of ERK1/2 activation promotes macrophage spreading. WT BMMs were plated on glass coverslips in the presence or absence of 1 μg/ml U0126. (A) Representative images of cells stained for F-actin. (B) Cell spread area was quantified at the indicated time points. The mean spread area ± s.e.m. from three separate experiments is shown. ***P<0.001 comparing WT+U0126 to untreated WT; n=~50 cells per time point per experiment. Scale bars: 5 μm.

et al., 1998) as well as phosphorylate FAK, a key component of adhesion regulation (Jung et al., 2004). PAK1$^{-/-}$ BMMs did not show a defect in adhesion (S.D.S. and A.J.R., unpublished), but PAK1 could be critical for the formation of new adhesions in lamellipodia that would be required for stable attachment of lamellipodia to the substratum.

Our findings indicate that PAK1-mediated activation of ERK1/2 was required for the stable extension of lamellipodia during spreading. A link between PAK and ERK1/2 activation has been previously observed in COS cells and fibroblasts (Beeser et al., 2005; Eblen et al., 2002; Frost et al., 1997), and our results indicate that PAK1 specifically contributes to ERK1/2 activation in macrophages in response to CSF1 stimulation and adhesion. ERK1/2 localises at the cell membrane (Glading et al., 2001) and to focal adhesions (Fincham et al., 2000), and was present at the cell periphery in spreading BMMs. PAK1 did not regulate ERK1/2 localisation, but active ERK1/2 levels were reduced at the plasma membrane in PAK1$^{-/-}$ BMMs, indicating that PAK1 promotes ERK1/2 activation rather than localisation at the cell periphery. It was not possible to investigate whether PAK1 and ERK1/2 colocalised because of the lack of PAK1 antibodies suitable for immunofluorescence. The most widely reported mechanism for PAK1-mediated activation of ERK1/2 is via direct phosphorylation of the MAPK kinase MEK1 by PAK1 on Ser298 (Beeser et al., 2005; Frost et al., 1997; Park et al., 2007); however, no changes in MEK1 Ser298 phosphorylation levels or MEK1/2 activity were observed in PAK1$^{-/-}$ BMMs, suggesting that there is an alternative mechanism for regulation of ERK1/2 by PAK1. One possibility is that PAK1 regulates other members of the MEK family of kinases (Gallagher et al., 2002; Lee et al., 2001) or that it acts as a scaffold protein for ERK and MEK (Pullikuth et al., 2005; Sundberg-Smith et al., 2005).

Chemical inhibition of MEK to reduce ERK1/2 activity induced a similar phenotype during macrophage adhesion to loss of PAK1. A function for ERK1/2 during adhesion has previously been observed in epithelial cells, and ERK1/2 can phosphorylate the adhesion adaptor protein paxillin, providing a binding site for FAK (Ishibe et al., 2004). Mutation of this paxillin site inhibited spreading and migration. Interestingly, PAK1 is also capable of phosphorylating paxillin (at a separate site to ERK1/2), which is reported to localise a PAK-PIX-GIT complex that is critical for adhesion turnover (Nayal et al., 2006). PAK has also been implicated in regulating focal adhesion turnover in endothelial cells (Kiosses et al., 1999). PAK1 and ERK1/2 regulation of paxillin phosphorylation and subsequent adhesion formation and/or turnover might therefore be responsible for the reduced lamellipodial stability observed in PAK1$^{-/-}$ BMMs. It would therefore be interesting to investigate adhesion dynamics during macrophage spreading.

