Kubo et al., http://www.jcb.org/cgi/content/full/jcb.201505011/DC1

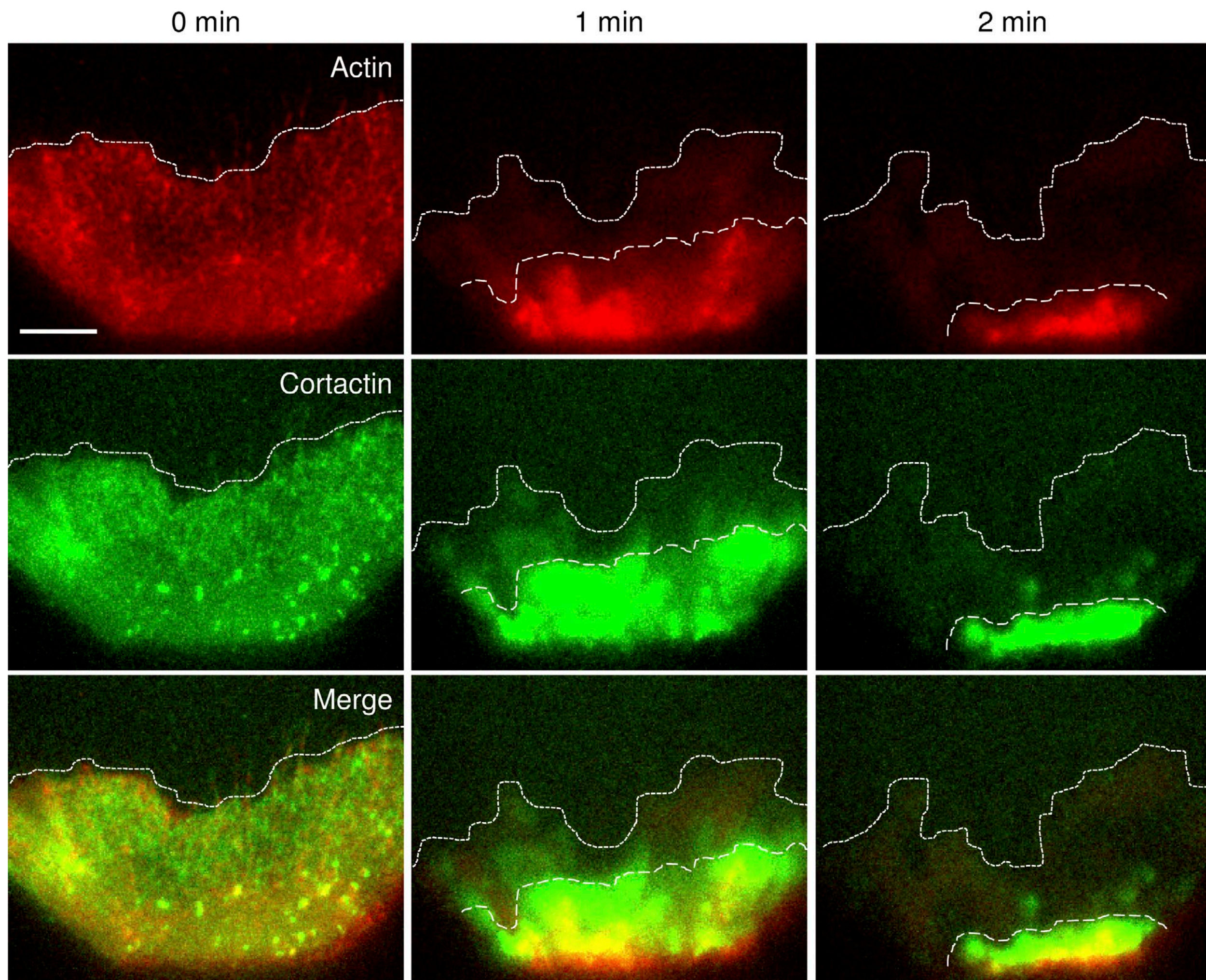


Figure S1. **The effect of cytochalasin D on retrograde movement of EGFP-cortactin in XTC fibroblasts.** Time-lapse fluorescent feature images of EGFP-cortactin and mCherry-actin in an XTC cell treated at 0 min with 1 µM cytochalasin D (see Video 3). Dotted lines indicate the cell's eading edge and boundary of fluorescent features. Bar, 5 µm.

