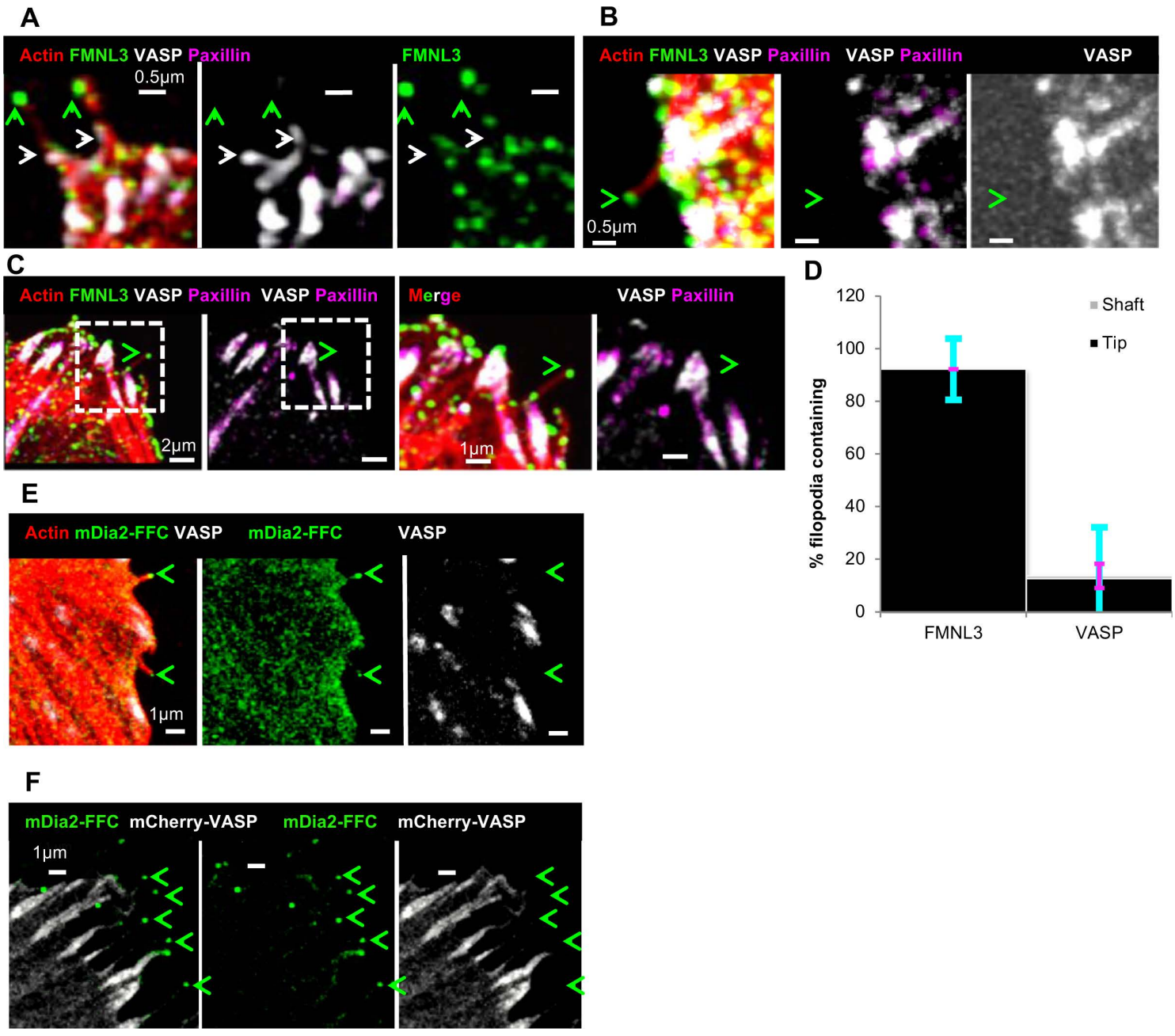
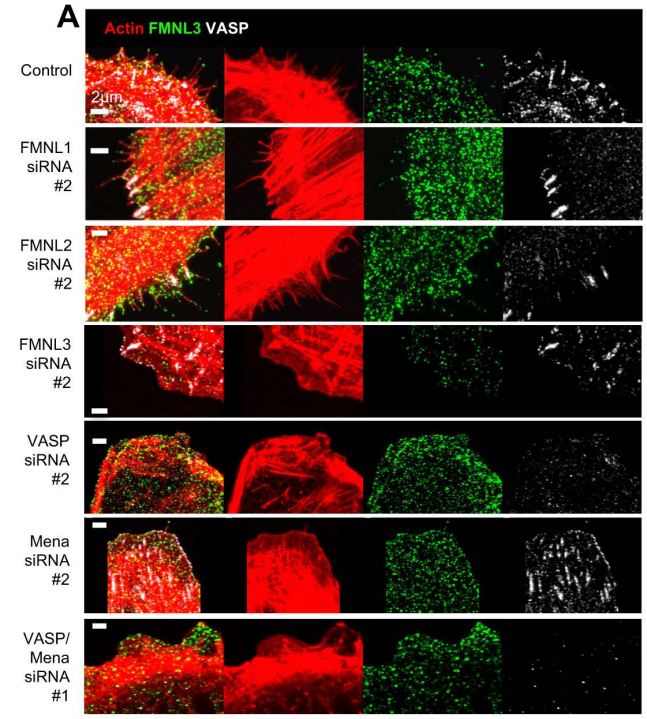
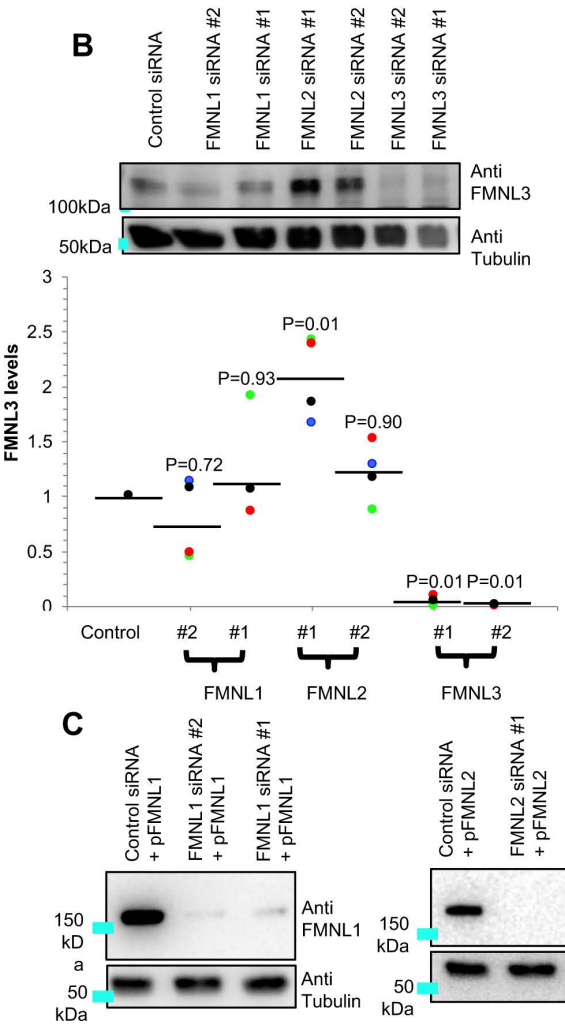
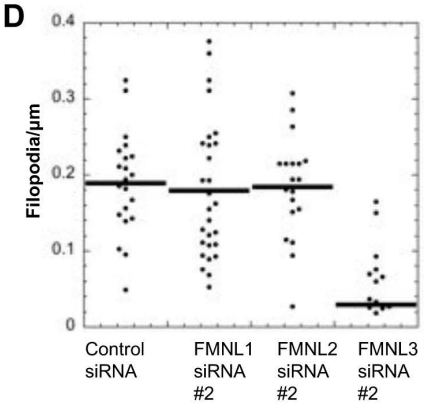


Supplementary Figure 1. Formin-enriched filopodia tips are largely devoid of VASP.

