

Supplementary Figures

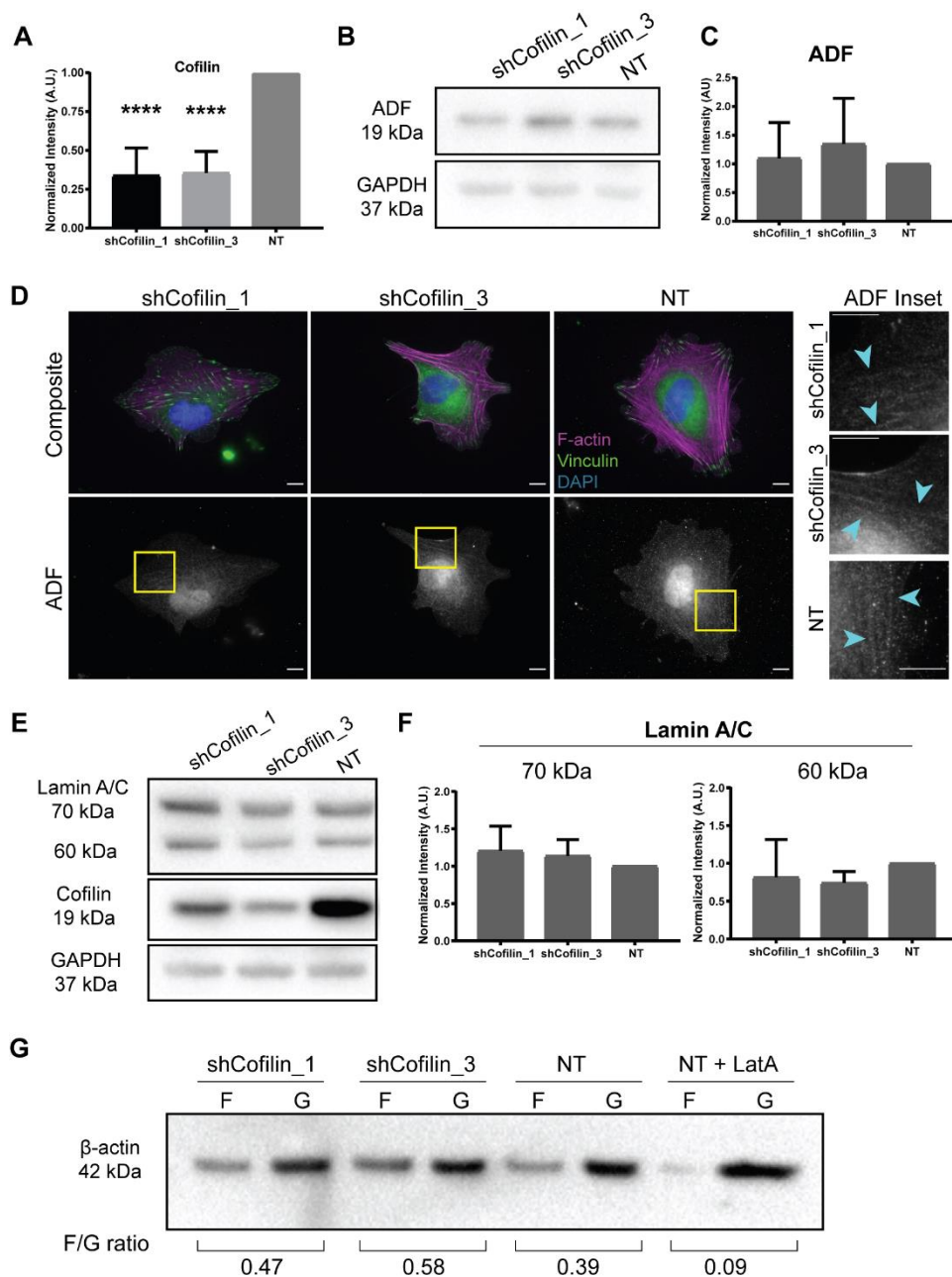


Figure S1. Cofilin depletion does not affect ADF or lamin A/C expression levels. (A) Average cofilin KD efficiency normalized to GAPDH loading controls. **** $p < 0.0001$, ANOVA, followed by post-hoc Holm-Sidak test. $N = 7$ independent experiments. (B) Western blot probing for ADF in cofilin-depleted cells. (C) Average ADF expression normalized to GAPDH loading control. $N = 4$ replicates across 2 independent experiments. (D) Cofilin KD or NT cells stained for F-actin (magenta), vinculin (green), nuclei (DAPI, blue), and ADF (gray). Right panel shows inset

indicated in ADF image. Blue arrows point to ADF localization to SFs. Scale bars: 10 μ m. N = 20, 22, and 23 shCofilin_1, shCofilin_3, and NT cells across 1 experiment. (E) Western blots probing for Lamin A/C, cofilin, and loading control GAPDH. (F) Average expression of 70 and 60 kDa forms of Lamin A/C normalized to GAPDH loading control. N = 4 replicates across 2 independent experiments. (G) Western blots probing for ratio of F-actin and G-actin in cell lysates in cofilin KD, NT controls, and NT cells treated with 2 μ M Latrunculin A to sequester actin in the G-actin form. Values indicate ratio of band intensities. Plots in (A), (C), and (F) show the average normalized integrated density, with error bars representing the standard deviation.

