Supplemental Materials

Methods

siRNA

siRNA oligonucleotide duplexes targeting human *ARHGAP22*, *ARHGAP25*, and *Rac1* were purchased from Invitrogen (Carlsbad, CA). The targeting sequences were as follows: *ARHGAP22*, KD#1 5'-GCAUACUCAAAUGUCAACAAGAUGA-3' (nt 886-910) and KD#2 5'-GGAAAUAAAGCUGCGGAACUCUGAA-3' (nt 1956-1980); *ARHGAP25*, KD#1 5'-AAACACUCCCUAACCGGAAAUGUUU-3' (nt 1301-1325) and KD#2 5'-UGGAGAAGAGGAAAUUGAUUCUUUG-3' (nt 1614-1638); *Rac1*, KD#1 5'-CCGCAAACAGAUGUGUUCUUAAUUU-3' (nt 217-241) and KD#2 5'-CAAACAGAUGUGUUCUUAAUUUGCU-3' (nt 220-244).

Cell culture and transfection

A549 and SW620 cells (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS) and 50 U/ml penicillin/streptomycin at 37°C/5% CO_2. SKMEL2 cells (ATCC) were cultured in Minimum essential medium Eagle (MEM, Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) and 50 U/ml penicillin/streptomycin at 37°C/5% CO_2. PC-3 cells (RIKEN Cell bank, Tsukuba, Japan) were cultured in RPMI 1640 supplemented with 10% (v/v) FBS and 50 U/ml penicillin/streptomycin at 37°C/5% CO_2. M2 and A7 cells were cultured as described previously (Ohta *et al.*, 2006). A549 and PC-3 cells were transfected with siRNA for 48 h using Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. SW620, SKMEL2, M2, and A7 cells were transfected with siRNA for 48 h using Lipofectamine RNAiMAX (Invitrogen) according to the manufacture's reverse transfection protocols. The efficiency of ARHGAP22 and ARHGAP25 depletions was confirmed by RT-PCR (see below).

Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted from carcinoma cell lines using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed from 500 ng of total RNA using AccuScript

High Fidelity RT-PCR System (Stratagene). The obtained cDNAs were amplified by PCR using recombinant *Taq* DNA polymerase according to the standard protocols (Takara Bio, Shiga, Japan). PCR conditions were 30 cycles of 98°C for 10 sec, 56°C for 30 sec and 72°C for 60 sec. The PCR-amplified products were analyzed by agarose gel electrophoresis. The sequences of primers for ARHGAP22, ARHGAP25, and β-actin (internal control) were as follows; ARHGAP22, 5'-AGACTCTCCACCTACGAC-3' (nt 1462-1479) and 5'-TTACTTTGGGGCCCTGGC-3' (nt 2080-2097); ARHGAP25, 5'-TGTGATTGGTGTGAATCTC-3' (nt 921-939) and 5'-TTAAGCCTCGGTCTTGGGT-3' (nt 1899-1917); β-actin, 5'-ACAGGATGCAGAAGGAGATC-3' (nt 932-951) and 5'-ATCCACATCTGCTGGAAGG-3' (nt 1050-1070). To confirm primer specificity between GAPs, the plasmid DNA containing human FilGAP (NM_001025616), ARHGAP22 (BC126444), or ARHGAP25 (NM_014882) cDNA was first used as a template of PCR to determine primer specificity. The primer sets for ARHGAP22 and ARHGAP25 produced specific PCR-amplicons, which corresponded to the expected size of ARHGAP22 (636 bp) and ARHGAP25 (997 bp), respectively (Supplemental Figure 1B, top panel). The primer set for β-actin also generated amplicons of the expected size (139 bp) (Supplemental Figure 1B, middle and bottom panels).

Antibodies and regents

Mouse anti-Rac monoclonal antibody was purchased from Millipore (Billerica, MA). Mouse anti-Myc (9E10) monoclonal and rabbit anti-Myc polyclonal antibodies were purchased from Sigma and MBL (Nagoya, Japan), respectively. NSC23766 were also purchased from Merck Biosciences (Nottingham, UK).

References

Ohta Y, Hartwig JH, Stossel TP. (2006). FilGAP, a Rho- and ROCK-regulated GAP for Rac binds filamin A to control actin remodelling. Nat Cell Biol 8, 803-814.

