Supporting Information

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SI Materials and Methods

Antibodies. Antibodies purchased from commercial suppliers are as follows: FLNA mouse monoclonal (AbNova; H00002316-M01), FLNA mouse monoclonal (USBiological; F4510), HA rat monoclonal (Roche; 3F10), Myosin IIA (Sigma; M-8064), and GFP mouse monoclonal (AbCam; ab-1218). Myc hybridomas were homemade. Chicken antibody against RefilinA was produced by Eurogentec, using a 15-aa-long peptide immunogen (QLTLEPRPRALRFGS). Guinea pig antibody against GST-fusion 1–120 N-terminal amino acid of RefilinB was produced by Proteogenix. Specific RefilinA and RefilinB antibodies were affinity purified and showed no cross-reactivity. Alexa-Fluor 546 phalloidin (Invitrogen; A22283) and secondary antibodies conjugated with Alexa488 (Molecular Probe), Cyanine3, or Cyanine5 (Jackson ImmunoResearch) were purchased from vendors as noted.

Plasmid Constructs. Plasmids encoding RefilinA N-terminal or C-terminal deletion mutants were obtained by PCR amplification of the FAM101A sequence using Phusion polymerase (Finnzymes). PCR products were cloned in the pcDNA myc His vector (Invitrogen) using XhoI and EcoRI restriction sites.

RefilinA deletion mutants (ΔBD1–ΔBD4) were obtained using QuikChange Site-Directed Mutagenesis (Stratagene) in accordance with the manufacturer's protocol using RefilinA–Myc plasmid as template.

Plasmids encoding HA-tagged FLNA deletion mutants were obtained by PCR amplification of the FLNA sequence (1) using Phusion polymerase (Finnzymes). PCR products were cloned in the pcDNA HA vector (Invitrogen) using BamH1 and Xho1 restriction sites. Plasmids encoding GFP-tagged FLNA (pEGFP–FLNA) deletion mutants were engineered using the QuikChange Site-Directed Mutagenesis kit.

For Expression of FLNA–RefilinA complex in sf9 insect cells, pFASTBAC–Flag–FLNA was constructed as previously described (1). Mouse RefilinA cDNA was amplified by PCR and ligated into pFASTBAC–HTa or pFASTBAC–HTa–EGFP using EcoRI/NotI sites.

All constructs were verified by DNA sequencing.

Yeast 2 hybrid screening and yeast two-way mating. A custom-designed human fetal cartilage/chondrocyte pretransformed Matchmaker cDNA library in pGADT7–Rec/Y187 (Clontech Laboratories) was mated to baits pGBAD-BD RefilinA (amino acids 11–216)/AH109 or pGBAD-BD RefilinA (amino acids 66–216)/AH109 on YPAD medium for 6 h. A total of 5×10^8 library and bait cells were used for each mating. Mated yeast were then plated onto SD/−Leu/−Trp/−His media for primary reporter selection and colonies were picked to SD/−Leu/−Trp/−Ade plates for secondary selection. Plasmid preparations were made from colonies that remained after these selective steps and the inserts were sequenced using standard techniques.

Directed yeast two-hybrid matings were carried out using the same yeast two-hybrid GAL4 reporter system. Bait, pGBAD-BD RefilinA (amino acids 11–216), pGBAD-BD RefilinA (amino acids 66–216), and pGBAD-BD FLNA were transfected into yeast strain AH109 and mated directly to the preys pACT2B FLNA R21, pACT2B RefilinA (amino acids 11–216) transfected into yeast strain PJ69-4α. Mating was carried out on YPAD medium for 6 h before replica plating to selective medium SD/−Leu/−Trp/−Ade. pGBAD-BD and pJ694α vectors and yeast strain PJ694α were gifted by David Markie (Dunedin School of Medicine, Dunedin, New Zealand).

ShRNA vector constructions. RefilinB-targeting shRNA sequences were designed from DESIR software as follows:

5′-CCGGCGATGGGCTAGCCGATGAATTCAAGAGATTCATCGGCTAGCCCATCG TTTTTG-3′ and 5′ AATTCAAAAACGATGGGCTAGCCGATGAATCTCTTGAATTCATCGGCTAGCCCATCG-3′ for shRNA #3; 5′-CCGGCCACCACTCTGGATTACAATTCAAGAGATTGTAATCCAGAGTGGTGGTTTG-3′ and 5′ AATTCAAAAACCACCACTCTGGATTACAATCTCTTGAATTGTAATCCAGAGTGGTGG-3′ for shRNA #5. ShRNA sequences were synthesized, pair annealed, and subcloned into pLKO.1 vector (Addgene) containing U6 human shRNA promoter, HIV packaging Psi sequence, and LTR repeats. This vector also contains the puromycin resistance genes for selection of stable cell lines.

