SUPPLEMENTAL MATERIALS AND METHODS

Antibodies, and reagents. Antibodies and their sources are as follows: β1 integrin (MAB1997, Millipore), β-tubulin (E7, Developmental Studies Hybridoma Bank, University of Iowa), ELMO2 (sc21655, Santa Cruz Biotechnology, and nb100-879, Novus Biologicals), FLAG-M2 (Sigma), GFP (sc8334, Santa Cruz Biotechnology), Na^+/K^+-ATPase (ab7671, Abcam), Rac1 (610651, BD Transduction Laboratories), V5 (R96025, Invitrogen). Epidermal growth factor (E4127), AG-1478 and all other chemicals were from Sigma. Transforming growth factor-β1 (100-21) and KGF (100-19) were purchased from Preprotech. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratories. AlexaFluor-conjugated goat anti-mouse IgG and AlexaFluor-conjugated phalloidin were purchased from Molecular Probes/Invitrogen. Protein A/G UltraLink resin was from Pierce Laboratories.

Plasmids. Vectors encoding mCherry-tagged ILK with a C-terminal V5 epitope, FLAG- and GFP-tagged ELMO2 and GFP-tagged RhoG have been described (Ho *et al.*, 2009). Plasmids encoding FLAG- and GFP-tagged ELMO2 mutants were generated by site directed mutagenesis, based on the presence of conserved, important residues in putative Armadillo repeats (Andrade *et al.*, 2001; deBakker *et al.*, 2004). The sequences of the primers used for each ELMO2 mutant are summarized in Supplemental Table 1. The shRNA backbone vector, pLKO.1, was obtained from Addgene (10878). A collection of vectors encoding ELMO2 shRNAs cloned in pLKO.1 was obtained from Openbiosystems (RHS4533). The sequences for the ELMO2 and control, scrambled shRNAs are summarized in Supplemental Table 2. The scrambled ELMO2 shRNA sequence was derived from shRNA1, using siRNA Wizard 3.1 (Invitrogen), and was cloned into pLKO.1. The shRNA vectors used also encode β-galactosidase under the control of the PGK

promoter. This modification was introduced by substituting puromycin resistance cDNA sequences in pLKO.1 with a cDNA encoding β-galactosidase and 3' bovine growth hormone poly-adenylation sequences.

Immunoblot analysis, cell fractionation and immunoprecipitation. Protein lysates were prepared and analyzed as described (Ho *et al.*, 2009). For immunoprecipitation experiments with whole cell lysates, keratinocytes were harvested and lysed in Co-IP buffer (1% Triton X–100, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) for 10 min at 4° C. Cell debris were removed by centrifugation and 1 mg of protein was pre-cleared with 10 μl protein A/G UltraLink resin for 2 h at 4°C. Pre-cleared lysates were incubated with antibodies indicated in individual experiments (16 h, 4°C). Immune complexes were isolated by addition of protein A/G resin (30 min, 4°C), followed by denaturation by boiling in Laemmli buffer. The proteins in the complexes were resolved by SDS-PAGE; they were transferred to nitrocellulose membranes and were analyzed by immunoblot.

Cell lysates were fractionated using a Subcellular Fractionation Kit (Cytoskeleton, Denver, Colorado) following the manufacturer's protocol. Cytosolic and crude membrane fractions were resolved by SDS-PAGE, and analyzed by immunoblot. Immunoprecipitation experiments using fractionated extracts were conducted as described above, with 1 mg protein per sample. All results shown are representative of at least four experiments.

X-gal staining. Keratinocytes were transfected with vectors encoding β-galactosidase and shRNAs. Forty-eight hours later, the cells were fixed in a solution containing 0.05% glutaraldehyde, 0.1 M phosphate buffer pH 7.3, 0.02 M EGTA, and 2 mM $MgCl_2$ for 20 min. After washing in Rinse buffer (0.1M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 0.02% sodium

deoxycholate, and 0.05% (v/v) NP-40), the cells were incubated at room temperature in Stain solution (Rinse solution supplemented with 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal) until a light blue colour developed. Staining reactions were terminated with two 10-min washes with Rinse solution.

Cell migration assays. Primary keratinocytes cultured for two days in low-calcium medium (0.05 mM $CaCl_2$) were transfected with plasmids encoding mCherry/V5-tagged ILK and/or GFP-tagged ELMO2, as indicated in the experiment of Fig. 1D. The cells were cultured in growth medium for 24 h, trypsinized, washed with PBS, and resuspended at 1.0 x 10^5 cells/ml in serum- and growth-factor-free EMEM. A 500-µl aliquot of this cell suspension was added to tissue culture inserts (Transwell ™, 8-µm pore size; 353097, BD Falcon). The test medium in the lower chamber consisted of EMEM supplemented with either BSA (2.5%) or EGF (10 ng/ml). The cells were cultured at 37°C for 6 h. Cells in the upper chamber were removed, and those keratinocytes that migrated through the membrane were fixed with 4% freshly diluted paraformaldehyde and stained with Hoescht 33528 (10 µg/ml, Sigma). The number of GFP- and mCherry-double positive cells that migrated through was determined by microscopic examination of the lower surface of the insert, and normalized to the total number of GFP- and mCherry-double positive cells that had been originally added to the upper chamber. In experiments with ILK-deficient cells, *Ilk*$^{f/f}$ keratinocytes were cultured for 2 days, and then incubated for 4-6 h in serum- and additive-free EMEM with Ad-βgal or Ad-Cre, added at a multiplicity of infection of 50. The cells were then cultured in normal growth medium for 72 h, at which time they were trypsinized and used in migration assays as described above. Each experiment was conducted with duplicate samples.

