

VASP cooperates with zyxin to organize mechanically competent adhesions

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Supplementary figures

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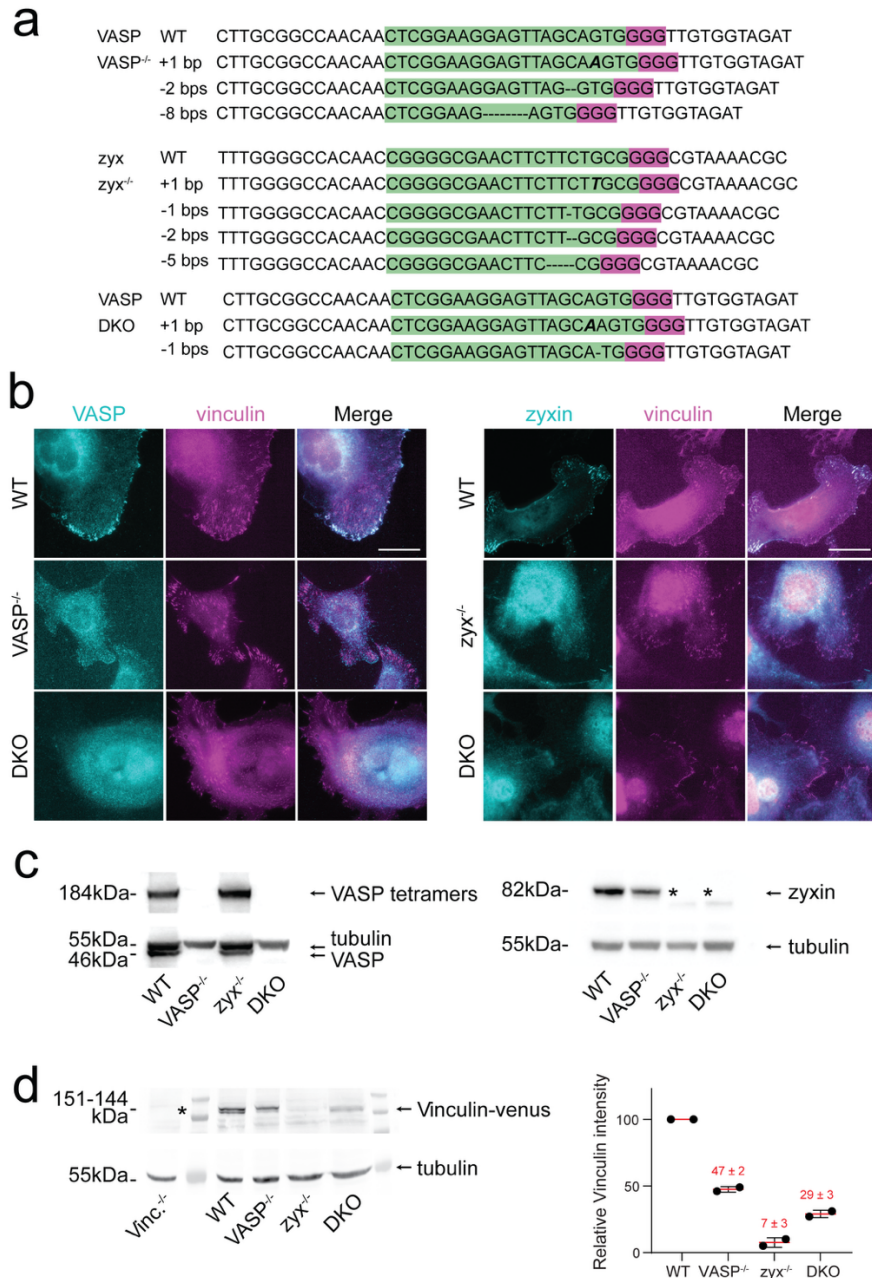


Figure S1. Genotyping and validation of VASP^{-/-}, zyxin^{-/-}, and DKO MEFs. **a**, Genomic sequences of WT and KO cells at the CRISPR-Cas9 target sites. Guide RNA binding regions and Protospacer Adjacent Motif (PAM) sequences following the target site are highlighted in green and magenta, respectively. **b**, Representative immunofluorescence images showing VASP and zyxin expression in WT and KO cells. Scale bar, 10 μ m. **c**, Representative immunoblots of VASP and zyxin expression in WT and KO cells. α -tubulin served as a loading control. * : zyxin fragments are detectable due to truncated expression resulting from a nonsense mutation downstream of the N-terminal knockout sites. **d**, A representative immunoblot of vinculin-Venus expression in WT and KO cells. Tubulin served as a loading control. Quantification from two independent experiments showed that loss of zyxin markedly impaired vinculin expression compared to the loss of VASP alone or to the combined loss of VASP and zyxin. Red horizontal lines represent mean values, bars, and standard deviations (related to Figure 1)