Adenovirus infection. Recombinant adenovirus particles expressing GFP or GFP–RefilinA were obtained from ViraQuest. A total of 4 10^4 cells were plated on polylysine-coated glass coverslips the day prior to infection. Cells were incubated with 15 multiplicity of infection (MOI) viral particles diluted in minimal volume of growth medium for 4 h, then completed with fresh medium, and placed in an incubator overnight. The next day, cells were washed and incubated in fresh medium.

Production and transduction of lentiviral vectors. To accomplish transgene incorporation into lentiviral capsids, pLKO.1 vectors were cotransfected with pSPAX.2 packaging vector (encoding HIV Gag, Pol, and Rev proteins) in combination with pMD2.G (encoding VSV-G) into 293T cells using Lentiphos HT (Clontech). The medium was then replaced with complete DMEM containing 10% FCS and the virus-containing supernatants were harvested 48 h posttransfection. All virus stocks were filtrated at 0.45 μm, aliquoted, and frozen at −80 °C. For infection, NIH 3T3 and NMuMG cells were plated into 6-well plates (2×10^5 in 2 mL of serum-supplemented DMEM). The next day, adherent cells were incubated with lentiviral particles (1–5 MOI) diluted in 2 mL of serum-supplemented medium with 8 μg/mL polybrene (Sigma). Transduction was maintained for 16 h, the cells were then washed, and the medium replaced. For stable transduction, puromycin selection (5 or 0.5 μg/mL for NIH 3T3 or NmuMG cells, respectively; Sigma) was performed starting 36 h post-infection and maintained during the time course of our experiments. Silencing efficiency was evaluated usingWestern blotting and immunofluorescence.

Immunofluorescent Staining. Cells were grown at a 4×10^4 density on poly-L-lysine–coated coverslips, and then fixed with either 4% paraformaldehyde in PBS followed by permeabilization with 0.2% Triton X-100 or fixed using methanol. Cells were incubated with blocking buffer (5% newborn goat serum-TBS) and incubated with primary antibodies overnight at 4 °C. Cells were washed in TBS and stained with the appropriate secondary antibodies.

Images were obtained with a Zeiss (Axiovert 200M) microscope or with a Leica (TCS SP2) confocal microscope. Hoescht, Alexa488, Cyanine3 (Cy3), and Cyanine5 (Cy5) fluorescence were excited and collected sequentially (400 Hz line by line).

Coimmunoprecipitation. Cells grown in 10-cm dishes were transfected with 6 μg of the appropriate plasmid using Lipofectamine 2000 Reagent (Invitrogen). After 18 h, cells were lysed in 500 μL lysis buffer (40 mM Tris pH 7.5, 150 mM NaCl, 0.3% Triton X-100, 4 mM EDTA, 4 mM EGTA) supplemented with anti-proteases and antiphosphatases. Protein G sepharose beads

(Sigma) coupled to mouse anti-myc antibody were incubated with the lysate for 2 h at 4 °C. Precipitates were washed four times in lysis buffer, solubilized in SDS sample buffer, fractionated by SDS/PAGE, and analyzed by Western blotting.

Purification of FLNa–RefilinA Complex Expressed in sf9 Insect Cells. The fusion proteins were expressed in sf9 insect cells in accordance with manufactures' instructions and the insect cells (5×10^8 cells) were harvested 72 h postinfection. After washing with PBS, the cells were frozen with liquid N2 and stored at −80 °C. Frozen cells were lysed in 40 mL of 20 mM sodium phosphate, pH 8.0, 100 mM NaCl, 20 mM imidazole, 1% Triton X-100, 1 mM β-mercaptoethanol (ME), 2 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, at 4 °C. The extracts were centrifuged at 20,000 × *g* for 20 min at 4 °C and loaded onto a Ni-NTA column (1 mL; Qiagen). The column was washed with washing solution I (20 mM sodium phosphate, pH 8.0, 20 mM imidazole, 1 mM β-ME, 200 mM NaCl, 0.1% Triton X-100) followed by washing solution II (20 mM sodium phosphate, pH 8.0, 20 mM imidazole, 100 mM NaCl, 1 mM β-ME), and bound recombinant proteins were eluted with 5 mL of 20 mM sodium phosphate, pH 8.0, 200 mM imidazole, 1 mM β-ME. Purified proteins were mixed with 20 mL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM EGTA, 5 mM EDTA, 1 mM β-ME, 2 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin and incubated with 1.0-mL anti-FLAG M2 agarose in a column (Sigma-Aldrich) for 1 h at 4 °C. The beads were washed with 20 mL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EGTA, and 0.1% Triton X-100. The bound fusion proteins were eluted with the washing buffer containing 100 μg/mL FLAG peptide (Sigma-Aldrich). Purified proteins were concentrated using an Amicon Ultra-15 (Millipore) with a molecular-weight cutoff of 50,000 and gel filtered on Superose 6 column (GE Healthcare). His–DsRed and His–EGFP were purified using Ni-NTA column followed by Superdex 200 gel filtration column (GE Healthcare).

