The atypical Rho family GTPase Wrch-1 regulates focal adhesion formation and cell migration

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Summary

Wrch-1 (Wnt-regulated Cdc42 homolog) is a new member of the Rho family that was identified as a gene transcriptionally upregulated by Wnt-1. Wrch-1 has no detectable GTPase activity and displays very high intrinsic guanine nucleotide exchange, implying that it is constitutively GTP-bound. The biological functions of Wrch-1 largely remain to be characterized. Here, we report that Wrch-1 prominently localizes to focal adhesions. Depletion of Wrch-1 by small interfering RNA increases focal adhesion formation, whereas Wrch-1 overexpression disassembles focal adhesions. Wrch-1 depletion inhibits myosin-light-chain phosphorylation, which in turn leads to an increase in the number of focal adhesions and inhibits cell migration in response to wound healing. Depletion of Wrch-1 also inhibits Akt and JNK activation. Although pharmacological inhibitors of Akt and JNK inhibit cell migration, they do not affect focal adhesions. Thus, our data suggest that Wrch-1 regulates cell migration by multiple mechanisms: on the one hand Wrch-1 controls focal adhesions by regulating myosin light chain and on the other hand Wrch-1 stimulates the activation of Akt and JNK.

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Key words: Wrch-1, Rho, Focal adhesion, Cell migration, Myosin

Introduction

Rho family GTPases are crucial regulators of diverse functions, including actin dynamics, cell migration, vesicular trafficking and cell proliferation (Jaffe and Hall, 2005). Wrch-1 (Wnt-regulated Cdc42 homolog) is a novel member of the Rho family that was originally identified as a gene transcriptionally upregulated by Wnt-1 (Tao et al., 2004).

Several features distinguish Wrch-1 from other Rho proteins. Wrch-1 has no detectable GTPase activity but its intrinsic guanine nucleotide exchange activity is extremely high, implying that it is constitutively in the GTP-bound, active state (Saras et al., 2004; Shutes et al., 2004). Wrch-1 displays extensive sequence homology to Cdc42-related proteins and has both N- and C-terminal extensions. The N terminus has three PxxP motifs that can serve as docking sites for SH3-domain-containing proteins. The N-terminal domain negatively regulates Wrch-1 function and this internal inhibition can be released by binding of the N-terminus to SH3-domain-containing proteins such as Grb2 (Shutes et al., 2004). Wrch-1 also is unique in its post-translational modifications and mode of membrane anchorage, being palmitoylated rather than prenylated (Berzat et al., 2005).

The biological functions of Wrch-1 largely remain to be characterized. Like Cdc42, activation of Wrch-1 stimulates the formation of filopodia (Saras et al., 2004) and, similar to other Rho family proteins including Cdc42, Rac and Rho, overexpression of Wrch-1 promotes cell transformation (Shutes et al., 2004). In this work we employ RNA interference (RNAi) to study Wrch-1 function. We show that small interfering RNA (siRNA)-mediated depletion of Wrch-1 increases focal adhesion and stress fiber formation, and inhibits cell migration. We also examine the molecular mechanisms that underlie the role of Wrch-1 in the regulation of focal adhesions and cell migration. This is the first study that investigates the cellular functions of endogenous Wrch-1.

Results

Wrch-1 regulates focal adhesions

The high similarity between Wrch-1 on the one hand and Cdc42 and Rac proteins on the other suggested that Wrch-1 may also function in the organization of the actin cytoskeleton. To examine this, we first determined the intracellular localization of Wrch-1. Although we have generated a polyclonal antibody that specifically recognizes recombinant Wrch-1, it failed to detect the endogenous protein in a number of cell lines we had examined, probably because of the low expression levels of endogenous Wrch-1. We therefore used a GFP-Wrch-1 fusion protein and expressed it in HeLaS3 cells, which express relatively high levels of endogenous Wrch-1 mRNA (Kirikoshi and Katoh, 2002). Interestingly, in cells that express low levels of GFP-Wrch-1, we observed that this protein is strongly enriched in focal adhesions, as indicated by...
its colocalization with vinculin (Fig. 1). Punctate staining in the center of the cell can also be discerned, in line with the earlier documented localization of Wrch-1 to an endosomal compartment (Berzat et al., 2005). A Myc-epitope-tagged version of Wrch-1 displays a similar localization pattern compared with that of GFP-Wrch-1 (data not shown). Taken together, these data strongly suggest that Wrch-1 resides at focal adhesions.