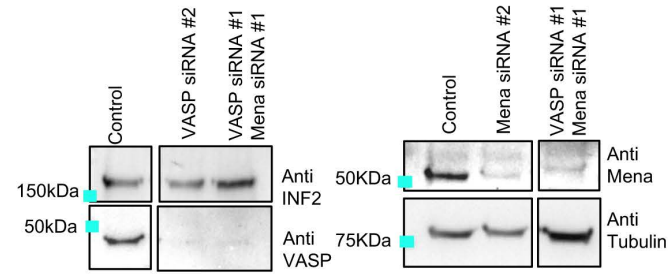
U2OS cells were plated on FN- or collagen-treated coverslips for 18 h before fixation and staining with TRITC-phalloidin (red), VASP (white), FMNL3 (green), and paxillin (magenta). **(A)** Example of over-processing the VASP signal, but still no detection of VASP at filopodial tip. Green arrowhead indicates tip. Over-saturated image indicates no background fluorescence removal from the VASP channel, and increasing brightness to over-saturate VASP signal at focal adhesions. Micrographs are MIP of 0.18 µm Z-slices, 15 slices. Plating on FN. **(B)** Representative micrograph of VASP localization to shaft (white arrowheads) for U2OS cell plated on FN. FMNL3-tip enriched filopodia shown with green arrowheads. **(C)** Example of collagen-plated cell. FMNL3-enriched tip filopodia (green arrowhead) and VASP-enriched focal adhesion. Left panels show expanded view, right panels show zoom. **(D)** Quantification of filopodia containing FMNL3 or VASP within their shafts or tips for collagen-plated cells. N = 114 filopodia, 19 cells, 3 experiments. ***p-value < 0.0001 (comparative tip localization for FMNL3 and VASP) or NS (not significant, comparative shaft localization for FMNL3 and VASP), as calculated by two sample t-test. Error bars, SD: cyan indicates tip, magenta indicates shaft. **(E)** Endogenous VASP absence at tips of mDia2-containing filopodia. U2OS cells were transfected with GFP-mDia2-FFC (green) and plated on FN-treated coverslips for 18 h before fixing and staining for TRITC-phalloidin (red) and VASP (white) . Green arrowheads indicate mDia2-FF enriched filopodia tips devoid of detectable VASP. Micrographs are MIP of 0.18 µm Z-slices, 4 slices. **(F)** VASP mCherry fusion absence at tips of mDia2-containing filopodia. U2OS cells were co-transfected with GFP-mDia2-FFC (green) and mCherry-VASP (white) and plated on FN-treated coverslips for 18 h before fixation. Green arrowheads indicate mDia2-FFC enriched filopodial tips, but devoid of detectable VASP. Micrographs are MIP of 0.18 µm Z-slices, 5 slices.

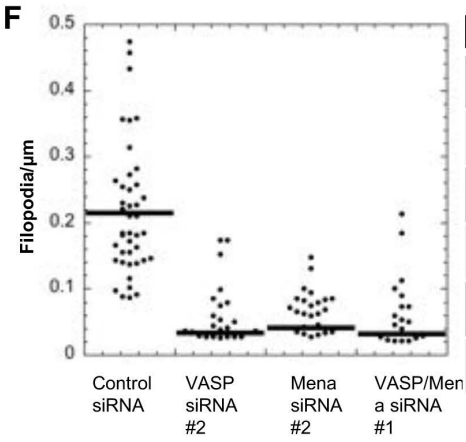






P-value	Comparison set
1.0000	Control vs FMNL1 siRNA #2
0.2762	Control vs FMNL2 siRNA #2
<0.0001	Control vs FMNL3 siRNA #2
1.0000	FMNL1 siRNA #1 vs FMNL2 siRNA #1
<0.0001	FMNL1 siRNA #2 vs FMNL3 siRNA #2
<0.0001	FMNL2 siRNA #2 vs FMNL3 siRNA #2





P-value	Comparison set
<0.0001	Control vs VASP siRNA #2
<0.0001	Control vs Mena siRNA #2
<0.0001	Control vs VASP/Mena siRNA #1
1.0000	VASP siRNA #2 vs Mena siRNA #2
1.0000	VASP siRNA #2 vs VASP/Mena siRNA #1
1.0000	Mena siRNA #2 vs VASP/Mena siRNA #1

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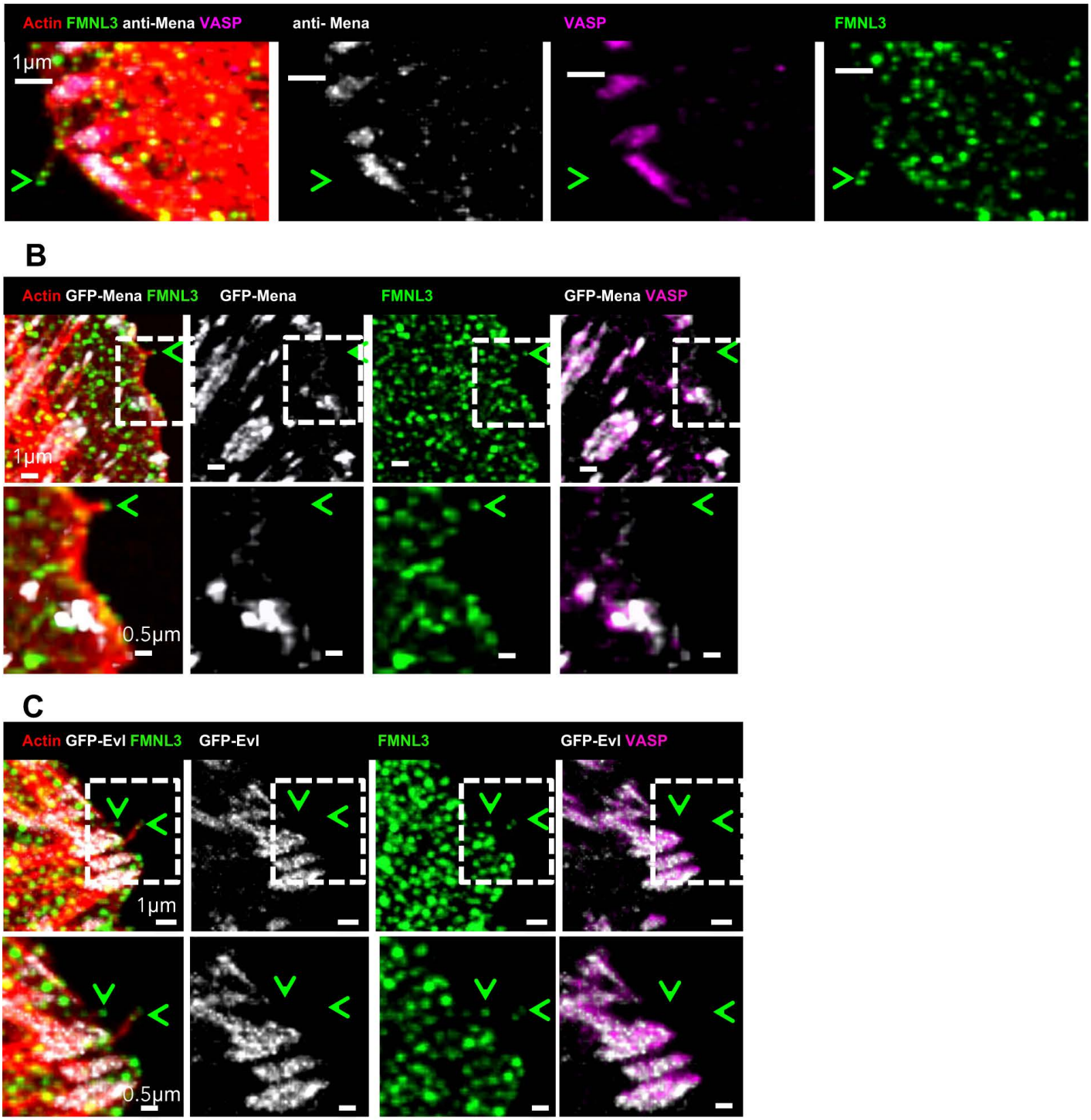
Supplementary Figure 2. FMNL3, Mena, and VASP are required for optimal filopodia assembly