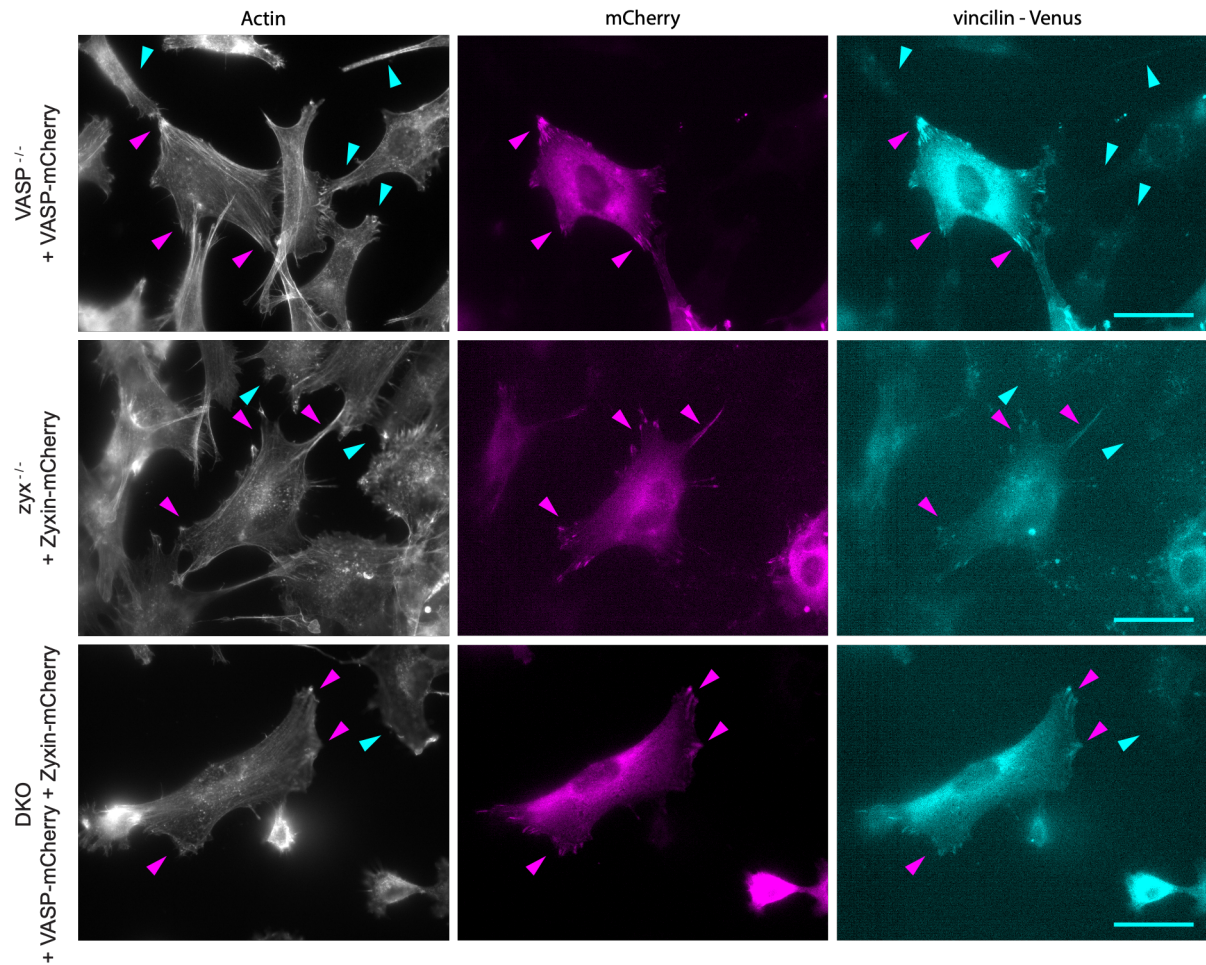


Figure S2. Rescue of VASP and/or zyxin expression in knockout cell lines restores the organization of the actin cytoskeleton, cell spreading and vinculin to the adhesion complexes. Representative fluorescence images of VASP^{-/-}, zyxin^{-/-}, and VASP/zyxin DKO cells following transient rescue with VASP-mCherry and/or zyxin-mCherry. Cells were stained for F-actin with Alexa Fluor 647-phalloidin, while adhesion complexes were visualized by vinculin-Venus. Rows correspond to rescue with VASP-mCherry (top), zyxin-mCherry (middle), or co-expression of VASP-mCherry and zyxin-mCherry (bottom). Magenta arrowheads indicate transfected, well-spread cells exhibiting VASP-mCherry- or zyxin-mCherry-enriched adhesion complexes. Cyan arrowheads indicate neighboring non-transfected KO cells, which display reduced vinculin recruitment to adhesion complexes and a less organized actin cytoskeleton, particularly in zyxin^{-/-} and DKO cells. Scale bar, 20 μ m.

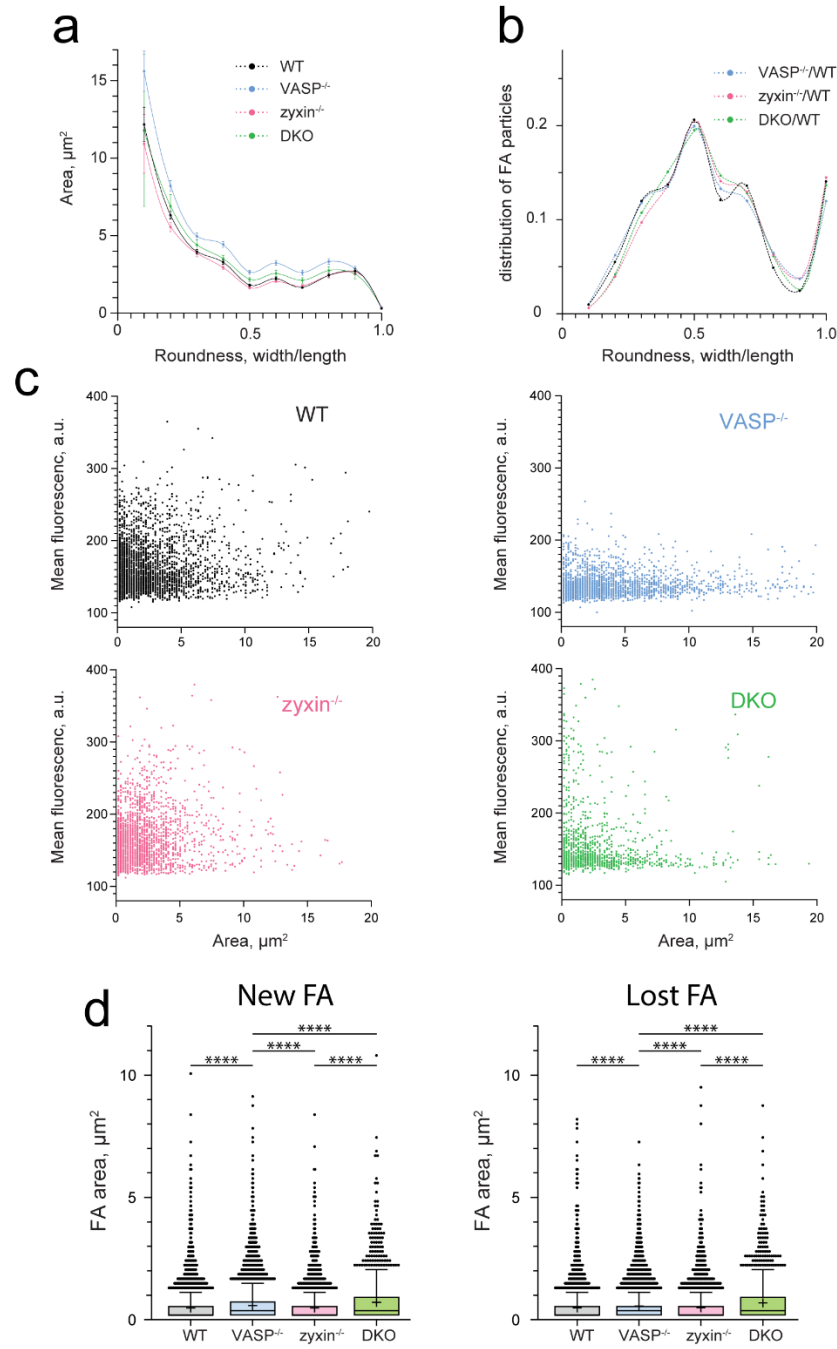


Figure S3. Quantitative analysis of adhesion particles in wildtype and KO cells. **a-c**, Adhesion particles were segmented using Ilastik (Berg et al., 2019) and built-in plugins in Fiji (ImageJ), and analyzed in MATLAB. No significant differences were observed between cell lines in the variation of adhesion area as a function of roundness (**a**), the distribution of adhesion roundness (**b**), or vinculin mean fluorescence relative to adhesion area (**c**). **d**, Comparison of the distribution of newly formed or lost adhesion particles across WT and KO cell lines revealed statistically significant differences, although changes in median (cross) and mean (bar) values were negligible. Statistical analysis was performed using one-way ANOVA and Holm-Šídák multiple comparisons test; p-values are indicated as **** < 0.0001 . (Related to Figure 1)

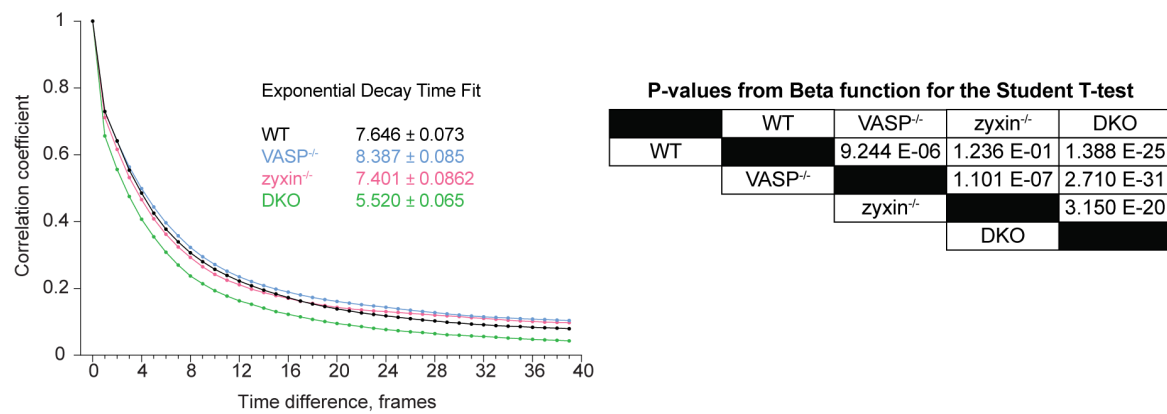


Figure S4. Dynamics of adhesion-associated vinculin assessed by autocorrelation analysis. Relative intensities of all adhesions pixels at two time points were compared. Autocorrelation analysis was performed on segmented images using Ilastik (Berg et al., 2019). Values represent the average of six independent movies per phenotype. Exponential decay times are reported $\pm$ standard deviation, and statistical significance was determined using the Beta function for the Student's t-test (Zaidel-Bar et al., 2007b; related to Figure 1).