The prominent localization of Wrch-1 to focal adhesions suggests that Wrch-1 can regulate the dynamics of these structures. To examine this, we knocked down endogenous Wrch-1 using siRNA and determined the effect of depleting Wrch-1 on focal adhesions. Wrch-1 knockdown modestly, but significantly, increases the number of focal adhesions per cell (Fig. 2A,D). Wrch-1 knockdown was confirmed by quantitative RT-PCR (Fig. 2B). Similar results were obtained with two independent oligos, indicating that the siRNAs act in a specific fashion to interfere with the generation of Wrch-1 protein.

We also note that depletion of Wrch-1 does not significantly alter the length or brightness of focal adhesions, indicating that the increase in the number of focal adhesions in Wrch-1-depleted cells is not compensated for by a decrease in focal adhesion size or density of focal adhesion proteins. Wrch-1 depletion also slightly, but significantly, decreases the steady-state cellular spread area (Fig. 2C), indicating that the increase in the number of focal adhesions per cell is not due to an increase in cell spreading. Remarkably, whereas the focal adhesions in cells transfected with control oligonucleotides directed against luciferase tend to be localized in the periphery of the cell, cells depleted of Wrch-1 display a marked increase in the number of centrally localized focal adhesions. Concomitant with the increase in focal adhesions, Wrch-1-depleted cells also show an increase in the formation of stress fibers.

We also examined the effect of overexpressing Wrch-1 on focal adhesions. Cells that express medium to high levels of GFP-Wrch-1 (see Materials and Methods, Immunofluorescence)
display a strong decrease in the number of focal adhesions (Fig. 3A,B) and cell rounding (Fig. 3A,C). Similar findings have been described previously (Saras et al., 2004). Most of the staining in these cells accumulates towards the center of the cell and does not colocalize with focal adhesions. We note that completely rounded cells, most of which displayed condensed chromatin, were excluded from the quantitative analysis. Focal adhesions in cells that express high levels of GFP itself are not affected, however (Fig. 3A). Thus, the results from both Wrch-1 depletion and overexpression studies strongly indicate a role of Wrch-1 in the control of focal adhesions.

Depletion of Wrch-1 inhibits cell migration

The importance of Wrch-1 in regulating focal adhesions suggested that Wrch-1 can also regulate cell migration. We therefore examined the role of Wrch-1 in cell migration using the monolayer wound healing assay (Valster et al., 2005). We observed that depletion of endogenous Wrch-1 significantly slows down cell migration (Fig. 4A).

The level of activated Wrch-1 is thought to be primarily controlled at the level of transcription rather than GTP loading, because its intrinsic guanine nucleotide exchange activity is very high (Saras et al., 2004; Shutes et al., 2004). We therefore examined the level of Wrch-1 mRNA during wound healing. Wrch-1 mRNA levels increase significantly and very rapidly after wounding, reaching half-maximum levels within 5 minutes of wounding (Fig. 4B). The increase in Wrch-1 mRNA is sustained for at least two hours. Although the increase in wounding-induced Wrch-1 mRNA we were able to detect was only approximately twofold, this is likely to be a strong underestimate, because we expect that only a relatively small fraction of the monolayer cells will be close enough to a wound edge to be able to respond to wounding.