(A) U2OS cells treated with control-siRNA, FMNL1-, FMNL2, FMNL3-, VASP, or Mena siRNA #2, or VASP/Mena siRNA #1 were fixed and stained with TRITC-phalloidin (red), FMNL3 and VASP . Micrographs are MIP of 0.5 µm Z-slices (3 slices). **(B)** FMNL3 western blot analysis of U2OS cells treated with control siRNA, FMNL1 siRNA#1 and 2, FMNL2 siRNA #1 and 2, or FMNL3 siRNA #1 and 2 then probed for FMNL3 or tubulin (loading control). Dot plot below shows FMNL3 levels quantified from 4 blots (2 independent knock-downs). P-value are calculated by one-way ANOVA analysis followed by post hoc Dunnett multiple comparison test. Blot shows red points in plot. **(C)** Left: Western blot of FMNL1 KD in U2OS cells, transfected with a plasmid expressing human FMNL1 (pFMNL1) and either a control siRNA or FMNL1-directed siRNA #2. Blots probed for FMNL1 (top) or tubulin (loading control). Right: Western blot of FMNL2 KD in U2OS cells, transfected with a plasmid expressing human FMNL2 (pFMNL2) and with either control siRNA or FMNL2-directed siRNA #2. Blots probed for FMNL2 (top) or tubulin (loading control). All lanes are from one gel, with intervening lanes cut out. Image processing of all lanes is identical. **(D)** Dot plot of filopodial density from U2OS cells treated with control siRNA (mean 0.211 ± 0.084), FMNL1 siRNA #2 (mean 0.196 ± 0.095), FMNL2 siRNA #2 (mean 0.172 ± 0.071), or FMNL3 siRNA #2 (mean 0.023 ± 0.041) fixed and stained with TRITC-phalloidin (red), anti-FMNL3 and anti-VASP. N = 31 (control), 34 (FMNL1 siRNA #2), 30 (FMNL2 siRNA #2) 38 (FMNL3 siRNA #2) cells, 3 experiments. Table to right shows p-values calculated by one-way ANOVA analysis followed by post hoc Dunn's multiple comparison test. **(E)** Left: western blot of U2OS cells treated with control siRNA, VASP siRNA#2, or VASP siRNA #1+Mena siRNA #1 combined, then probed for VASP or INF2 (loading control). All lanes are from one gel, with intervening lanes cut out. Image processing of all lanes is identical. Right: Western blot analysis of U2OS cells treated with control siRNA, Mena siRNA#2, or VASP siRNA #1+Mena siRNA #1 combined, then probed for Mena or tubulin (loading control). All lanes are from one gel, with intervening lanes cut out. Image processing of all lanes is identical **(F)** Dot plot of filopodial density from U2OS cells treated with control siRNA (mean 0.215 ± 0.098), VASP siRNA #2 (mean 0.033 ± 0.046), Mena siRNA #2 (mean 0.041 ± 0.040), or VASP siRNA #1 and Mena siRNA #1 combined (mean 0.031 ± 0.050) fixed and stained with TRITC-phalloidin (red), anti-FMNL3 and anti-VASP. N = 41 (control), 43 (VASP siRNA #2), 40 (Mena siRNA #2) 39 (Vasp/Mena siRNA #1) cells, 3 experiments. Table to right shows p-values calculated by one-way ANOVA analysis followed by post hoc Dunn's multiple comparison test.



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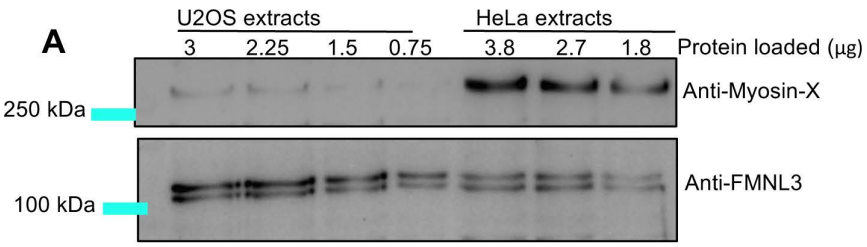
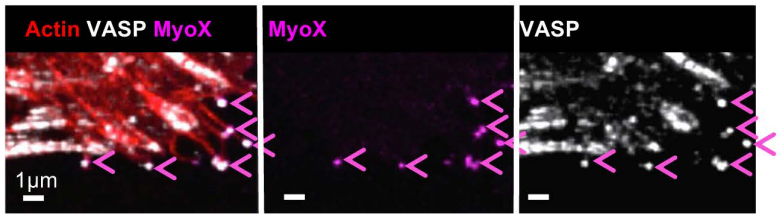
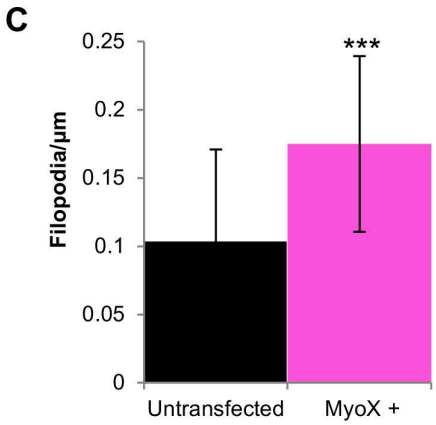
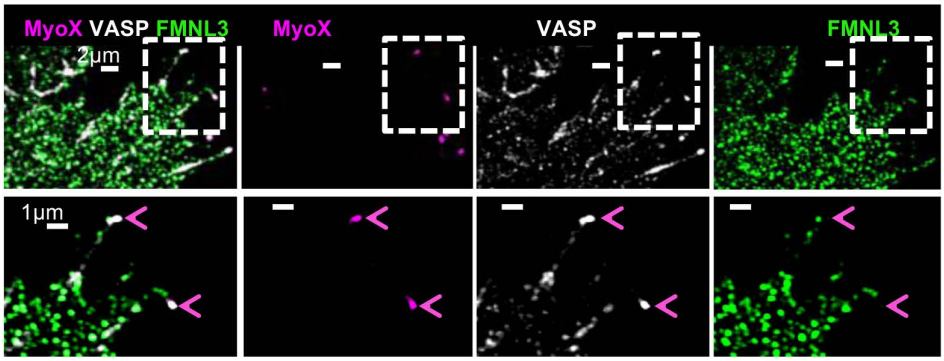
Supplementary Figure 3. Mena and Evl are not enriched at filopodial tips

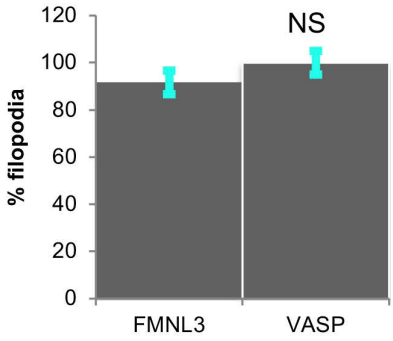
(A) U2OS cells were plated on FN-treated coverslips for 18 h before fixation and staining with TRITC-phalloidin (red), anti-Mena (white), anti-FMNL3 (green), and anti-VASP (magenta). Green arrowhead indicates FMNL3-enriched filopodial tips. **(B)** U2OS cells were transfected with GFP-Mena (white), plated on FN-treated coverslips for 18 h before fixation and staining with TRITC-phalloidin (red), anti-FMNL3 (green) and anti-VASP (magenta). Green arrowhead indicates FMNL3-enriched filopodial tip. **(C)** U2OS cells were transfected with GFP-EVL (white), plated on FN-treated coverslips for 18 h before fixation and staining with TRITC-phalloidin (red), anti-FMNL3 (green) and anti-VASP (magenta). Green arrowheads indicates FMNL3-enriched filopodial tip.