References for Supplemental Materials and Methods

Andrade, M.A., Petosa, C., O'Donoghue, S.I., Muller, C.W., and Bork, P. (2001). Comparison of ARM and HEAT protein repeats. J Mol Biol *309*, 1-18.

deBakker, C.D., Haney, L.B., Kinchen, J.M., Grimsley, C., Lu, M., Klingele, D., Hsu, P.K., Chou, B.K., Cheng, L.C., Blangy, A., Sondek, J., Hengartner, M.O., Wu, Y.C., and Ravichandran, K.S. (2004). Phagocytosis of apoptotic cells is regulated by a UNC-73/TRIO-MIG-2/RhoG signaling module and armadillo repeats of CED-12/ELMO. Curr Biol *14*, 2208-2216.

Ho, E., Irvine, T., Vilk, G.J., Lajoie, G., Ravichandran, K.S., D'Souza, S.J., and Dagnino, L. (2009). Integrin-linked Kinase Interactions with ELMO2 Modulate Cell Polarity. Mol Biol Cell *20*, 3033-3043.

Supplemental Table 1

Sequence of Primers used to generate ELMO2 mutants*

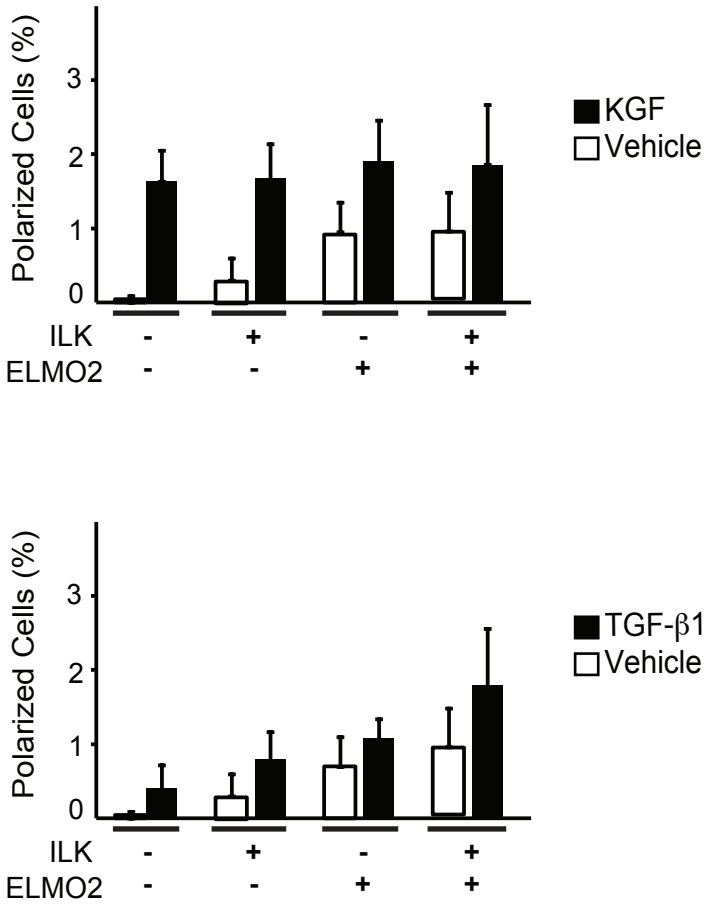
Mutant	Primer Sequence (Coding strand)
I12S	5'-CGTCTGACATTGTCAAAGTGGCC**TC**CGAGTGGCCAGGTGCTAACGCCCAG-3'
G72A	5'-CAGACCCGAAATGACATTAAGAACG**C**GACAATCTTACAACTGGCCGTCTCC-3'
L156S	5'-GCATTCACCCTGACTGCCTTCTTAGAGAGCATGGATCATGGCATTGTCTCCTG-3'
G218A	5'-CAGAAGATAGCGGAGGAGATCACCGTGG**CA**CAGCTCATCTCCCACCTGCAG GTC-3'