Wrch-1 mediates phosphorylation of myosin regulatory light chain

Actomyosin contractility plays a crucial role in the regulation of focal adhesion assembly and turnover (Burridge and Chrzanowska-Wodnicka, 1996; Webb et al., 2002). Myosin II activity is controlled by the phosphorylation status of its regulatory light chain (MLC). We therefore examined the effect of depleting Wrch-1 on MLC phosphorylation using an antibody specific for MLC phosphorylated at residues Thr18 and Ser19. Wrch-1 depletion strongly inhibits MLC phosphorylation in HeLaS3 cells growing in the presence of serum (Fig. 5A). Levels of total MLC are not affected by Wrch-1 depletion (Fig. 5A). These results indicate that Wrch-1 is necessary for maintaining myosin II activity. Rho subfamily members play key roles in MLC phosphorylation (Burridge and Wennerberg, 2004; Jaffe and Hall, 2005). Thus, one possible mechanism for the role of Wrch-1 in MLC phosphorylation is that Wrch-1 regulates the activity of Rho proteins. However, we did not find any significant effect on Rho activation upon depleting Wrch-1. Moreover, the ROCK inhibitor Y27632 stimulates – rather than inhibits – cell migration in HeLaS3 cells (data not shown).

The phosphorylation status of MLC is controlled by a number of MLC kinases and the myosin phosphatase (Matsumura, 2005). Of these kinases, myosin light-chain kinase (MLCK) appears to play the key role in regulating focal adhesion turnover, at least in fibroblasts (Totsukawa et al., 2004; Webb et al., 2004). To examine the hypothesis that MLCK mediates the function of Wrch-1 in the regulation of...
focal adhesions in HeLaS3 cells, we used ML-7, a small molecule that inhibits both MLCK and zipper-interacting protein kinase (ZIPK) (Saitoh et al., 1987; Komatsu and Ikebe, 2004). We found that, similar to depletion of Wrch-1, low concentrations of ML-7 cause an increase in the formation of focal adhesions and stress fibers (Fig. 5B,C), supporting the view that Wrch-1 stimulates the disassembly of focal adhesions by regulating an MLCK-related kinase.

Inhibition of MLCK and/or ZIPK by ML-7 also slows down cell migration (Fig. 5D), consistent with the role of focal adhesion site turnover in cell migration (Totsukawa et al., 2004). Importantly, addition of ML-7 to Wrch-1-depleted cells does not further decrease cell migration (Fig. 5D), suggesting that Wrch-1 acts on the same pathway as MLCK and/or ZIPK in the regulation of cell migration.

The Wrch-1 effector PAK regulates focal adhesions and cell migration
Currently, the only known effector of Wrch-1 is the serine-threonine kinase PAK (p21-activated protein kinase) (Tao et al., 2001; Saras et al., 2004; Aspenstrom et al., 2004). We therefore also examined the effect depletion of PAK kinases has on focal adhesions and cell migration. Combined depletion of PAK1 and PAK2 significantly increases the number of focal adhesions per cell (Fig. 6A,B). Depletion of PAK1/2 also inhibits MLC phosphorylation and cell migration (Fig. 6C,D). Moreover, addition of ML-7 to PAK1/2-depleted cells does not further decrease cell migration (Fig. 6D), similar to the lack of any effect of ML-7 on Wrch-1-depleted cells (Fig. 5D). Thus, these results support the notion that Wrch-1 acts upstream of PAK1 and MLC to regulate focal adhesions and cell migration.

Wrch-1 regulates JNK activation
Overexpression of Wrch-1 has been shown to activate Jun N-terminal kinase (JNK) (Tao et al., 2001). Furthermore, JNK phosphorylates paxillin, a protein that is associated with focal adhesions, and this phosphorylation event is thought to contribute to focal adhesion turnover and cell migration (Huang et al., 2003). To examine whether Wrch-1 regulates focal adhesions and cell migration via JNK, we first determined the effect of Wrch-1 depletion on JNK activation induced during the wound healing response of HeLaS3 cell cultures. As observed for airway epithelial cells (White et al., 2005), JNK is activated within 30 minutes after wound healing, and depletion of Wrch-1 inhibits JNK activation to below baseline levels (Fig. 7A). Interestingly, although inhibition of JNK activity using the specific small molecule inhibitor SP600125 does not alter the number of focal adhesions (Fig. 7C), it does significantly inhibit cell migration in control cells, but not in Wrch-1-depleted cells (Fig. 7B). These data suggest that Wrch-1-mediated JNK activation plays a role in cell migration, and that this role is independent from the role of Wrch-1 in focal adhesions.