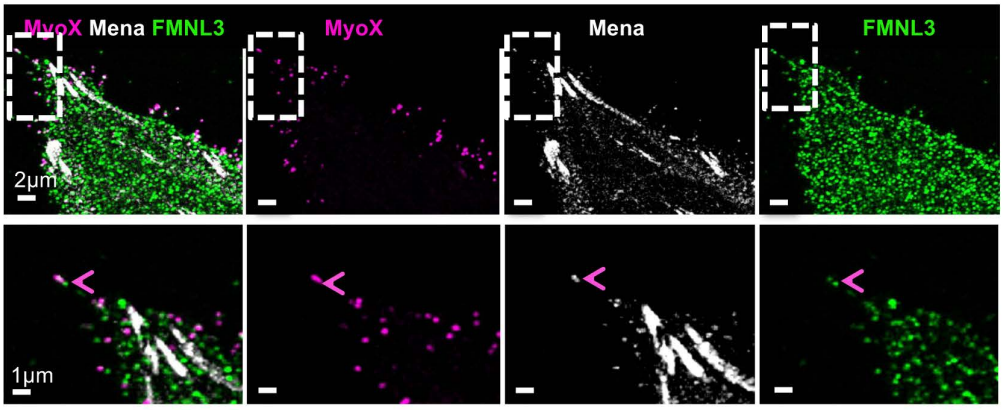


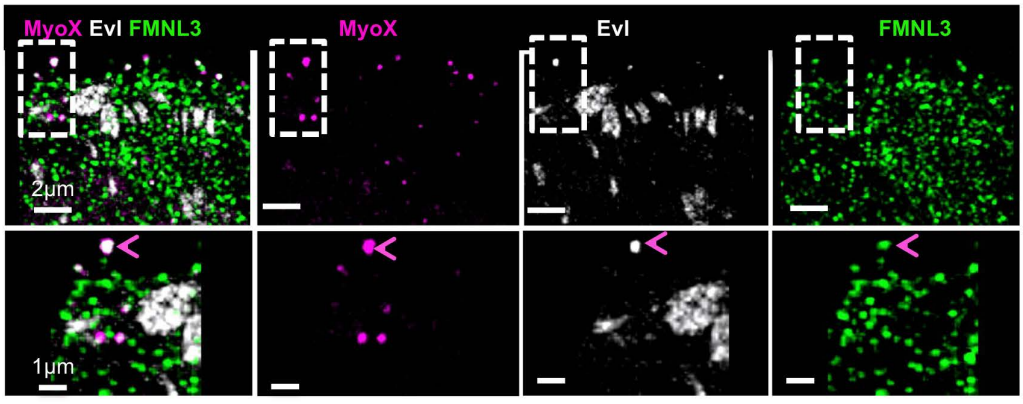






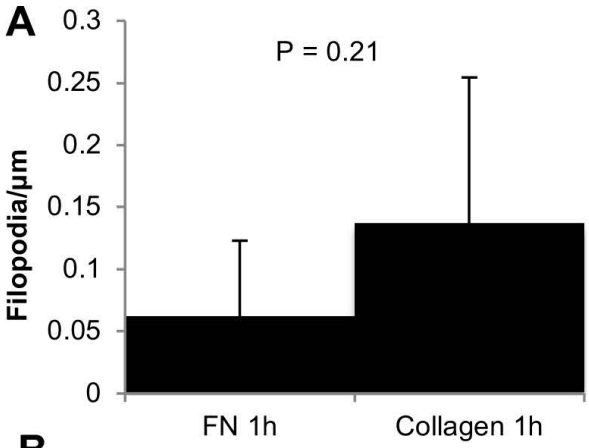


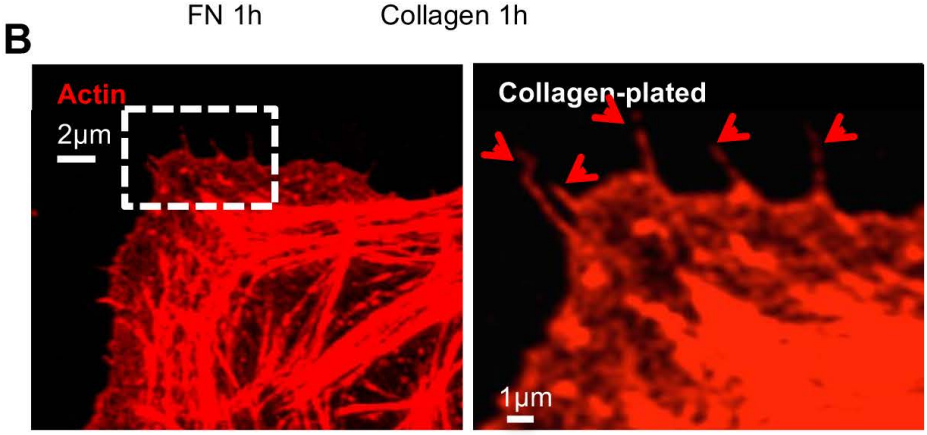


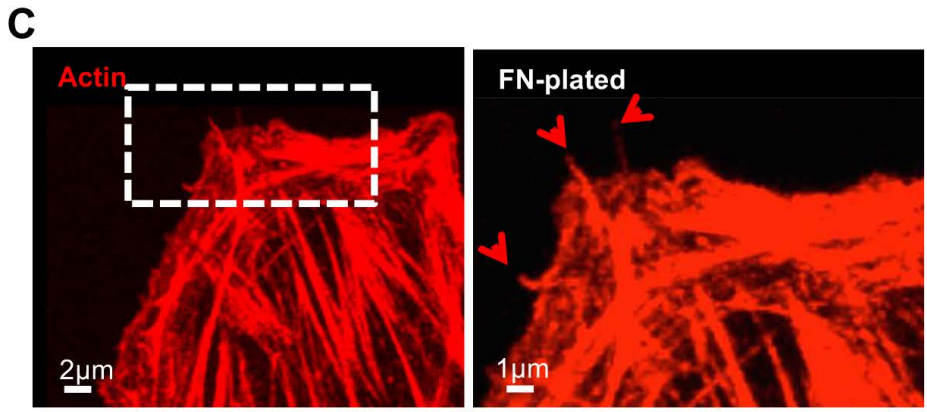


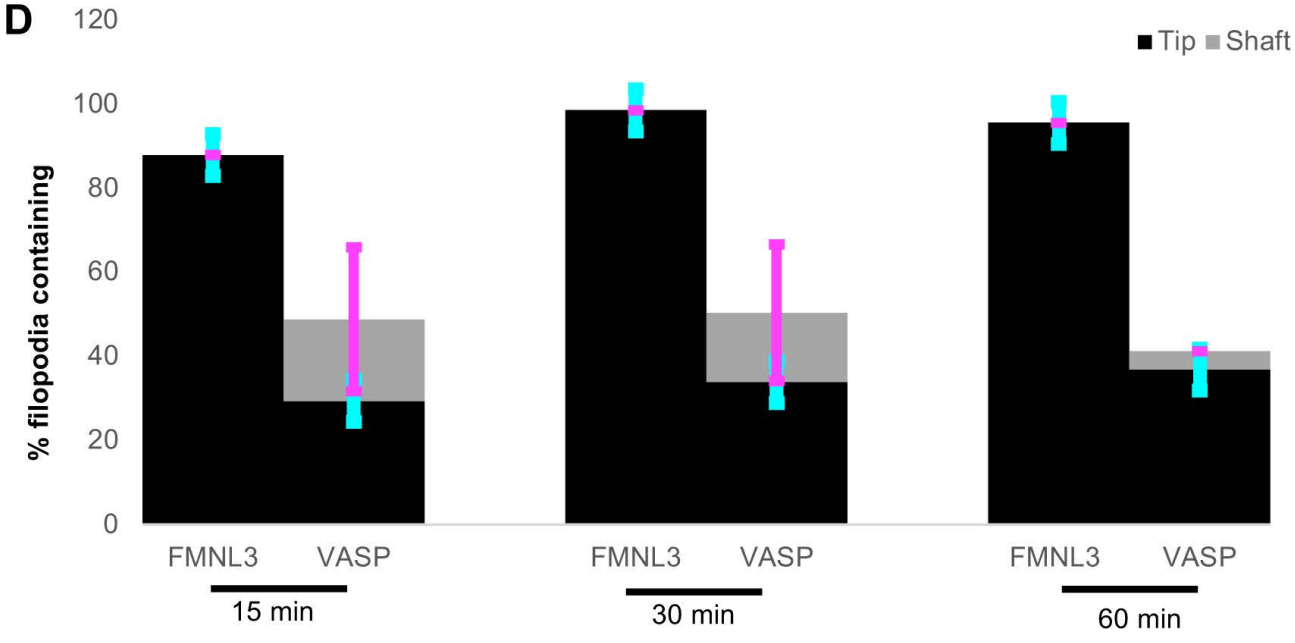
Supplementary Figure 4. ENA/VASP proteins enrich at filopodial tips upon myosin-X expression

(A) Western blot of U2OS cells and HeLa cells probed for myosin-X and FMNL3 (loading control). Dilution series for each extract (μg extract protein indicated). **(B)** U2OS cells were transfected with GFP-myosin-X (magenta) and plated on FN-treated coverslips for 18 h before fixation and staining with anti-VASP (white) and TRITC-phalloidin (red). Magenta arrowheads indicate myosin-X enriched filopodia tips. MIP of 0.18 μm Z-slices, 8 slices. **(C)** Filopodial density at leading edge of GFP-myosin-X transfected cells fixed and stained in B. N = 9 cells. ***p-value < .0001 as calculated by two sample t-test. Error bars, SD. **(D)** Example in collagen-plated cells. U2OS cells were transfected with GFP-myosin-X (magenta) and plated on collagen-treated coverslips for 18 h before fixation and staining anti-VASP (white) and