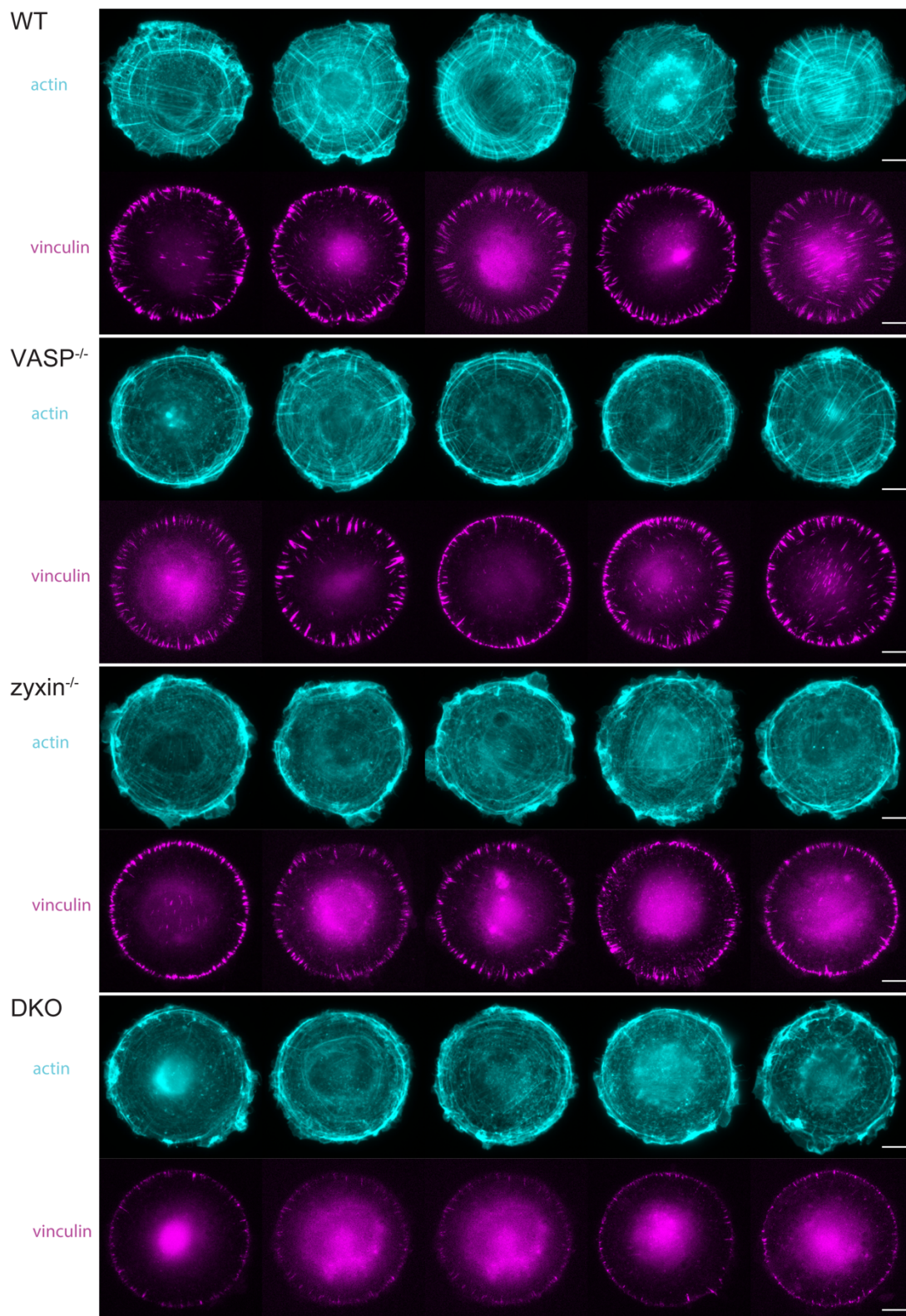


Figure S5. Loss of VASP and zyxin affects FA, actin network organization, and membrane protrusions of cells on disk patterned surfaces. Five representative images of MEFs spread on circular micropatterns (48 μm in diameter, $\sim 1810 \mu\text{m}^2$), selected from a total of 112 for WT, 104 for VASP^{-/-}, 129 for zyxin^{-/-}, 96 for DKO cells analyzed. Vinculin and actin are shown in magenta and cyan, respectively. Scale bar, 10 μm . (related to Figure 2 and supplementary movies).

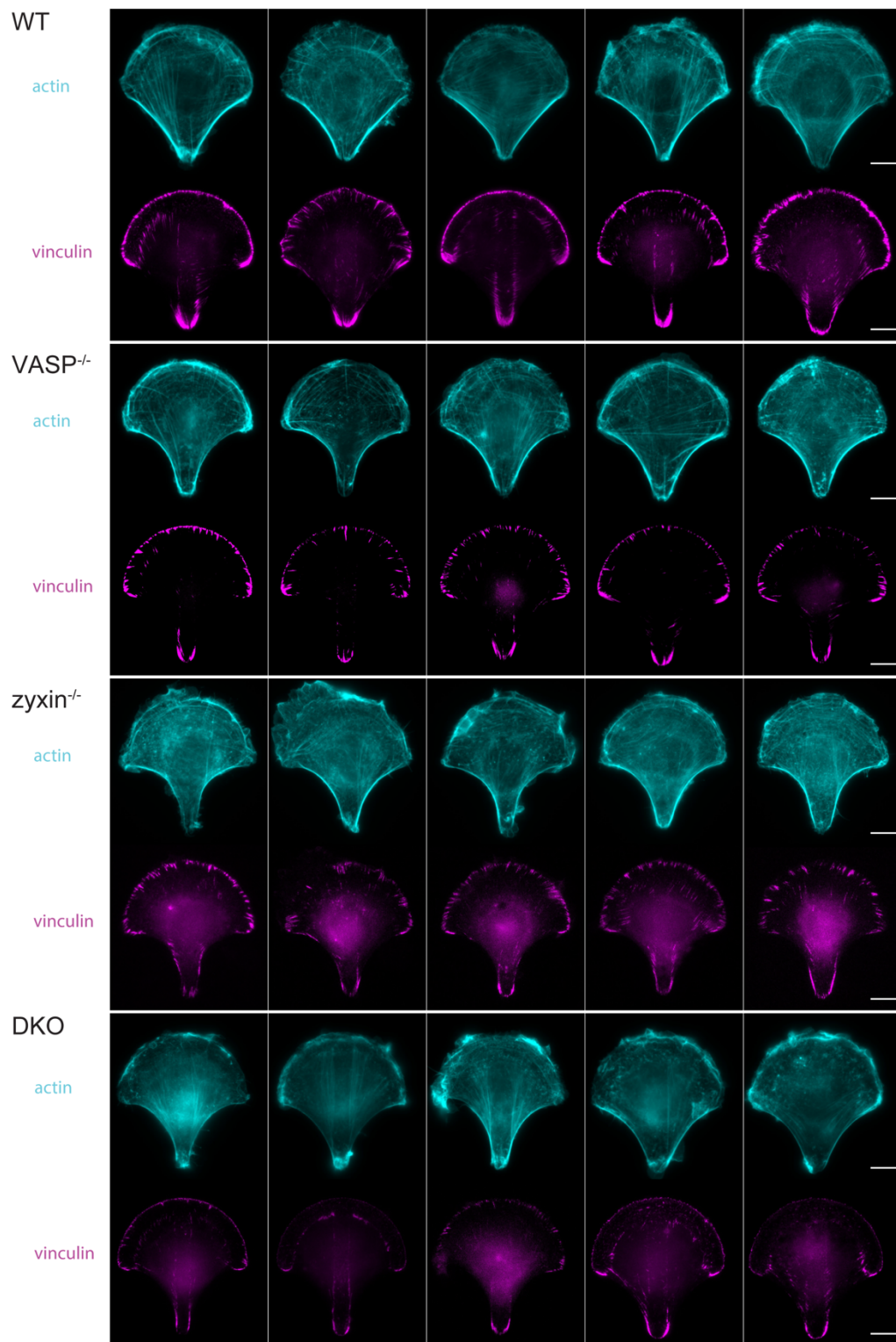


Figure S6. Loss of VASP and zyxin affects FA, actin network organization, and membrane protrusions of cells on crossbow patterned surfaces. Five representative images of MEFs spread on crossbow micropatterns (50 μm in height, 48 μm width, $\sim 940 \mu\text{m}^2$ maximum spread area), selected from a total of 66 for WT, 62 for VASP^{-/-}, 68 for zyxin^{-/-}, 49 for DKO cells analyzed. Vinculin and actin are shown in magenta and cyan, respectively. Scale bar, 10 μm . (related to Figure 2 and supplementary movies).