Despite the clear involvement of PAK1 in the regulation of macrophage spreading and lamellipodial stability following

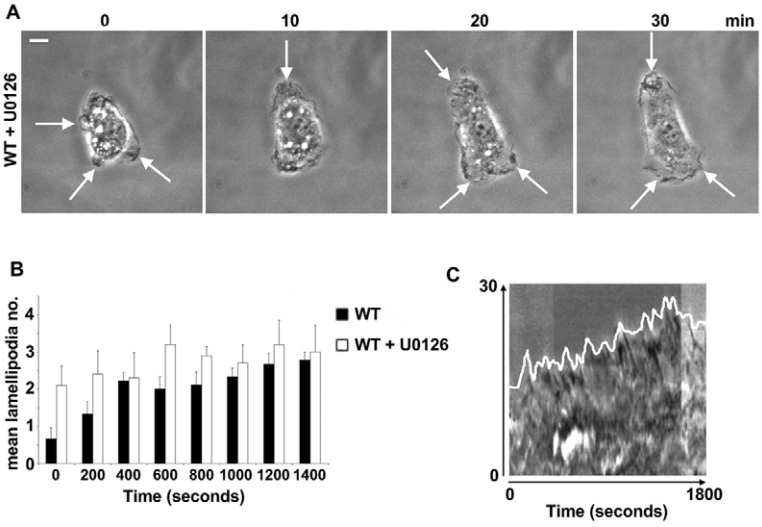

Fig. 6. Inhibition of ERK1/2 activation reduces lamellipodial stability. (A) WT BMMs in growth medium were pretreated with 1 μg/ml U0126 for 1 hour and plated on to glass-bottomed tissue culture dishes. Time-lapse microscopy was used to monitor cell spreading. Frames from the indicated time points are shown. Arrows indicate lamellipodia. Scale bar: 20 μm. (B) Quantification of the number of lamellipodia per cell observed in the time-lapse movie frames specified. Data are the mean ± s.e.m. of three separate experiments. (C) Representative kymograph of a lamellipodium in a U0126-treated cell. The membrane edge is highlighted (white line).

adhesion, PAK1 did not affect macrophage migration. Although PAK1 has previously been linked to cell migration and chemotaxis in other cell types (Adam et al., 1998; Li et al., 2003; Rousseau et al., 2006; Sells et al., 1999; Wang et al., 2002; Zhou et al., 2003), no differences between WT and PAK1$^{-/-}$ macrophages were observed under the conditions we tested. Group 1 PAKs (PAK1-PAK3) have been suggested to affect fibroblast migration by recruiting β-PIX to the leading edge (Cau and Hall, 2005), but β-PIX localisation was not affected by lack of PAK1 in macrophages (S.D.S. and A.J.R., unpublished). Although it is possible that PAK2 might fulfill this function, macrophages appear to be more flexible in the signalling pathways they use for migration than some other hematopoietic cells. For example, lack of Rac1 and Rac2 does not affect their migration speed (Wheeler et al., 2006), whereas neutrophils lacking Rac2 have a much reduced migration speed (Gu et al., 2003; Roberts et al., 1999). This may reflect the ability of macrophages to migrate in diverse environments and conditions in vivo. It is therefore likely that under certain conditions the role of PAK1 in regulating lamellipodial stability will affect their migratory behaviour.

Materials and Methods

Isolation and culture of bone marrow-derived macrophages

Wild-type (WT) femoral bone marrow cells were isolated from 6- to 8-week-old C57B6 mice and PAK1$^{-/-}$ bone marrow cells from 6- to 8-week-old C57B6 or C57B6/Sv129 mice. Bone-marrow-derived macrophages (BMMs) were obtained as previously described (Wells et al., 2004). No difference was observed in the behaviour of macrophages from C57B6 or C57B6/Sv129 mice. BMMs were maintained in macrophage growth medium consisting of RPMI 1640 medium containing L-glutamine (Invitrogen), supplemented with 10% FCS (Sigma), 1 mM sodium pyruvate (Invitrogen), 1% (v/v) non-essential amino acids (Invitrogen), 10 μM β-mercaptoethanol (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen). 10% L-cell conditioned medium was added as a source of CSF1. Macrophage starvation medium consisted of growth medium without L-cell conditioned medium.

Transfection

PAK1$^{-/-}$ BMMs were transfected with pEGFP-C1 or pEGFP-PAK1 using the Amaxa Nucleofector apparatus (Amaxa Biosystems), according to the manufacturer's instructions. Transfected BMMs were incubated at 37°C for 24 hours in growth medium before use in experiments.