anti-FMNL3 (green). Magenta arrowheads indicate myosin-X enriched filopodial tips. Top panels show expanded region, bottom panels show zoomed regions. Micrographs are MIP of 0.18 μm Z-slices, 9 slices. **(E)** Quantification of FMNL3 or VASP enrichment at tips of filopodia in GFP-myosin-X transfected cells (collagen-plated). N = 87 filopodia, 16 cells, 3 experiments. NS (not significant), as calculated by two sample t-test. Error bars, SD. **(F)** U2OS cells were co-transfected with mCherry-myosin-X (magenta) and GFP-Mena (white), then plated on FN-treated coverslips for 18 h before fixation and staining for FMNL3 (green). Magenta arrowheads indicate myosin-X enriched filopodia tips. Top panels show expanded regions, bottom panels show zoomed regions. Micrographs are MIP of 0.18 μm Z-slices, 7 slices. **(G)** U2OS cells were co-transfected with mCherry-myosin-X (magenta) and GFP-Evl (white), then plated on FN-treated coverslips for 18 h before fixation and staining for FMNL3 (green). Magenta arrowheads indicate myosin-X enriched filopodia tips. Top panels show expanded regions, bottom panels show zoomed regions. Micrographs are MIP of 0.18 μm Z-slices, 6 slices.