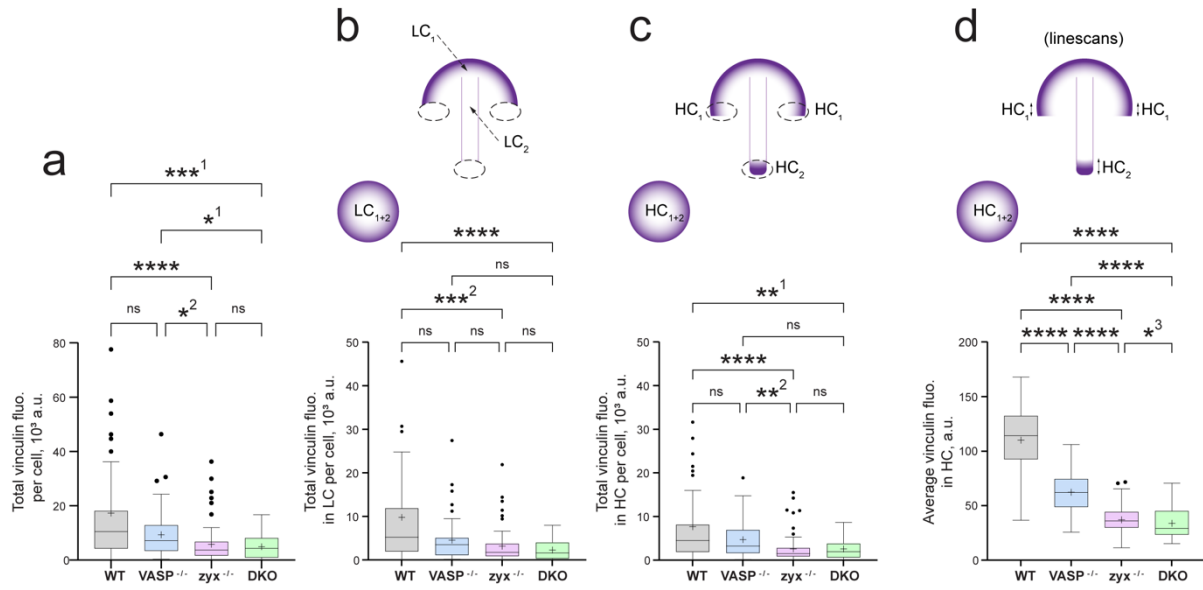


Figure S7. Quantitative analysis of vinculin density in low- and high-curvature regions of cells spread on crossbow micropatterns. **a**, Total vinculin fluorescence intensity measured in segmented adhesion particles for each cell line. Each dot represents the total vinculin fluorescence per cell. A total of 65 for WT, 55 for VASP^{-/-}, 68 for zyxin^{-/-}, 37 for DKO cells were analyzed. Cells with vinculin fluorescence barely distinguishable from background could not be reliably segmented and were excluded from the analysis. **b,c**, Quantification of total vinculin fluorescence intensity in low-curvature (LC1 and LC2, patterned region excluding encircled HC areas) and high-curvature (HC1 and HC2, as defined by the circled areas) regions. Although zyxin-deficient and DKO cells exhibited a significant reduction in vinculin recruitment, VASP-deficient cells showed no significant difference compared with WT, indicating that the localized phenotype observed in average maps (**Fig 2a,d**) is diluted when the full high-curvature region is analyzed. **d**, Quantification of vinculin fluorescence associated with segmented focal adhesions within a restricted high-curvature region corresponding to the line-scan analysis shown in Fig. 2d. Under these conditions, VASP-deficient cells displayed a significant reduction in vinculin recruitment, consistent with the averaged fluorescence maps. These data indicate that VASP primarily regulates the spatial enrichment of vinculin at the most mechanically loaded adhesions rather than the total adhesion-associated vinculin pool within the entire high-curvature region. **a-d**, Box-and-whisker plots were generated using the Tukey method. Boxes represent the interquartile range (25th–75th percentiles), the horizontal line indicates the median, and the cross shows the mean. Whiskers extend to the most extreme values within $1.5 \times$ the interquartile range; values beyond this range are shown as individual outliers. Statistical significance was assessed using the Kruskal–Wallis test or one-way analysis of variance (ANOVA), as indicated. The p values for **c-g**: **** = <0.0001 / ***_{1,2} = 0.0004 / **₁ = 0.0094 / **₂ = 0.0026 / *₁ = 0.0490 / *₂ = 0.0215 / *₃ = 0.0157 / n.s.>0.05. (related to Figure 2)