Wrch-1 binds to the regulatory p85 subunit of PI3K and regulates Akt
We also examined the effect of Wrch-1 depletion on additional signaling elements that have been implicated in cell migration. Whereas depletion of Wrch-1 does not have any significant effect on ERK phosphorylation in wounded monolayers (data not shown), it significantly inhibits wound-induced Akt phosphorylation (Fig. 8A). Akt has been shown to play a crucial role in HeLaS3 cell migration (Li et al., 2005). We
confirmed this in HeLaS3 cells using an inhibitor of phosphatidylinositol 3-kinase (PI3K), a signaling element that acts directly upstream of Akt (Fig. 8B). Notably, the PI3K inhibitor LY294002 does not further decrease the migration of Wrch-1-depleted cells, suggesting that Wrch-1 acts upstream of PI3K, and probably Akt, in the regulation of cell migration. LY294002 does not significantly affect the organization of the actin cytoskeleton, however, and does not alter the number of focal adhesions (supplementary material Fig. S1), indicating that the roles of PI3K and Akt in cell migration do not involve the regulation of focal adhesions.

Wrch-1 displays a high degree of sequence homology with Cdc42 and Rac (Tao et al., 2001), both of which can activate PI3K by binding to its p85 regulatory subunit (Zheng et al., 1994; Tolia et al., 1995). A GST-fusion protein of the N-terminal domain (a.a. 1-333) of p85 (Hill et al., 2001) efficiently pulls down full-length Wrch-1 from cell lysates (Fig. 8C). p85N also interacts with a Wrch-1 deletion mutant that misses the N-terminal proline-rich domain, but in a less efficient manner. These observations suggest that Wrch-1 interacts with p85 both via its effector domain – presumably with the Bcr domain of p85 (Zheng et al., 1994) – and its proline-rich domain (probably with the SH3 domain of p85).

**Discussion**

Here, we used RNAi to examine the role of Wrch-1 in the organization of the actin cytoskeleton and cell migration. Our data suggest that Wrch-1 regulates cell migration by multiple mechanisms, by stimulating Akt and JNK activation on the one hand and, on the other hand, by stimulating focal adhesion disassembly in a manner that is independent of Akt and JNK. This is the first study that analyzes the cellular functions of endogenous Wrch-1.

Wrch-1 is unique among the Rho family of GTPases in several respects, including its very high intrinsic guanine nucleotide exchange activity and its post-translational modification by a palmitoyl group (Saras et al., 2004; Shutes et al., 2004; Berzat et al., 2005). Adding to these unique features, we found that Wrch-1 prominently localizes to focal adhesions, although vesicular perinuclear staining is also present.
Fig. 8. Wrch-1 binds to the regulatory p85 subunit of PI3K and is necessary for Akt activation. (A) Wrch-1 depletion inhibits wound-induced Akt phosphorylation. Lysates were prepared as in Fig. 7A. Akt phosphorylation was visualized using an against antibody phosphorylated Akt (Ser473). An antibody against dynamin 2 was used to demonstrate equal loading. Levels of phosphorylated Akt from three independent experiments were quantified and normalized to levels of dynamin. **P<0.001, two-tailed t-test. (B) PI3K is necessary for cell migration. PI3K activity was inhibited by LY294002 (4 μM) (with overnight pre-treatment). Other conditions are as described in Fig. 5D. *P<0.0005 and **P<5×10^-6, two-tailed t-test. Data are representative of two independent experiments. (C) Wrch-1 binds to p85. Wrch-1 proteins were expressed in HelaS3 cells and pulled down using GST-p85 beads, as described in Materials and Methods.
migration of control HeLaS3 cells but not of Wrch-1-depleted cells. Furthermore, we showed that Wrch-1 directly interacts with the p85 regulatory subunit of PI3K. Together, these observations strongly suggest that Wrch-1 acts upstream of PI3K and Akt in the regulation of cell migration. Moreover, the PI3K-Akt branch of Wrch-1 signaling does not appear to be involved in regulating focal adhesions. Since Akt-1 and Akt-2 have been shown to play negative and positive roles, respectively, in cell migration (Yoeli-Lerner et al., 2005; Irie et al., 2005), it will be of great interest to examine whether Wrch-1 selectively mediates the activation of Akt2 versus Akt1.

