SUPPLEMENTARY INFORMATION

METHODS

Cell size determination. Determination of diameter or volume of BMMs was performed with a CASY Model TT cell counter and analyzer (Schärfe System GmbH).

[^3]H]Thymidine incorporation assay. Cells were seeded at $2.10^5$ cells per well in 96-well plate, starved for 16 h, followed by stimulation as indicated in medium containing[^3]H]Thymidine. 24 or 48 h later the cells were harvested and the[^3]H]Thymidine incorporated in DNA measured by scintillation counting.

Immunofluorescence microscopy. Cell fixation and direct fluorescence staining of microfilaments by rhodamine-phalloidin was done by incubation of cells with 3.7% formaldehyde for 10 min, followed by permeabilisation with 0.2% Triton X-100 for 20 min. The cells were then incubated for 30 min with 2% BSA, followed by TRITC-phalloidin (Sigma) staining of filamentous actin. Slides were mounted using Slow Fade Antifade kit. All specimens were examined on a Zeiss LSM510 confocal laser-scanning microscope, using the accompanying LSM510 software.

Anti-PIP3 staining was performed by 20 min fixation of the cells in 3.7% formaldehyde followed by 10 min permeabilisation with 0.2% saponin, 40 min blocking with 2% BSA and overnight incubation with anti-PIP3 antibody at 4°C, detected by 3-4 h treatment using Cy3-anti mouse IgG (H+L) (Jackson ImmunoResearch Labs).

Quantification of adhesive area, elongation ratio and ruffling response. Cells were stimulated with CSF-1 (30 ng/ml) or vehicle, followed by staining for F-actin as described above. For quantification of cell adhesive area and elongation ratio images acquired by confocal microscope were analyzed by Image Pro Plus as described previously (Wells et al., 2004). Quantification of ruffling is presented as the % of cells with no ruffling, with isolated areas of ruffling covering no more than 50% of the dorsal surface and that with extensive ruffling covering more than 50% of the dorsal surface (relatively to the total number of cells observed).

Determination of GTP-loading on Rac1 and RhoA. The Rac1 activation assay with GST-PBD (p21-binding domain of PAK, expressed as a GST-fusion protein) was based on the assay method provided by Upstate (www.upstate.com). Cells were lysed in Mg2+ lysis buffer, provided in the assay kit, mixed with 8 μg GST-PBD bound to glutathione-agarose and incubated for 1 h at 4°C. Precipitates were washed 3 times with Mg2+ lysis buffer and suspended in Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE, transferred to PVDF membrane and blotted with anti-Rac1 antibody.

RhoA activation assay was performed using GST-RBD (Rho binding domain of Rhotekin expressed as a GST fusion protein) (Cytoskeleton Inc). Cells were lysed in RIPA buffer (50 mM Tris.HCl pH 7.2, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 10% glycerol supplemented with protease inhibitors). Cleared cell lysates were incubated at 4°C for 1 h with 50 μl glutathione-Sepharose-bound GST-RBD. Precipitates were washed 3 times with washing
buffer (50 mM Tris.HCl pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, supplemented with protease inhibitors) and suspended in Laemmli sample buffer followed by SDS-PAGE and WB for RhoA using a monoclonal antibody.

**IP and WB.** Unless otherwise indicated (in the case of IP/WB for detection of phosphorylation of PTEN and p190RhoGAP), cells were lysed in lysis buffer containing 50 mM Tris.HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 50 mM NaF and 1% Triton X-100 supplemented with 10 μg/ml aprotinin, 1 μM pepstatin, 10 μg/ml leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate, followed by clearing of the lysate by centrifugation in a cooled microcentrifuge. Supernatants were directly used for analysis by SDS-PAGE, or immunoprecipitated at 4°C overnight using the indicated antibodies. Immune complexes were collected with 50 μl of 50% slurry of protein A- or protein G-Sepharose after incubation for 2 h, washed with lysis buffer, resolved on 10% SDS-PAGE, and transferred onto PVDF membranes. The blots were probed with the indicated antibodies, followed by detection using enhanced chemiluminescence (Amersham).