Visual Assay for F-Actin Bundling and Recruitment of FLNa–RefilinA Complex. A total of 12 μM G-actin was mixed with 0.12 μM Flag–FLNA or Flag–FLNA/His–EGFP–RefilinA complex and polymerization of actin was initiated by adding 10× actin polymerization buffer (final: 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM ATP) in the presence of 2 units of Alexa-Fluor 568 phalloidin. The samples were incubated at 37 °C for 1 h. For transmission electron microscopy, samples were diluted to 2 μM F-actin in actin polymerization buffer and a drop was briefly placed on the surface of a glow-discharged carbon and formvar-coated copper grid. The grid was washed in polymerization buffer, negatively stained with 2% uranyl acetate, air dried, and viewed in a JEOL-1200 EX electron microscope at an accelerating voltage of 80 kV.

Electron Microscopy. U373A cells, attached to 5-mm round glass coverslips, were infected with RefilinA–GFP viral particles for 48 h. The cells were permeabilized with 0.75% Triton X-100 in PHEM buffer (2) containing 1 μM phallacidin and 0.05% glutaraldehyde for 2 min at 37 °C, washed with PHEM buffer, and then fixed with 1% glutaraldehyde in PHEM buffer for 10 min. Coverslips were washed extensively in distilled water, rapidly frozen on a helium-cooled copper block, freeze dried at −80 °C, and rotary coated with 1.5 nm of tantalum/tungsten at a 25° angle and 5 nm of carbon at 90° without rotation. The metal replicas were floated off the coverslip using 25% hydrofluoric acid, washed in water, and collected on carbon-coated formvar 200-mesh copper grids. Grids were photographed in a JEOL 1200-EX electron microscope at 80 kV. Digital "anaglyph" 3D images from ±12° tilted stereo paired micrographs were prepared as described (3).

1. Nakamura F, Osborn TM, Hartemink CA, Hartwig JH, Stossel TP (2007) Structural basis of filamin A functions. *J Cell Biol* 179:1011–1025.
2. Schliwa M, van Blerkom J, Porter K (1981) Stabilization of the cytoplasmic ground substance in detergent-opened cells and a structural and biochemical analysis of its composition. *Proc Natl Acad Sci USA* 78:4329–4333.
3. Heuser JE (2000) Membrane traffic in anaglyph stereo. *Traffic* 1:35–37.

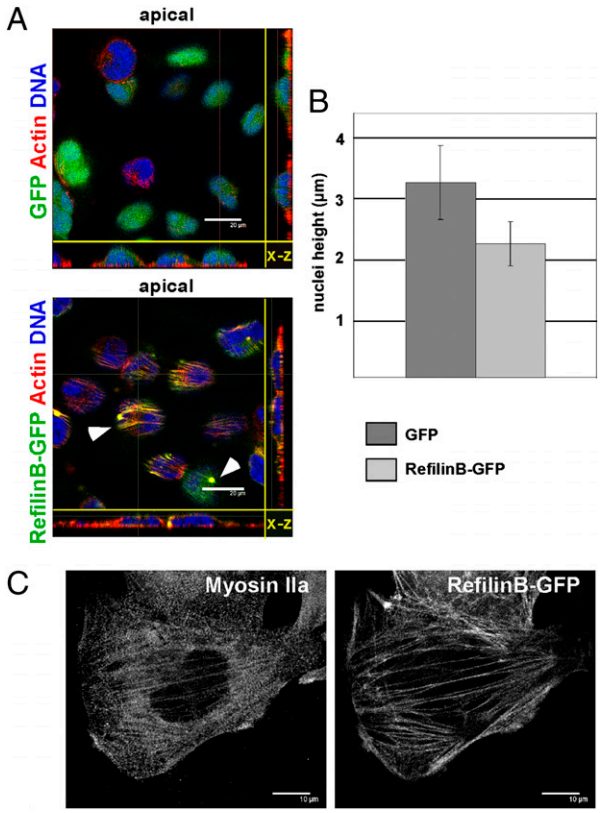

Fig. S1. RefilinB promotes perinuclear actin bundles in U373A cells. (*A* and *B*) U373A cells were infected with recombinant adenovirus expressing GFP–control or GFP–RefilinB. (*A*) Cells were fixed with PFA, stained with Alexa 546 phalloidin (red) and Hoechst (blue) for actin and the nucleus, respectively. Merged confocal microscopy sections at the apical surface and profile view (*xz*) of full reconstitution are shown. Arrowheads point to actin superstructures. (Scale bar, 20 μm.) See also corresponding Movies S1 and S2. (*B*) Nuclei height of 250 cells expressing GFP or RefilinB–GFP was measured using confocal microscopy. Mean height is represented in *B*. (*C*) U373A infected with recombinant adenovirus expressing RefilinB–GFP were fixed and immunostained with rabbit anti-Myosin IIa as indicated. (Scale bar, 10 μm.)