Overexpression of activated versions of Wrch-1, generated either by a point mutation that interferes with GTP hydrolysis, by truncation of the N-terminal domain or by both, has been shown to transform NIH 3T3 cells using anchorage-independent growth as a read-out (Shutes et al., 2004). We therefore also examined the potential role of Wrch-1 in cell proliferation. However, we did not find any effect on the proliferation of HeLaS3 cells in limiting serum concentrations upon depletion of Wrch-1 (supplementary material Fig. S2), arguing for a specific role for Wrch-1 in the migratory behavior of these cells.

Several Wnt family members, including Wnt1, Wnt3a, Wnt4 and Wnt11 have been shown to stimulate cell migration (De Calisto et al., 2005; Endo et al., 2005; Qiang et al., 2005; Kuphal et al., 2006), suggesting that induction of Wrch-1 contributes to Wnt-regulated cell migration. However, whether Wnt signaling is involved in wound healing in HeLaS3 monolayers remains to be determined. Notably, we did indeed observe that Wrch-1 message is significantly induced immediately after wounding, although the very fast kinetics of this increase appears to argue against the involvement of Wnt release and subsequent Wnt-mediated induction of Wrch-1. Thus, it is possible, if not likely, that Wrch-1 message levels can also be controlled in a Wnt-independent fashion.

In conclusion, we describe a new function of Wrch-1 in cell migration that is likely to be mediated by the concerted action of multiple Wrch-1-regulated signaling mechanisms respectively controlling focal adhesions, and the activation of Akt and JNK.

Materials and Methods
Reagents
Small interfering RNA (siRNA) oligonucleotide duplexes were obtained from Dharmacon. The siRNA target sequences are: GL2 luciferase (5'-AACGTA-CGCCGAAATCTTACCTGGA-3'), Wch-1 (5'-AAGTACGCTGTTTGGATATCA-3'), W-1, Dharmacon sGENOME E-D-009882-01 and 5'-AAGAAGCTGCAATGGAGAACATTGGA-3', W-2, D-009882-02), PAK1 (5'-GAAGAAATACAGCGGT3'-3') and PAK2 (5'-GAAAGAAGCTGCAATGAA-3'). The following antibodies were used: anti-phosphorylated MLCK1/S19), anti-MLC2, anti-PAK2, anti-phosphorylated Akt, PAK4 (7473) and anti-phosphorylated Jun(S63) 54B3 (Cell Signaling), anti-human vinculin (clone hVIN-1) (Sigma), anti-dynamin 2 (Santa Cruz), anti-PAK1 (Zymed), anti-Wrch-1 (Abcam), anti-HA (Roche) and Texas-Red donkey anti-mouse (Jackson ImmunoResearch). The inhibitors ML-7, SP600125 (JNK inhibitor II) and LY294002 (PI3K inhibitor) were obtained from Calbiochem and FITC-phalloidin was from Sigma. The GFP-Wrch-1 plasmid was obtained from W. Tao (Merk), pCGN-HA-Wrch-1Q107L, a point mutant of Wrch-1 that is defective in GTP hydrolysis, was obtained from A. Levine (Cancer Institute of New Jersey, New Brunswick, NJ); pCGN-HA-ΔN-Wrch-1Q107L, a version of Wrch-1 that lacks the first 4 N-terminal amino acids, was generated in the same vector.