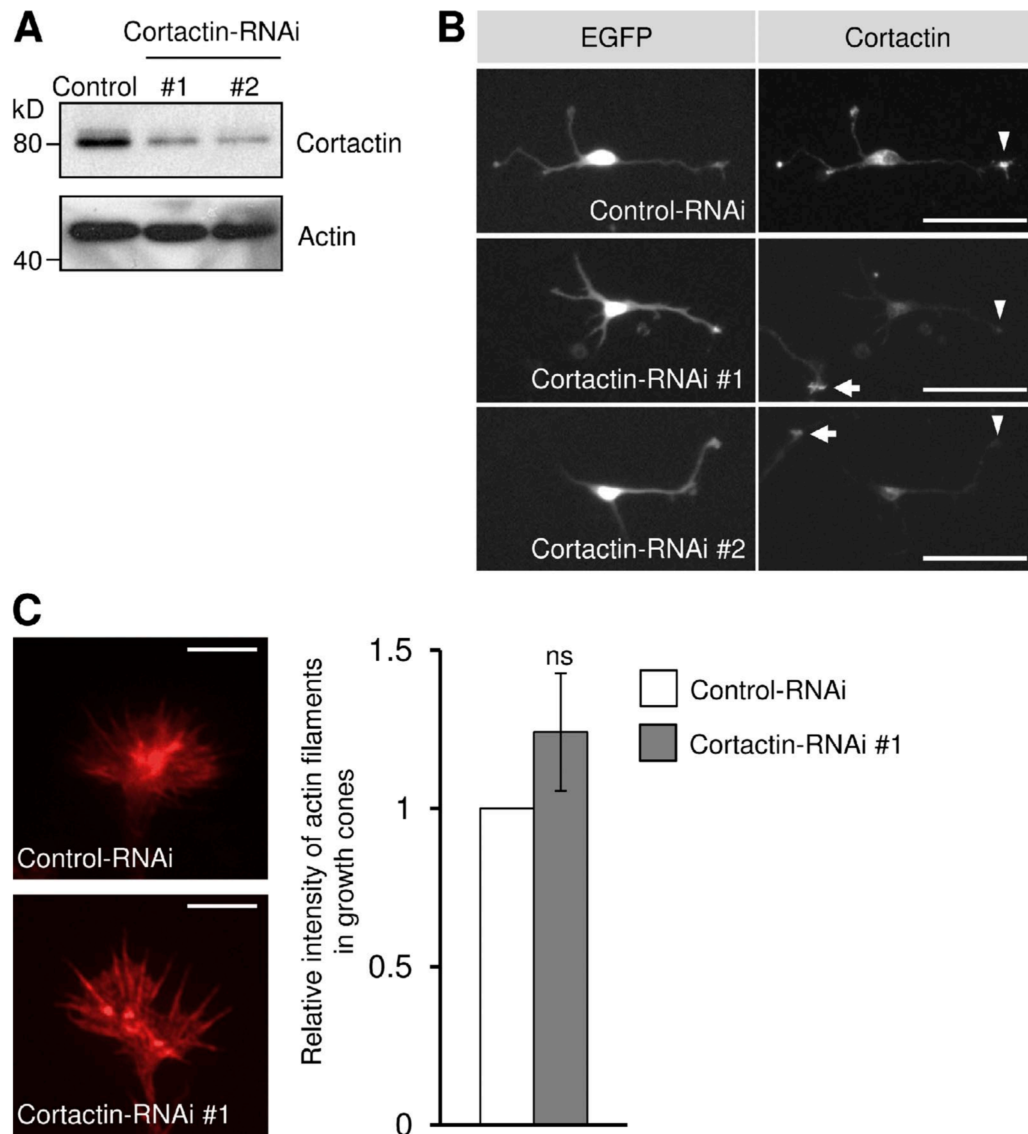


Figure S2. **Suppression of cortactin by RNAi and effect of cortactin suppression on F-actin level in axonal growth cones.** (A and B) Hippocampal neurons transfected with a control miRNA or miRNA against cortactin (#1 or #2) were cultured on polylysine for 48 h. Then, proteins extracted from the neurons were analyzed by immunoblotting with anti-cortactin and anti-actin antibodies (A), or the cells were fixed and immunostained with anti-cortactin antibody (B). The vector for miRNA expression is designed to coexpress EGFP. Axonal growth cones of transfected and untransfected cells are indicated by arrowheads and arrows, respectively. (C) Neurons transfected with control miRNA or cortactin miRNA were cultured on polylysine for 48 h, then fixed and stained with Alexa Fluor 594 phalloidin (left). The relative fluorescence intensity of F-actin at axonal growth cones (arrows) was measured (right; n = 110 cells for control miRNA and 110 cells for cortactin miRNA). Data represent means ± SEM; ns, nonsignificant. Bars: (B) 50 µm; (C) 5 µm.