Immunoblotting

Adherent BMMs were stimulated with 33 ng/ml recombinant murine CSF1 (R&D systems). Alternatively BMMs in suspension were stimulated by adhesion to tissue culture plastic (Nunc). Cells were harvested in lysis buffer containing 50 mM Tris-HCl pH 7.6, 2 mM EDTA, 150 mM NaCl, 10 mM $MgCl_2$, 1% (v/v) NP-40, 10% (v/v) glycerol, 10 μg/ml DTT, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 0.72 IU aprotinin and 1 mM NaF. Protein concentrations were determined using the Bradford protein assay dye (Bio-Rad) and equal amounts of protein were separated by SDS-PAGE. Proteins were then transferred onto polyvinylidene fluoride membrane (Schleicher & Schuell) and membranes were blocked in 5% non-fat dried milk in PBS. Membranes were then incubated at 4°C for 16 hours in 0.5% non-fat dried milk with rabbit polyclonal anti-PAK1, anti-PAK2 and anti-PAK3 (C19, Santa Cruz), anti-PAK1, anti-Thr423-*P*-PAK, anti-Ser473-*P*-Akt, anti-Thr202/Tyr204-*P*-ERK1/2, anti-ERK1/2, anti-Ser298-*P*-MEK1, anti-S217/221-*P*-MEK1, anti-Ser338-*P*-Raf (all from Cell Signaling Technology), anti-Thr180/Tyr182-*P*-p38 antibodies (New England Biolabs), anti-Rac1 (Upstate Biotechnology) or mouse monoclonal anti-β-actin (Sigma). After washing, membranes were incubated with horseradish-peroxidase-conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare) for 1 hour at room temperature. Membranes were developed using enhanced chemiluminescence (ECL, GE Healthcare).

Immunofluorescence

BMMs (1×10^4) were seeded onto 13-mm-diameter glass coverslips. They were fixed in 4% paraformaldehyde, permeabilised in 0.5% Triton X-100 and then blocked with 20% goat serum for 30 minutes at room temperature. ERK1/2 were visualised using rabbit anti-ERK1/2 (1:100) and anti-ERK1/2-*P* with rabbit anti-Thr202/Tyr204-*P*-ERK1/2 antibodies (1:200; Cell Signaling Technology) followed by Cy5-conjugated anti-rabbit antibody (Jackson ImmunoResearch) and TRITC-phalloidin (Sigma) to visualise F-actin. Images were acquired with a Zeiss LSM510 confocal microscope and a ×40/1.30 NA Plan Neofluar objective using LSM510 software.

Flow cytometry

BMMs were suspended in 5% BSA in PBS at 5×10^5 cells/ml. They were stained on ice for 30 minutes with rat polyclonal FITC-conjugated anti-F4/80 or rat polyclonal FITC-conjugated IgG2b negative control antibody (1:100; Serotec). BMMs were washed twice in 5% BSA in PBS and then fixed in 0.4% paraformaldehyde for 10 minutes. Cell surface fluorescence was measured using a BD FACS Canto flow cytometer and analysed using FACS Diva software (Becton Dickinson).

Transwell chemotaxis assay

The upper and lower chambers of a 5 μm-pore polycarbonate Transwell filter (Corning) were incubated for 30 minutes at 37°C in macrophage starvation medium. The medium in the lower chamber was replaced with starvation medium containing 33 ng/ml recombinant CSF1, and 1×10^5 BMMs were added to the upper chamber in starvation medium. After 24 hours, transmigrated cells were fixed and stained using the REASTAIN Quick-Diff kit (Reagena). For each filter, 10 random images were acquired using a Zeiss Axiophot microscope and a ×100 Zeiss Plan-Neofluar 1.30 NA oil objective, and the cell number in each image was determined.