Cell culture and transfection
HeLaS3 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine, supplemented with 5% fetal bovine serum, 5% bovine growth serum (Hyclone) and penicillin/streptomycin. Transient transfection of siRNA was carried out using Lipofectamine 2000 (Invitrogen). Cells were plated in a 24-well plate. Control and Wrch-1 siRNA-transfected cells were further maintained in DMEM without antibiotics. Twenty nM of siRNA were transfected using the protocol provided by manufacturer. Wrch-1 levels were determined by quantitative PCR on the day of the assay. Transient transfection of plasmids was carried out using Effectene (Qiagen). Cells were plated at 30% confluency in a 24-well plate with growth medium. Once fully spread (approximately 4 hours after plating), cells were transfected following the protocol supplied by the manufacturer (0.4 μg of plasmid, 3.2 μl of enhancer and 4 μl of Effectene).

GST pull-down assays
Plasmids were transfected into HelaS3 cells with Effectene (Qiagen) as described above for siRNAs. Cells were lysed in RIP buffer with Protease Inhibitor Cocktail without EDTA (Roche) and total protein concentration was determined with Protein Assay reagent (Bio-Rad). Equal amounts of purified GST-p85N (Hill et al., 2001) were incubated with equal concentrations of cell lysates containing either ΔNWrch-1Q107L or Wrch-1Q107L at 4°C for 16 hours. 50 μl of glutathione beads (GE) were added at 4°C for another 2 hours. Subsequently, the beads were washed five times and eluted with 80 μl of 2× sample buffer. Samples were run in either 10% LongLife gels (Life Therapeutics) or 4-20% Precise gels (Pierce) and transferred to PVDF membrane (Bio-Rad). Proteins were detected with antibodies against HA (Wrch-1) or GST (GST-p85N).

Quantitative PCR
Total RNA was isolated using the RNeasy kit (Qiagen) and one-step quantitative PCR of Wrch-1 RNA was performed using the RT qPCR MasterMix kit (Eurogentec, San Diego, CA) and read out using the ABI TaqMan system (forward primer: 5'-CAGCGAAGATTGCAGTCG-3', reverse primer: 5'-GAGATCGACTCGTTC-3' and probe: 5'-CTGTCCTACAAAGCCCCATCATCC-3'). Wounding-induced induction of Wrch-1 mRNA was determined using the multiple wounding protocol described below.

Wound healing assay
Wound healing assays were performed essentially as described in (Valster et al., 2005). In brief, a confluent monolayer of HelaS3 cells was wounded with a P1000 tip in 1× PBS without Ca2+ and Mg2+ and subsequently returned to full growth medium. Wounds were imaged at various times at the same spots using an IX70 Olympus inverted microscope equipped with a 10× NA=0.3 objective, an Ora II cooled charge coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ) and Esee image analysis software (Inovision, Raleigh, NC). Wound widths were measured for at least eight spots. Distance of cell migration was calculated as half of the wound width at a given time point and by subtracting this value from the initial half-width.

Immunofluorescence and quantification of focal adhesions
Cells were plated sparsely on laminin-coated coverslips (BD Falcon) for 24 or 48 hours. Subsequently, cells were fixed in 4% formaldehyde/PBS, permeabilized with 0.1% Triton X-100 dissolved in PBS, followed by staining with FITC-conjugated phalloidin and indirect immunofluorescence using an anti-vinculin antibody. Images were collected with the set-up described under Wound healing assay, using a 60×, NA=1.4 objective.

Focal adhesions were manually traced using Esee image analysis software. The average length of focal adhesion was calculated by dividing the total length of all focal adhesions in a given cell by the number of focal adhesions in that cell. The average focal adhesion brightness was then calculated as the total focal adhesion brightness divided by the number of focal adhesions.