* Mutated residues are underlined

Supplemental Table 1

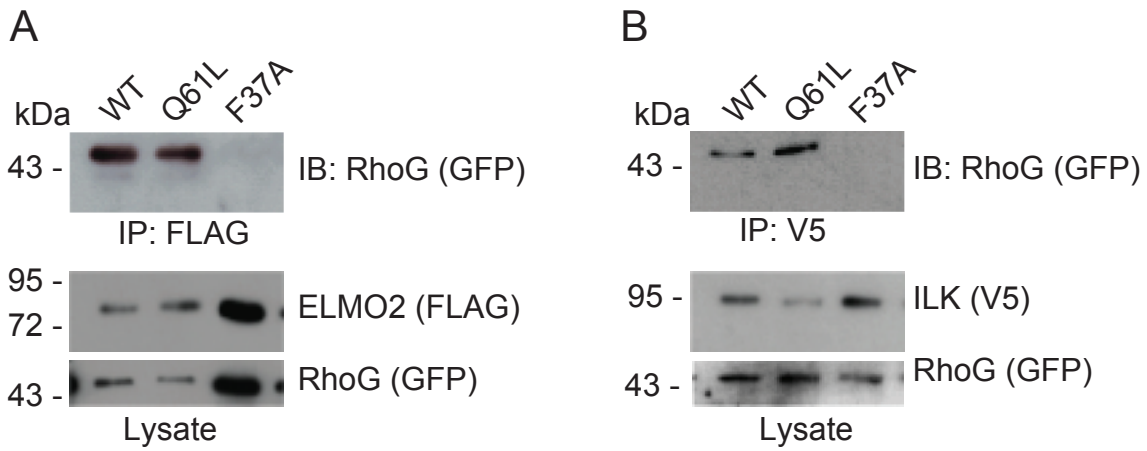
Sequence of mature ELMO2 shRNA

shRNA	Sequence	ID No.
# 1	5'-CGGTCTATAATCCTGAATCAT-3	TRCN0000029064
# 2	5'-GCTGGGATTTACCAACCACAT-3'	TRCN0000029065
# 3	5'-GCATCCATTATCAAGGAAGTT-3'	TRCN0000029066
Scrambled	5'-GACCTCTCTCCATACAACTCT-3'	