Quantification of cell area

BMMs (1×10^4) were seeded onto 13-mm-diameter glass coverslips in macrophage growth medium. Where indicated, WT BMMs were incubated with 1 μg/ml U0126 (Promega) or 50 μM PD98059 (Calbiochem) in growth medium for 1 hour prior to seeding. At various time points, BMMs were fixed, permeabilised and stained with TRITC-phalloidin to visualise F-actin. The basal planes of BMMs were imaged using confocal microscopy. Images were pre-processed using Adobe Photoshop 6.0 and ImageJ (NIH) and then quantified using Metamorph 5.01 (Universal Imaging Systems). Each image was passed through a median filter using a 3×3 kernel to remove background light interference, then converted into a binary threshold image, and the cell area was determined using the integrated morphometry analysis function.

Time-lapse microscopy

For analysis of migration speed, BMMs (2×10^5) in macrophage growth medium were plated onto 35 mm^2 tissue culture plastic dishes (Nunc). During acquisition of movies, BMMs were incubated in a humidified chamber at 37°C with 10% CO_2. Phase-contrast micrographs were acquired using a Hitachi Denshi KPM1E/K-S10 768×576 pixel, eight-bit CCD camera using a ×10 Plan NeoFluar 0.30 N.A. objective (Zeiss). An image frame was collected every 10 minutes for 8 hours using Kinetic Imaging Motion Analysis software (Andor Technology). Migration speed was analysed using Kinetic Imaging Motion Analysis software and Mathematica 5.0 (Wolfram Research Institute) workbooks.

For analysis of membrane dynamics during cell adhesion, 2 ml macrophage growth medium was added to a glass-bottomed dish (MatTek) and incubated in a humidified chamber at 37°C, 10% CO_2 on an Eclipse TE 2000-E microscope (Nikon). BMMs (1×10^5) in macrophage growth medium (with or without prior incubation with 1 μg/ml U0126) were added and images captured every 10 seconds for 30 minutes with AQM Advance 6 software (Andor Technology) and a Hamamatsu Photonics ORCA-ER CCD camera using a ×40 Plan Fluor 1.30 NA oil objective (Nikon). From these movies, the number of lamellipodia was quantified at different time points, and the perimeter length of individual lamellipodia was measured using Metamorph 5.01 (Universal Imaging Systems). The dynamics of lamellipodial extension was analysed on kymographs generated from the time-lapse movies (Metamorph 5.0.1).

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References

Adam, L., Vadlamudi, R., Kondapaka, S. B., Chernoff, J., Mendelsohn, J. and Kumar, R. (1998). Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. *J. Biol. Chem.* **273**, 28238-28246.

Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J. and Cowley, S. (1994). Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J.* **13**, 1610-1619.

Beeser, A., Jaffer, Z. M., Hofmann, C. and Chernoff, J. (2005). Role of group A p21-activated kinases in activation of extracellular-regulated kinase by growth factors. *J. Biol. Chem.* **280**, 36609-36615.

Cau, J. and Hall, A. (2005). Cdc42 controls the polarity of the actin and microtubule cytoskeletons through two distinct signal transduction pathways. *J. Cell Sci.* **118**, 2579-2587.

Ching, Y. P., Leong, V. Y., Lee, M. F., Xu, H. T., Jin, D. Y. and Ng, I. O. (2007). P21-activated protein kinase is overexpressed in hepatocellular carcinoma and enhances cancer

metastasis involving c-Jun NH2-terminal kinase activation and paxillin phosphorylation. *Cancer Res.* **67**, 3601-3608.

Choma, D. P., Pumiglia, K. and DiPersio, C. M. (2004). Integrin α3β1 directs the stabilization of a polarized lamellipodium in epithelial cells through activation of Rac1. *J. Cell Sci.* **117**, 3947-3959.

Daub, H., Gevaert, K., Vandekerckhove, J., Sobel, A. and Hall, A. (2001). Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16. *J. Biol. Chem.* **276**, 1677-1680.

del Pozo, M. A., Schwartz, M. A., Hu, J., Kiosses, W. B., Altman, A. and Villalba, M. (2003). Guanine exchange-dependent and -independent effects of Vav1 on integrin-induced T cell spreading. *J. Immunol.* **170**, 41-47.

