

VASP cooperates with zyxin to organize mechanically competent adhesions

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Focal adhesions (FA) are key cellular assemblies that support connectivity between the intracellular cytoskeleton and extracellular matrix. They thereby control cell shape and movement and are key components of cells' mechanosensing machinery. Their organisation and dynamics must be precisely integrated with the dynamics of the actin cytoskeleton but the mechanisms involved in this integration remain to be fully understood.

From among a number of important FA proteins, the authors in this study focus their investigation on zyxin and VASP. They use advanced light microscopy and cryo-electron tomography (cryo-ET) to study CRISPR/Cas9 modified mouse embryonic fibroblast (VASP^{-/-}; zyxin^{-/-} and knock-out of both ("DKO")) to investigate the role of zyxin and VASP in organisation of actin within FAs. FA size and turnover together with overall cell shape and actin organisation are quantified in different genetic backgrounds. In addition, individual actin filament arrangements in portions of FAs in each condition are visualised using cryo-ET. The resolution of these cryo-ET data, supported by sub-volume averaging, allowed the authors to analyse actin filament polarity and also to identify a subset of actin filaments bound by tropomyosin, with each filament population being mapped back into the context of the FA sub-volume.

In general, although the approach taken in each individual experiment is well explained and the findings potentially of interest, as outlined below, there are currently major limitations in the presentation, completeness and analysis of the data, as well as with the internal coherence and consistency, that undermine confidence in the authors' conclusions.

1) The introduction could be more focused to allow the reader to clearly appreciate what questions the authors were seeking to answer and thereby more clearly appreciate their findings – given the complexity of FA dynamics and regulation, it is important to clearly understand the unique contribution of each component that is being tested and the extent to which functional redundancy is observed.

2) The plot in Fig 1b is not properly explained/annotated – what are the individual points compared to the box plots? Although the number of cells for each condition is stated in the legend, what is the n for each condition in the plot, what do the whiskers correspond to and is the dataset size appropriate given the expected effect size – ie is the statistical significance of the observations being driven primarily by the very large dataset of whatever is being measured? The authors should consider using SuperPlots to more effectively analyze their data and its variation (PMID: 32346721) to more robustly ensure this analysis is capturing mechanistically important differences in FA behavior in each cell line.

3) In the micropatterned cellular imaging experiments, the authors state that FA size and density of vinculin within adhesions appears diminished when zyxin is knocked out. Could this result from reduced vinculin expression? A western blot to show total protein levels of vinculin across the different cell lines would clarify this point.

4) Given the classification of different types of FAs presented in Fig 1a, how did the authors approach differentiating between FA type and region during cryo-ET data collection to ensure equivalent comparison across different cell lines?

5) Evidence in support of the process and quality of the data from the cryo-ET averaging experiments is incomplete: a more detailed description of the cryo-ET reconstruction and subtomogram averaging pipeline, possibly illustrated with a schematic or workflow diagram, alongside the confidence score associated with the success of polarity determination in each dataset

and local resolution depiction, should be included. At the quoted resolution, the distinct subdomains of actin should be visible at higher thresholds and such views should be provided in support of their analysis – the numerical resolution achieved is not sufficient to judge the success of the reconstruction process.

6) Given the unavoidable distortion in z in cryo-ET data, what is the effect of the cryo-ET missing wedge on the authors' measurements of the distance of actin filaments to substrate (Fig 5a) – how do the height differences compare with this intrinsic error in these data?

Minor

- title for Fig 1 does not reflect the experimental content of the figure
- lines 373/374 – do the authors mean “Essay” or rather “assay”
- scale bar is missing in Figure S7

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

This paper looking at the effects of Zyxin and VASP (Z/V) has some interesting data and sophisticated analysis, but seems a little bit uncoherent, talking about Z/V then a structure of actin/tropomyosin thrown in. Tomography looks nice and provides good imaging of actin structures in cells.

A few comments.

Why use circular patterns that mimic a non-polarized cell? This seems the least interesting shape to study as without polarization you mainly just see the amorphous adhesions structures; it might have been more interesting on a crossbow or similar to get the reproducibility between cells but also to see the structure of adhesions that resemble better those seen in culture and in migration. I guess the regions where the cell breaks past the symmetry but then these are expanding beyond the ECM pattern so are not engaged to anything?