Figure Legends

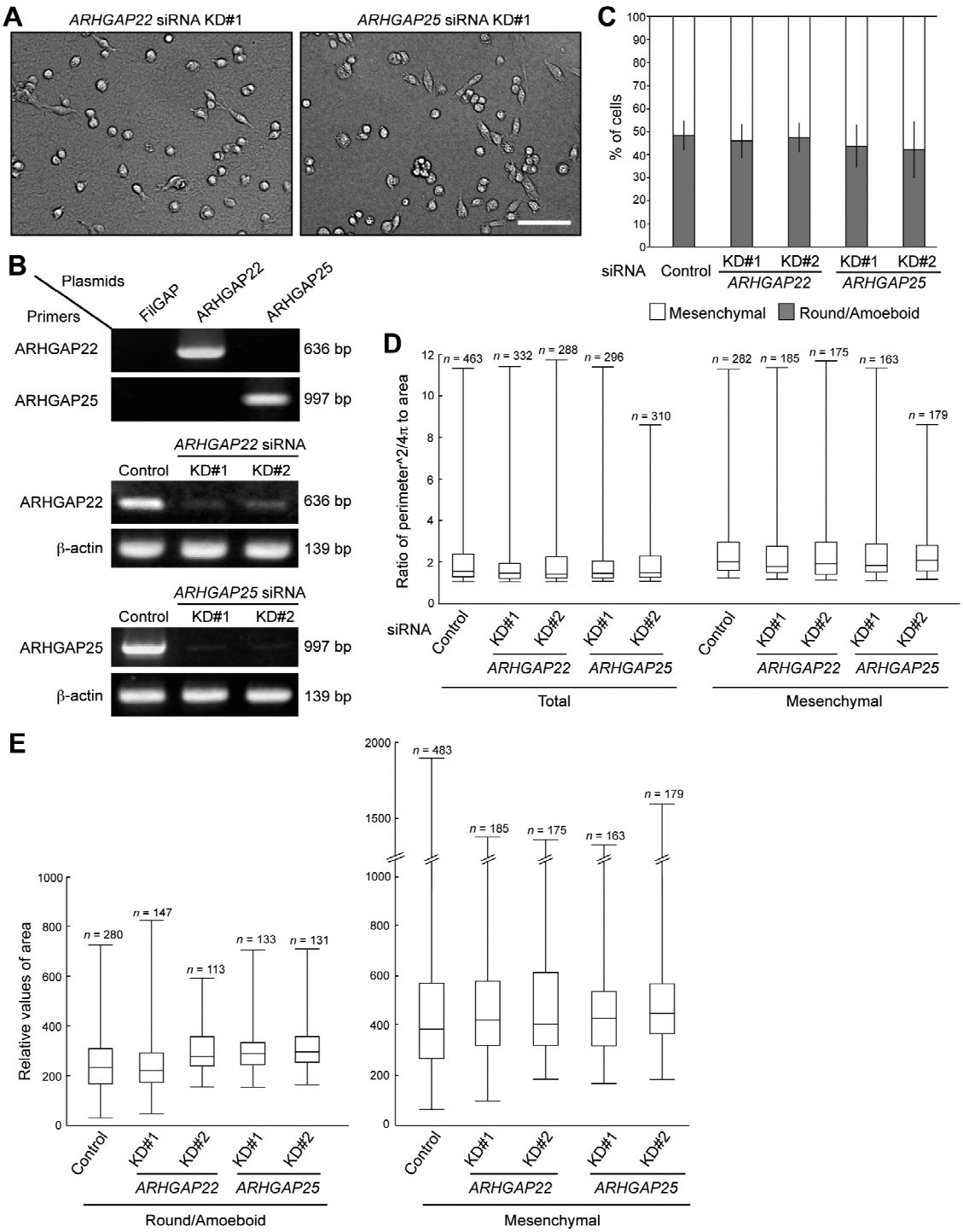

Figure S1: Depletion of ARHGAP22 and ARHGAP25 does not affect cell morphology of MDA-MB-231 cells on collagen gels. MDA-MB-231 cells are transfected with control or siRNAs targeting *ARHGAP22* or *ARHGAP25*, and cultured on plastic plates for 2 d. The control or transfected cells were trypsinized, and then plated on collagen gels and