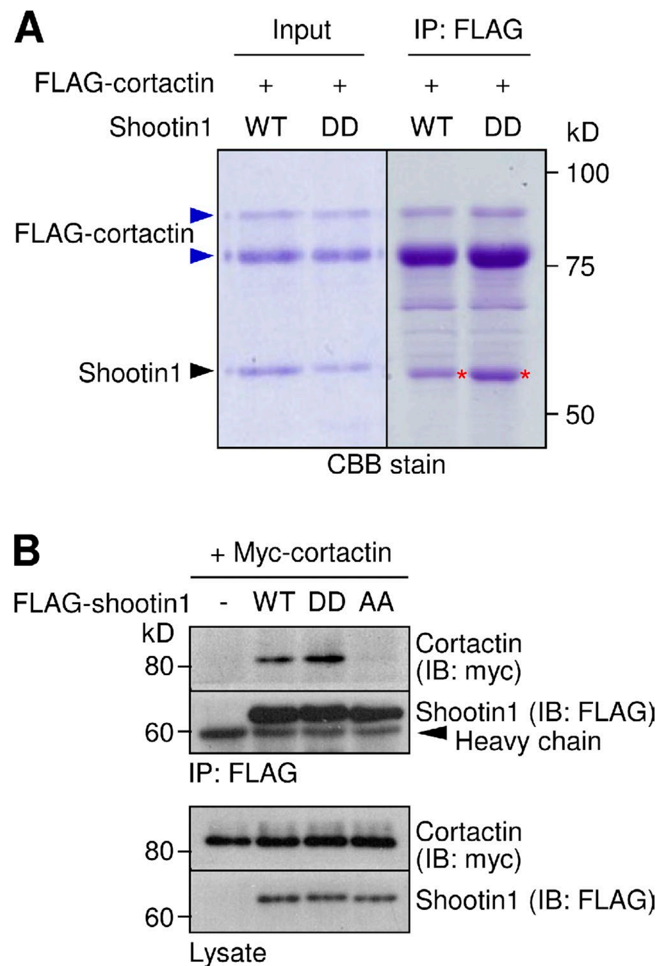


Figure S3. **Pak1-mediated shootin1 phosphorylation enhances shootin1–cortactin interaction.** (A) In vitro binding assay using purified shootin1 (2 µM) and purified cortactin (2 µM). Shootin1-WT or shootin1-DD were incubated with FLAG-cortactin and anti-FLAG antibody. The immunoprecipitates were then analyzed by SDS-PAGE and CBB staining. 0.125% of the input proteins were also analyzed. As reported previously (Wu and Parsons, 1993; MacGrath and Koleske, 2012), purified cortactin is composed of a single major band at 80 kD and an additional upper band (blue arrowheads). Asterisks denote shootin1-WT and shootin1-DD. (B) Coimmunoprecipitation of shootin1 mutants and cortactin in COS7 cells. Cells were cotransfected with FLAG-shootin1 (WT, DD, or AA) and myc-cortactin, and lysates were incubated with anti-FLAG antibody. The immunoprecipitates were immunoblotted with anti-FLAG or anti-myc antibody.