Dharmawardhane, S., Brownson, D., Lennartz, M. and Bokoch, G. M. (1999). Localization of p21-activated kinase 1 (PAK1) to pseudopodia, membrane ruffles, and phagocytic cups in activated human neutrophils. *J. Leukoc. Biol.* **66**, 521-527.

Diakonova, M., Bokoch, G. and Swanson, J. A. (2002). Dynamics of cytoskeletal proteins during Fcγ receptor-mediated phagocytosis in macrophages. *Mol. Biol. Cell* **13**, 402-411.

Eblen, S. T., Slack, J. K., Weber, M. J. and Catling, A. D. (2002). Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Mol. Cell. Biol.* **22**, 6023-6033.

Edwards, D. C., Sanders, L. C., Bokoch, G. M. and Gill, G. N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat. Cell Biol.* **1**, 253-259.

Fincham, V. J., James, M., Frame, M. C. and Winder, S. J. (2000). Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. *EMBO J.* **19**, 2911-2923.

Frost, J. A., Xu, S., Hutchison, M. R., Marcus, S. and Cobb, M. H. (1996). Actions of Rho family small G proteins and p21-activated protein kinases on mitogen-activated protein kinase family members. *Mol. Cell. Biol.* **16**, 3707-3713.

Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E. and Cobb, M. H. (1997). Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *EMBO J.* **16**, 6426-6438.

Gallagher, E. D., Xu, S., Moomaw, C., Slaughter, C. A. and Cobb, M. H. (2002). Binding of JNK/SAPK to MEKK1 is regulated by phosphorylation. *J. Biol. Chem.* **277**, 45785-45792.

Ge, X., Fu, Y. M. and Meadows, G. G. (2002). U0126, a mitogen-activated protein kinase kinase inhibitor, inhibits the invasion of human A375 melanoma cells. *Cancer Lett.* **179**, 133-140.

Glading, A., Uberall, F., Keyse, S. M., Lauffenburger, D. A. and Wells, A. (2001). Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. *J. Biol. Chem.* **276**, 23341-23348.

Gu, Y., Filippi, M. D., Cancelas, J. A., Siefring, J. E., Williams, E. P., Jasti, A. C., Harris, C. E., Lee, A. W., Prabhakar, R., Atkinson, S. J. et al. (2003). Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. *Science* **302**, 445-449.

Gujdar, A., Sipeki, S., Bander, E., Buday, L. and Farago, A. (2003). Phorbol ester-induced migration of HepG2 cells is accompanied by intensive stress fibre formation, enhanced integrin expression and transient down-regulation of p21-activated kinase 1. *Cell Signal.* **15**, 307-318.

Hamelers, I. H., Olivo, C., Mertens, A. E., Pegtel, D. M., van der Kammen, R. A., Sonnenberg, A. and Collard, J. G. (2005). The Rac activator Tiam1 is required for α3β1-mediated laminin-5 deposition, cell spreading, and cell migration. *J. Cell Biol.* **171**, 871-881.

Harding, A., Tian, T., Westbury, E., Frische, E. and Hancock, J. F. (2005). Subcellular localization determines MAP kinase signal output. *Curr. Biol.* **15**, 869-873.

Hirsch, S., Austyn, J. M. and Gordon, S. (1981). Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J. Exp. Med.* **154**, 713-725.

Hofmann, C., Shepelev, M. and Chernoff, J. (2004). The genetics of Pak. *J. Cell Sci.* **117**, 4343-4354.

Honma, N., Genda, T., Matsuda, Y., Yamagiwa, S., Takamura, M., Ichida, T. and Aoyagi, Y. (2006). MEK/ERK signaling is a critical mediator for integrin-induced cell scattering in highly metastatic hepatocellular carcinoma cells. *Lab. Invest.* **86**, 687-696.

Ishibe, S., Joly, D., Liu, Z. X. and Cantley, L. G. (2004). Paxillin serves as an ERK-regulated scaffold for coordinating FAK and Rac activation in epithelial morphogenesis. *Mol. Cell* **16**, 257-267.