For detection of p190RhoGAP (total and phosphorylated) and for RhoGAP assays, cells were lysed in the RIPA buffer used for RhoA activity assay and IP performed for 90 min at 4°C followed by 2 h incubation with protein G-Sepharose. The proteins were resolved on 7.5% SDS-PAGE or used for the RhoGAP assay as described below.

For detection of Tyr-phosphorylated PTEN cells pre-incubated with 30 μM pervanadate for 10 min and after stimulation lysed in lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 25 mM β-glycerol phosphate, 1 mM DTT (Lu et al., 2003). IP was for 90 min at 4°C followed by 2 h incubation with protein G-Sepharose. The proteins were resolved on 8% SDS-PAGE.

**PTEN lipid phosphatase activity assay.** The PTEN lipid phosphatase activity was measured as previously described (Leslie et al., 2000; Snaddon et al., 2001) with some modification. In brief, PTEN was immunoprecipitated from 100-200 μg of BMM lysate as described above and then immune complexes washed twice in lysis buffer, twice in lysis buffer containing 500 mM LiCl and twice in washing buffer (10 mM Tris.HCl pH 8.0, 50 mM NaCl). Phospholipid vesicles were prepared using 0.425 mM L-α-phosphatidylserine, dipalmitoyl-heptammonium salt (Calbiochem) and 2 mM DOPS (L-α-phosphatidyl-L-serine, dioleoyl sodium, Sigma) in 10 mM Tris.HCl pH 7.4, 1 mM EGTA and sonicated. The phosphatase assay was performed in 50 μl of PTEN enzyme reaction buffer (50 mM Tris.HCl pH 8.0, 50 mM NaCl, 10 mM MgCl₂ and 10 mM DTT) containing 1 μg L-α-phosphatidylinositol 3,4,5-triphosphate, at 37°C for 60 min. The phosphate released from the substrate was measured using Green Reagent (Biomol, Plymouth Meeting, PA) according to the manufacturer’s instructions. In brief, the supernatant of the reaction was transferred to a 96-well plate, followed by addition of 100 μl of the Green Reagent, incubation for 20 min at room temperature to allow color development and measurement of absorbance at 620 nm. Released phosphate
was determined, in triplicate experiments, by comparison to a standard curve consisting of phosphate standards diluted in PTEN enzyme reaction buffer.

Where indicated, PTEN immunoprecipitates were washed in SHP1 phosphatase buffer (50 mM Tris.HCl, pH 7.5, 0.1 mM EGTA, 0.03 % (v/v) Brij and 10 mM DTT), incubated with or without 0.4 mg/ml recombinant GST-SHP1 for 30 min at 30°C under agitation, followed by determination of PTEN activity and its phosphorylation on Tyr as described above.

In some experiments, PTEN was immunoprecipitated from 300 μg total cell lysate and its lipid phosphatase activity was measured using an ELISA kit from Echelon, according to the manufacturer's instructions. In this case, the PIP2 produced was determined, in triplicate experiments, by comparison to a standard curve consisting of PIP2 standards bound to the ELISA plate.

For both assays, different amounts of proteins of cell lysates, from which PTEN was immunoprecipitated, were tested to ensure that the appropriate amount of enzyme were used so that the produced Pi or PIP2 was in the linear range of the respective standard curves.

**RhoGAP assay.** For GTP loading, RhoA-His protein (final concentration 31.25 μM) was loaded with 88 μM GTP in reaction buffer consisting of 12.7 mM Tris.HCl pH 7.5, 15.9 mM NaCl, 5 mM EDTA, 0.68 mg/ml BSA and 63.5 μM DTT in a 19.2 μl volume. After 10 min at 37°C, the exchange reaction was stopped by addition of MgCl₂ to a final concentration of 20 mM and incubation of the reaction vessel on ice.