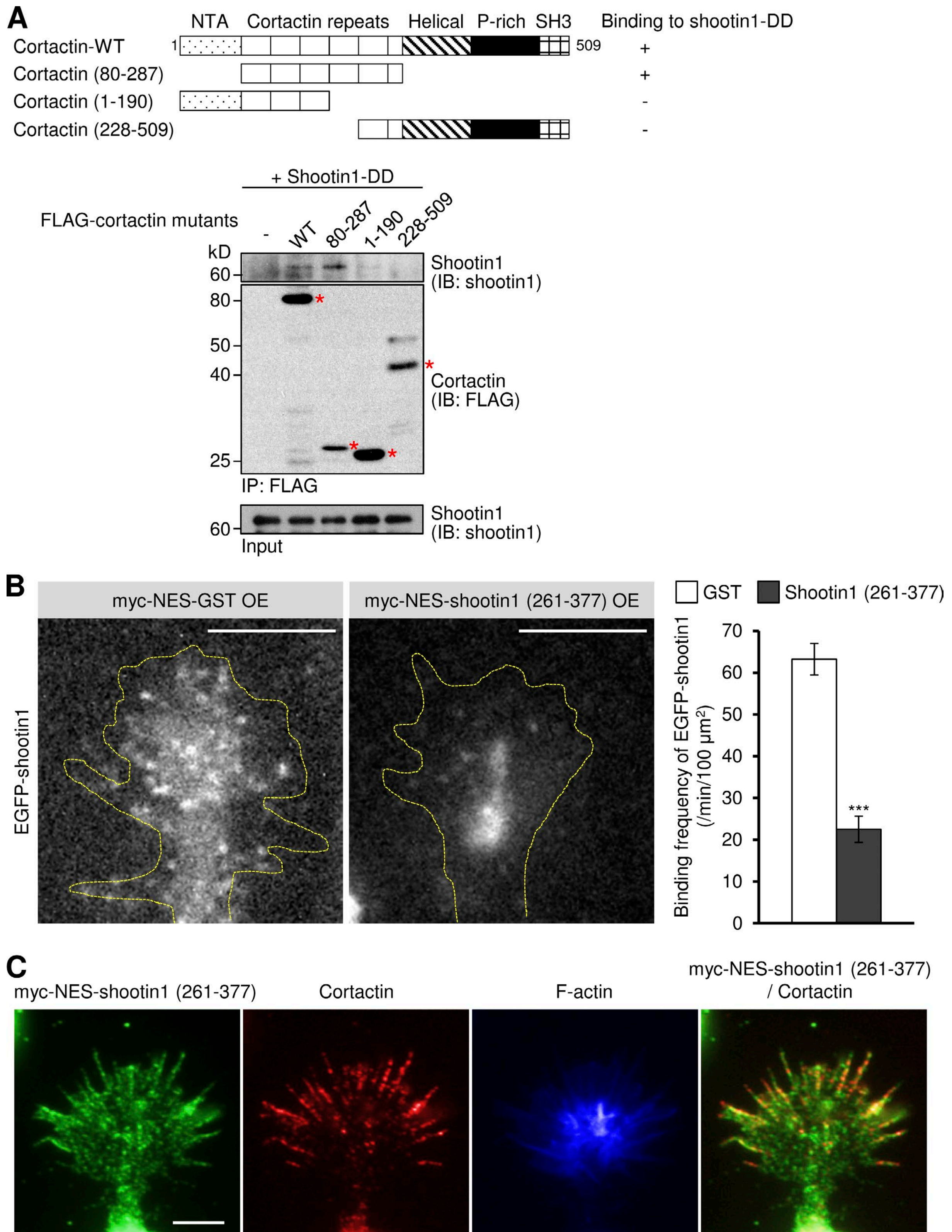
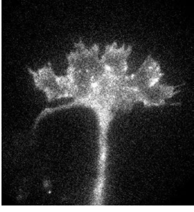
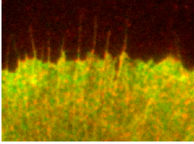


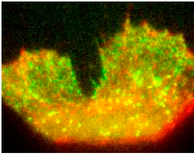
Figure S4. **Shootin1 (261–377) disrupts shootin1-cortactin interaction and colocalizes with F-actins in axonal growth cones.** (A, top) Schematic representation of WT and cortactin deletion mutants, and their abilities to interact with shootin1-DD. (A, bottom) In vitro binding assay using purified FLAG-tagged cortactin mutants and purified shootin1-DD. FLAG-cortactin mutants (80 nM) were incubated with shootin1-DD (80 nM) and anti-FLAG antibody. The immunoprecipitates were immunoblotted with anti-FLAG or anti-shootin1 antibody. Asterisks denote FLAG-tagged cortactin mutants. (B) Fluorescent feature images of EGFP-shootin1 expressed in hippocampal neurons (left). Myc-NES-GST (B) or myc-NES-shootin1 (261–377) was also overexpressed in the cell. See Videos 8 and 9. Broken lines indicate the leading edge of the growth cones. The right graph shows the signal binding frequency of EGFP-shootin1. *n* = 15 growth cones. (C) Hippocampal neurons overexpressing myc-NES-shootin1 (261–377) were cultured for 48 h, and immunostained with anti-myc antibody (green), anti-cortactin antibody (red), and phalloidin (blue). Data represent means ± SEM (error bars); ***, $P < 0.01$. Bars, 5 µm.