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

Jones, S. L., Knaus, U. G., Bokoch, G. M. and Brown, E. J. (1998). Two signaling mechanisms for activation of alphaM beta2 avidity in polymorphonuclear neutrophils. *J. Biol. Chem.* **273**, 10556-10566.

Jung, I. D., Lee, J., Lee, K. B., Park, C. G., Kim, Y. K., Seo, D. W., Park, D., Lee, H. W., Han, J. W. and Lee, H. Y. (2004). Activation of p21-activated kinase 1 is required for lysophosphatidic acid-induced focal adhesion kinase phosphorylation and cell motility in human melanoma A2058 cells. *Eur. J. Biochem.* **271**, 1557-1565.

Kermorgant, S., Zicha, D. and Parker, P. J. (2004). PKC controls HGF-dependent c-Met traffic, signalling and cell migration. *EMBO J.* **23**, 3721-3734.

King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S. and Marshall, M. S. (1998). The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature* **396**, 180-183.

Kiosses, W. B., Daniels, R. H., Otey, C., Bokoch, G. M. and Schwartz, M. A. (1999). A role for p21-activated kinase in endothelial cell migration. *J. Cell Biol.* **147**, 831-844.

Lee, S. H., Eom, M., Lee, S. J., Kim, S., Park, H. J. and Park, D. (2001). BPix-enhanced p38 activation by Cdc42/Rac/PAK/MKK3/6-mediated pathway. Implication in the regulation of membrane ruffling. *J. Biol. Chem.* **276**, 25066-25072.

Li, Z., Hannigan, M., Mo, Z., Liu, B., Lu, W., Wu, Y., Smrcka, A. V., Wu, G., Li, L., Liu, M. et al. (2003). Directional sensing requires Gβγ-mediated PAK1 and PIXα-dependent activation of Cdc42. *Cell* **114**, 215-227.

Meng, J., Meng, Y., Hanna, A., Janus, C. and Jia, Z. (2005). Abnormal long-lasting synaptic plasticity and cognition in mice lacking the mental retardation gene Pak3. *J. Neurosci.* **25**, 6641-6650.

Nayal, A., Webb, D. J., Brown, C. M., Schaefer, E. M., Vicente-Manzanares, M. and Horwitz, A. R. (2006). Paxillin phosphorylation at Ser273 localizes a GIT1-PIX-PAK complex and regulates adhesion and protrusion dynamics. *J. Cell Biol.* **173**, 587-589.

Park, E. R., Eblen, S. T. and Catling, A. D. (2007). MEK1 activation by PAK: a novel mechanism. *Cell Signal.* **19**, 1488-1496.

Pixley, F. J. and Stanley, E. R. (2004). CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol.* **14**, 628-638.

Price, L. S., Leng, J., Schwartz, M. A. and Bokoch, G. M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol. Biol. Cell* **9**, 1863-1871.

Pullikuth, A., McKinnon, E., Schaeffer, H. J. and Catling, A. D. (2005). The MEK1 scaffolding protein MP1 regulates cell spreading by integrating PAK1 and Rho signals. *Mol. Cell. Biol.* **25**, 5119-5133.

Roberts, A. W., Kim, C., Zhen, L., Lowe, J. B., Kapur, R., Petryniak, B., Spaetti, A., Pollock, J. D., Borneo, J. B., Bradford, G. B. et al. (1999). Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity* **10**, 183-196.

Rose, D. M., Alon, R. and Ginsberg, M. H. (2007). Integrin modulation and signaling in leukocyte adhesion and migration. *Immunol. Rev.* **218**, 126-134.

Rousseau, S., Dolado, I., Beardmore, V., Shpiro, N., Marquez, R., Nebreda, A. R., Arthur, J. S., Case, L. M., Tessier-Lavigne, M., Gaestel, M. et al. (2006). CXCL12 and C5a trigger cell migration via a PAK1/2-p38α MAPK-MAPKAP-K2-HSP27 pathway. *Cell Signal.* **18**, 1897-1905.