It is not clear how Z/V and tropomyosin are connected, the authors claim there are changes in tropomyosin at FAs when Z/V are knocked out but they need to show this, I did not see any tropomyosin fluorescent imaging so beyond the structure they show of the actin/tropomyosin complex the rest is inferred somewhat. Strengthening this aspect would improve the manuscript. With the complexity of the tropomyosin isoform possibilities, is it definite that these slightly more dorsal filaments are the same population?

In Figure 5, it would be useful to see representative images as it appears there is minimal change to any of the measures of filaments in this figure.

Again, in Figure 6, are these changes visible in imaging, and how do they compare to the numerous other studies that have looked at Z/V using more conventional markers of adhesion dynamics?

Showing how the tomography and fluorescence microscopy look when looking at same structures would be useful.

Overall, the data looks excellent, and the experiments look to be performed to a high standard, but the study is not that informative in what it shows due to the experimental design, and the very modest changes in actin filament behavior when Z/V are knockout, so the novel insights are somewhat limited.

Reviewer #4

(Remarks to the Author)

In this work, the authors build on a model of focal adhesions (FAs) that proposes the existence of an actin-regulatory layer physically coupled to actomyosin stress fibers and enriched in Ena/VASP proteins, zyxin, and tropomyosins. Although these proteins have been implicated in coordinating actin elongation, tension sensing, and filament stabilization, direct structural evidence for their interplay and for how they shape adhesion-associated actin networks has been limited. Zyxin has been proposed to recruit Ena/VASP and to maintain stress-fiber integrity.

To address this structural gap, the authors generated VASP^{-/-}, zyxin^{-/-}, and double-knockout (DKO) MEFs and described FA morphology, adhesion dynamics, and actin architecture relative to WT cells. Using 186 tomograms from 45 WT, 8 VASP^{-/-}, 29 zyxin^{-/-}, and 13 DKO cells, actin filament polarity was determined with the Actin Polarity Toolbox, supported by ~12–13 Å subtomogram averages of actin and tropomyosin–actin filaments, respectively.

Key findings include:

- WT FAs exhibit uniformly oriented bundles with ~71% forward-oriented (barbed-end-toward-membrane) filaments. This proportion decreases progressively in VASP-/- (68%), zyxin-/- (65%), and DKO (63%) cells, with corresponding increases in backward-oriented filaments.
- Filament density remains high in VASP-/- (~4000 filaments/ μm^3) but is substantially reduced in zyxin-/- (~3000/ μm^3) and DKO (~2500/ μm^3), consistent with reduced vinculin signal and impaired FA maturation.
- Tropomyosin-decorated actin (~6% of annotated filaments) localizes exclusively to the dorsal aspect of FA-associated bundles (~110 nm above the substrate) and remains unaffected by loss of VASP or zyxin, suggesting that tropomyosin defines a stable “contractile” layer independent of VASP/zyxin-mediated regulation.