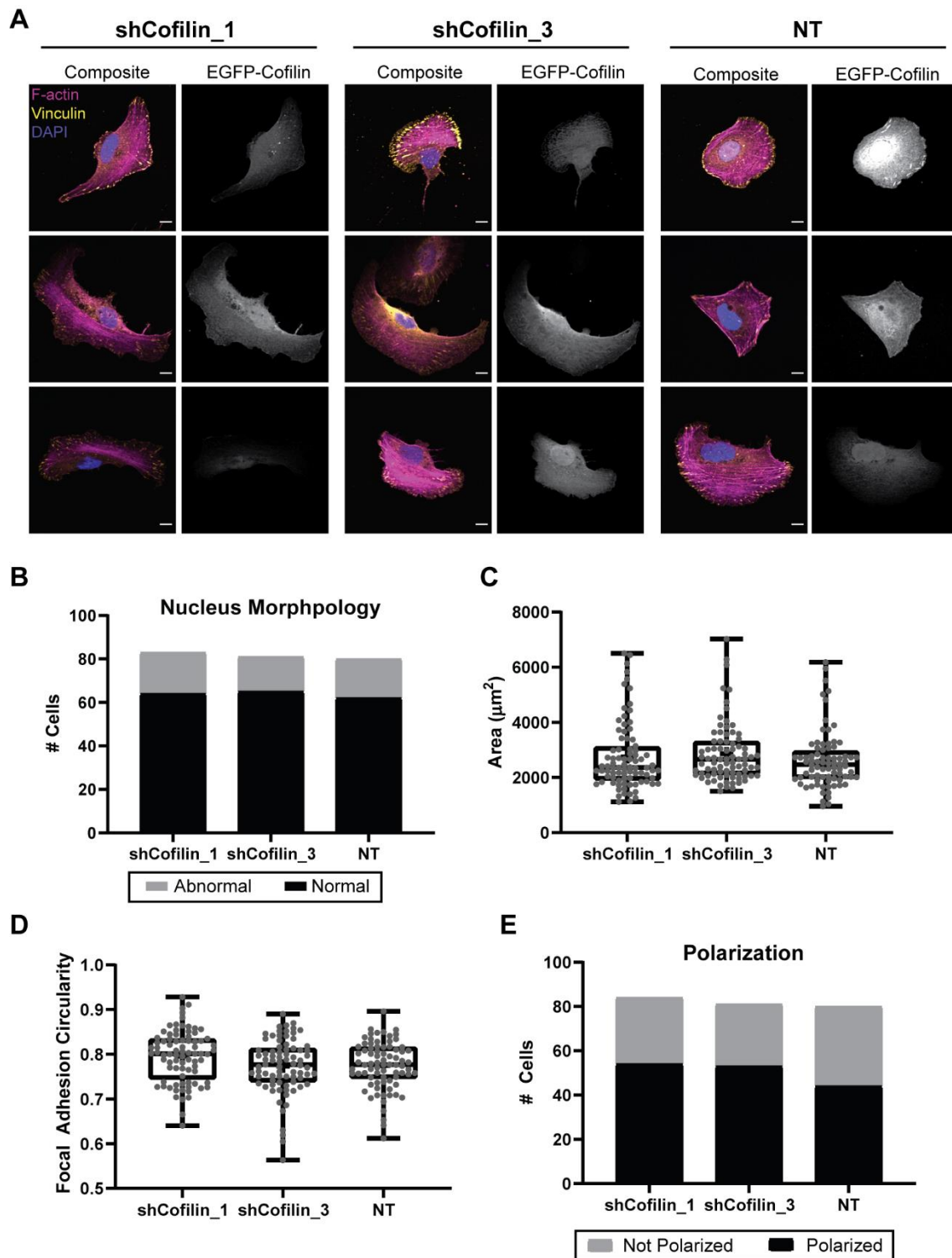


Figure S2. Transfection with cofilin-WT construct rescues phenotypic differences in cofilin KD cells. (A) Representative images of cofilin KD or NT cells transfected with EGFP-cofilin WT. Magenta: F-actin/phalloidin, yellow: vinculin, blue: DAPI, gray: EGFP-cofilin. Scale bar: 10 μ m. (B) Distribution of transfected cells with abnormal or normal nuclei. (C) Distribution of cell spread

areas of transfected cells. (D) Distribution of focal adhesion circularity in cells expressing EGFP-cofilin. Each point represents the average focal adhesion circularity for one cell. (E) Distribution of cell polarities in cells expressing EGFP-cofilin. In all panels, distributions across conditions are not statistically significant. N = 84, 81, and 80, shCofilin_1, shCofilin_3, and NT cells, respectively, across 3 independent experiments. Boxes show the 25th, 50th, and 75th percentiles, with whiskers extending to the maximum and minimum. The cross indicates the mean.