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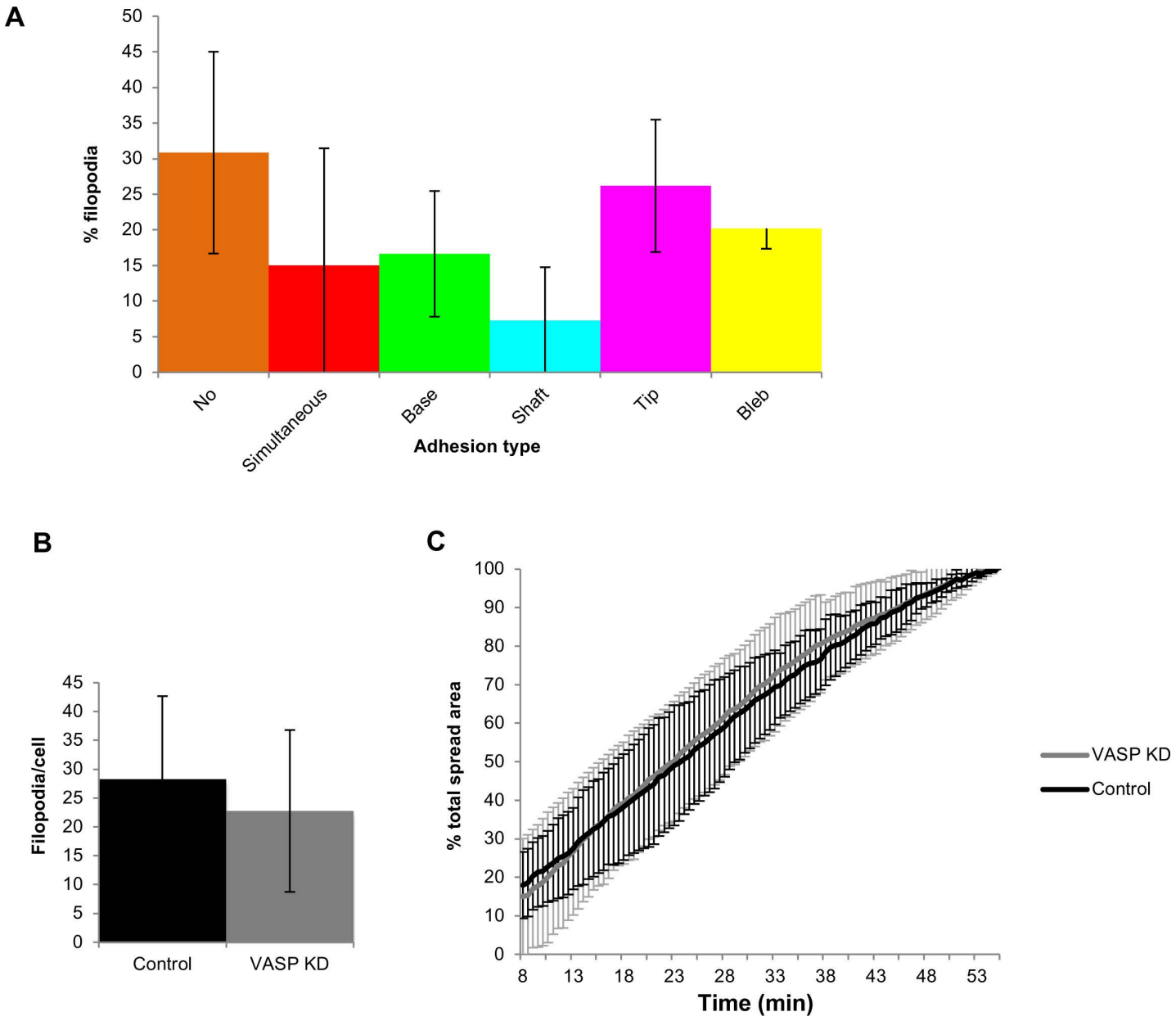
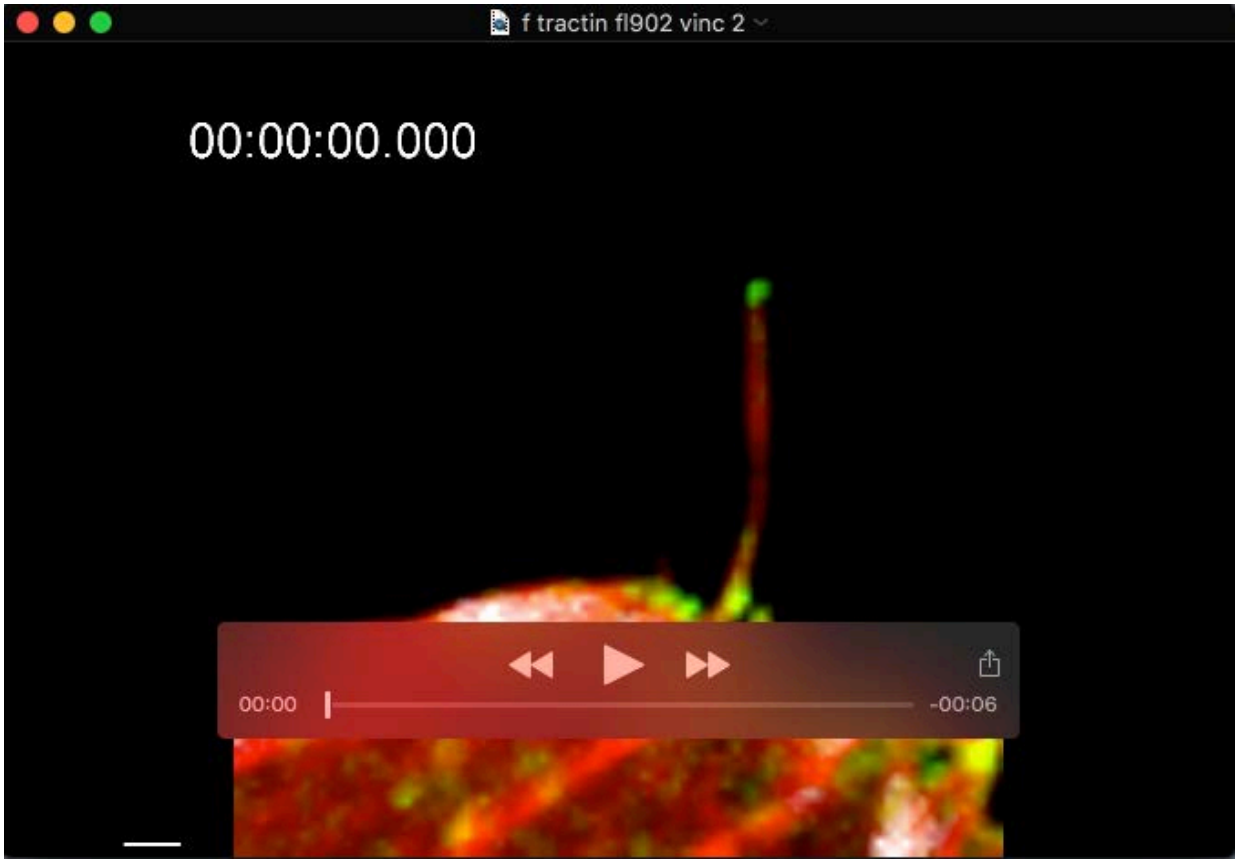
Supplementary Figure 5. U2OS spreading on collagen causes increased filopodia.

(A) U2OS cells were plated on collagen-treated or FN-treated coverslips for 1 h before fixation and staining with TRITC-phalloidin (red). FN: N= 19 filopodia, 9 cells; Collagen: N = 76 filopodia, 10 cells. P-value calculated by two sample t-test. Error bars, SD. **(B) and (C)** examples of collagen-based and FN-based spreading, respectively. Red arrows indicate filopodia. Micrographs are MIP of 0.18 µm Z-slices, 13 and 17 slices, respectively. **(D)** U2OS cells plated on FN-treated coverslips for varying times (15 min, 30 min, and 1 h) before fixation and staining with TRITC-phalloidin, anti-VASP, and anti-FMNL3. Graph shows percentage of filopodia at leading edge of spreading cells containing FMNL3 or VASP within their tip or shafts. 15 min: N = 123 filopodia, 15 cells; 30 min: N = 49 filopodia, 10 cells; 60 min = 67 filopodia, 17 cells. All *** p-value < 0.0001 (comparative tip and shaft localization for FMNL3 and VASP), NS (comparative shaft localization for FMNL3 and VASP at 60 min), as calculated by two sample t-test. Error bars, SD: cyan indicate tip, magenta indicate shaft.



Supplementary Figure 6. Effects of VASP suppression on spreading cells
(A) Quantification of filopodia/adhesion relationship in VASP-depleted U2OS cells (siRNA #2-treated) spreading on collagen. N = 116 filopodia, 9 cells, 2 experiments. Error bars, SD. **(B)** Effect on filopodial number during cell spreading. U2OS cells treated with scrambled siRNA or VASP siRNA, co-expressing GFP-paxillin and mApple–F-tractin were imaged for filopodial number over a 10 min time-course, within the first 28 min during spreading on collagen. Whole cell edge was analyzed. N = 182-226 filopodia, 8 ROI, 4 cells, 3 experiments. P-value = 0.45. Error bars, SD**. (C)** Percentage of total spread area, control and VASP depleted cells. N = 5 cells, 3 experiments. Error bars, SD**.**



Movie 1: Association of FMNL3-associated filopodia with focal adhesions U2OS cell co-transfected with FMNL3-GFP (green), mApple-F-tractin (red), and BFP-vinculin (white), plated on fibronectin-treated coverslip for 18 h. Confocal microscopy with a single ventral image acquired every 20 sec for 10 min. Corresponds with Fig. 1B. Scale bar 1 μm.