Major Comments

1. The authors conclude that loss of VASP alters FA morphology, reduces actin density, and disrupts filament polarity near the plasma membrane, with these defects more pronounced in zyxin-/- and DKO cells. They propose that VASP and zyxin are mechanistically linked but carry out distinct roles in coupling FAs to the actin cytoskeleton. However, as noted in the literature, zyxin-/- mice exhibit no overt physiological phenotype. The authors therefore need to provide a clear explanation for why nanoscale defects they report do not translate into detectable physiological consequences, and how these structural perturbations should be interpreted in light of the apparent organism-level redundancy or compensation.
2. The study relies on thin peripheral lamellipodial regions for cryo-ET, but differences in lamellipodial morphology between genotypes may affect sampling or tilt-series quality. The manuscript should address how sampling biases were controlled or normalized. Variance should be quantified within each condition (across multiple cells, multiple regions per cell, and multiple grids), and these variances should be compared across conditions using appropriate statistical tests.
3. CrYOLO-based tracing is central to the analysis. However, performance data for the 3D filament tracing mode of crYOLO is not available in the literature. An analysis of error rates and potential picking bias using manual validation would strengthen confidence in the data.
4. The shift in polarity between WT (71%) and VASP-/- (68%) is modest. The authors attribute this to compensation by other Ena/VASP proteins, but this possibility is not directly tested. Additional experimental evidence is needed to support this interpretation.
5. The subtomogram averages used for polarity assignment are at ~12–13 Å. Although the authors claim reliable polarity determination at this resolution, the manuscript does not include adequate controls or validation. Given that polarity of individual filaments can be ambiguous in the tomograms at this resolution, the robustness of these assignments needs further support.
6. Although the knockout lines are well-validated, rescue experiments (e.g., re-expression of VASP or zyxin) are essential to establish causality. Without rescue, the conclusions remain correlative and cannot fully exclude secondary compensatory effects.
7. The interpretation that altered polarity contributes to defects in adhesion maturation is primarily correlative. The authors should provide direct measurements of FA dynamics or cytoskeletal behavior (e.g., retrograde flow) to substantiate the proposed mechanism.
8. Tropomyosin decoration is presented as isoform-independent. However, different isoforms have distinct biochemical and regulatory roles at FAs. Without isoform-level resolution, the functional conclusions are limited.

Minor Comments

1. The manuscript states that 186 tomograms were collected, but the number used for final polarity analysis varies across regions and genotypes. A clear flowchart or table of inclusion/exclusion criteria would improve transparency.
2. Only ~6% of annotated filaments show tropomyosin decoration. Additional discussion of whether this fraction is expected, and how detection sensitivity was evaluated, would be helpful.
3. The dorsal localization (~110 nm above substrate) is interesting, but the manuscript could benefit from clearer figures or schematic illustrations to highlight this spatial compartmentalization.
4. Some comparisons (e.g., density differences) lack explicit p-values or effect-size reporting. Adding these would improve scientific rigor.
5. Terms such as “forward,” “barbed-end-toward-membrane,” and “oriented toward the plasma membrane” should be standardized for clarity.

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have added considerably more data, methodological detail and analyses in support of their conclusions. They have thus addressed the previous queries to produce an interesting and insightful study.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The authors have done an excellent job of revising this manuscript,

Reviewer #4

(Remarks to the Author)

The authors invested significant effort in revising the manuscript. The revised version is stronger, with the addition of rescue experiments, expanded methodological descriptions, complementary imaging, live-cell analyses, and improved statistical presentation. These revisions address several of the original concerns and improve the overall quality of the manuscript.

The remaining concerns are as follows:

1. Validation of actin polarity assignments remains incomplete. While the authors expanded the description of the APT workflow and introduced confidence metrics, these constitute internal validation rather than independent confirmation of polarity assignments. The confidence metrics increase confidence in the proposed assignments but do not establish whether the polarity assignments are correct. Moreover, the biological application of the APT workflow remains relatively limited outside the work of its developers, with comparatively little independent validation across other biological systems. Because filament polarity underpins the principal biological conclusions of this study, the absence of orthogonal validation remains a significant limitation.
2. The proposed compensation by other Ena/VASP family members remains speculative. The modest polarity difference between WT and VASP-null cells is attributed to compensation by Mena/EVL; however, this hypothesis is not experimentally tested. The literature on redundancy makes this explanation biologically plausible but does not demonstrate that such compensation occurs in the cells examined in this study.
3. Sampling bias and statistical independence remain insufficiently addressed. Although the authors clarify their sampling strategy and substantially increase the number of analyzed filaments, larger datasets increase statistical precision but do not address potential sampling bias or pseudoreplication. The manuscript still does not quantify variability across cells, regions, or grids, nor does it clearly distinguish biological from technical replication. Consequently, the underlying concerns regarding statistical independence remain unresolved.
4. The proposed mechanism remains primarily correlative. The additional live-cell imaging strengthens the manuscript, but direct quantitative evidence linking altered actin polarity to focal adhesion maturation is still lacking.

Collectively, these revisions improve the manuscript and make the proposed model more consistent and biologically plausible. However, they do not resolve the principal methodological limitations or provide the independent experimental validation and quantitative evidence needed to substantiate the central conclusions.