To determine the effect of overexpressing Wrch-1 on focal adhesions, we chose cells that expressed medium to high levels of GFP-Wrch-1, including cells that displayed GFP-Wrch-1 at 1 levels visible through the microscope ocular, in contrast to low expression levels that were only detectable by the CCD camera.

Multiple wounding assay
This method was modified from a previously described protocol (White et al., 2005). Confluent monolayers in 60-mm dishes were wounded in four directions using a micro metal nit comb (Bayer), with a tip width of 0.35-0.40 mm and tip to tip distance of 0.6-0.7 mm. Monolayers of HeLaS3 cells were returned to growth medium at 37°C. Once fully spread (approximately 4 hours after plating), cells were transfected following the protocol supplied by the manufacturer (0.4 μg of plasmid, 3.2 μl of enhancer and 4 μl of Effectene).

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kinase assay was performed in 50 μl of kinase buffer supplemented with 200 μM ATP for 30 minutes at 30°C. Fifty μl of 2× SDS sample buffer was added to terminate reactions after spinning down beads and removal of kinase buffer. Samples were run in 10% LongLife Gels (Life Therapeutics), transferred onto PVDF membrane (Bio-Rad) and detected with anti-phosphorylated Jun(Ser63). For the detection of activated Akt, 5% sample buffer was added to the cell lysates and phosphorylated Akt was detected by western blotting. For the Wrch-1 mRNA induction experiments, at various times after wounding, RNA was extracted using RNeasy (Qiagen) and Wrch-1 mRNA levels were determined by quantitative PCR.

Quantification of western blots
Western blots were scanned using ScanMaker i900 (Microtek) and analyzed using ImageJ software (NIH).

Cell proliferation assay
Cell proliferation was determined using the sulforhodamine B colorimetric assay (Skehan et al., 1990). 4×10^4 siRNA-transfected cells were plated into six wells of a 96-well plate in growth medium supplemented with 1% serum. Plates were processed as previously described (Chan et al., 2005).