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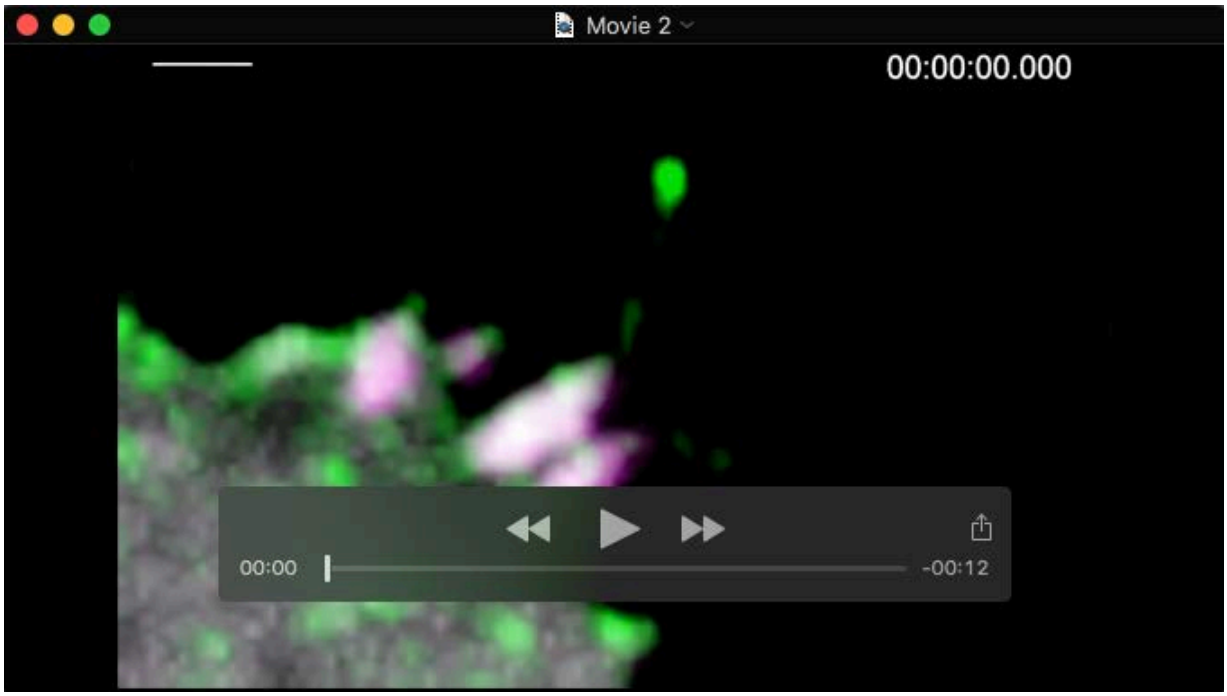
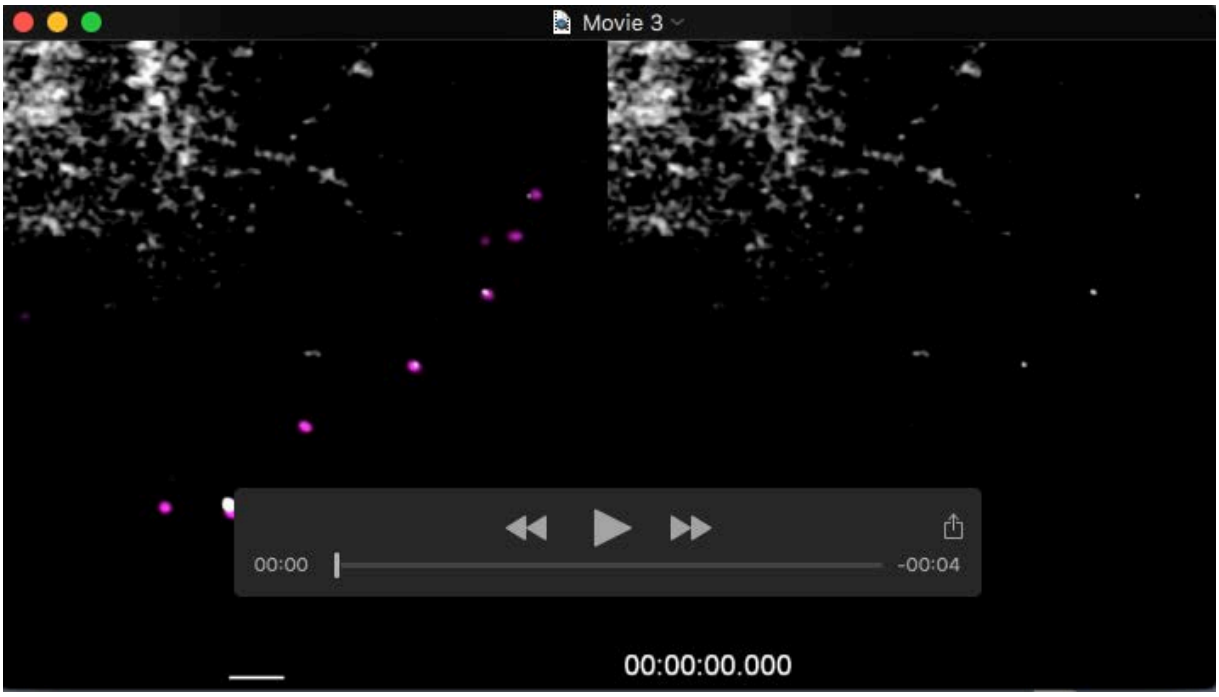


Movie 2: VASP is not enriched in FMNL3-mediated filopodia

U2OS cell co-transfected with FMNL3-GFP (green), mCherry-VASP (white), and BFP-vinculin (magenta), plated on fibronectin-treated coverslip for 18 h. Confocal microscopy with a single ventral image acquired every 20 sec for 10 min. First part of movie shows all three channels, second part shows mCherry-VASP (white) alone. Corresponds with Fig. 2D. Scale bar 1 μm.



Movie 3: VASP is enriched at filopodial tips in myosin-X expressing cells

U2OS cell co-transfected with GFP-myosin-X (magenta) and mCherry-VASP (white), plated on fibronectin-treated coverslip for 18 h. Airyscan microscopy with a single ventral image acquired every 1 min for 20 min. Movie contains overlay of both channels on left hand side, and VASP alone on right hand side. Corresponds with Fig. 5C. Scale bar 2 µm.

Movie 4: Examples of filopodial shaft and base adhesions during cell spreading

U2OS cell co-transfected with mApple-F-tractin (red) and GFP-paxillin (white), plated on fibronectin-treated coverslip for 18 min before imaging. Airyscan microscopy with a single ventral image acquired every 30 sec for 12 min. First part of movie shows both channels, second part shows GFP-paxillin (white) alone. Cyan arrow indicates shaft adhesion, green arrow indicates base adhesion. Corresponds with Fig. 7A. Scale bar 2 μm.

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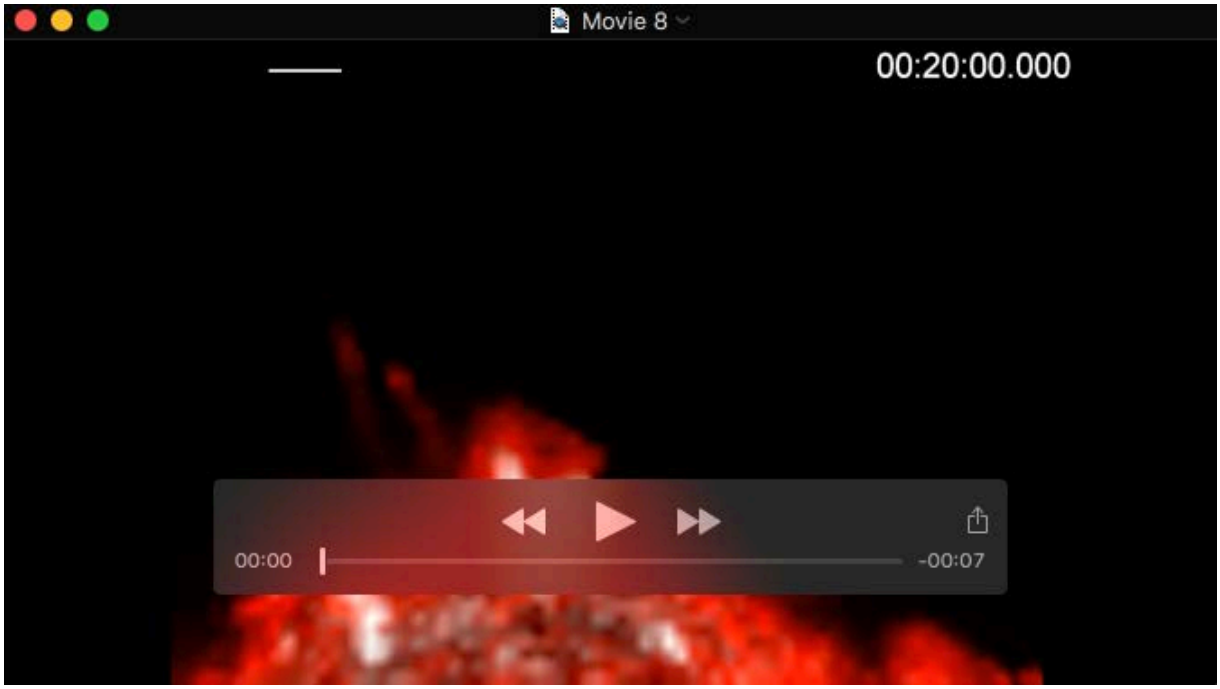

Movie 5: Example of simultaneous filopodial and adhesion assembly during cell spreading

U2OS cell co-transfected with mApple-F-tractin (red) and GFP-paxillin (white), plated on fibronectin-treated coverslip for 20 min before imaging. Airyscan microscopy with a single ventral image acquired every 30 sec for 4 min. First part of movie shows both channels, second part shows GFP-paxillin (white) alone. Red arrow indicates location of simultaneous filopodia and adhesion assembly. Corresponds with Fig. 7B. Scale bar 1 μm.