cultured for 24 h. (A) Differential interference contrast (DIC) images of *ARHGAP22* or *ARHGAP25* siRNA-transfected cells on collagen gels. Scale bar, 100 μm. (B) Reverse transcriptase-PCR (RT-PCR) analysis of *ARHGAP22* and *ARHGAP25* mRNA expression in siRNA-transfected cells that were cultured on plastic plates. To determine primer specificity, RT-PCR was performed on plasmid DNA containing *FilGAP*, *ARHGAP22*, or *ARHGAP25* cDNA using the ARHGAP22/25-specific primers (top panels). Silencing efficiency of *ARHGAP22* and *ARHGAP25* siRNAs is shown in middle and bottom panels, respectively. β-actin was used as an internal control. (C) Cells ($n > 500$) were counted and categorized as mesenchymal or round/amoeboid. (D) Quantification of cell morphology. Ratio calculated as perimeter^2/4πarea ($n > 200$ total cells) (**E**) Relative values of area ($n >$ 100 total cells).

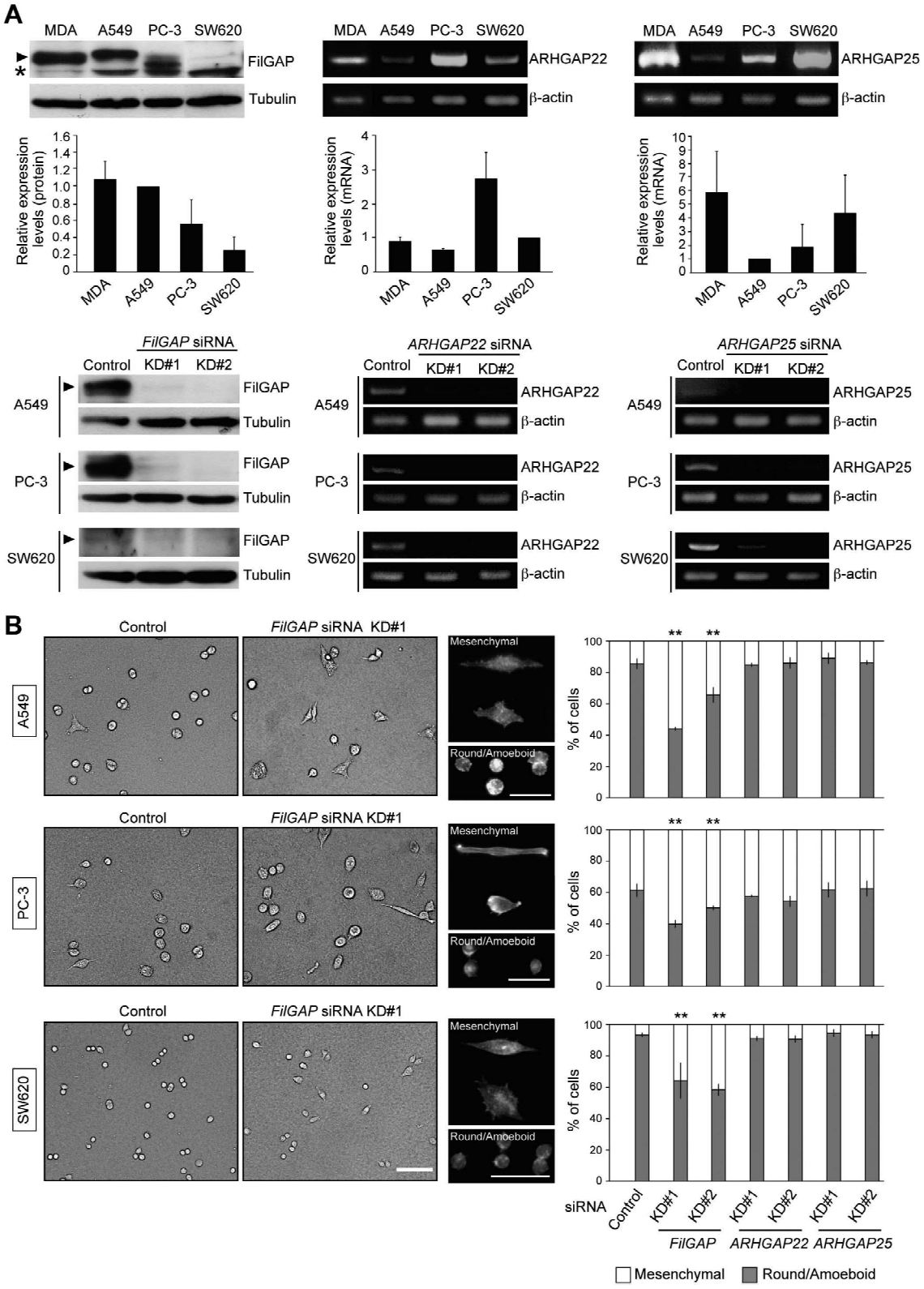

Figure S2: Effects of depletion of FilGAP, ARHGAP22, or ARHGAP25 on cell morphology of carcinoma cell lines on collagen gels. A549, PC-3, and SW620 cells were transfected with the indicated siRNAs and cultured on plastic plates for 2 d. The control

or transfected cells were trypsinized, and then plated on collagen gels and cultured for 24 h. (A) Expression (upper panels) and siRNA silencing efficiency (lower panels) of FilGAP (left), ARHGAP22 (middle), and ARHGAP25 (right) were analyzed by immunoblotting using anti-FilGAP antibody (left) and RT-PCR using ARHGAP22/25-specific primers (middle and right). Tubulin (left) and β-actin (middle and right) were used as a loading control and internal control, respectively. Asterisk (left) indicates a non-specific band. Relative protein and mRNA expression levels were normalized using tubulin and β-actin levels, respectively. (B) DIC images of control and *FilGAP* siRNA-transfected cells on collagen gels (left). Scale bar, 100 μm. Representative images of F-actin staining of cells with mesenchymal or round/amoeboid morphology are shown (middle). Scale bars, 50 μm. Cells ($n > 500$) were categorized as mesenchymal or round/amoeboid (right). $^{**}P < 0.01$. Statistical significance was determined by Student's *t*-test.