Finally, the comparison with a major cytoskeletal intermediate filament protein such as vimentin is not particularly informative in this context. A more relevant comparison is the well-established functional redundancy within the zyxin/LIM-domain focal adhesion family. While such redundancy provides a biologically plausible explanation for the relatively mild phenotype, it does not constitute evidence that compensatory mechanisms are responsible for the observations reported here.

Overall, the manuscript represents a valuable technical contribution and has been substantially improved through revision. However, the principal mechanistic conclusions continue to rely, in part, on inference rather than direct experimental validation, and key methodological uncertainties remain unresolved. As a result, the study does not yet provide the level of mechanistic rigor and broad biological insight expected for publication in Communications Biology.

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To the editor:

Re: VASP cooperates with zyxin to organize mechanically competent adhesions

GENERAL COMMENTS:

We would like to thank you and the reviewers for your time and constructive comments that improved substantially the manuscript. We have modified the manuscript according to the reviewers' comments. Therefore, we have performed the suggested experiments, and added additional results to the figures, as well as the requested analysis.

Below, we address one-by-one **all the specific comments** raised by the referees.

RESPONSES TO SPECIFIC COMMENTS OF THE REFEREES:

Reviewers' comments:

Reviewer #1 and associated reviewer #2 (Remarks to the Author):

Focal adhesions (FA) are key cellular assemblies that support connectivity between the intracellular cytoskeleton and extracellular matrix. They thereby control cell shape and movement and are key components of cells' mechanosensing machinery. Their organisation and dynamics must be precisely integrated with the dynamics of the actin cytoskeleton but the mechanisms involved in this integration remain to be fully understood.

From among a number of important FA proteins, the authors in this study focus their investigation on zyxin and VASP. They use advanced light microscopy and cryo-electron tomography (cryo-ET) to study CRISPR/Cas9 modified mouse embryonic fibroblast (VASP^{-/-}; zyxin^{-/-} and knock-out of both ("DKO")) to investigate the role of zyxin and VASP in organisation of actin within FAs. FA size and turnover together with overall cell shape and actin organisation are quantified in different genetic backgrounds. In addition, individual actin filament arrangements in portions of FAs in each condition are visualised using cryo-ET. The resolution of these cryo-ET data, supported by sub-volume averaging, allowed the authors to analyse actin filament polarity and also to identify a subset of actin filaments bound by tropomyosin, with each filament population being mapped back into the context of the FA sub-volume.

In general, although the approach taken in each individual experiment is well explained and the findings potentially of interest, as outlined below, there are currently major limitations in the presentation, completeness and analysis of the data, as well as with the internal coherence and consistency, that undermine confidence in the authors' conclusions.

We thank the reviewer for the time and constructive comments. In the revised manuscript, we have complied to all the reviewer comments which improved the manuscript and made it substantially clearer. It also includes the requested analysis and tests. Most importantly, we conducted the required experiments and the analysis suggested by the reviewer.

1) The introduction could be more focused to allow the reader to clearly appreciate what questions the authors were seeking to answer and thereby more clearly appreciate their findings – given the complexity of FA dynamics and regulation, it is important to clearly

understand the unique contribution of each component that is being tested and the extent to which functional redundancy is observed.

We modified the introduction to be more focused on the questions and results. We have clearly formulated the questions that are addressed by our study and to which the data has answered.

As such, we added:

line 64 “... whether and how they cooperate [VASP and zyxin] to coordinate FA maturation, force transmission, and turnover remains unresolved.”

Line 92 “These observations prompted us to focus on VASP and ask whether its contribution to focal adhesion maturation primarily reflects its actin polymerization activity at adhesion sites or its function, together with zyxin, in the tension-dependent stress fiber repair machinery.”

2) The plot in Fig 1b is not properly explained/annotated – what are the individual points compared to the box plots? Although the number of cells for each condition is stated in the legend, what is the n for each condition in the plot, what do the whiskers correspond to and is the dataset size appropriate given the expected effect size – ie is the statistical significance of the observations being driven primarily by the very large dataset of whatever is being measured? The authors should consider using SuperPlots to more effectively analyze their data and its variation (PMID: 32346721) to more robustly ensure this analysis is capturing mechanistically important differences in FA behavior in each cell line.