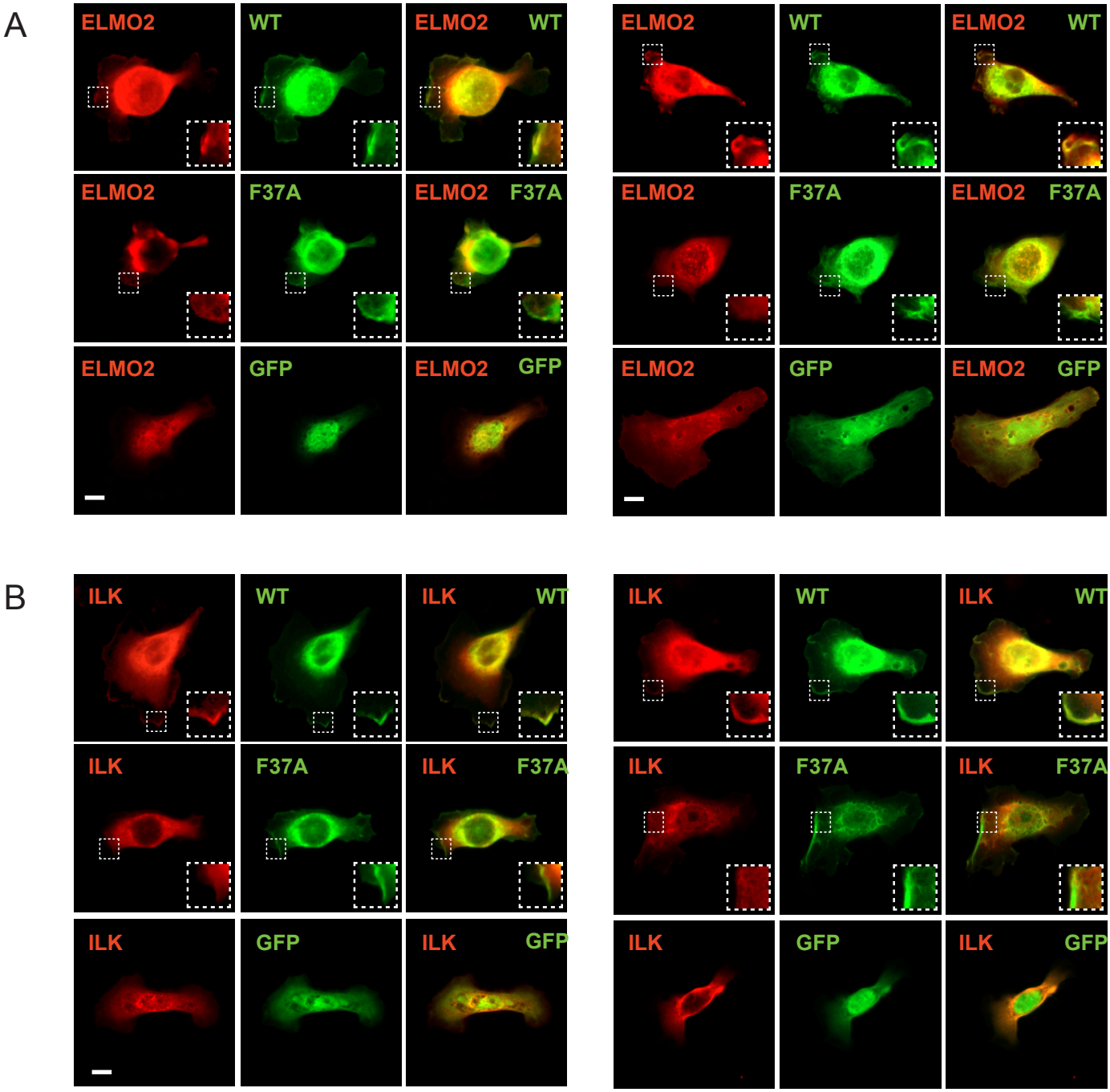
Supplemental Figure 1

Primary cultured keratinocytes were transfected with vectors encoding mCherry/V5-tagged ILK and/or GFP-tagged ELMO2. Twenty-four hours after transfection, the cells were incubated in FBS- and growth factor-free medium for 4 h, followed by stimulation with KGF (20 ng/ml) or TGF-β1 (10 ng/ml), as indicated in individual graphs, for 2 min. The cells were processed for direct fluorescence microscopy, to visualize and quantify the fraction of cells that exhibited front-rear polarity. The results are expressed as the mean + SEM (n=3). The results were analyzed by ANOVA, and no statistically significant differences were found in growth factor-treated cells compared with the corresponding vehicle-treated controls.



