

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☐ ☐ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☐ ☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection

- For cryo-EM : SerialEM
- For fluorescence microscopy : LAS X software (Leica), CellSens software (Olympus), SoftWoRx and Resolve3D 352 software (Applied Precision LLC)

Data analysis

- For cryo-EM :
ATP code is available at https://github.com/WChung2/actin_polarity_toolbox_adhesion
MATLAB (R2019b)
Actin Polarity Toolbox (APT, 1.0)
IMOD (4.9.12)
crYOLO (1.9.9)
RELION(3.0.8, 4.0.0)
UCSF Chimera (1.9)
- For fluorescence microscopy :
MATLAB (R2019b)
Fiji/ImageJ2 software
GraphPad Prism 10
Ilastik (Berg et al., 2019. doi: 10.1038/s41592-019-0582-9)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The EM structures of actin filament and tropomyosin-actin filament were uploaded on the Electron Microscopy Data Bank with the accession codes EMD-17742 and EMD-17744, respectively. A tomogram of WT, zyxin-/-, VASP-/-, and DKO were uploaded on the Electron Microscopy Data Bank with the accession codes EMD-17745, EMD-17746, EMD-17750, and EMD-17752, respectively.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- ☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Describe how sample size was determined, detailing any statistical methods used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.
Data exclusions	- For cryo-ET : Individual actin subtomograms, which did not contribute to an improvement of resolution in subtomogram averaging, were excluded based on unsupervised 2D classification and 3D classification.
Replication	- For live cell imaging on pattern-free surfaces : more than 3 biologically independent replications were performed for each cell line - For autocorrelation analyses using cells on pattern-free surfaces : the autocorrelation values analyses were the average of 6 independent movies for each phenotype, from three3 biologically independent experiments. - For patterning quantifications, 3 biologically independent replications were performed for each cell line. - For live cell imaging on patterned surfaces : 2 or 4 biologically independent replications were performed for WT and DKO or VASP-/- and Zyxin -/- respectively. - For cryo-ET : 3 biologically independent replications were performed for all EM measurements.
Randomization	- For cryo-ET : For subtomogram averaging and particle analysis the 3D refinement and averaging procedures were performed with random individual half sets. - For fluorescence microscopy, analyses were performed with random sets of cells.
Blinding	N/A

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☐	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

- rabbit polyclonal anti-p34-Arc (Sigma-Aldrich 07-227)
- goat-anti-rabbit IgG H&L Alexa Fluor 488 (Abcam ab150077)
- rabbit polyclonal anti-VASP - from Jan FAIX - Institute for Biophysical Chemistry, Hannover Medical School, Hannover, Germany
- mouse monoclonal anti-zyxin clone 164D4 - from Clemens Rottner - Molecular Cell Biology Group, Helmholtz Centre for Infection Research, Braunschweig, Germany
- Alexa Fluor 647 donkey anti-rabbit (Thermo Fisher Scientific A31573)
- Alexa Fluor 647 donkey anti-mouse (Jackson ImmunoResearch 715-605-150)
- anti- α -tubulin (Sigma-Aldrich T5168)
- Peroxidase AffiniPure donkey anti-mouse (Jackson Immuno Research 715-035-150)
- Peroxidase AffiniPure donkey anti-rabbit (Jackson Immuno Research 711-035-152)
- mouse monoclonal anti-Vinculin hVIN-1 (Sigma-Aldrich V9131)
- goat anti-mouse IgG H&L Alexa Fluor 647 (Abcam ab150115)
- mouse monoclonal anti-tropomyosin 1 and 2 antibody (TM311, Merck)
- Star Orange goat anti-mouse IgG (STORANGE-1001, Abberior)

Validation

- For anti-p34-Arc : https://www.merckmillipore.com/CH/en/product/Anti-p34-Arc-ARPC2,MM_NF-07-227-I-100UG
- For goat anti-rabbit IgG H&L Alexa Fluor 488 : https://www.abcam.com/en-us/products/secondary-antibodies/goat-rabbit-igg-h-l-alex-fluor-488-ab150077?srltid=AfmBOoqv-O3pyXD0DCP-yFBXRJvb5OkLr_9D3-5o87_Y-l2b_r-98HC
- For anti-VASP : Elife. 2020 May 11;9:e55351. doi: 10.7554/eLife.55351.
- For anti-zyxin : Mol Biol Cell. 2001 Oct;12(10):3103-13. doi: 10.1091/mbc.12.10.3103.
- For Alexa Fluor 647 donkey anti-rabbit : <https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-31573>
- For Alexa Fluor 647 donkey anti-mouse : <https://www.jacksonimmuno.com/catalog/products/715-605-150>
- For anti- α -tubulin : https://www.sigmaaldrich.com/CH/fr/product/sigma/t5168?utm_source=google&utm_medium=cpc&utm_campaign=21480226247&utm_content=164812866133&gad_source=1&gad_campaignid=21480226247&gbraid=0AAAAAD8kLQTHQeq9vDYyHGb6PPkFnr-g3&gclid=CjwKCAjwisnGBhAXEiwA0zEOR6hQZPzWD_QdIJH5e-NafC3RYSNWYqSSewoQXjRtQ3d7fr5zBfRCZxoCIRAQAvD_BwE
- For Peroxidase AffiniPure donkey anti-mouse : <https://www.jacksonimmuno.com/catalog/products/715-035-150>
- For Peroxidase AffiniPure donkey anti-rabbit : <https://www.jacksonimmuno.com/catalog/products/711-035-152>
- mouse monoclonal anti-Vinculin hVIN-1 : <https://www.sigmaaldrich.com/CH/fr/product/sigma/v9131?srltid=AU7gw4U7LCSVxkHVx4pXg-dvpBMmP4QykXUizhFcWsnouOJ6sYkknDXF>
- goat anti-mouse IgG H&L Alexa Fluor 647 : <https://www.abcam.com/en-us/products/secondary-antibodies/goat-mouse-igg-h-l-alex-fluor-647-ab150115>
- mouse monoclonal anti-tropomyosin 1 and 2 antibody : <https://www.merckmillipore.com/EG/en/product/sigma/t2780>
- Star Orange goat anti-mouse IgG : <https://abberior.shop/abberior-STAR-ORANGE-goat-anti-mouse-IgG-500-ul-1-mg-ml>

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)

- the parental MEF cell line, MEF VV, is vinculin-/- reconstituted with vinculin-venus, was received from Prof. Carsten Grashoff, Institute for Integrative Cell Biologie und Physiology, Munster, Germany
- VASP-/-, zyxin-/-, and double-knockout (DKO) MEFs were generated from the parental MEF VV line using the CRISPR/Cas9, by sequentially knocking out VASP, zyxin, and both genes respectively.

Authentication

- For MEF VV line : Grashoff et al, Nature. 2010 Jul 8;466(7303):263-6. doi: 10.1038/nature09198.
- For VASP-/-, zyxin-/-, and double-knockout (DKO) MEFs : this study

Mycoplasma contamination

All cell lines used were tested negative for mycoplasma contamination

Commonly misidentified lines (See [ICLAC](#) register)

N/A

Palaeontology and Archaeology

Specimen provenance

N/A

Specimen deposition

N/A

Dating methods

N/A

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight

N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks

N/A

Novel plant genotypes

N/A