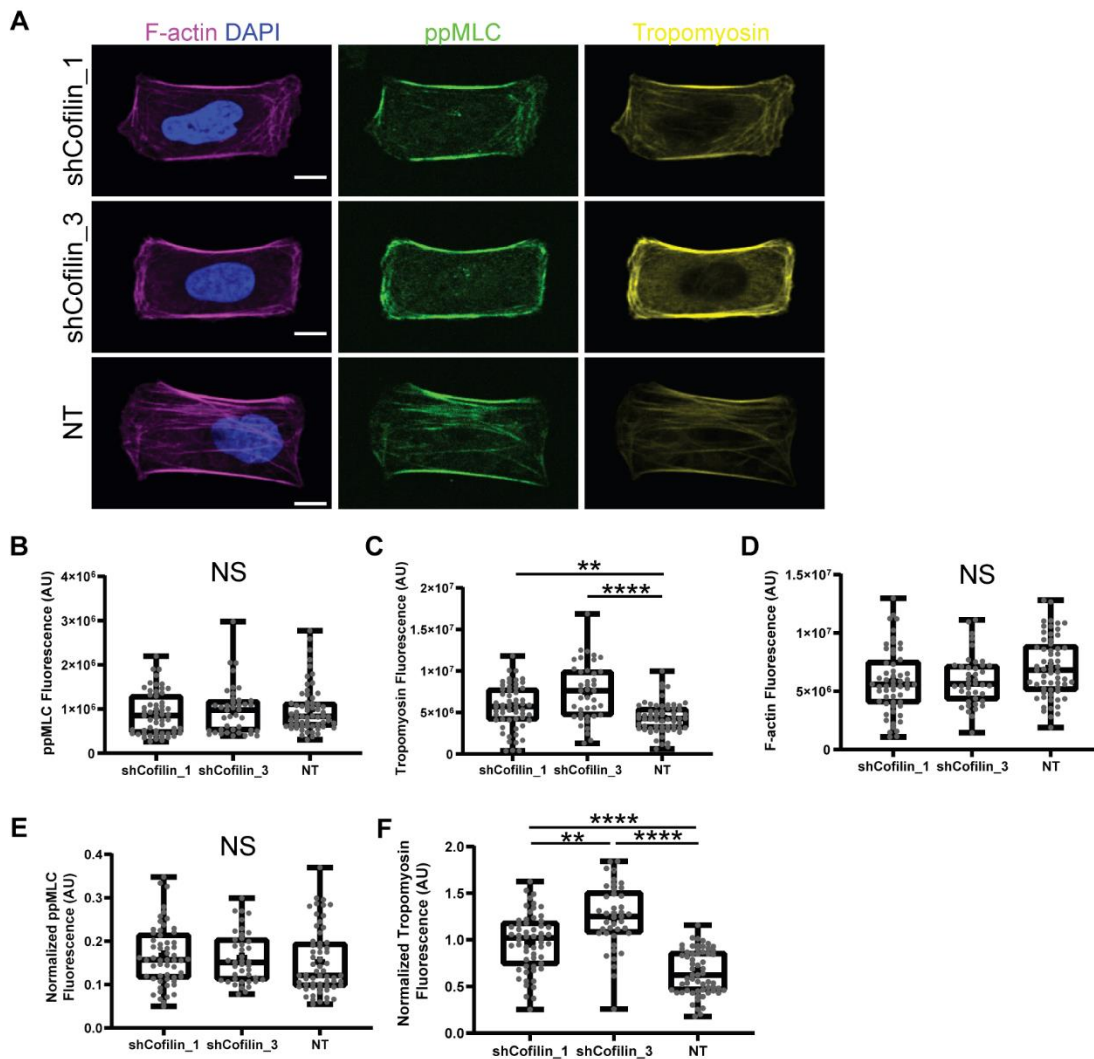


Figure S3. SFs in U-patterned cells contain more tropomyosin. (A) U-patterned cells fixed and stained for F-actin (magenta), DAPI (blue), ppMLC (green), and tropomyosin (yellow). Scale bar: 10 μ m. Representative cells are shown from 2 independent experiments. (B) Distribution of integrated ppMLC fluorescence intensities along the top SF spanning the gap of the U-pattern. (C) Distribution of integrated tropomyosin fluorescence intensities along the top SF spanning the gap of the U-pattern. (D) Distribution of integrated F-actin fluorescence intensities along the top SF spanning the gap of the U-pattern. (E) Distribution of ppMLC fluorescence intensities normalized to F-actin fluorescent intensity. (F) Distribution of tropomyosin fluorescence intensities normalized to F-actin fluorescent intensity. ** $p < 0.01$, **** $p < 0.0001$, NS: not significant. Kruskal-Wallis test, post-hoc Dunn's test. N = 57, 41, and 61 stress fibers from different shCofilin_1, shCofilin_3, and NT cells, respectively, across 2 independent experiments. Boxes