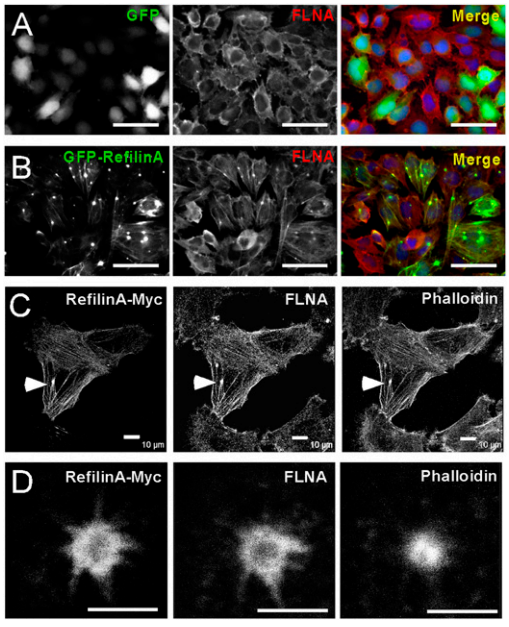

Fig. S2. RefilinA promotes the recruitment of FLNA on actin bundles and the formation of actin superstructures. (*A* and *B*) U373A were infected with recombinant adenovirus expressing GFP–control (*A*) or GFP–RefilinA (*B*). Cells were fixed and immunostained with mouse anti-FLNA (red). (*C* and *D*) U373A cells, transfected with RefilinA–Myc plasmid were fixed with PFA and coimmunostained with rabbit anti-Myc, mouse anti-FLNA antibodies, and actin was stained with Alexa 486 phalloidin. In *C*, arrowhead points to an actin superstructure. *D* shows that RefilinA–Myc and FLNA are concentrated at the periphery of the actin superstructure. [Scale bar, 10 μm (*C*) and 2 μm (*D*).]

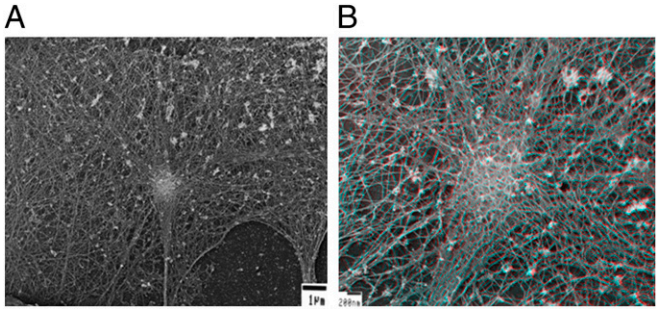

Fig. S3. Representative electron micrographs showing the structure of cytoskeletal F-actin foci generated in U373A cells by the expression of RefilinA. (*A*) Low magnification view of the cortical cytoskeleton. (Scale bar, 1 μm.) (*B*) Higher magnification 3D anaglyph of stereo paired images taken at +12° tilt of the F-actin ultrastructure. (Scale bar, 200 nm.)

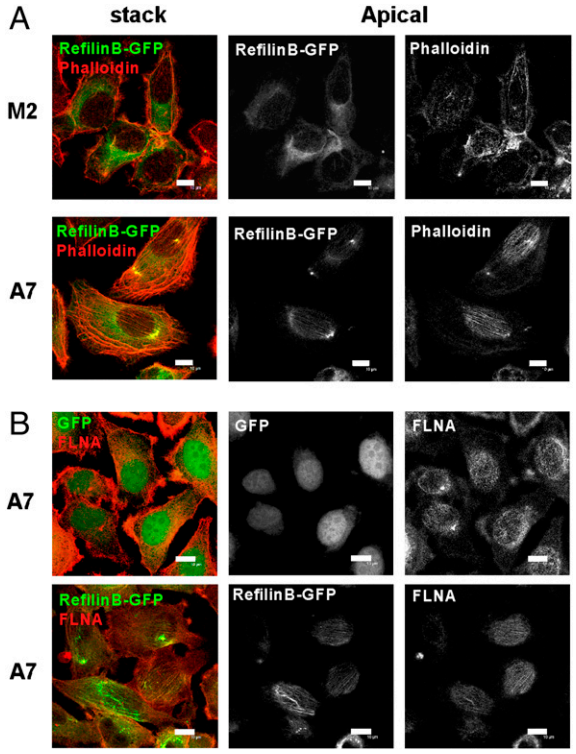

Fig. S4. Perinuclear actin stress fibers strictly depend on interaction of Refilin with FLNA. (*A* and *B*) M2 cells or A7 cells, as indicated in the *Left* margin, infected with recombinant adenovirus expressing RefilinB–GFP (*A*). (*B*, *Lower*) or control GFP (*B*, *Upper*) were fixed and stained with Alexa 486 phalloidin (*A*) or immunostained with anti FLNA (*B*). Full merged confocal reconstitution (*Left*) and individual black and white staining at the apical surface are shown. (Scale bar, 10 μm.)