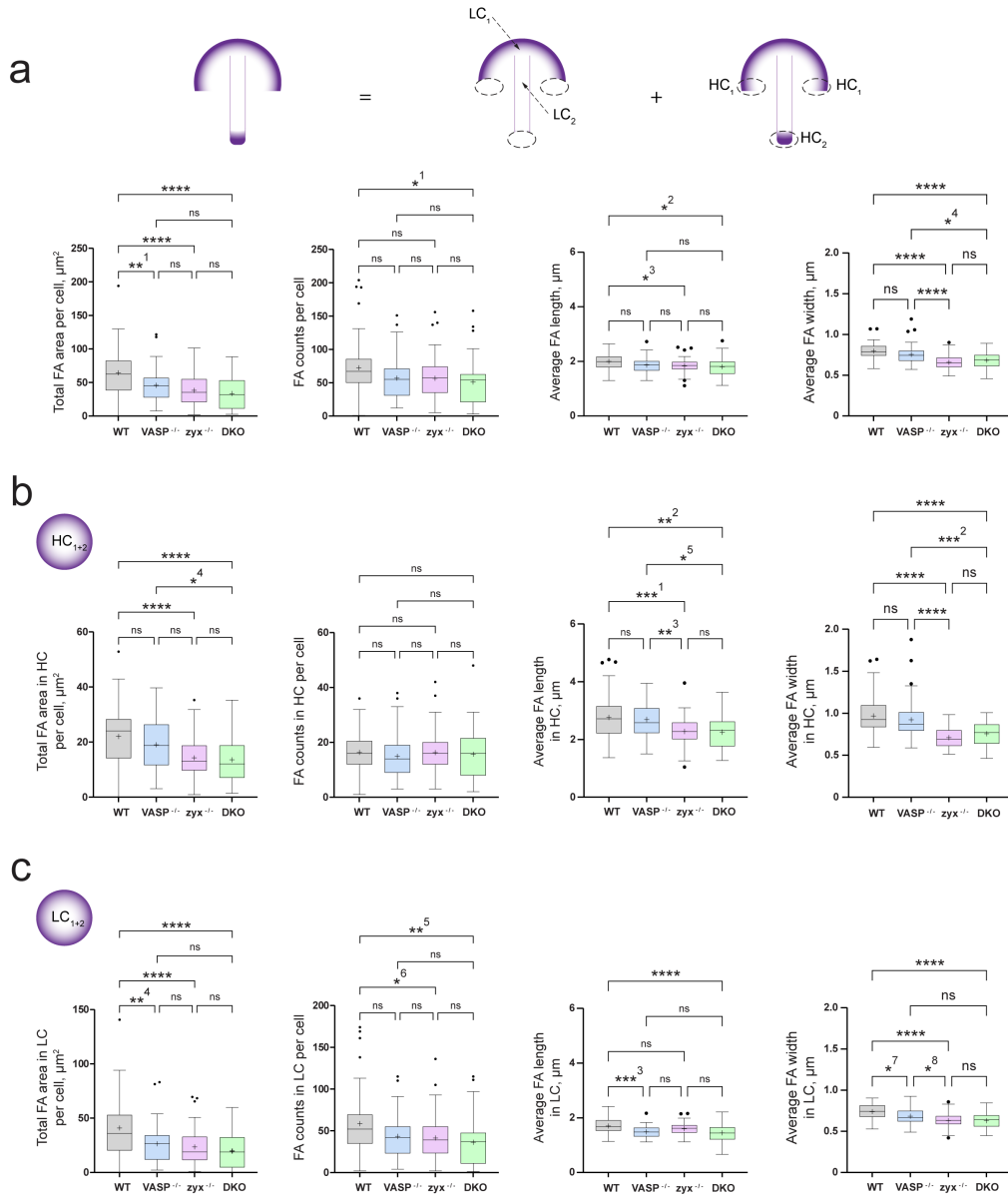


Figure S8. Quantitative morphological analysis of vinculin density in low- and high-curvature regions of cells spread on crossbow micropatterns. **a**, Total focal area, count, average length and width of adhesions were measured using segmented particles for each cell line. Each dot represents the measurements per cell. A total of 65 for WT, 55 for VASP^{-/-}, 68 for zyxin^{-/-}, 37 for DKO cells were analyzed. Cells with vinculin fluorescence barely distinguishable from background could not be reliably segmented and were excluded from the analysis. **b and c**, Quantifications were performed in high-curvature (HC1 and HC2, **b**) and low-curvature (LC1 and LC2, **c**) regions, as defined by the dotted lines. The most significant morphological effects were observed in the high-curvature regions of zyxin^{-/-} and DKO cells, where adhesions are expected to experience higher mechanical tension. **a-c**, Box-and-whisker plots were generated as in Fig. S7. The p values for **c-g**: **** = <0.0001 / ***1 = 0.0002 / ***2 = 0.0007 / ***3 = 0.0001 / ** 1 = 0.0068 / *2 = 0.0013 / ***3 = 0.0043 / **4 = 0.0022 / **5 = 0.0037 / *1 = 0.0455 / *2 = 0.0192 / *3 = 0.0187 / *4 = 0.0106 / *5 = 0.0150 / *6 = 0.0116 / *7 = 0.0112 / *8 = 0.0154 / n.s.>0.05. (related to Figure 2).

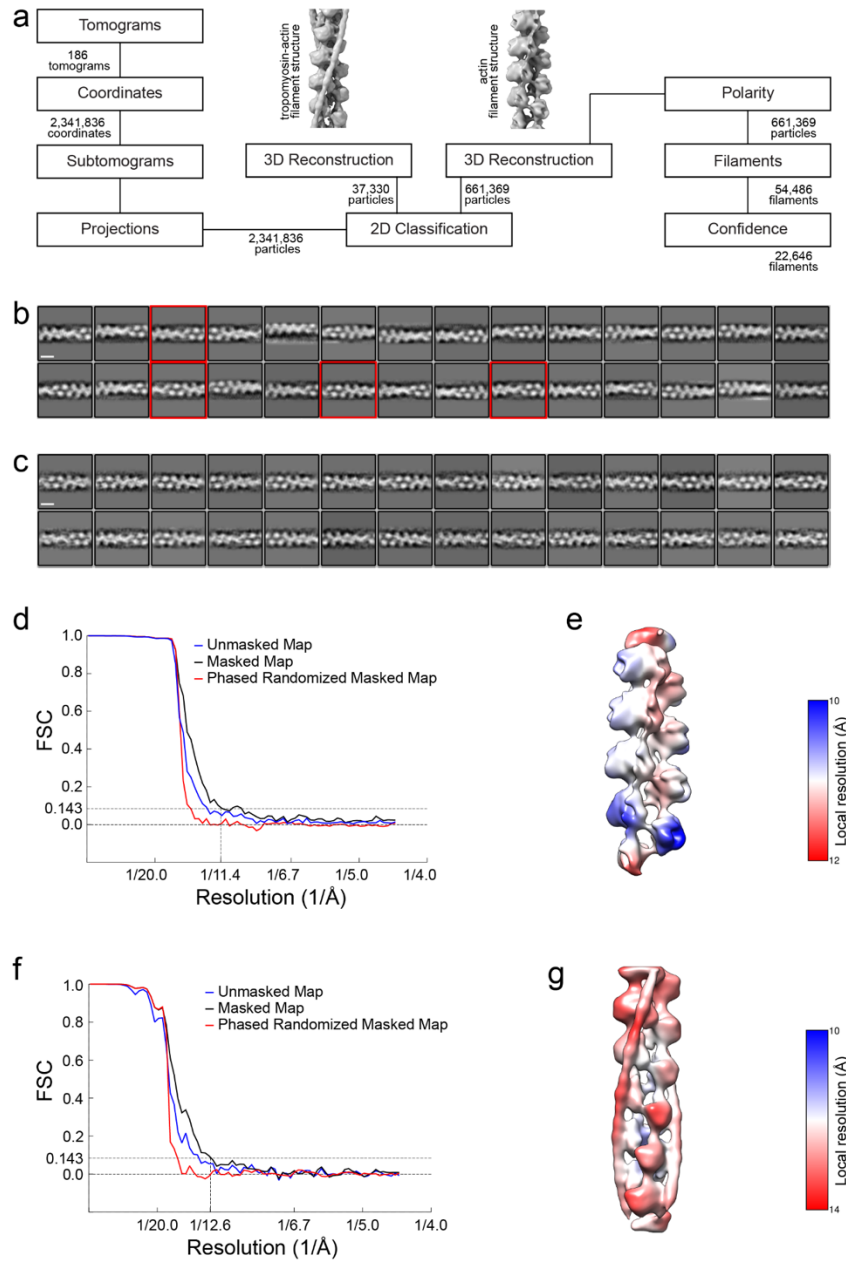


Figure S9. **Reconstruction of actin and tropomyosin-actin filaments.** **a**, Schematic overview of the subtomogram averaging pipeline based on APT. **b**, Representative two-dimensional (2D) class averages of actin segments. Classes displaying tropomyosin features are highlighted in red and used for reconstruction of tropomyosin-actin. Box size, 35 nm; scale bar, 8 nm. **c**, Representative two-dimensional class averages of tropomyosin-actin segments. Box size, 35 nm; scale bar, 8 nm. **d**, FSC plot of the actin filament structure indicating a resolution of 11.4 Å, as determined using the 0.143 threshold criterion. **e**, Actin filament structure colored according to local resolution, which ranges from 10 Å (dark blue) to 12 Å (red), as indicated by the color bar. **f**, FSC plot of the refined tropomyosin-actin filament structure indicating a resolution of 12.6 Å, as determined using the 0.143 threshold criterion. **g**, Tropomyosin-actin filament structure colored according to local resolution, which ranges from 10 Å (dark blue) to 14 Å (red), as indicated by the color bar.

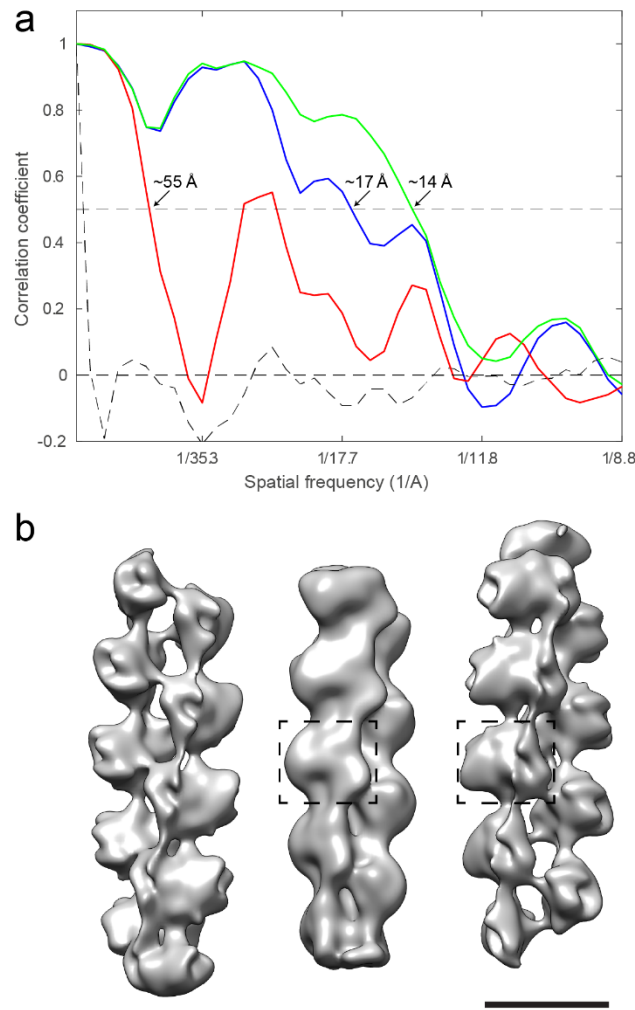


Figure S10. Determination of polarity of the actin filament reconstruction. **a**, FSC curves between the actin filament reconstruction (blue curve) and the tropomyosin-actin filament reconstruction (green curve), each docked into EMD-15106 as an external high-resolution reference. Using the FSC = 0.5 criterion, the upper resolution limits are ~17 Å for the actin filament reconstruction and ~14 Å for the tropomyosin-actin filament reconstruction. Reversing the orientation of the actin filament reconstruction (red curve) reduces the agreement with the external reference to ~55 Å. The dashed line shows the correlation between two masked noise volumes, using the same mask that was used for the calculation of the FSC curves. This indicates that the used mask has only a minor effect on the estimated upper resolution limits. **b**, Three actin filament structures low-pass filtered to 17 Å are shown. The central structure displays EMD-15106 as a reference with known polarity, while the left and right structures show the actin filament reconstruction docked in the two possible polarity orientations. In the orientation shown on the right, the shape of individual actin monomers agrees well with the reference (dashed boxes). Scale bar, 8 nm.