show the 25th, 50th, and 75th percentiles, with whiskers extending to the maximum and minimum. The cross indicates the mean.

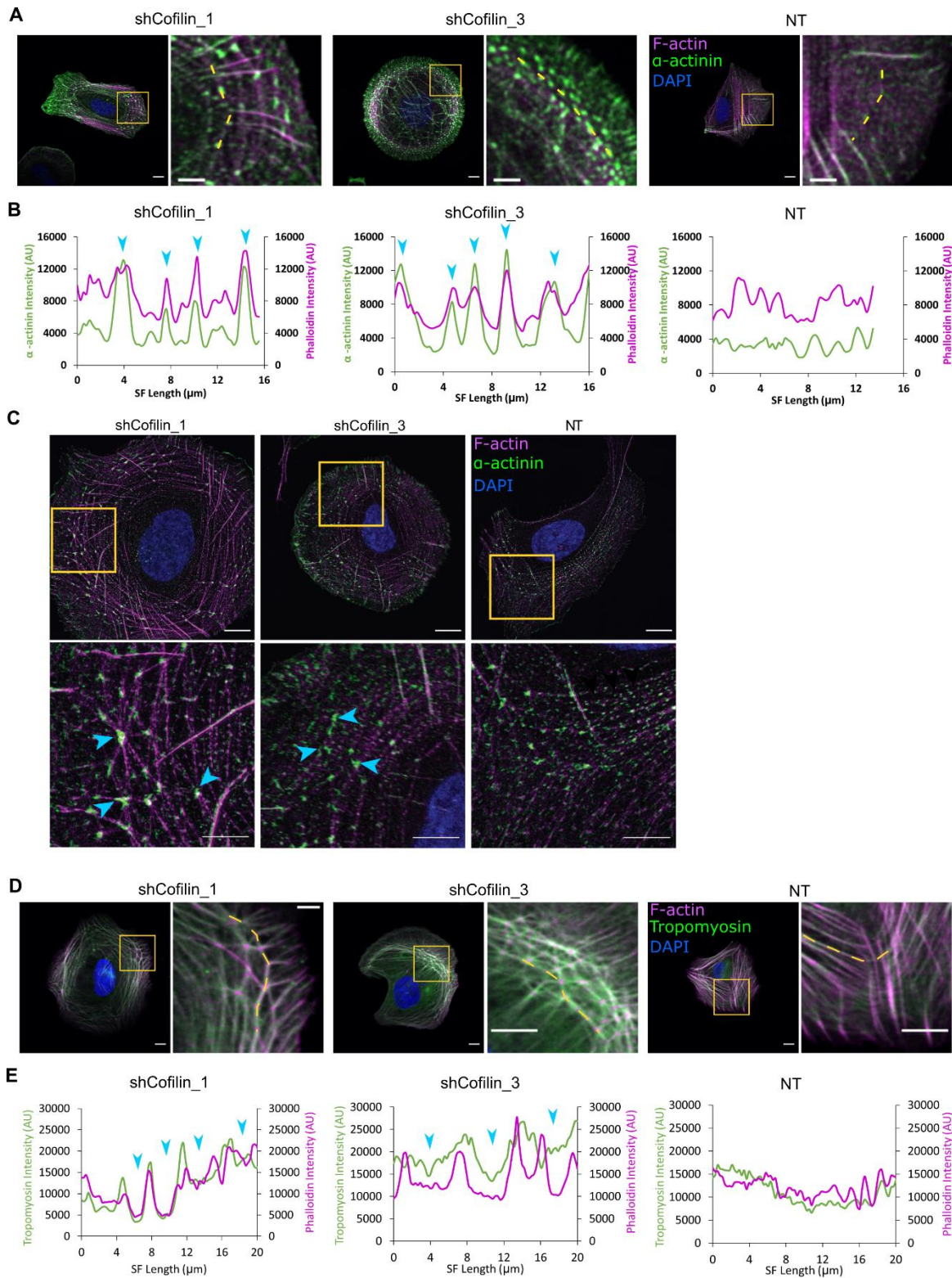


Figure S4. Transverse arc nodes are enriched in α -actinin and devoid of tropomyosin. (A) Confocal images of cofilin KD and NT cells stained for F-actin (magenta), α -actinin (green), and DAPI (blue). Images reproduced from Fig. 4C. (B) Linescans along transverse arcs (indicated by the dashed yellow line in panel A) in the α -actinin (green) and phalloidin (magenta) channels. Blue arrow heads indicate nodal regions of α -actinin clusters. (C) SIM images of cofilin KD and NT cells stained for F-actin (magenta), α -actinin (green), and DAPI (blue). Blue arrowheads point to α -actinin clusters at nodal points. White arrows point to small, periodic α -actinin clusters. (D) Confocal images of cofilin KD and NT cells stained for F-actin (magenta), tropomyosin (green), and DAPI (blue). Images reproduced from Fig. 4D. (E) Linescans along transverse arcs (indicated by the dashed yellow line in panel A) in the tropomyosin (green) and phalloidin (magenta) channels. Blue arrow heads indicate nodal regions devoid of tropomyosin. Scale bars for all panels: 10 μ m, Inset: 5 μ m.