We thank the reviewers for this point and apologize for the missing information in the original submission. As suggested, we replaced Fig. 1b with SuperPlot and we increased the data sets for all cell types and used the suggested SuperPlots for the analysis.

3) In the micropatterned cellular imaging experiments, the authors state that FA size and density of vinculin within adhesions appears diminished when zyxin is knocked out. Could this result from reduced vinculin expression? A western blot to show total protein levels of vinculin across the different cell lines would clarify this point.

We have now analysed the expression levels in all cells (supplementary Fig. S1) and expanded the pattern surfaces experiments.

4) Given the classification of different types of FAs presented in Fig 1a, how did the authors approach differentiating between FA type and region during cryo-ET data collection to ensure equivalent comparison across different cell lines?

We thank the reviewer for raising this point. All cryo-ET was conducted using correlative microscopy in which we focused on membrane proximal regions of peripheral FAs, with elongated vinculin signal. We used rather large number of FA (186 FAs) to reduce an intentionally subjective biased. We mention this in the text to explain better how the study was conducted.

5) Evidence in support of the process and quality of the data from the cryo-ET averaging experiments is incomplete: a more detailed description of the cryo-ET reconstruction and subtomogram averaging pipeline, possibly illustrated with a schematic or workflow diagram, alongside the confidence score associated with the success of polarity determination in each dataset and local resolution depiction, should be included. At the quoted resolution, the distinct subdomains of actin should be visible at higher thresholds and such views should be provided in support of their analysis – the numerical resolution achieved is not sufficient to judge the success of the reconstruction process.

In line with the reviewer's suggestion, we added a schematic representation of the subtomogram averaging pipeline based on the actin polarity toolbox (APT) (revised Fig. S9a). In addition, we substantially expanded the Materials and Methods section to provide a detailed description of the subtomogram reconstruction of the actin filament and tropomyosin–actin filament structures (lines 685–773) in the revised manuscript). As suggested, we also stratified the confidence analysis for polarity determination by cellular condition (lines 763–773; new Supplementary Fig. S11). Furthermore, we included local resolution maps for both the actin filament (new Supplementary Fig. S9e) and the tropomyosin–actin filament (new Supplementary Fig. S9g) structures.

In the original manuscript, nominal resolution values of the subtomogram averages were estimated in RELION using the gold-standard Fourier shell correlation (FSC) approach. In the revised manuscript, we additionally assessed the upper resolution limit of the subtomogram averages (new Supplementary Fig. S10a; lines 725–739) and revisited the polarity determination. This analysis demonstrates that the polarity of the actin filament structure can be reliably assigned even at the estimated upper resolution limit of ~17 Å (new Supplementary Fig. S10b; lines 740–754).

6) Given the unavoidable distortion in z in cryo-ET data, what is the effect of the cryo-ET missing wedge on the authors' measurements of the distance of actin filaments to substrate (Fig 5a) – how do the height differences compare with this intrinsic error in these data?

The reviewers are right that the accuracy in parallel to the electron beam is not as high as in the x-y plain of a tomogram. Therefore, when we saw that the measured differences between the cell lines were rather small, we have not interpreted these differences but rather wrote: “While the positions and angles of the actin filaments relative to the substrate **were similar** across all cell lines...”

Minor

- title for Fig 1 does not reflect the experimental content of the figure

The title was rephrased in line with the reviewer comment.

- lines 373/374 – do the authors mean “Essay” or rather “assay”

We apologize for this typo, which was corrected.

-scale bar is missing in Figure S7

A scale bar was added to the original Fig. S7, named Fig. S12 in the revised manuscript.

Reviewer #3 (Remarks to the Author):

This paper looking at the effects of Zyxin and VASP (Z/V) has some interesting data and sophisticated analysis, but seems a little bit uncoherent, talking about Z/V then a structure of actin/tropomyosin thrown in. Tomography looks nice and provides good imaging of actin structures in cells.