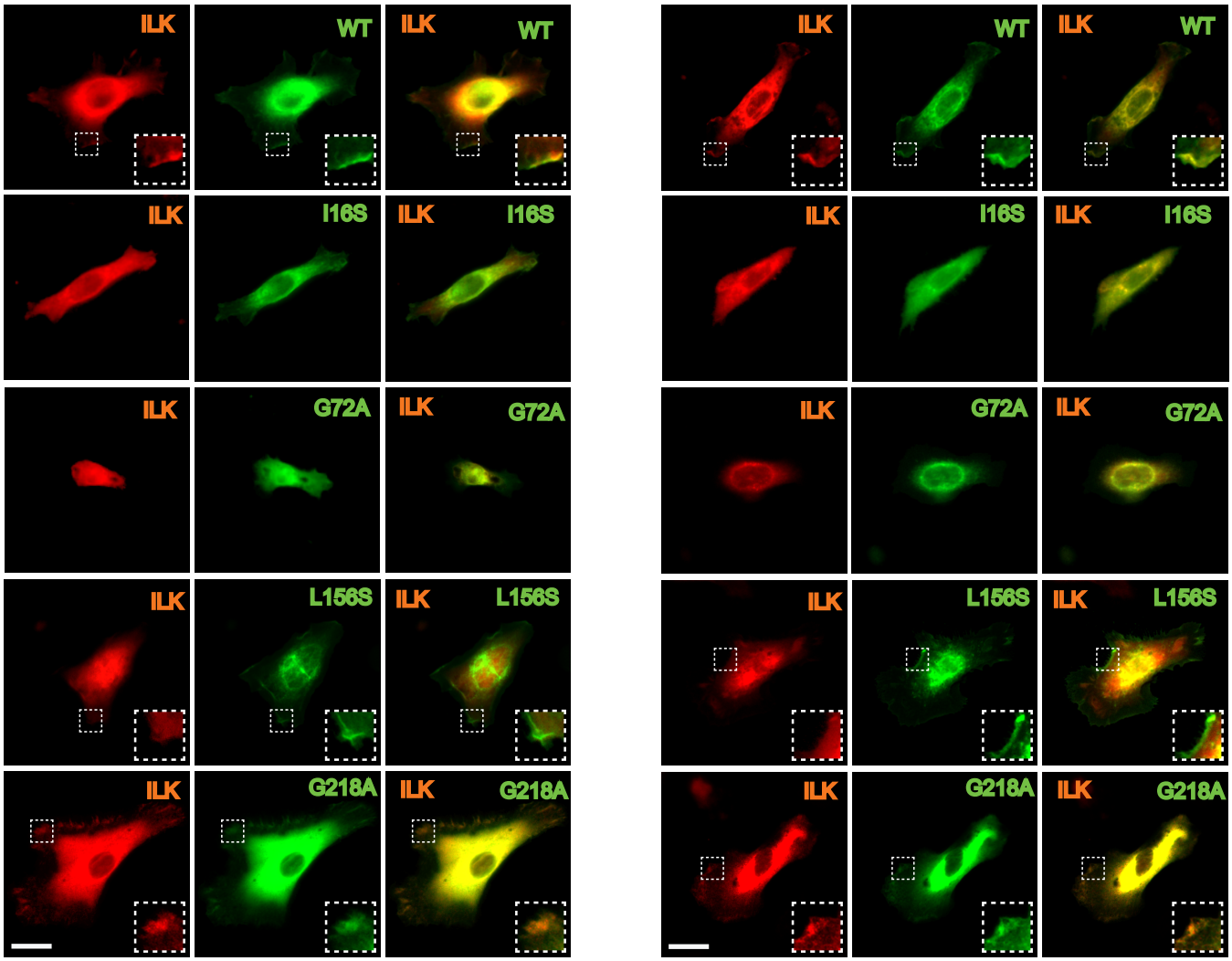
Supplemental Figure 2

IMDF cells were transiently transfected with vectors encoding GFP-tagged RhoG proteins and either FLAG-tagged ELMO2 (Panel A) or mCherry/V5-tagged ILK (panel B). The lysates were subjected to immunoprecipitation with anti-FLAG (panel A) or anti-V5 (panel B) antibodies, and the immunecomplexes were resolved by SDS-PAGE and analyzed by immunoblot with anti-GFP antibodies to detect RhoG proteins. Samples of the cell lysates were also analyzed by immunoblot with the antibodies indicated in parentheses.



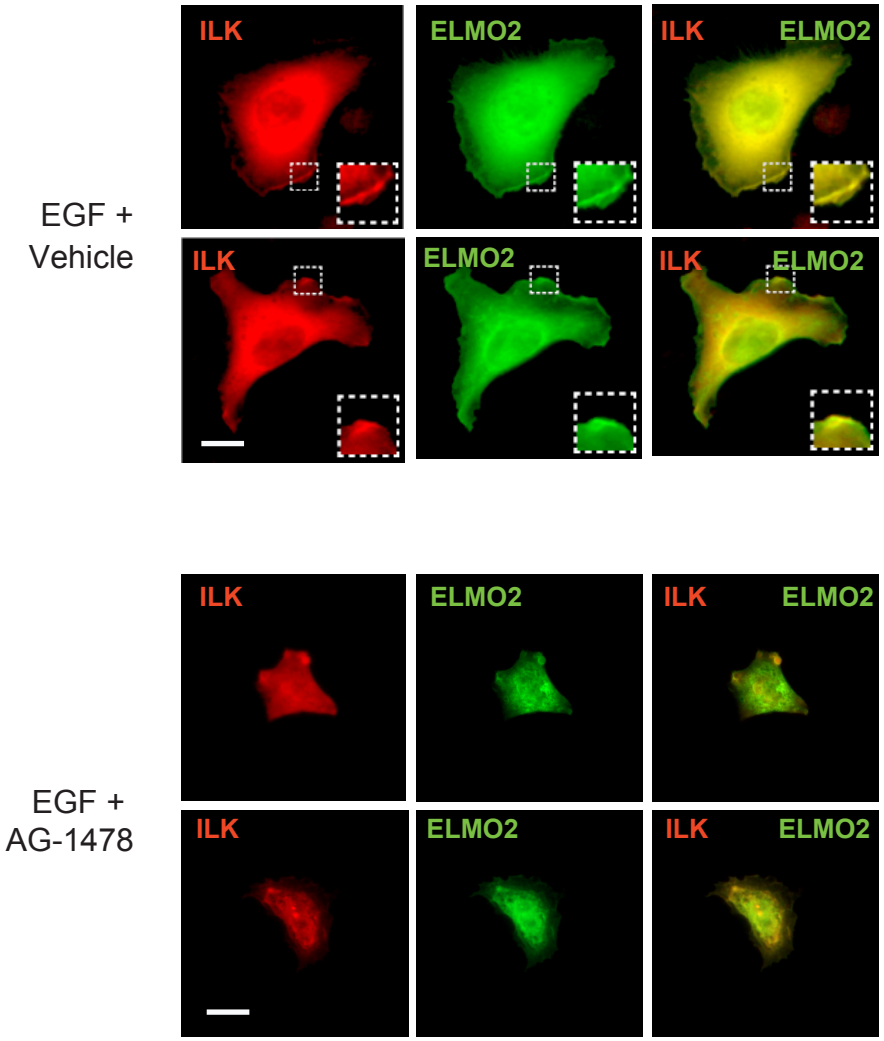
Supplemental Figure 3

Primary mouse keratinocytes were transfected with vectors encoding WT or F37A GFP-tagged RhoG proteins and either FLAG-tagged ELMO2 (panel A) or mCherry/V5-tagged ILK (panel B), as indicated. Twenty-four hours after transfection, the cells were trypsinized and replated onto a laminin 332 matrix. The cells were processed for microscopy 3 hours after replating. Exogenous ELMO2 was visualized using anti-FLAG antibodies, RhoG and ILK were visualized using, respectively, GFP and mCherry fluorescence. Boxed areas are shown at higher magnification in insets. Bar, 20 μm.