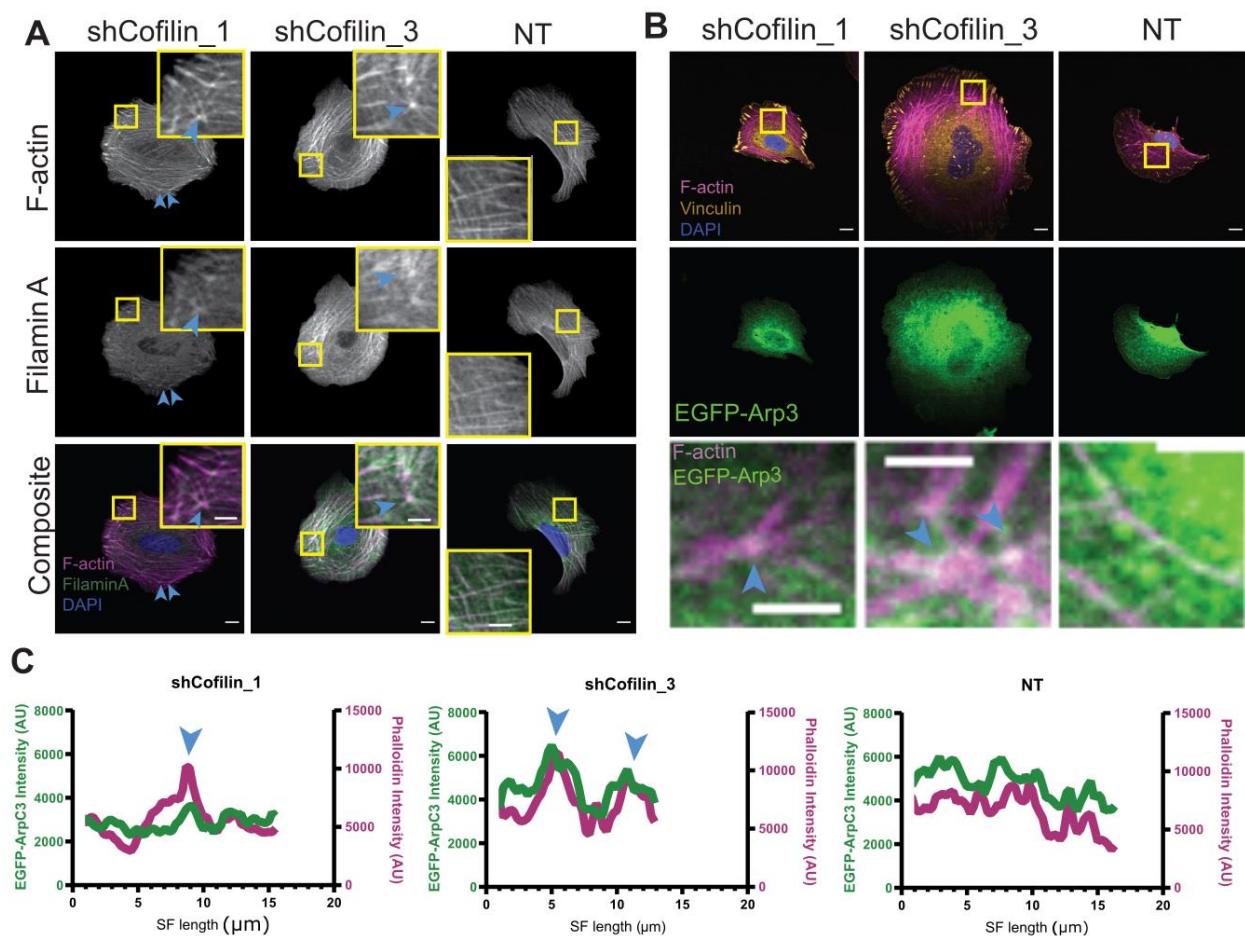


Figure S5. Transverse arc nodes are enriched in crosslinkers filamin A and Arp3. (A) Cells stained for F-actin (magenta), filamin A (green), and DAPI (blue). Blue arrowheads point to nodes. (B) Cells transfected with EGFP-Arp3 subunit (green) and stained for F-actin (magenta), vinculin (yellow), and nuclei (DAPI, blue). Blue arrowheads point to nodes. (E) Example linescans along transverse arcs for F-actin (magenta) and Arp3 (green). Blue arrowheads point to nodes. Scale