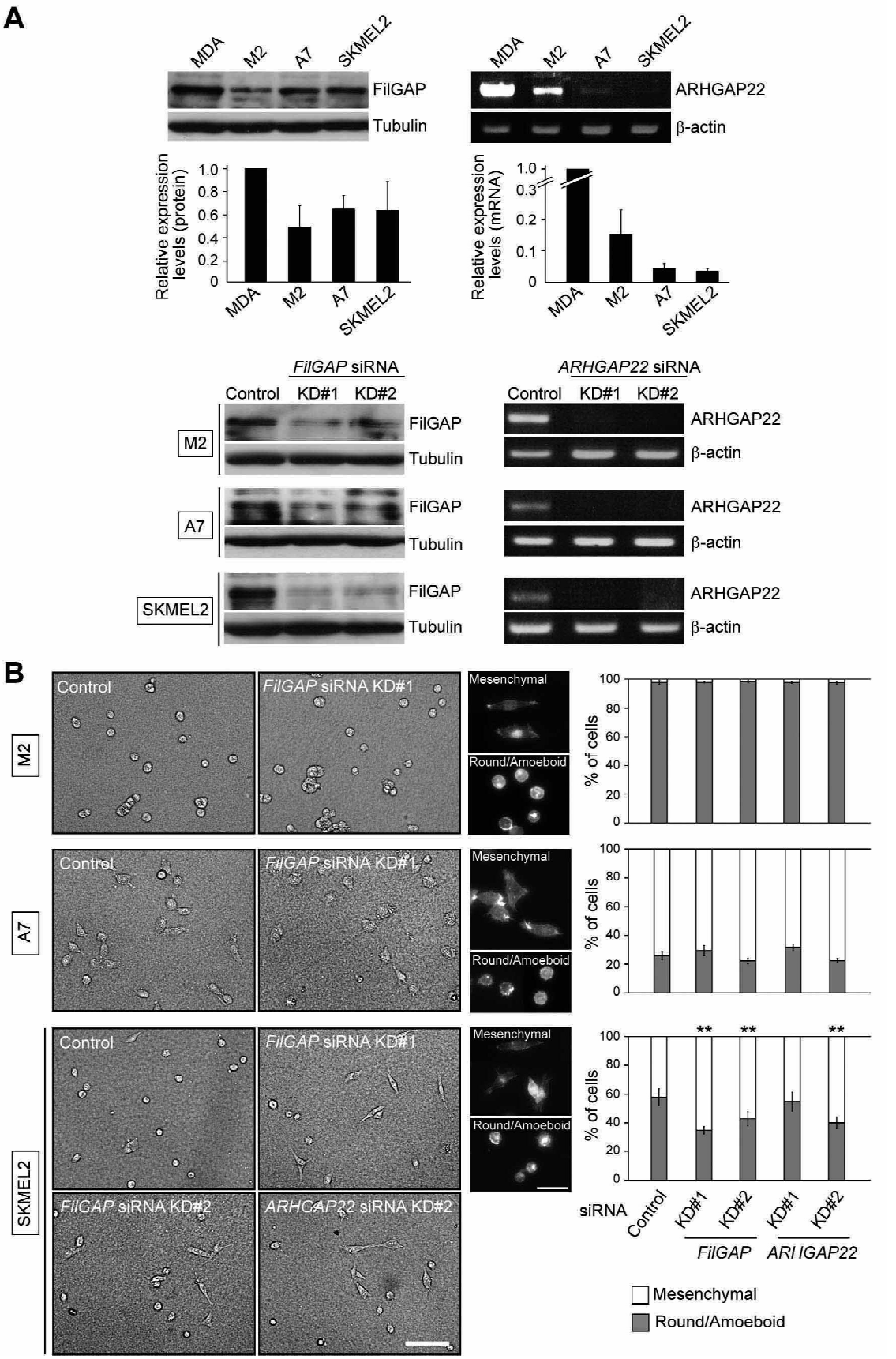
A
MDA
M2
A7
SKMEL2
FilGAP
Tubulin
ARHGAP22
β-actin
Relative expression levels (protein)
Relative expression levels (mRNA)
FilGAP siRNA
ARHGAP22 siRNA
Control
KD#1
KD#2
B
Control
FilGAP siRNA KD#1
FilGAP siRNA KD#2
ARHGAP22 siRNA KD#2
Mesenchymal
Round/Amoeboid
% of cells
siRNA
FilGAP
ARHGAP22
Mesenchymal
Round/Amoeboid

Figure S3: Effects of depletion of FilGAP and ARHGAP22 on cell morphology of melanoma cell lines on collagen gels. M2, A7, and SKMEL2 cells were transfected with the indicated siRNAs and cultured on plastic plates for 2 d. The control or transfected cells were trypsinized, and then plated on collagen gels and cultured for 24 h. (A) Expression (upper panels) and siRNA silencing efficiency (lower panels) of FilGAP (left) and ARHGAP22 (right) were analyzed as in Supplemental Figure 1A. (B) DIC images of control and indicated siRNA-transfected cells on collagen gels (left). Scale bar, 100 μm. Representative images of F-actin staining of cells with mesenchymal or round/amoeboid morphology are shown (middle). Scale bar, 50 μm. Cells ($n > 500$) were categorized as mesenchymal or round/amoeboid (right). $^{**}P < 0.01$. Statistical significance was determined by Student's *t*-test.