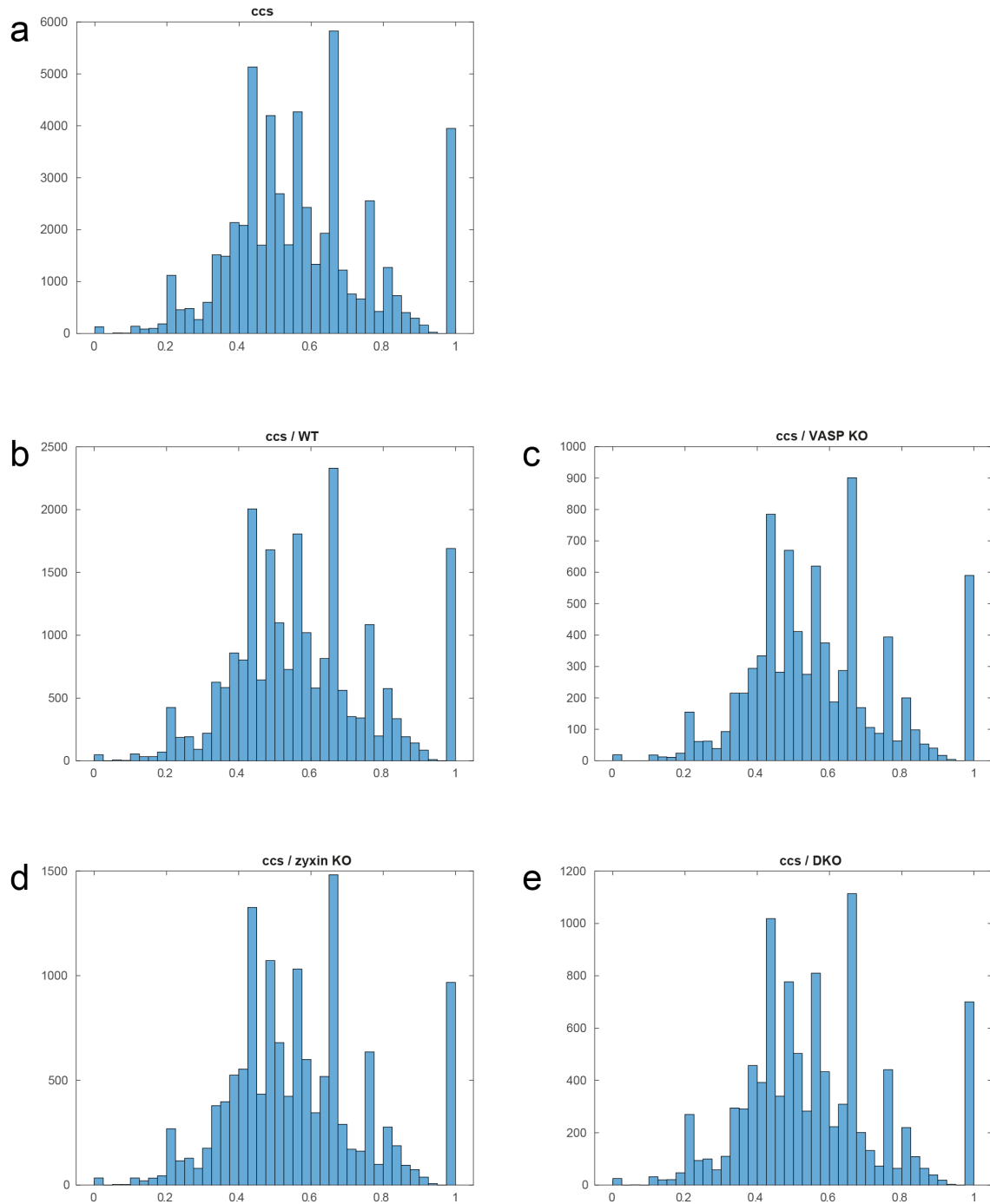


Figure S11. Confidence analysis of actin polarity determination. **a**, In total 54,486 actin filaments were extracted from 141 tomograms of all cellular conditions. The histogram shows the ccs values of these filaments. A number of 22,646 filaments pass the $\text{ccs} \geq 0.6$ confidence criterion for polarity determination. **b**, Histogram of ccs values from 9,735 filaments extracted from 57 tomograms of WT cells. **c**, Histogram of ccs values from 3,363 filaments extracted from 19 tomograms of VASP KO cells. **d**, Histogram of ccs values from 5,626 filaments extracted from 44 tomograms of zyxin KO cells. **e**, Histogram of ccs values from 3,922 filaments extracted from 21 tomograms of DKO cells.