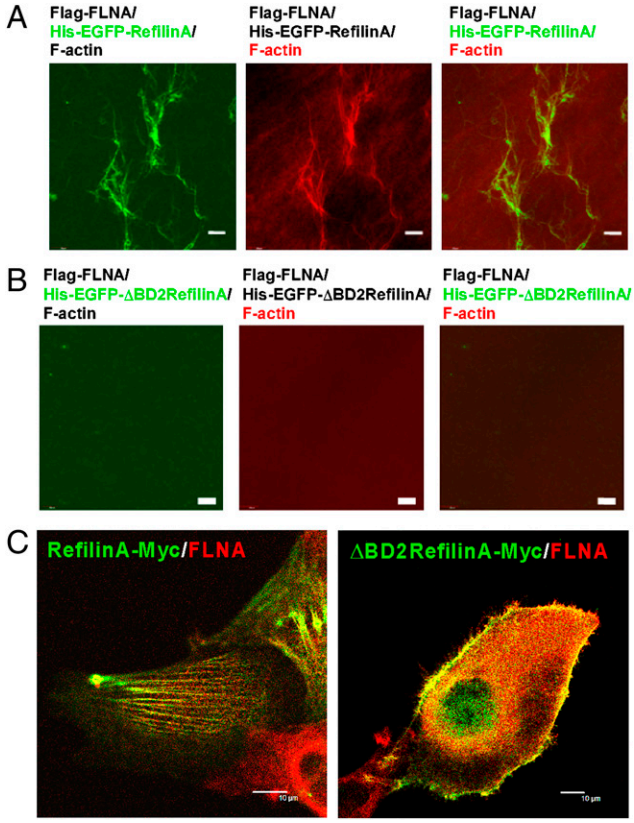

Fig. S5. Deletion of BD_2 of RefilinA abrogates its bundling activity in vitro and in vivo. (*A* and *B*) Confocal fluorescent microscopy of 12 μM G-actin mixed with 0.12 μM Flag–FLNa/His–EGFP–RefilinA complex (*A*) or 0.12 μM Flag–FLNa/His–EGFP–ΔBD_2 RefilinA complex (*B*) stained with Alexa-Fluor 568 phalloidin. (Scale bar, 200 μm.) (*C*) U373A cells, transfected with Myc-tagged RefilinA (*Left*) and ΔBD_2 RefilinA mutant (*Right*) were fixed and immunostained with mouse anti-FLNA antibody (red) and rabbit anti-Myc (green). (Scale bar, 10 μm.)

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GPIbα		-	**F**	**R**	**S**	**S**	**L**	**F**	**L**	**W**	**V**	-	571---610
RefilinBD2	1---137	-	**Y**	**R**	**S**	**Q**	**L**	**T**	**L**	**E**	**P**	-	145---204
Integrinβ1A		-	**Y**	**K**	**S**	**A**	**V**	**T**	**T**	**V**	**V**	-	771---778
Integrinβ7		-	**Y**	**K**	**S**	**A**	**I**	**T**	**T**	**T**	**I**	-	740---747
Integrinβ2		-	**F**	**K**	**S**	**A**	**T**	**T**	**T**	**V**	**M**	-	755---762
Integrinβ3		-	**Y**	**K**	**E**	**A**	**T**	**S**	**T**	**F**	**T**	-	767---779
Migfilin		-	**V**	**A**	**S**	**S**	**V**	**F**	**I**	**T**	**L**	-	17---373
RefilinBD4	1---163	-	**A**	**R**	**S**	**S**	**F**	**R**	**T**	**T**	**L**	**H**	172---204
FilGAP		-	**F**	**S**	**T**	**F**	**G**	**E**	**L**	**T**	**V**	-	734---748
CFTR		-	**V**	**V**	**S**	**K**	**L**	**F**	**F**	**S**	**W**	-	19---1480

Fig. S6. Amino acid sequence alignment of the FLNA-binding sites of human glycoprotein Ibα (GPIbα), β integrins, FilGAP, migfilin, and CFTR and comparison with putative RefilinA binding sites BD_1 and BD_4. Sequence alignment of RefilinA BD_2 and BD_4 with proteins that interact with filamin on domain 21 was done using Clustal W2 multisequence alignment program distributed by EMBL. Amino acids indicated with asterisks are mainly involved in binding interaction.