bars for all panels: 10 μm , Inset: 5 μm . Representative images shown taken from samples sizes of N = 36, 39, and 37 shCofilin_1, shCofilin_3, and NT cells, respectively, across 2 independent experiments.

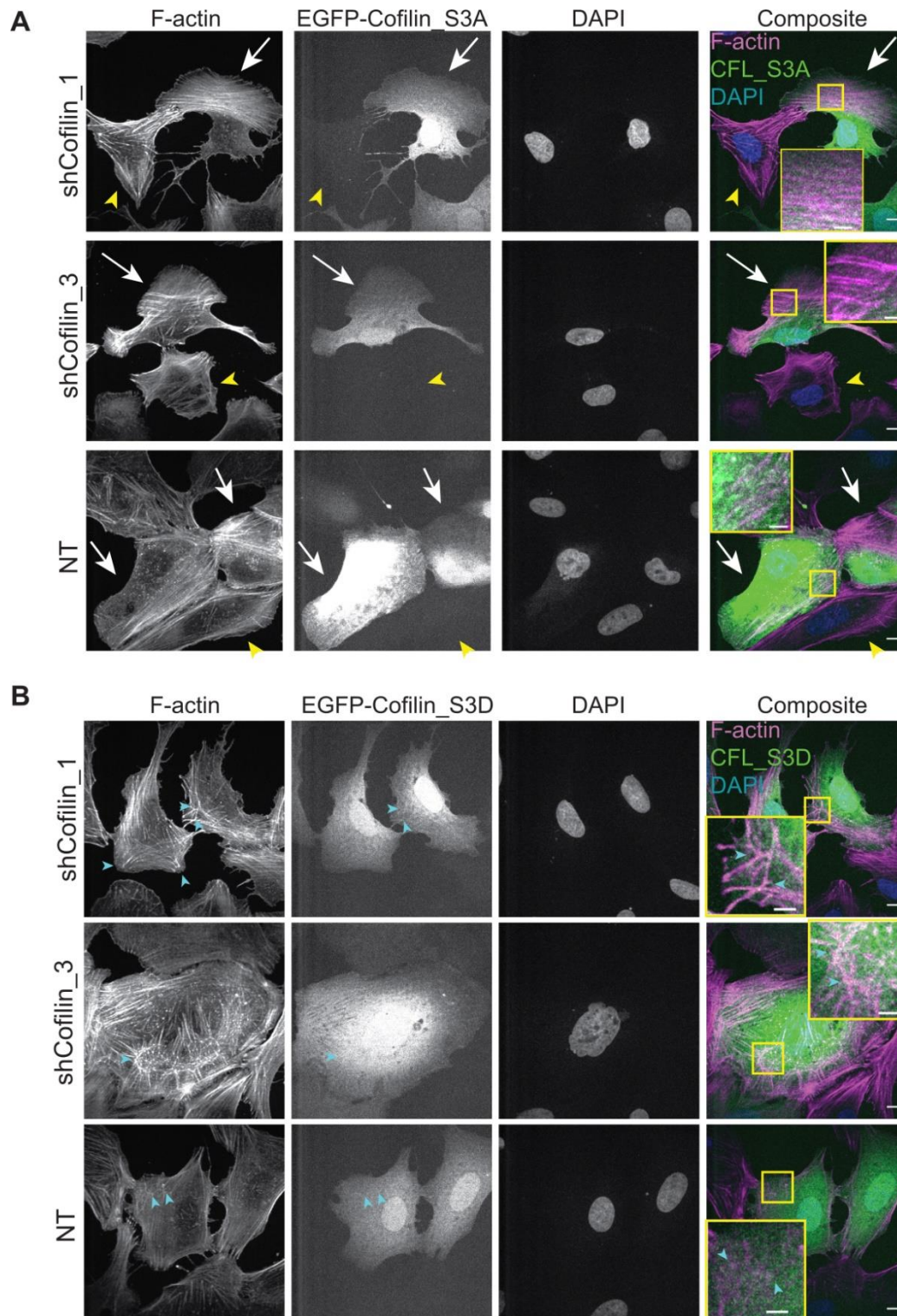


Figure S6. Cofilin KD cells regain a polarized SF phenotype when transfected with constitutively active cofilin. (A) shCofilin_1, shCofilin_3, and NT cells transfected with cofilin_S3A (constitutively active, non-phosphorylatable), indicated by white arrows. Yellow