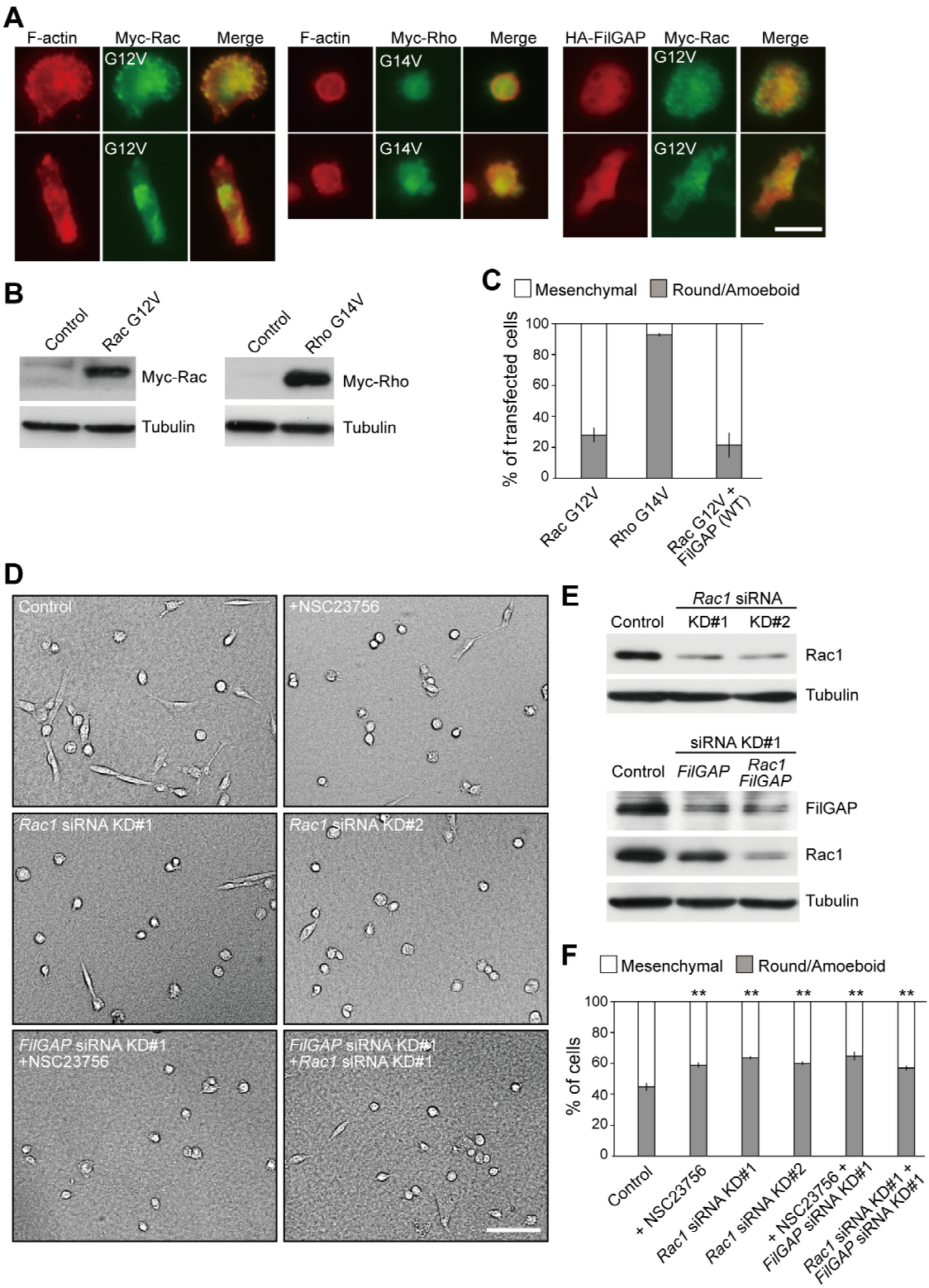

Figure S4: Activation and inactivation of Rac and Rho regulate cell morphology of MDA-MB-231 cells on collagen gels. (A) MDA-MB-231 cells were transfected with pCMV5-Myc plasmids encoding Rac (G12V) or Rho (G14V), and cultured on plastic

plates for 24 h. In addition, cells were co-transfected with pCMV5-HA-FilGAP and Myc-Rac (G12V) plasmids. The transfected cells were trypsinized and then plated on collagen gels. After 24 h, cells were fixed and stained with anti-Myc antibody for Rac and Rho (green) and anti-HA antibody for FilGAP (red) or phalloidin for F-actin (red). Merged fluorescent images are also shown. Scale bar, 50 μm. (B) Ectopic expression of Rac and Rho mutants was detected by immunoblotting using anti-Myc antibody. Tubulin was used as a loading control. (C) Transfected cells were categorized as mesenchymal or round/amoeboid. The data are the mean ± S.D. of three independent experiments ($n > 100$ cells for each experiment). (D) MDA-MB-231 cells were transfected with control, *Rac1* siRNAs, *FilGAP* siRNA KD#1, or both *Rac1* siRNA KD#1 and *FilGAP* siRNA KD#1, and cultured on plastic plates for 2 d. The control or transfected cells were trypsinized, and then plated on collagen gels and cultured for 24 h in the absence or presence of 100 μM NCS23756. (E) Immunoblot showing that Rac1 and FilGAP are depleted after 2 d of siRNA treatment on plastic plates. Rac1 and FilGAP were detected by immunoblotting using anti-Rac1 and anti-FilGAP antibodies, respectively. Tubulin was used as a loading control. (F) Cells were categorized as mesenchymal or round/amoeboid. The data are the mean ± S.D. of three independent experiments ($n > 500$ cells for each experiment). $^{**}P < 0.01$. Statistical significance was determined by Student's *t*-test.