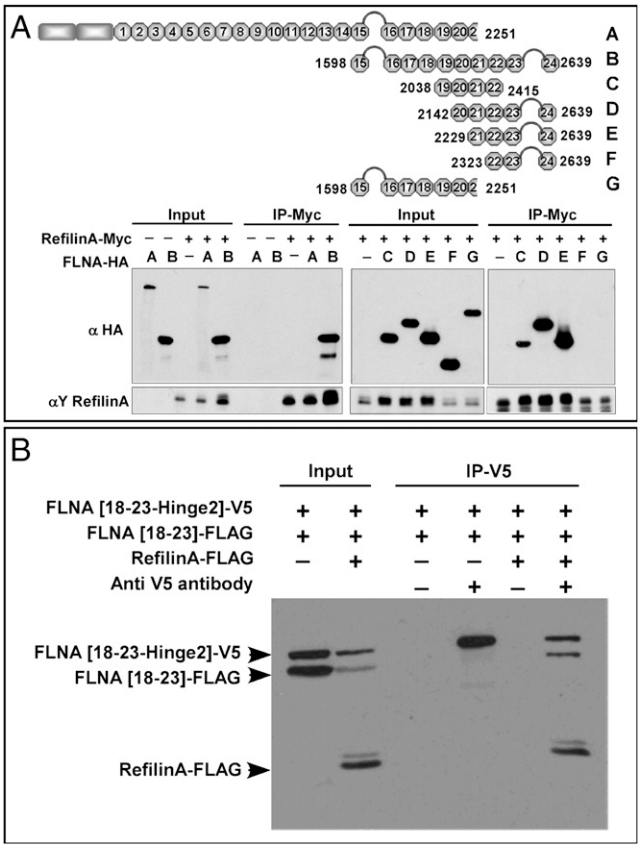


Fig. S7. The mechanism of Refilin-FLNA interaction. (*A*) Coimmunoprecipitation confirms that RefilinA interacts with repeat 21 of FLNA. Upper panel, schematic representation of HA-tagged FLNA fragments used. Lower panel, interaction between RefilinA and HA-tagged FLNA fragments were determined by coimmunoprecipitation in FLNA-deficient M2 cells cotransfected with RefilinA–Myc and different HA-tagged FLNA fragments. Total cell extracts (input) and immunoprecipitates (IP-Myc) were analyzed by Western blot using rat anti-HA (αHA) and chicken anti-RefilinA (αY RefilinA) antibodies. (*B*) Refilin is able to associate as a ternary complex with FLNA mutant proteins containing domains 18–23 and hinge 2 with a V5 tag (FLNA[18-23-Hinge2]-V5) or domains 18–23, with no hinge 2 and with a FLAG tag (FLNA[18-23]-FLAG). Hek293 cells were cotransfected with either FLNA[18-23-Hinge2]-V5 and FLNA[18-23]-FLAG or FLNA [18-23-Hinge2]-V5, FLNA[18-23]-FLAG and RefilinA (residues 11–216)-FLAG. V5 tagged proteins were immunoprecipitated with mouse anti V5 antibody. Total cell extracts (Input) and immunoprecipitates (IP-V5) were analyzed by Western blot using mouse anti FLAG and V5.

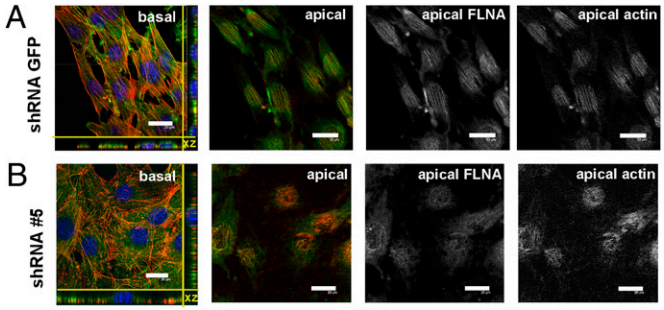


Fig. S8. RefilinB promotes formation of perinuclear actin cap in NIH 3T3 cells. (*A* and *B*) Stably transfected NIH 3T3 cells with recombinant lentivirus expressing GFP-control shRNA (*A*) or RefilinB-shRNA #5 (*B*) were fixed with PFA and double labeled with FLNA antibody (green) and Alexa 546 Phalloidin for actin (red). Nuclei were stained with Hoechst (blue) In the left panels are shown merged sections at basal level and profile view (xz). In right panels are shown merged and individual FLNA and F-actin staining at the apical surface. (Scale bar, 20 μm.)