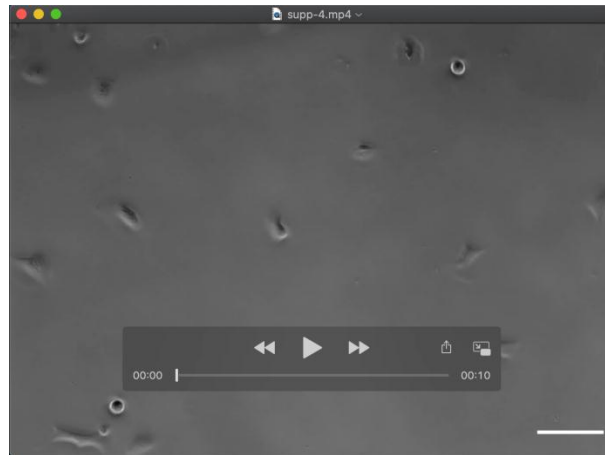
arrows indicate non-polarized cells not transfected with cofilin_S3A. Representative images shown from a sample size of 10 cells for each condition across 1 independent experiment. (B) shCofilin_1, shCofilin_3, and NT cells transfected with cofilin_S3D (dominant negative, phosphomimetic). Blue arrows point to multiple protrusions or nodes present in transfected cells. Cells are stained for F-actin (magenta) and DAPI (blue) and express an EGFP-fused cofilin mutant. Representative images shown from a sample size of 9, 10, and 10 shCofilin_1, shCofilin_3, and NT cells, respectively, across 1 independent experiment.



Movie 1. shCofilin₁ cells migrating on a surface uniformly-coated with fibronectin. Movie corresponds to Figure 1G. Colored lines indicate tracks used to determine migration speeds. Time: hh:mm:ss. Scale bar: 10 μ m.



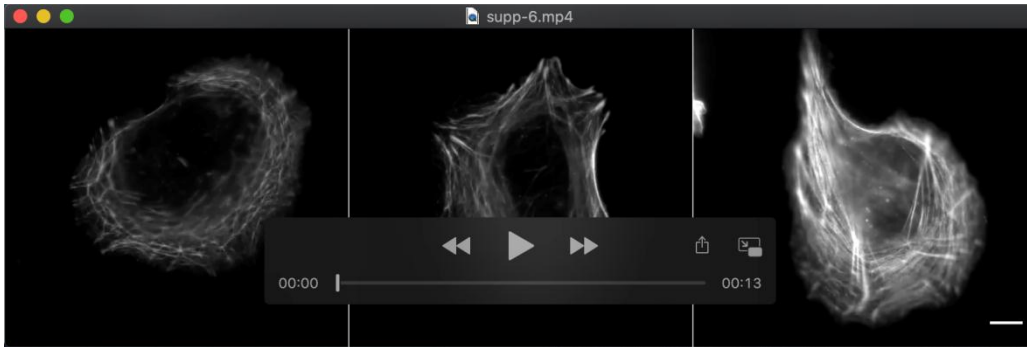
Movie 2. shCofilin₃ cells migrating on a surface uniformly-coated with fibronectin. Movie corresponds to Figure 1G. Colored lines indicate tracks used to determine migration speeds. Time: hh:mm:ss. Scale bar: 10 μ m.



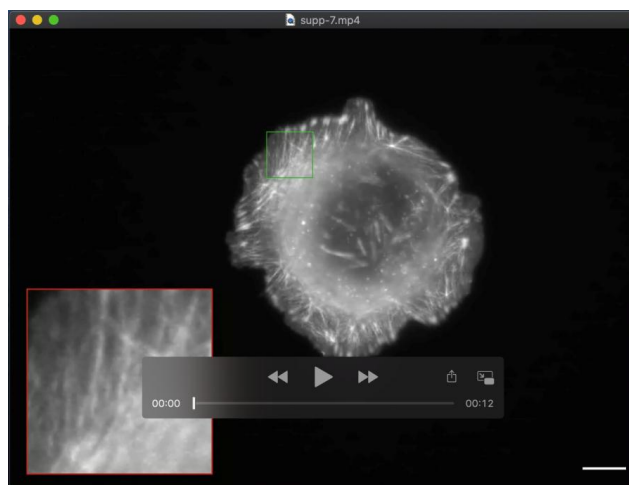
Movie 3. NT cells migrating on a surface uniformly-coated with fibronectin. Movie corresponds to Figure 1G. Colored lines indicate tracks used to determine migration speeds. Time: hh:mm:ss. Scale bar: 10 μ m.