To detect RhoA GAP activity associated with p190RhoGAP the latter protein was immunoprecipitated as described above, followed by GAP assay on the beads. The GAP assay was performed in 30 μl reaction volume containing 3 μl GTP-loaded RhoA, 1 mM GTP, 16 mM Tris.HCl pH 7.5, 0.87 mg/ml BSA and 80 μM DTT. As a blank, a sample containing only GTP-loaded RhoA was used and as a positive control the GAP activity of purified p50RhoGAP was measured. The samples were incubated for 15 min at 30°C, followed by transfer to ice and transfer of the supernatant into 96-well plates. Phosphate generated by hydrolysis of GTP was determined by addition of 120 μl of cytophos reagent (Cytoskeleton) to each well, followed 10 min later by measurement of the absorbance at 650 nm.

**Extraction of cytosolic and nuclear proteins.** The extraction of cytosolic and nuclear proteins based on the method of (Dignam et al., 1983) with minor modifications. Briefly, the cells harvested in chilled PBS, centrifuged at 6000 rpm for 5 min, resuspended in buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.5% NP-40 supplemented with protease inhibitors) and left on a rotator for 15 min. After centrifugation at 6000 rpm for 6 min, the supernatant which corresponds to cytosolic fraction removed and the pellet of nuclei resuspended in buffer C (20 mM Hepes pH 7.9, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol) and incubated for 30 min on ice. Then, buffer D (20 mM Hepes pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol) added and the samples centrifuged for 10 min at 13000 rpm. The nuclear proteins in the supernatant removed in new vials. Whenever indicated, RhoA was immunoprecipitated from the cytosolic fraction and its association (co-
immunoprecipitation) with p27 examined by western blotting with an antibody against p27.

**Time-lapse microscopy.** Cells were seeded on hydrochloric acid-washed 22x22 mm coverslips at 2.5x10⁴ cells/ml in macrophage growth medium, incubated overnight followed by an 8 h starvation of CSF-1 in macrophage starvation medium. Dunn chemotaxis chambers were set up as previously described (Allen et al., 1998; Zicha et al., 1991) using recombinant murine CSF-1 (30 ng/ml) as the chemoattractant. Cells were filmed at 37°C and images collected with a KPM1E/K-S10 CCD camera (Hitachi Denshi, Japan) every 5 min for 16 h using Tempus software (Kinetic Imaging Ltd, Liverpool, UK – now Andor Technology, Northern Ireland). All the time lapse sequences were displayed as a movie and cells from the first frame were tracked for the whole of the time-lapse sequences using Motion Analysis software (Andor Kinetic Imaging Ltd, Liverpool, UK) and the resulting tracks were tested for directional bias using Mathematica 6.0 workbooks (Allen et al., 1998; Wells et al., 2004).

To study random migration, cells were seeded on 35 mm dishes in macrophage growth medium for 24 h, starved of CSF-1 overnight followed by stimulation with CSF-1 (30 ng/ml) and filming for 16 h as described above. The mean migration speed of the population was derived after tracking of the cells and mathematical analysis using the software described above.

For the wound healing assays, cells were seeded at high density in macrophage growth medium and 1 day after the cells had formed confluent monolayer they starved of CSF-1 and wounded. The wound was made by scraping a pipette tip across the cell monolayer. When indicated, 10 μM Y27632 was added 30 min before wounding.

**Flow cytometry.** Cells were detached using EDTA (Versene), counted using a haemocytometer and divided into aliquots of 3x10⁵ cells per FACS tube. Cells were incubated for 30 min in 5% BSA, washed three times with PBS and incubated for 1h either with PE-conjugated rat IgG2a anti-mouse CSF-1R antibody, FITC-conjugated rat IgG2B anti-mouse F4/80 antibody or isotype-matched control antibodies. Fluorescence was quantified on a FACScan and analyzed using CellQuest software (BD Biosciences).

**References**