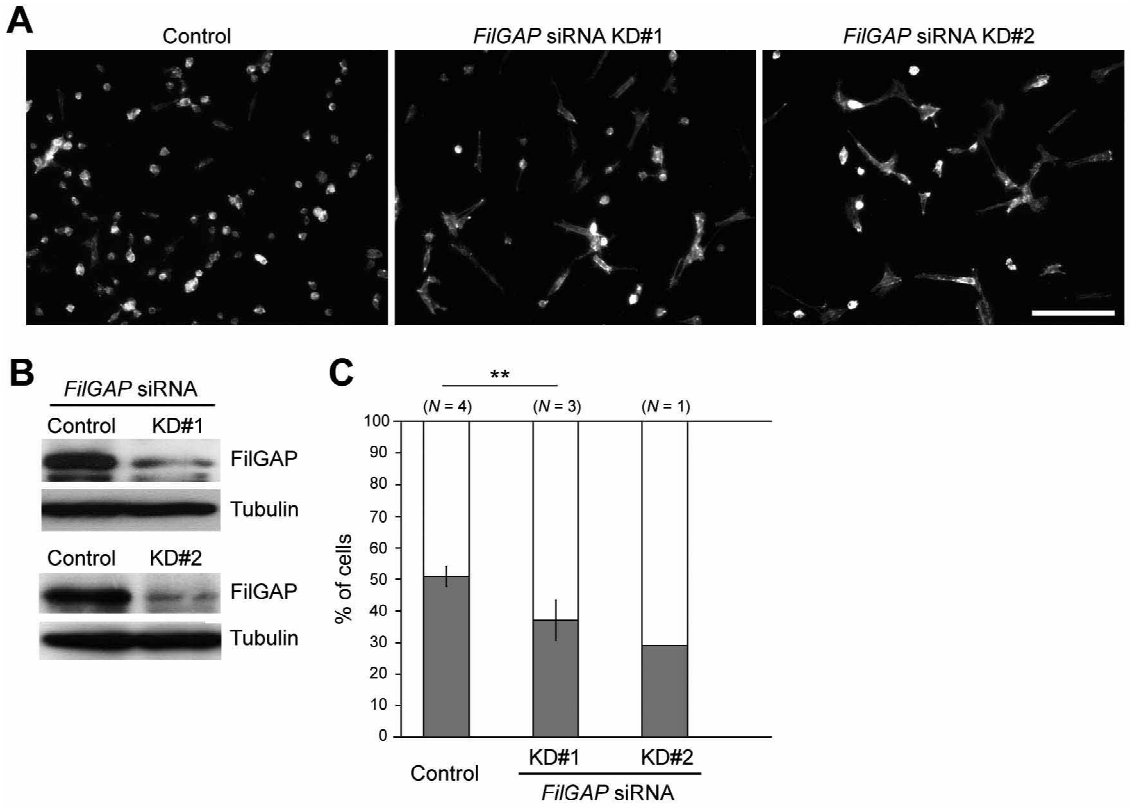

Figure S5: MDA-MB-231 cells stably expressing GFP exhibits a mesenchymal morphology on collagen gels when transfected with *FilGAP* siRNA. GFP-expressing stable cells were treated with *FilGAP* siRNA and cultured on collagen gels for 24 h. (A) Representative images of F-actin staining of cells on collagen gels. Scale bar, 100 μm. (B) Immunoblots showing that FilGAP is depleted after 2 d of siRNA treatment. (C) Cells ($n > 500$) were categorized as mesenchymal or round/amoeboid. $^{**}P < 0.01$. Statistical significance was determined by Student's *t*-test (N = number of experiments).