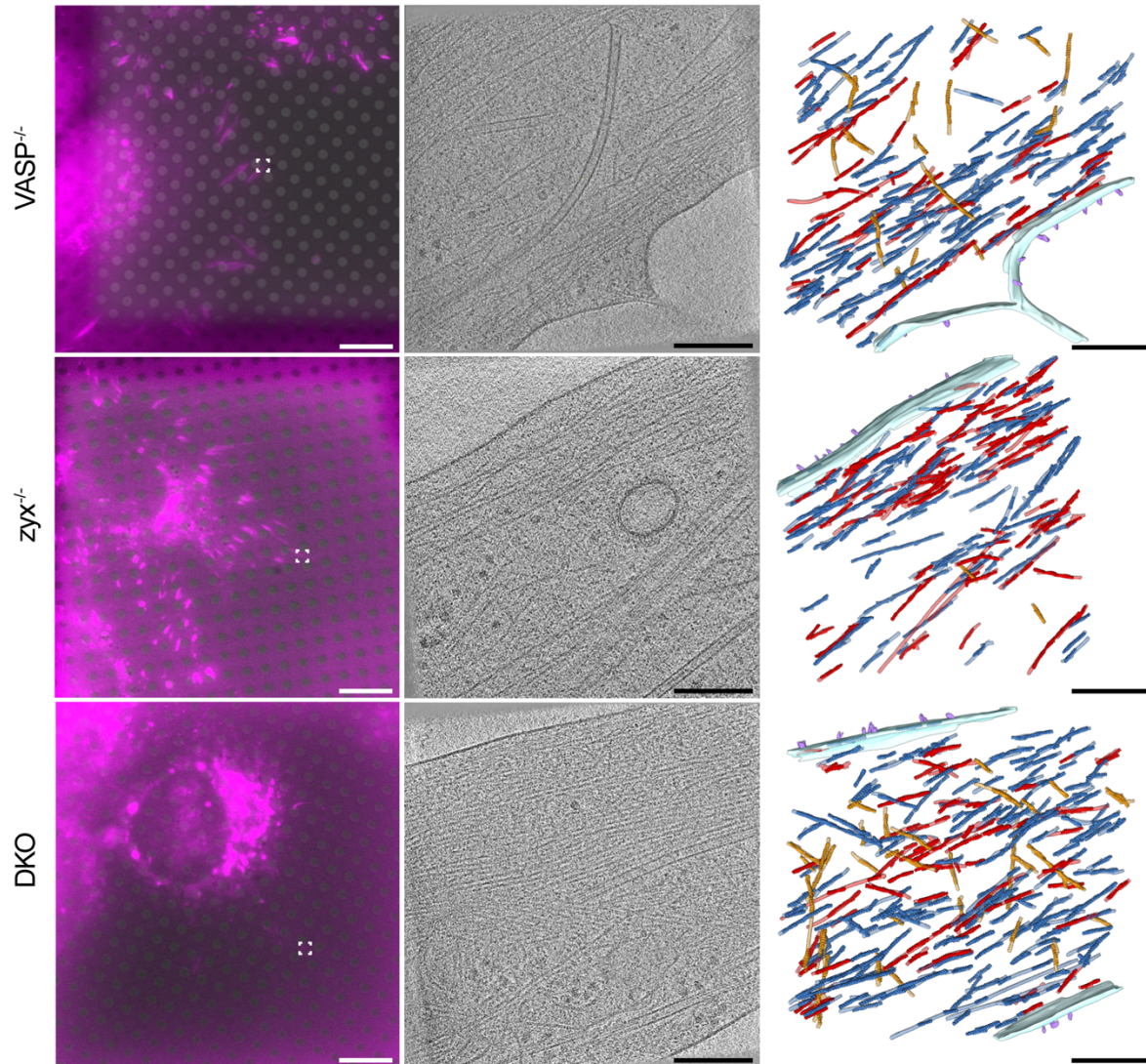


Figure S12. **Correlative light and electron microscopy (CLEM) of knockout (KO) cells.** Representative fluorescence images of KO cells expressing vinculin on electron microscopy (EM) grids are shown in the left panel. Scale bar, 10 μm . The middle panel presents an x-y slice of the tomogram acquired at the indicated regions (white boxes). The right panel displays isosurface renderings of the corresponding tomograms. Scale bar, 200 nm. Filaments are colored as follows: forward filaments (blue) have their barbed ends oriented toward the cell periphery along the FA axis; backward filaments (red) are oriented in the opposite direction; transverse filaments (brown) are not aligned with the FA axis. The plasma membrane was manually segmented and is shown in light blue. Scale bar, 200 nm.

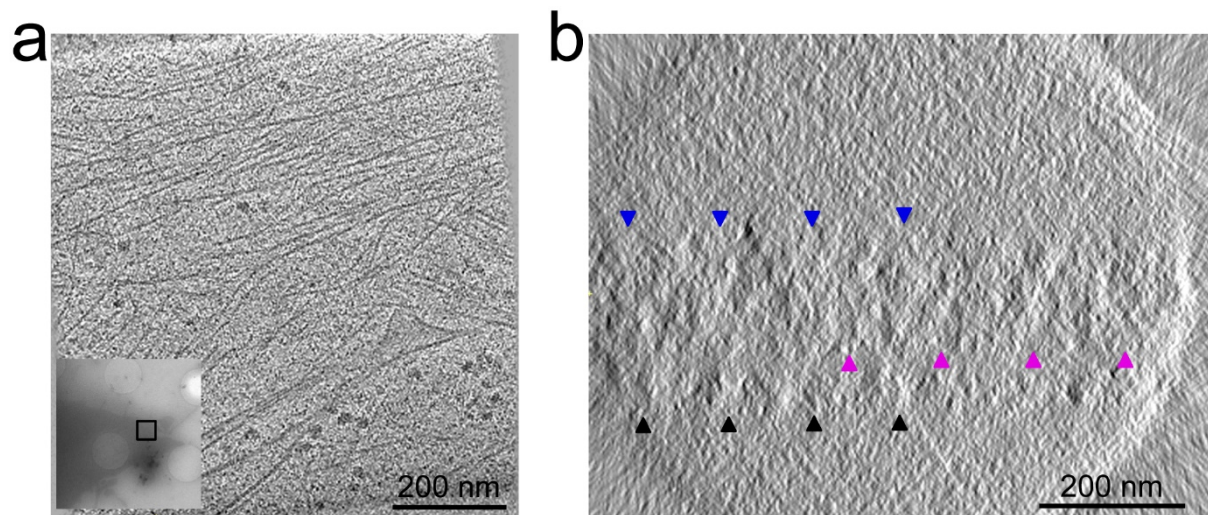


Figure S13. **Determination of substrates within tomograms.** **a**, Representative x-y slice of a tomogram at a focal adhesion. A corresponding low-magnification correlative image is shown at the bottom left. **b**, x-z slice of the tomogram shown in a. The substrate beneath the cell on the EM grid is marked by magenta arrowheads. Blue and black arrowheads indicate the top of the sample and the opposite side of the EM grid, respectively.

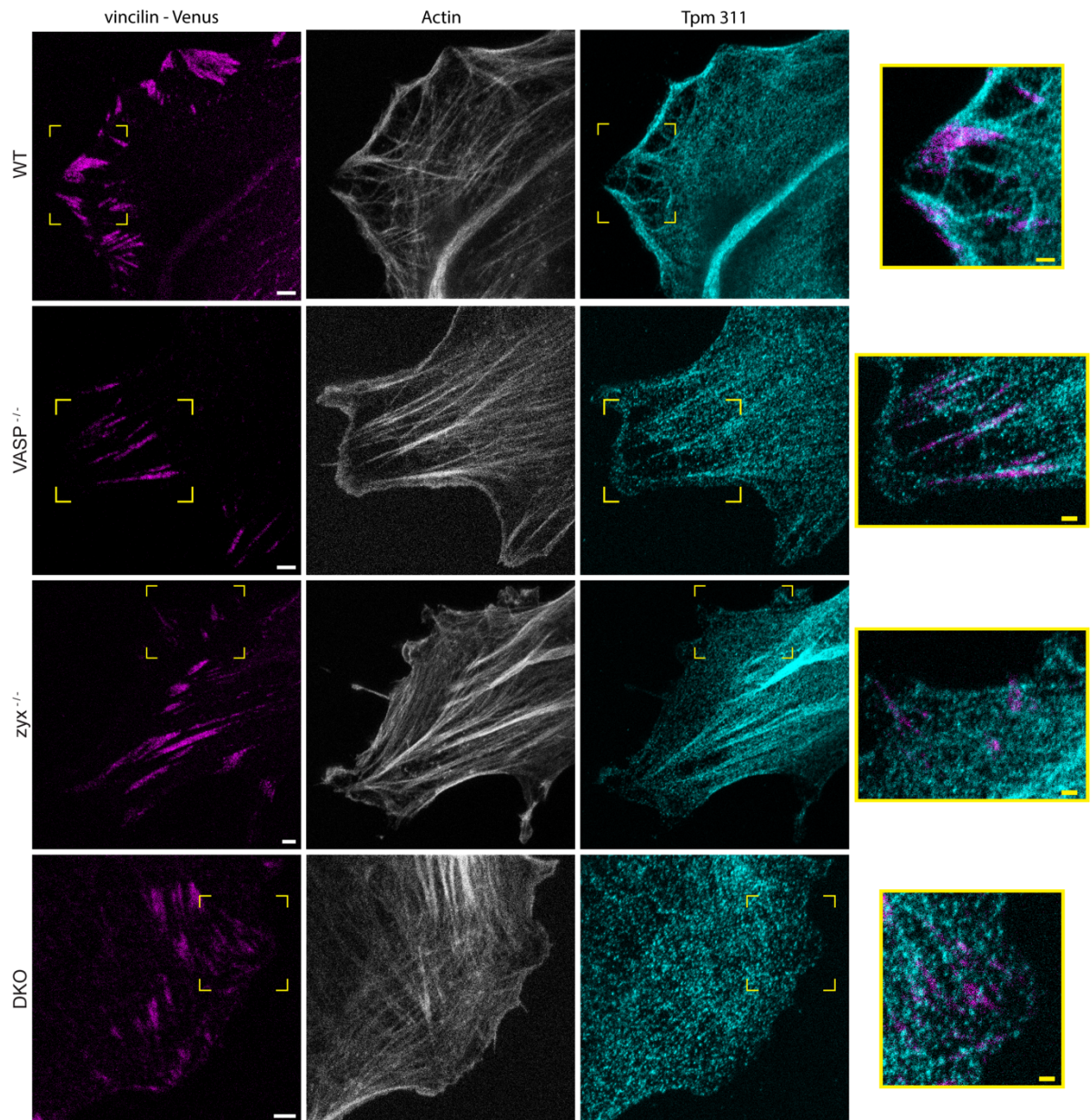


Figure S14. Distribution of vinculin, actin cytoskeleton, and tropomyosin at the protruding cell edge. Representative confocal images of vinculin and super-resolution STED (STimulated Emission Depletion) images of F-actin and tropomyosin at the protruding cell edge in wild-type and knockout cells. Scale bar, 2 μm . The yellow boxed region indicates the area enlarged in the rightmost images, highlighting the spatial relationship between vinculin-containing adhesions and tropomyosin. Scale bar, 1 μm . In the WT and VASP^{-/-} cells, tropomyosin immunostaining decorated the proximal region of the adhesions. In contrast, zyx^{-/-} and DKO cells exhibited smaller and less intense adhesions, accompanied by a more diffuse actin network and diffuse tropomyosin staining. (related to Figure 4)