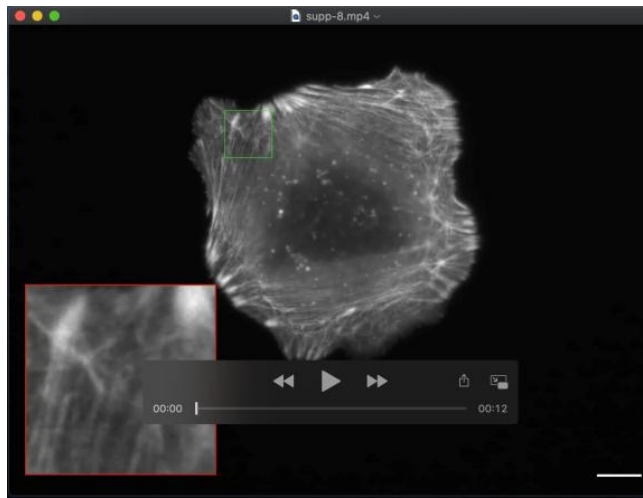
Movie 4. Ablation of single SF in patterned cofilin KD (left and middle panel) and NT cells. The SF on top of the fibronectin U-pattern is severed and its retraction is tracked over time. Movie corresponds to Figure 3A and B. SFs are visualized with Lifeact-RFP. Time: hh:mm:ss. Scale bar: 10 μ m.



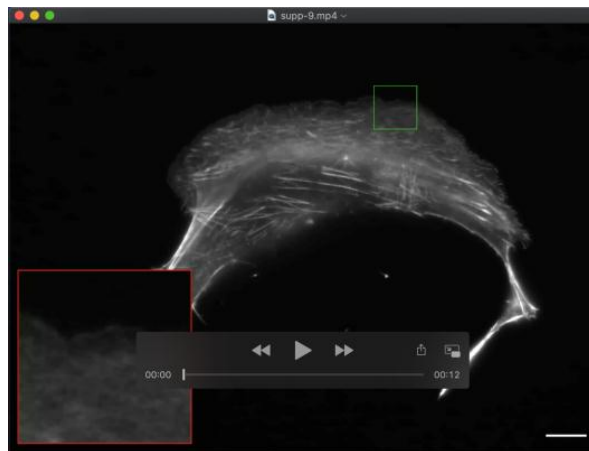
Movie 5. Stress fiber movement in cofilin KD and NT cells. Red arrows point to transverse arcs fusing together to form thicker SFs. Movie corresponds to Figure 4A. Cells are visualized with Lifeact-RFP. Time: hh:mm:ss. Scale bar: 10 μ m.



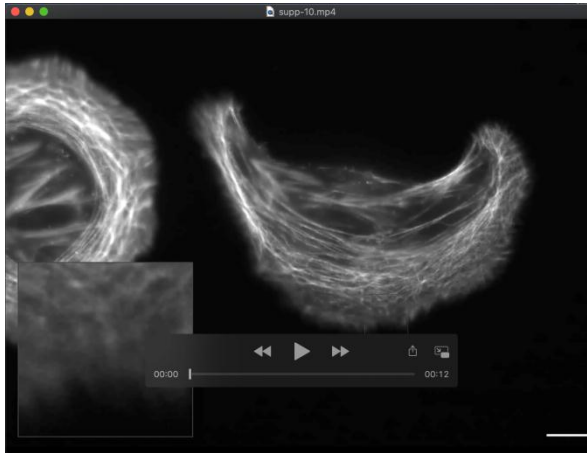
Movie 6. Node formation and movement in shCofilin_1 cell. Movie corresponds to Figure 4E. Cell is visualized with Lifeact-RFP. Time: hh:mm:ss. Scale bar: 10 μ m.



Movie 7. Node formation and movement in shCofilin₃ cell. Movie corresponds to Figure 4E. Cell is visualized with Lifeact-RFP. Time: hh:mm:ss. Scale bar: 10 μ m.



Movie 8. SF movement and transverse arc fusion in NT cell. Nodes do not form in this cell during the course of imaging. Movie corresponds to Figure 4E. Cell is visualized with Lifeact-RFP. Time: hh:mm:ss. Scale bar: 10 μ m.



Movie 9. Node formation, movement, and resolution in NT cell. Transient nodes form and are resorbed into transverse arcs during retrograde stress fiber movement. Movie corresponds to Figure 4E. Cell is visualized with Lifeact-RFP. Time: hh:mm:ss. Scale bar: 10 μ m.



Movie 10. Timelapse images tracking cofilin KD and NT cells before and after EGF treatment to induce a polarized phenotype. Movie corresponds to Figure 6C. Cells are visualized with Lifeact-RFP. Time: hh:mm:ss from addition of 100 ng/mL EGF. Scale bar: 10 μ m.