We thank the reviewer for this constructive comment and suggestion to add additional experiments, which we followed. We have revised the manuscript to improve the overall coherence and to more clearly articulate the central biological question. In the revised version, we show that VASP cooperates with zyxin to maintain and adapt the focal adhesion-actin network under high mechanical strain. Within this framework, we believe it is important to report that tropomyosin decoration of adhesion-associated stress fibres is unaffected by the loss of either VASP or zyxin. This finding demonstrates that tropomyosin defines a structurally distinct layer of the force-bearing actin bundle, independent of the VASP-zyxin pathway, while likely contributing to the actomyosin-generated tension required for focal adhesion strengthening and maturation.

A few comments.

Why use circular patterns that mimic a non-polarized cell? This seems the least interesting shape to study as without polarization you mainly just see the amorphous adhesions structures; it might have been more interesting on a crossbow or similar to get the reproducibility between cells but also to see the structure of adhesions that resemble better those seen in culture and in migration. I guess the regions where the cell breaks past the symmetry but then these are expanding beyond the ECM pattern so are not engaged to anything?

We are grateful the reviewer for this comment. In the original manuscript, we used circular patterning because they reproducibly organize the actin cytoskeleton into a stereotypical architecture composed of peripheral focal adhesions giving rise to dorsal stress fibres connected to centripetally flowing radial contractile fibres. This organisation enabled robust quantitative analyses of focal adhesions at the whole-cell scale.

In line with the reviewer suggestion, in the revised manuscript, we have complemented the above analyses with live-cell imaging of cells confined on crossbow micropatterns. The crossbow geometry spatially segregates high force-bearing adhesions located at the tips of the pattern, which are subjected to actomyosin-generated tension transmitted by ventral stress fibres spanning the bow and the stem. This experimental setup allowed us to specifically analyse mechanically loaded adhesions and revealed that VASP cooperates with zyxin to maintain and adapt high force-bearing focal adhesions under mechanical load.

It is not clear how Z/V and tropomyosin are connected, the authors claim there are changes in tropomyosin at FAs when Z/V are knocked out but they need to show this, I did not see any tropomyosin fluorescent imaging so beyond the structure they show of the actin/tropomyosin complex the rest is inferred somewhat. Strengthening this aspect would improve the manuscript. With the complexity of the tropomyosin isoform possibilities, is it definite that these slightly more dorsal filaments are the same population?

In Figure 5, it would be useful to see representative images as it appears there is minimal change to any of the measures of filaments in this figure.

Again, in Figure 6, are these changes visible in imaging, and how do they compare to the numerous other studies that have looked at Z/V using more conventional markers of adhesion dynamics?

Showing how the tomography and fluorescence microscopy look when looking at same structures would be useful.

We thank the reviewer for this helpful comment. We have added Supplementary Figure S14, which includes confocal images of vinculin together with super-resolution STED (stimulated emission depletion) images of F-actin and tropomyosin at the protruding cell edge in wild-type and knockout cells. These images illustrate the distribution of vinculin, the actin cytoskeleton, and tropomyosin at the protruding cell edge and provide complementary structural information supporting our findings.

Regarding the identity of the tropomyosin-decorated filaments, we agree that multiple tropomyosin isoforms are present in mammalian cells. However, distinguishing individual isoforms is beyond the scope of the present study and is not achievable by cryo-ET alone, as the different tropomyosin isoforms are structurally indistinguishable at the resolution obtained. Accordingly, we have refrained from assigning a specific tropomyosin isoform to the decorated actin filaments. Rather, our conclusion is limited to the observation that the population of tropomyosin-decorated actin filaments resolved in situ is similarly distributed in wild-type and VASP- and/or zyxin-deficient cells, indicating that their association with adhesion-associated stress fibres is largely independent of these proteins.

Overall, the data looks excellent, and the experiments look to be performed to a high standard, but the study is not that informative in what it shows due to the experimental design, and the very modest changes in actin filament behavior when Z/V are knockout, so the novel insights are somewhat limited.

We thank the reviewer for the insightful remarks. As detailed above, we believe that the revised and improved manuscript more clearly articulates its central message, namely that VASP is a key partner of the zyxin-mediated repair machinery operating at mechanically load-bearing focal adhesions.