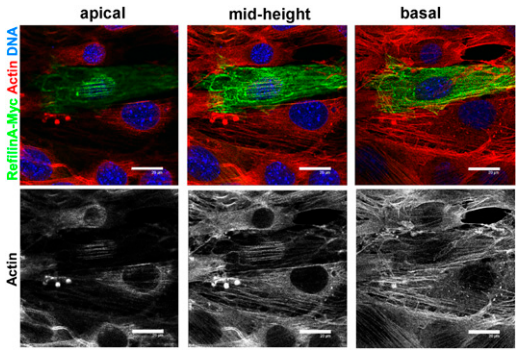
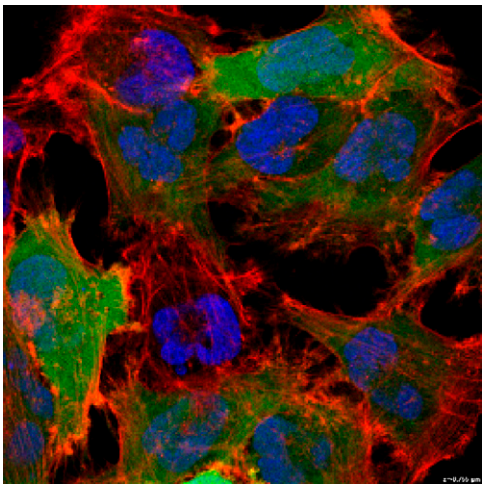
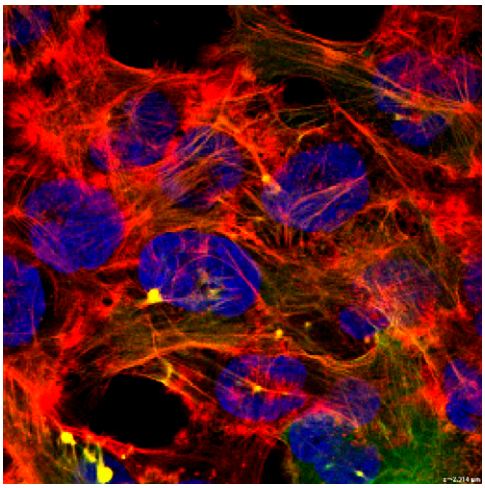


Fig. S9. RefilinA rescues actin cap formation in stably transfected NIH 3T3 cells with recombinant lentivirus expressing RefilinB-shRNA #5. Stably transfected 3T3 cells with recombinant lentivirus expressing RefilinB-shRNA #5 were transfected with recombinant RefilinA–Myc plasmid. Cells were fixed and labeled with rabbit anti-Myc antibody (green), Alexa 486 Phalloidin (red) and Hoechst (blue). Merged confocal microscopy images and actin staining at the apical, mid-height, and basal surface are shown. (Scale bar, 20 μm.) RefilinA–Myc promotes formation of an actin cap that correlates with decrease cell height.



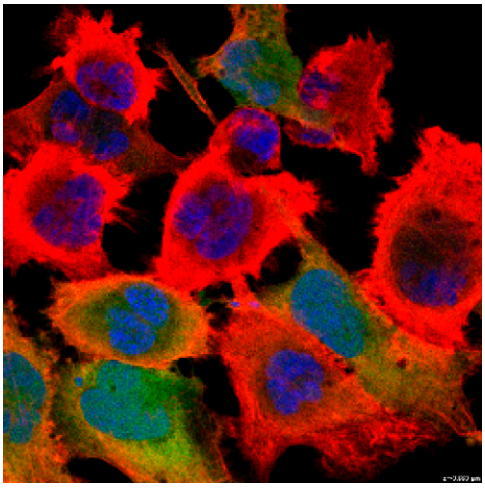
Movie S1. (related to Fig. S1*A*) Confocal (parallel) cross-sections of the actin filament (red) and GFP (green) in U373A cells shown from top to bottom.

Movie S1



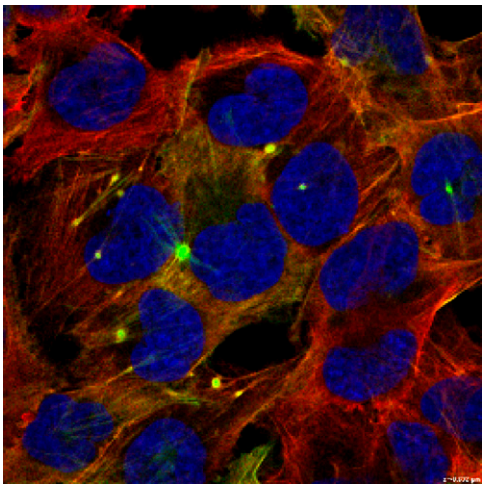
Movie S2. (related to Fig. S1*B*) Confocal (parallel) cross-sections of the actin filament (red) and RefilinB-GFP (green) organization in U373A cells shown from top to bottom.

Movie S2



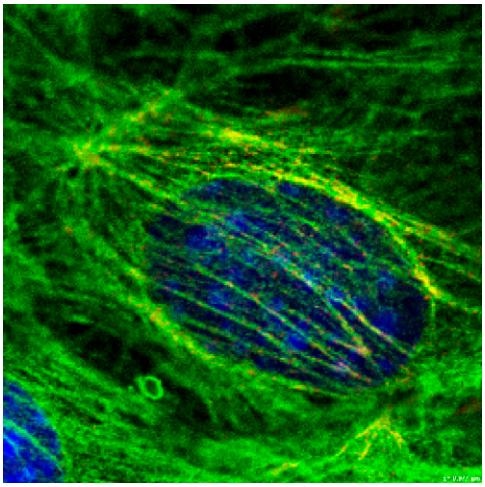
Movie S3. (related to Fig. 1*D*) Confocal cross-sections of the FLNA immunostaining (red) and GFP fluorecence (green) in U373A cells shown from the top to the bottom.