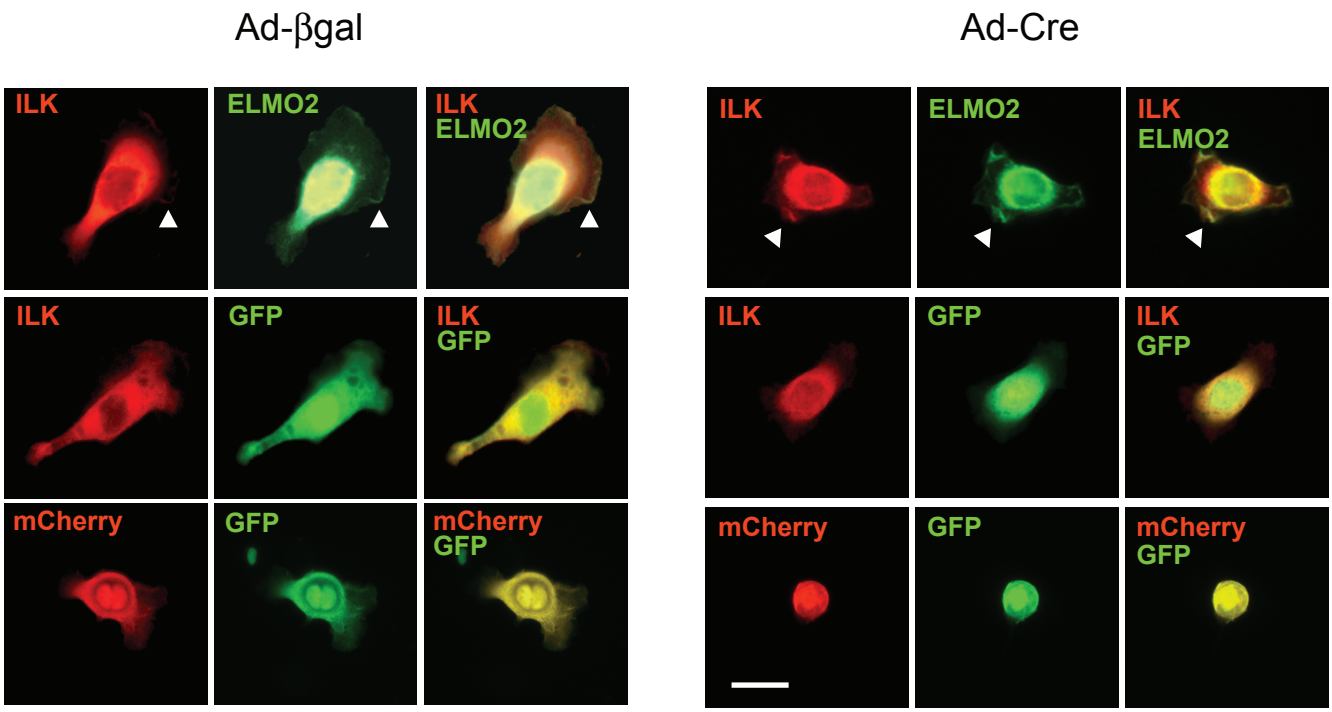
Supplemental Figure 4

Primary mouse keratinocytes were transfected with vectors encoding mCherry/V5-tagged ILK or the indicated GFP-tagged ELMO2 protein.Twenty-four hours after transfection, the cells were trypsinized and replated onto a laminin 332 matrix. The cells were processed for direct fluorescence microscopy 3 hours after replating. Boxed areas are shown at higher magnification in insets. Bar, 20 μm.