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References
Almeida, E. A., Ilic, D., Han, Q., Jin, F., Kawakatsu, H., Schlaeffer, D. D. and Damsky, C. H. (2000). Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH2-terminal kinase. J. Cell Biol. 149, 741-748.
Aspenstrom, P., Fransson, A. and Saras, J. (2004). Rho GTPases have diverse effects on the organization of the actin filament system. Biochem. J. 377, 327-337.
Berezat, A. C., Buss, J. E., Chenette, E. J., Weinaume, C. A., Shutes, A., Der, C. J., Minden, A. and Cox, A. B. (2005). Transforming activity of the Rho family GTPase, Wrch-1, a Wnt-regulated Cdc42 homolog, is dependent on a novel carboxy-terminal palmitoylation motif. J. Biol. Chem. 280, 33055-33065.
Burrage, K. and Wenerberger, K. (2004). Rho and rac take center stage. Cell 116, 167-179.
Burrage, K. and Chrzansowa-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol. 12, 463-518.
Chan, A. Y., Coniglio, S. J., Chuang, Y. Y., Michaelson, D., Knaus, U. G., Philips, M. R. and Symons, M. (2005). Roles of the Rac1 and Rac3 GTPases in human tumor cell invasion. Oncogene. 24, 7821-7829.
Chew, T. L., Masaracchia, R. A., Goeckeler, Z. M. and Wysolmerski, R. B. (1998). Regulation of myosin II during cytokinesis in higher eukaryotes. J. Biol. Chem. 273, 16374-16378.
Huang, C., Rajfur, Z., Borchers, C., Schaller, M. D. and Jacobson, K. (2003). JNK phosphorylates paxillin and regulates cell migration. Nature 424, 219-223.
Huang, C., Jacobson, K. and Schaller, M. D. (2004). MAP kinases and cell migration. J. Cell Sci. 117, 4619-4628.
Irie, H. Y., Pearline, R. V., Gruneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S. and Brugge, J. S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J. Cell Biol. 171, 1023-1034.
Jaffe, A. B. and Hall, A. (2005). Rho GTPases: biochemistry and biology. Annu. Rev. Cell Dev. Biol. 21, 247-269.
Kirikoshi, H. and Katoh, M. (2002). Expression of WRCH1 in human cancer and down-regulation of WRCH1 by beta-estradiol in MCF-7 cells. Int. J. Oncol. 20, 777-783.
Komatsu, S. and Ikebe, M. (2004). ZIP kinase is responsible for the phosphorylation of myosin II and necessary for cell motility in mammalian fibroblasts. J. Cell Biol. 165, 243-254.
Kuphal, S., Lodermeyer, S., Bataille, F., Schuierer, M., Huang, B. H. and Bosserhoff, A. K. (2006). Expression of Dickkopf genes is strongly reduced in malignant melanoma. Oncogene 25, 5027-5036.
Li, J., Ballif, B. A., Powelka, A. M., Dai, J., Gygi, S. P. and Hsu, V. W. (2005). Phosphorylation of ACAP1 by Akt regulates the stimulation-dependent recycling of rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of A431 cells. J. Cell Biol. 143, 1087-1099.
Matsumura, F. (2005). Regulation of myosin II during cytokinesis in higher eukaryotes. Trends Cell Biol. 15, 371-377.
Quang, Y. W., Walsh, K., Yao, L., Kedei, N., Blumberg, P. M., Rubin, J. S., Shuates, A., Berzat, A. C., Cox, A. D. and Der, C. J. (2004). Atypical mechanism of regulation of the Wrch-1 Rho family small GTPase. Curr. Biol. 14, 2025-2056.
Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kennedy, S. and Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer drug screening. J. Natl. Cancer Inst. 82, 1107-1112.
Stockton, R. A., Schafer, E. and Schwartz, M. A. (2004). p21-activated kinase regulates endothelial permeability through modulation of contractility. J. Biol. Chem. 279, 46621-46630.
Takino, T., Nakada, M., Miyamori, H., Watanabe, Y., Sato, T., Guntulga, D., Yoshikoa, K., Yamada, K. M. and Sato, H. (2005). JASPI/JIP3 cooperates with focal adhesion kinase to regulate c-Jun N-terminal kinase and cell migration. J. Biol. Chem. 280, 37772-37781.
Tao, W., Pennica, D., Xu, L., Kallil, R. F. and Levine, A. J. (2001). Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1. Genes Dev. 15, 1796-1807.
Tolias, K. F., Cantley, L. C. and Carpenter, C. L. (1995). Rho family GTPases bind to phosphoinositide kinases. J. Biol. Chem. 270, 17656-17659.
Totsukawa, G., Wu, Y., Sasaki, Y., Harshbome, D. J., Yamakita, Y., Yamashiro, S. and Matsumura, F. (2004). Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. J. Cell Biol. 164, 427-439.
Valster, A., Tran, N. L., Nakada, M., Berens, M. E., Chan, A. Y. and Symons, M. (2005). Cell migration and invasion assays. Methods 37, 208-215.
Webb, D. J., Parsons, J. T. and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. Nat. Cell Biol. 4, E97-E100.
Weinberg, K., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T. and Horwitz, A. F. (2004). FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat. Cell Biol. 6, 184-161.
White, S. R., Tse, R. and Marroquin, B. A. (2005). Stress-activated protein kinases mediate cell migration in human airway epithelial cells. Am. J. Respir. Cell Mol. Biol. 32, 301-310.
Xia, Y. and Karin, M. (2004). The control of cell motility and epithelial morphogenesis by jun kinases. Trends Cell Biol. 14, 94-101.
Yoeli-Lerner, M., Yiu, G. K., Rabinovitz, I., Erhardt, P., Jauliac, S. and Toker, A. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Nat. Cell Biol. 20, 539-550.
Zheng, Y., Bagrodia, S. and Cerione, R. A. (1994). Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J. Biol. Chem. 269, 18727-18730.