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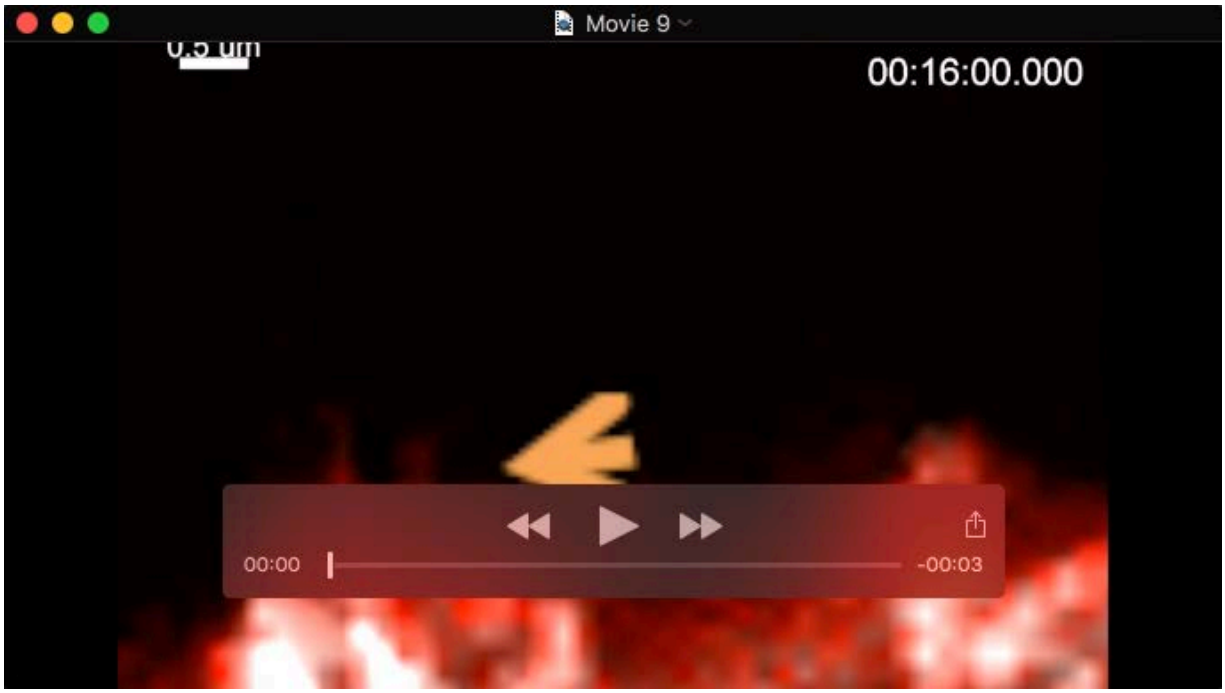

Movie 6: Example of filopodium assembles without an accompanying new adhesion assembly during cell spreading

U2OS cell co-transfected with mApple-F-tractin (red) and GFP-paxillin (white), plated on fibronectin-treated coverslip for 16 min before imaging. Airyscan microscopy with a single ventral image acquired every 30 sec for 2 min. First part of movie shows both channels, second part shows GFP-paxillin (white) alone. Orange arrow indicates location of filopodia assembly with no adhesion assembly. Corresponds with Fig. 7C. Scale bar 0.5 μm.

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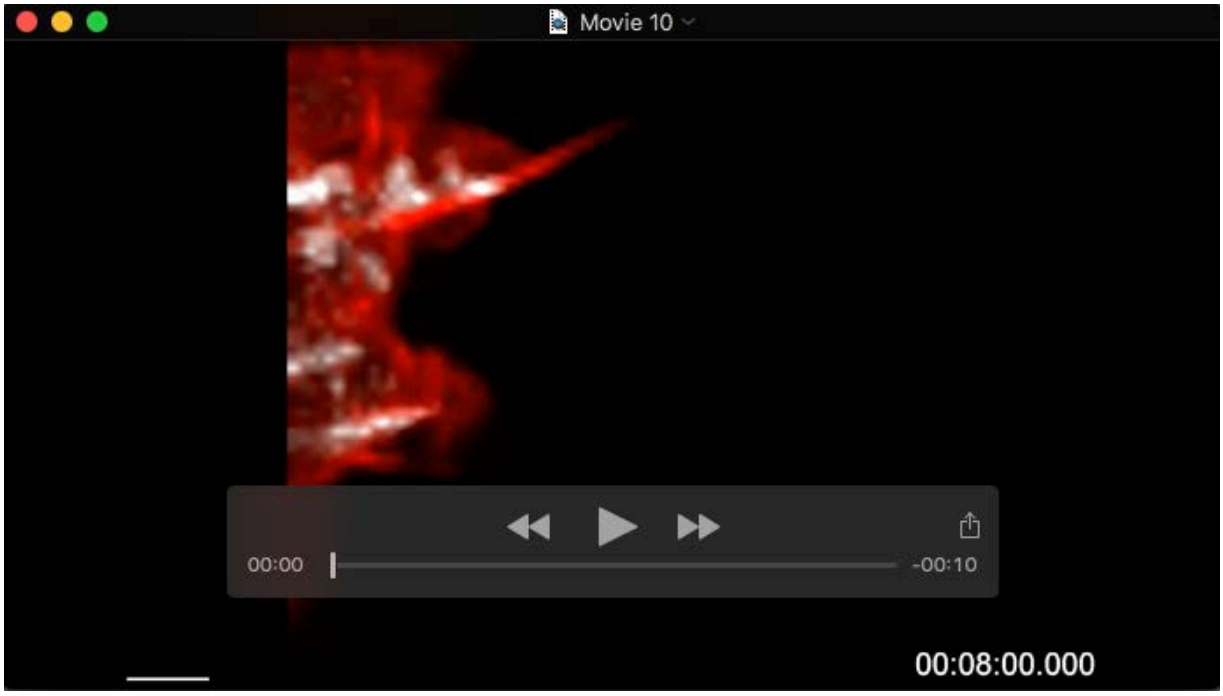

Movie 7: Tip adhesion assembly in VASP-depleted cell

VASP depleted U2OS cell co-transfected with mApple-F-tractin (red) and GFP-paxillin (white), plated on fibronectin-treated coverslip for 8 min before imaging. Airyscan microscopy with a single ventral image acquired every 30 sec for 11:30 min:sec. First part of movie shows both channels, second part shows GFP-paxillin (white) alone. Magenta arrows indicate two separate tip adhesions, which transition sequentially to shaft then base adhesions. Corresponds with Fig. 8A. Scale bar 2 μm.