Movie S3



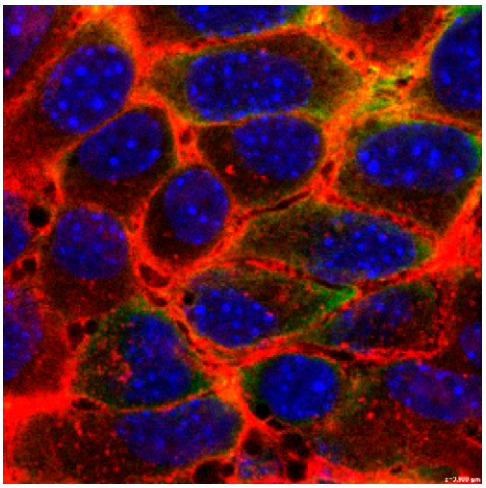
Movie S4. (related to Fig. 1*E*) Confocal cross-sections of the FLNA (red) and RefilinB–GFP (green) staining in U373A cells shown from top to bottom.

Movie S4



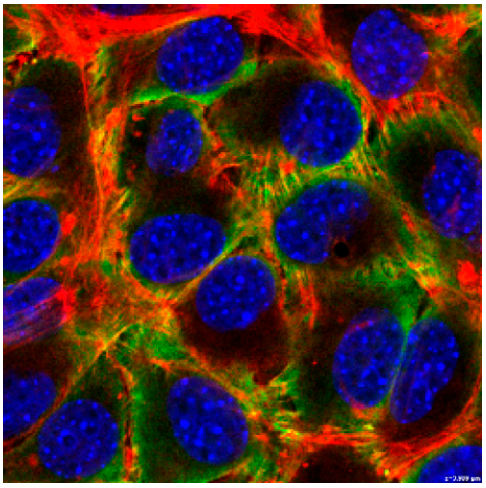
Movie S5. (related to Fig. 4*C*) Confocal cross-sections of the FLNA (red) and RefilinB (green) immunostaining in NIH 3T3 cells shown from top to bottom.

Movie S5



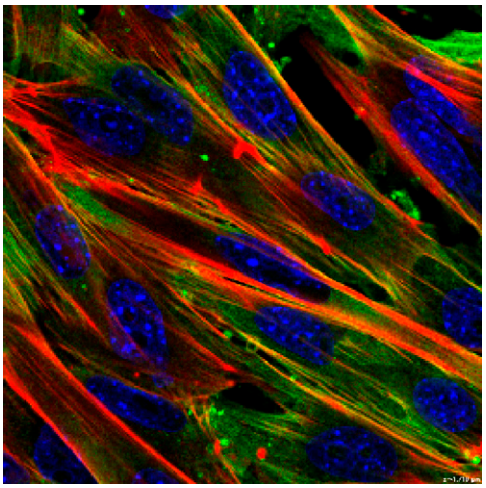
Movie S6. (related to Fig. 5*D*, control) Confocal cross-sections of the FLNA immunostaining (green) and phalloidin staining (red) in NMuMG cells shown from top to bottom.

Movie S6



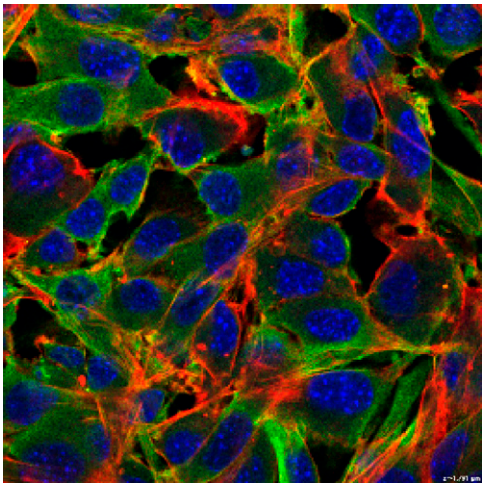
Movie S7. (related to Fig. 5*D*, TGF-β 20 h) Confocal cross-sections of the FLNA immunostaining (green) and phalloidin staining (red) in NMuMG cells treated with TGF-β for 20 h shown from top to bottom.

Movie S7



Movie S8. (related to Fig. 5*D*, TGF-β 48 h) Confocal cross-sections of the FLNA immunostaining (green) and phalloidin staining (red) in NMuMG cells treated with TGF-β for 48 h shown from top to bottom.

Movie S8



Movie S9. (related to Fig. 5*D*, shRefilinB TGF-β 48 h) Confocal cross-sections of the FLNA immunostaining (green) and phalloidin staining (red) in stably transfected NMuMG cells with RefilinB shRNA, treated with TGF-β for 48 h shown from top to bottom.

Movie S9