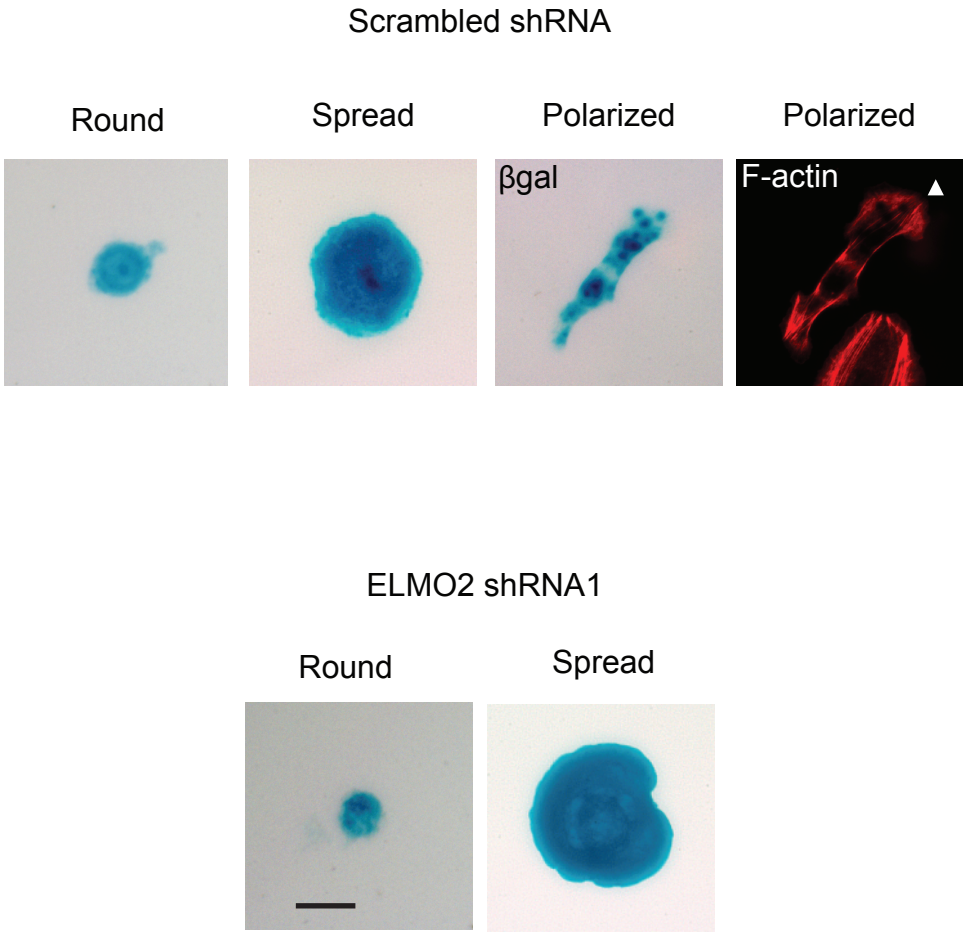
Supplemental Figure 5

Primary mouse keratinocytes were transfected with vectors encoding GFP-tagged ELMO2 and mCherry/V5-tagged ILK. Twenty-four hours after transfection, the cells were incubated in FBS- and EGF-free medium for 4 h, and in the presence of either DMSO (0.1%; Vehicle) or AG1478. The cells were then stimulated with EGF for 2 min, and processed for fluorescence microscopy. Boxed areas are examples of regions in which ELMO2 and ILK co-localize, and are shown at higher magnification in the insets. Bar, 20 μm.



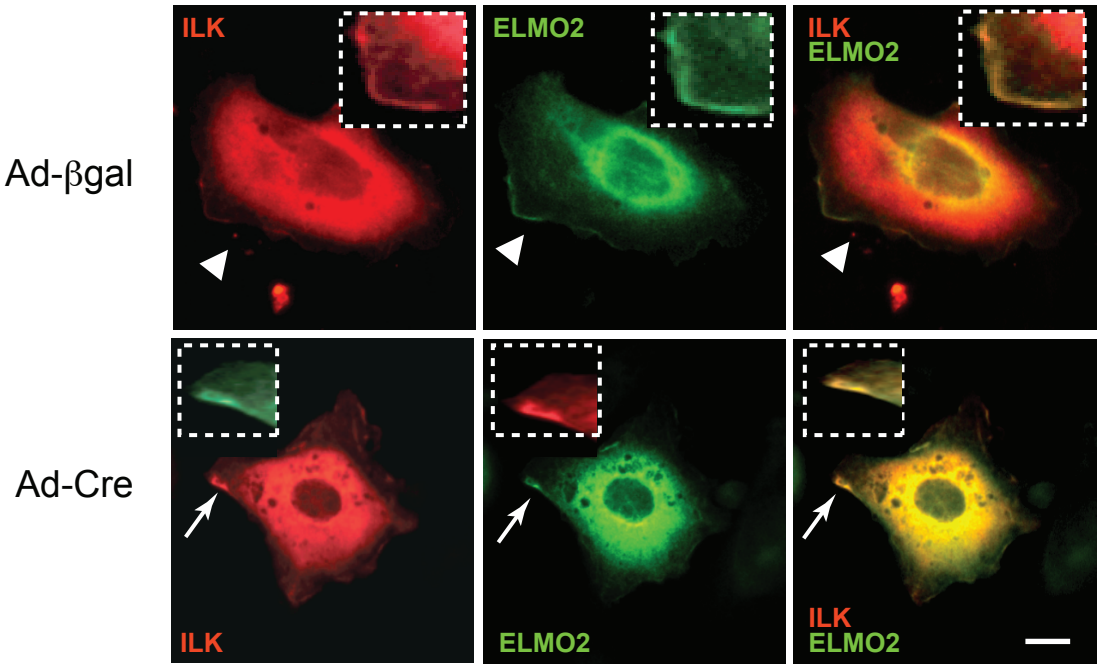
Supplemental Figure 6

Ilk$^{f/f}$ keratinocytes were infected with Ad-βgal or Ad-Cre, and 24 h later were transfected with vectors encoding mCherry, GFP, mCherry/V5-tagged ILK and/or GFP-tagged ELMO2. Twenty-four hours after transfection, the cells were incubated in FBS- and EGF-free medium for 4 h, followed by stimulation with EGF (10 ng/ml) for 2 min. The cells were processed for direct fluorescence microscopy. Polarized ILK-deficient cells were not detected in EGF-treated cultures. Arrowheads indicate edges of lamellipodia in cells exhibiting front-rear polarity. Bar, 100 μm.



