Supplementary materials

Immunofluorescence microscopy

Cells were seeded on glass cover slips (n.1 thickness) for 24 h and fixed with 2% paraformaldehyde at room temperature for 20 minutes. After rinsing with PBS containing 200 mM Glycine, cells were incubated for 2 h at 4°C with primary antibody. For our studies, the following antibodies were used: mouse monoclonal antibody anti-E-cadherin (BD Biosciences Pharmingen, San Jose, CA) diluted 1:100, anti-β-catenin (BD Biosciences Pharmingen, San Jose, CA) diluted 1:100, anti-p190 (Upstate Biotechnology, Lake Placid, NY) diluted 1:200, or rabbit polyclonal anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:40. After washing three times with PBS containing 0.1% Brij-58 (Sigma-Aldrich, St. Louis, MO), cells were incubated for 1 h with anti-mouse Alexa 488 or anti-rabbit Alexa 594 secondary antibodies (Invitrogen, Carlsbad, CA), diluted 1:1000 in PBS 0.1% Brij-58. Cover slips were then washed with PBS 0.1% Brij-58, inverted onto a glass microscope slide, in a drop of Prolong Gold mounting solution (Invitrogen), containing DAPI, and visualized with LSM510 confocal system interfaced with a Zeiss Axiovert 100 M microscope.

siRNA sequences

Small interfering RNAs (siRNAs) for RhoA (ON-TARGET-plus, SMARTpool siRNA: 5'-CGACAGCCCUGAUAGUUUA-3', 5'-GACCAAAGAUGGAGUGAGA-3', 5'-GCAGAGAUAUGGCAAACAG-3', 5'-GGAAUGAUGAGCACACAAG-3'), p190RhoGAP (5'-GGAGGAAUCUGUAUCAUG-3, 5'-GAACAGCGAUUUAAAGCAU-3', 5'-GAUGGGCUGUCUUUCAUUA-3', 5'-UCAGCGAGAUCCAAUGUAA-3'), DLC-1 (5'-UUAAGAACCUGGAGGACUA-3', 5'-GUACGAAAGAGGAGCGUUU-3',5'-UUAAAGAAGUCAAAGAGAA-3', 5'-GCAUGUACUUAGAGGGCUU-3'), and LARG/ARHGEF12 (5'-GAUCAAAUCUCGUCAGAAA-3', 5'-GAAAUGAGACCUCUGUUAU-3', 5'-GGACAUAUGCCCUUUAGAA-3', 5'-GGCAACAUUUCCCAAGAUA-3') were

purchased from Thermo Scientific Dharmacon (Lafayette, CO). RISC-free siRNA was used as control.

Cell growth and migration assays

For the soft agar assay, 1 x 10^5 cells were mixed with RPMI 1640 medium supplemented with 5% FBS and containing 0.4% agar (Sigma-Aldrich). This cellular suspension was placed over 0.6% of basal agar in 60 mm dishes. Cells were grown for 2 to 3 weeks. Colonies were analyzed by microscopy and counted with Artek Counter Model 880 (Artek Systems Corporation, Farmingdale, NY).

Cell migration experiments were performed using wound healing and transwell assays. For the wound healing assay, 1.5 x 10^5 cells were seeded in 12-well plates and incubated overnight at 37°C to generate confluent cultures. After achieving confluence, the cell layer in each well was scratched using a plastic pipette tip. The migration of the cells at the edge of the scratch was monitored at time 0 and 16 hours later, when cells were stained with crystal violet for 10 minutes, destained in PBS, visualized microscopically and photographed.

The transwell assay was performed using 6.5-mm-diameter Falcon cell culture inserts (8 μm pore size; Becton Dickinson, Franklin Lakes, NJ) precoated with 0.01% gelatin in 24-well plates. Cells were trypsinized and resuspended in serum-free RPMI 1640 medium in the upper chamber of the filter (10^5 cells in 500 μL). 800 μL of medium containing 10% FBS were added in the lower chamber, and after overnight incubation, cells remaining on the upper surface of the filter were removed with a cotton swab. Cells that had migrated to the lower surface were fixed with methanol, stained for 10 minutes with crystal violet, destained, photographed, and solubilized overnight with 1% Triton X-100. The protein lysates were quantified colorimetrically using a spectrophotometer Beckman DU640. The absorbance at λ = 600 nm is proportional to the migrated cells, and the average was calculated between three independent experiments. Error bars indicate S.D. of the mean.

Cell viability and growth rate were determined by Methyl-Thiazolyldiphenyl-Tetrazolium bromide (MTT) colorimetric assay. MTT (Sigma-Aldrich) is converted to water-insoluble formazan crystals of dark blue color by mitochondrial dehydrogenases of living cells. The assay was performed in 96-well plates. Cells were seeded at 1.5 x 10^4 per well and analyzed after 48 h or 72 h of incubation. The blue crystals were solubilized with DMSO and the intensity, which is proportional to the surviving cells, was measured colorimetrically at λ = 550 nm (Mosmann, 1983).

Immunoprecipitation

For immunoprecipitation, 2 mg of lysate were reacted with rabbit anti-EGFR polyclonal antibody (Santa Cruz Biotechnology) for 1 h on ice and incubated with protein A/G Agarose (Pierce, Rockford, IL) for 1 h at 4 °C. Immunoprecipitates were washed three times with 0.5 mL of washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and the same protease and phosphatase inhibitors as in the lysis buffer) and eluted with Laemmli buffer (Laemmli, 1970). Samples were boiled for 5 min at 95°C and proteins were analyzed by SDS–polyacrylamide gel electrophoresis. Western blot was performed using mouse monoclonal antibody specific for P-Tyr (Upstate Biotechnology) or rabbit anti-EGFR polyclonal antibodies (Santa Cruz Biotechnology).

Nuclear fractionation

Nuclear fractionation was performed to detect the nuclear level of β-catenin in H1299 cells and in the stable clones expressing E-cadherin. The preparation of pure nuclei from cellular lysates was carried out using Nuclei Pure Prep nuclei isolation kit (Sigma-Aldrich), in which centrifugation through a dense sucrose cushion was performed to protect nuclei and avoid cytoplasmic contaminants, according to the manufacturer's instructions. The level of E-cadherin, β-catenin, c-myc and β-actin was measured in the nuclear fraction and in the total extract by Western blot.

Statistical analysis

All data are the mean ± S.D. of three separate experiments. Levels of significance were determined by two-sided Student *t*-test. P values less than 0.05 were considered statistically significant.