Sanders, L. C., Matsumura, F., Bokoch, G. M. and de Lanerolle, P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science* **283**, 2083-2085.

Schurmann, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H. G., Reed, J. C. and Bokoch, G. M. (2000). p21-activated kinase 1 phosphorylates the death agonist bad and protects cells from apoptosis. *Mol. Cell. Biol.* **20**, 453-461.

Sells, M. A., Boyd, J. T. and Chernoff, J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. *J. Cell Biol.* **145**, 837-849.

Sundberg-Smith, L. J., Doherty, J. T., Mack, C. P. and Taylor, J. M. (2005). Adhesion stimulates direct PAK1/ERK2 association and leads to ERK-dependent PAK1 Thr212 phosphorylation. *J. Biol. Chem.* **280**, 2055-2064.

Suzuki-Inoue, K., Yatomi, Y., Asazuma, N., Kainoh, M., Tanaka, T., Satoh, K. and Ozaki, Y. (2001). Rac, a small guanosine triphosphate-binding protein, and p21-activated kinase are activated during platelet spreading on collagen-coated surfaces: roles of integrin α2β1. *Blood* **98**, 3708-3716.

Szczur, K., Xu, H., Atkinson, S., Zheng, Y. and Filippi, M. D. (2006). Rho GTPase CDC42 regulates directionality and random movement via distinct MAPK pathways in neutrophils. *Blood* **108**, 4205-4213.

ten Klooster, J. P., Jaffer, Z. M., Chernoff, J. and Hordijk, P. L. (2006). Targeting and activation of Rac1 are mediated by the exchange factor β-Pix. *J. Cell Biol.* **172**, 759-769.

Vidali, L., Chen, F., Cicchetti, G., Ohta, Y. and Kwiatkowski, D. J. (2006). Rac1-null mouse embryonic fibroblasts are motile and respond to platelet-derived growth factor. *Mol. Biol. Cell* **17**, 2377-2390.

Wang, D., Sai, J., Carter, G., Sachpatzidis, A., Lolis, E. and Richmond, A. (2002). PAK1 kinase is required for CXCL1-induced chemotaxis. *Biochemistry* **41**, 7100-7107.

Weiss-Haljiti, C., Pasquali, C., Ji, H., Gillieron, C., Chabert, C., Curchod, M. L., Hirsch, E., Ridley, A. J., van Huijsduijnen, R. H., Camps, M. et al. (2004). Involvement of phosphoinositide 3-kinase γ, Rac, and PAK signaling in chemokine-induced macrophage migration. *J. Biol. Chem.* **279**, 43273-43284.

Wells, C. M., Walmsley, M., Ooi, S., Tybulewicz, V. and Ridley, A. J. (2004). Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. *J. Cell Sci.* **117**, 1259-1268.

Wheeler, A. P., Wells, C. M., Smith, S. D., Vega, F. M., Henderson, R. B., Tybulewicz, V. L. and Ridley, A. J. (2006). Rac1 and Rac2 regulate macrophage morphology but are not essential for migration. *J. Cell Sci.* **119**, 2749-2757.

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

Zeng, Q., Lagunoff, D., Masaracchia, R., Goeckeler, Z., Cote, G. and Wysolmerski, R. (2000). Endothelial cell retraction is induced by PAK2 monophosphorylation of myosin II. *J. Cell Sci.* **113**, 471-482.

Zenke, F. T., King, C. C., Bohl, B. P. and Bokoch, G. M. (1999). Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. *J. Biol. Chem.* **274**, 32565-32573.

Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J. and Bokoch, G. M. (1995). Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.* **270**, 23934-23936.

Zhou, G. L., Zhuo, Y., King, C. C., Fryer, B. H., Bokoch, G. M. and Field, J. (2003). Akt phosphorylation of serine 21 on Pak1 modulates Nck binding and cell migration. *Mol. Cell. Biol.* **23**, 8058-8069.