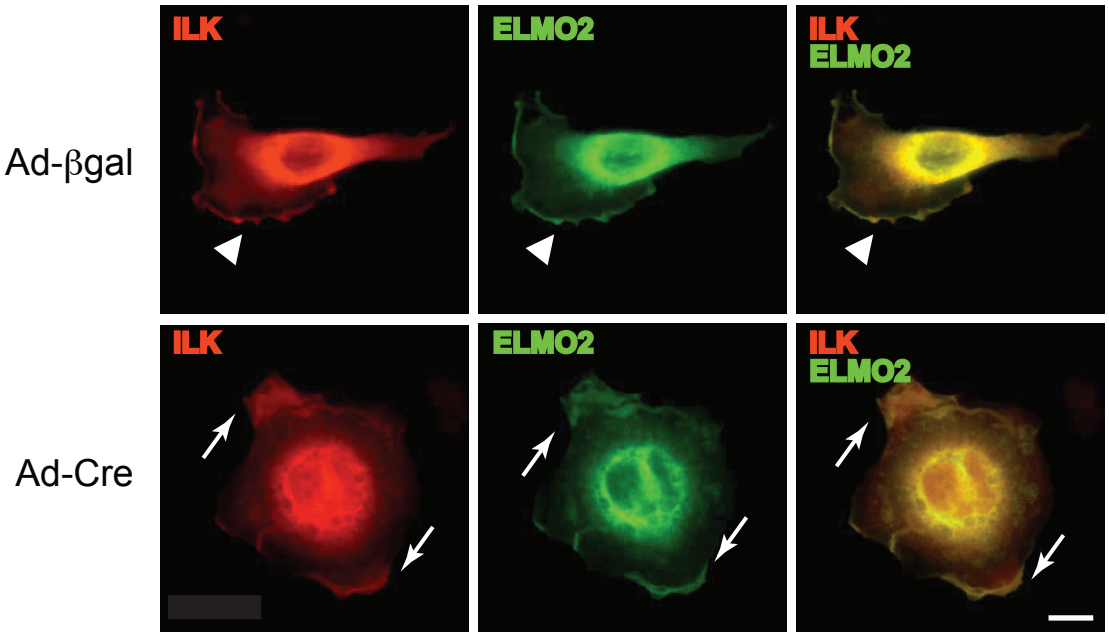
Supplemental Figure 7

Keratinocytes were transfected with vectors encoding the indicated shRNAs and 24 h later were were incubated in FBS- and EGF-free medium for 4 h, followed by stimulation with EGF (10 ng/ml) for 2 min. The cells were fixed and processed for X-gal staining and stained for F-actin with Alexa-594 conjugated phalloidin. Images represent cells transfected with the indicated shRNAs and stimulated with EGF. ELMO2 shRNA transfected cells treated with EGF did not show polarized phenotypes. The arrowhead shows F-actin staining at lamellipodia formed at the cell front in a polarized cell. F-actin-associated fluorescence in the cell body is less bright in areas with high concentration of X-gal precipitate. Bar, 50 μm.



Supplemental Figure 8

Rac1$^{f/f}$ keratinocytes were infected with Ad-βgal or Ad-Cre, and 24 h later were transfected with vectors encoding mCherry/V5-tagged ILK and GFP-tagged ELMO2. Twenty-four hours after transfection, the cells were incubated in FBS- and EGF-free medium for 4 h, followed by stimulation with EGF (10 ng/ml) for 2 min. The cells were processed for direct fluorescence microscopy. Rac1-deficient cells exhibiting front-rear polarity were not detected in EGF-free or EGF-treated cultures. Arrowheads indicate edges of lamellipodia in cells exhibiting front-rear polarity, and arrows indicate cell protrusions formed in Rac1-deficient cells in which ELMO2 and ILK fluorescence co-localized. These regions are shown at higher magnification in the insets. Bar, 20 μm.



Supplemental Figure 9

Intb1$^{f/f}$ keratinocytes were infected with Ad-βgal or Ad-Cre, and 48 h later were transfected with vectors encoding mCherry/V5-tagged ILK and GFP-tagged ELMO2.
Twenty-four hours after transfection, the cells were incubated in FBS- and EGF-free medium for 4 h, followed by stimulation with EGF (10 ng/ml) for 2 min. The cells were processed for epifluorescence microscopy. Arrowheads indicate lamellipodia in cells exhibiting front-rear polarity. Arrows indicate cell protrusions formed in integrin β1-deficient cells in which ELMO2 and ILK fluorescence co-localized. Bar, 40 μm.