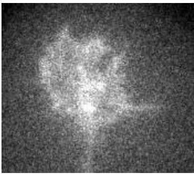
Video 1. **Movement of fluorescent features of EGFP-cortactin in an axonal growth cone.** Hippocampal neurons expressing EGFP-cortactin were cultured for 1 d. Images of a growth cone were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 3 min.



Video 2. **Movement of fluorescent features of EGFP-cortactin and mCherry-actin in an XTC fibroblast.** XTC fibroblasts were transfected with EGFP-cortactin and mCherry-actin. Images of EGFP-cortactin (green) and mCherry-actin (red) in the cell's leading edge were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 3 min.

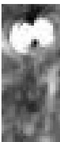


Video 3. **The effect of cytochalasin D on retrograde movement of EGFP-cortactin and mCherry-actin signals in XTC fibroblasts.** XTC fibroblasts were transfected with EGFP-cortactin and mCherry-actin. Images of EGFP-cortactin (green) and mCherry-actin (red) in the cell's leading edge were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 5 min. Cytochalasin D (1 µM) was applied at the indicated time.

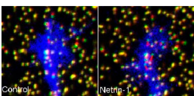


Video 4. **The effect of cytochalasin D on retrograde movement of EGFP-cortactin signals in the axonal growth cone.** Hippocampal neurons expressing EGFP-cortactin were cultured for 1 d. Images of a growth cone were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 8 min. Cytochalasin D (1 µM) was applied at the indicated time.

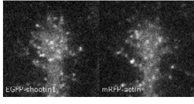
Video 5. **Retrograde movement of an L1-CAM-Fc–coated bead on an axonal growth cone expressing control miRNA**. Hippocampal neurons expressing control miRNA were cultured for 3 d. L1-CAM-Fc–coated beads were then placed on the growth cones. DIC images of beads were acquired using a fluorescence microscope (Eclipse TE2000-U; Nikon). Frames were taken every 1 s for 2 min.



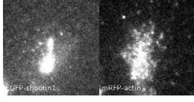
Video 6. **Retrograde movement of an L1-CAM-Fc–coated bead on an axonal growth cone expressing cortactin miRNA**. Hippocampal neurons expressing cortactin miRNA were cultured for 3 d. L1-CAM-Fc–coated beads were then placed on the growth cones. DIC images of beads were acquired using a fluorescence microscope (Eclipse TE2000-U; Nikon). Frames were taken every 1 s for 2 min.



Video 7. **Netrin-1–induced promotion of traction force under an axon outgrowth cone.** Hippocampal neurons expressing EGFP were cultured for 2 d on L1-CAM-Fc–coated polyacrylamide gels embedded with 200-nm fluorescent beads. Time-lapse imaging of fluorescent beads and growth cones was performed using a confocal microscope (LSM710; Carl Zeiss). The original and displaced positions of the beads in the gel are indicated by green and red colors, respectively. Fluorescence of EGFP is shown with blue coloring. Frames were taken every 3 s for 150 s before (control) and 60 min after netrin-1 (300 ng/ml) stimulation.



Video 8. **Movement of fluorescent features of EGFP-shootin1 and mRFP-actin in a hippocampal neuron overexpressing Myc-NES-GST.** Hippocampal neurons expressing EGFP-shootin1 and mRFP-actin and overexpressing Myc-NES-GST were cultured for 1 d. Images of a growth cone were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 3 min.



Video 9. **Movement of fluorescent features of EGFP-shootin1 and mRFP-actin in a hippocampal neuron overexpressing Myc-NES-shootin1 (261–377).** Hippocampal neurons expressing EGFP-shootin1 and mRFP-actin and overexpressing Myc-NES-shootin1 (261–377) were cultured for 1 d. Images of a growth cone were captured using a fluorescence microscope (Axioplan2; Carl Zeiss). Frames were taken every 5 s for 3 min.

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Wu, H., and J.T. Parsons. 1993. Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J. Cell Biol.* 120:1417–1426. http://dx.doi.org/10.1083/jcb.120.6.1417