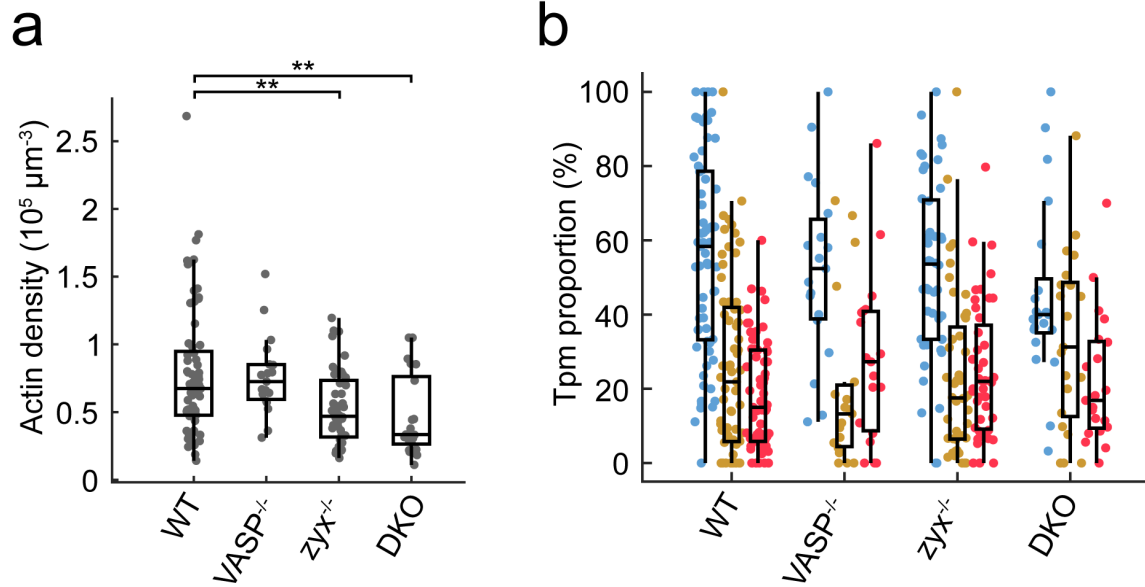


Figure S15. **Actin filament density and tropomyosin distribution at focal adhesions in wild-type (WT) and knockout (KO) cells.** **a**, Actin filament density at FAs in WT and KO cells. Parameters were measured using 57 tomograms for WT, 19 for VASP^{-/-}, 44 for zyx^{-/-}, and 21 for DKO. Statistical comparisons using the Tukey-Kramer method, and a one-way analysis of variance (ANOVA) showed significant variations among the measured parameters, **P < 0.01. **b**, Proportion of tropomyosin associated with forward (blue), parallel (brown), and backward (red) filaments at FAs in WT and KO cells.

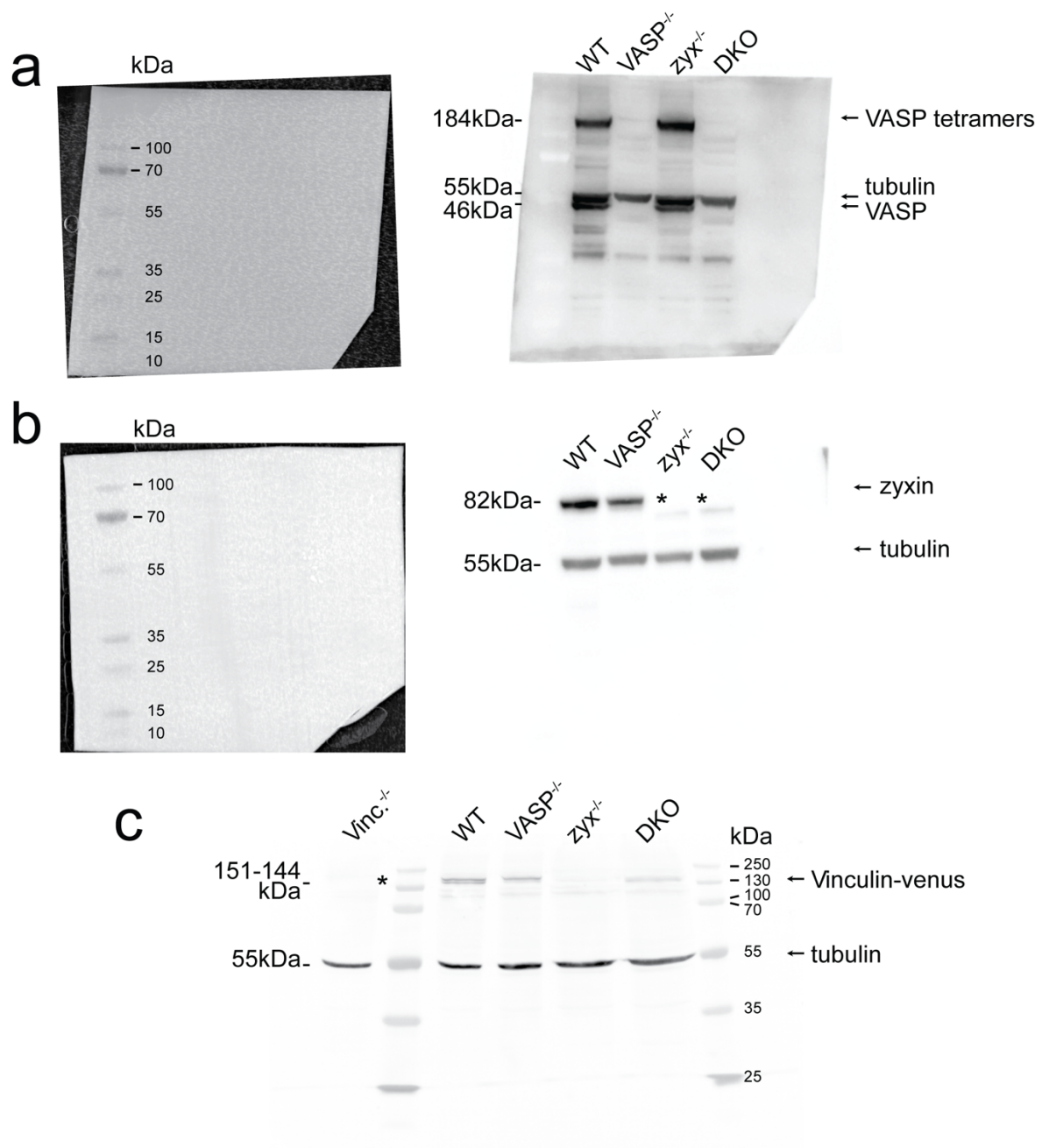


Figure S16. **Whole, uncut, unmodified immunoblot images used to validate VASP^{-/-}, zyxin^{-/-}, and DKO MEFs.** **a,b**, Representative immunoblots of VASP and zyxin expression in WT and KO cells. * : zyxin fragments are detectable due to truncated expression resulting from a nonsense mutation downstream of the N-terminal knockout sites. Molecular weights were imaged using transmitted light (leftmost images). **c**, A representative immunoblot of vinculin-Venus expression in WT and KO cells. a-c, α -tubulin served as a loading control. (related to Supplementary Figure S1)