Reviewer #4 (Remarks to the Author):

In this work, the authors build on a model of focal adhesions (FAs) that proposes the existence of an actin-regulatory layer physically coupled to actomyosin stress fibers and enriched in Ena/VASP proteins, zyxin, and tropomyosins. Although these proteins have been implicated in coordinating actin elongation, tension sensing, and filament stabilization, direct structural evidence for their interplay and for how they shape adhesion-associated actin networks has been limited. Zyxin has been proposed to recruit Ena/VASP and to maintain stress-fiber integrity.

To address this structural gap, the authors generated VASP^{-/-}, zyxin^{-/-}, and double-knockout (DKO) MEFs and described FA morphology, adhesion dynamics, and actin

architecture relative to WT cells. Using 186 tomograms from 45 WT, 8 VASP^{-/-}, 29 zyxin^{-/-}, and 13 DKO cells, actin filament polarity was determined with the Actin Polarity Toolbox, supported by ~12–13 Å subtomogram averages of actin and tropomyosin–actin filaments, respectively.

Key findings include:

- WT FAs exhibit uniformly oriented bundles with ~71% forward-oriented (barbed-end–toward-membrane) filaments. This proportion decreases progressively in VASP^{-/-} (68%), zyxin^{-/-} (65%), and DKO (63%) cells, with corresponding increases in backward-oriented filaments.
- Filament density remains high in VASP^{-/-} (~4000 filaments/ μm^3) but is substantially reduced in zyxin^{-/-} (~3000/ μm^3) and DKO (~2500/ μm^3), consistent with reduced vinculin signal and impaired FA maturation.
- Tropomyosin-decorated actin (~6% of annotated filaments) localizes exclusively to the dorsal aspect of FA-associated bundles (~110 nm above the substrate) and remains unaffected by loss of VASP or zyxin, suggesting that tropomyosin defines a stable “contractile” layer independent of VASP/zyxin-mediated regulation.

We thank the reviewer for the time and constructive comments.

Major Comments

1. The authors conclude that loss of VASP alters FA morphology, reduces actin density, and disrupts filament polarity near the plasma membrane, with these defects more pronounced in zyxin^{-/-} and DKO cells. They propose that VASP and zyxin are mechanistically linked but carry out distinct roles in coupling FAs to the actin cytoskeleton. However, as noted in the literature, zyxin^{-/-} mice exhibit no overt physiological phenotype. The authors therefore need to provide a clear explanation for why nanoscale defects they report do not translate into detectable physiological consequences, and how these structural perturbations should be interpreted in light of the apparent organism-level redundancy or compensation.

We thank the reviewer for pointing out that zyxin^{-/-} mice should not have deficiencies. We would like to point out a few important issues:

1. Similar early observation (no clear deficiencies) were published for vimentin^{-/-} mouse. But over the last ~30 years it turned out that many physiological processes

have shown to be affected. Therefore, it is likely to happen with the Zyxin $-/-$ mice. Below is an example.

2. There are already several phenotypes found in the deficient mice models, for example: Zyxin-deficient mice ($Zyx^{-/-}$) display macrothrombocytopenia (PMID: 34657146). Another preprint showed that Zyxin reduces endothelial inflammation and mitigates atherosclerosis (doi: <https://doi.org/10.1101/2024.10.21.619550>)
3. Important discoveries were conducted with cells from the deficient mice to explain the function of zyxin, for example, it was shown, this year, that force-activated zyxin assemblies coordinate actin nucleation. (PMID: 39952249)

Taking together the above, we (and others) consider zyxin an important canonical component of FAs.

2. The study relies on thin peripheral lamellipodial regions for cryo-ET, but differences in lamellipodial morphology between genotypes may affect sampling or tilt-series quality. The manuscript should address how sampling biases were controlled or normalized. Variance should be quantified within each condition (across multiple cells, multiple regions per cell, and multiple grids), and these variances should be compared across conditions using appropriate statistical tests.

The study was conducted on mature FAs with the canonical fluorescent signature. These FAs are not found in lamellipodial regions. In previous study, published in this journal, we used the current approach to study lamellipodial structure (Chung et al. 2022). In light of the reviewer comment, we mentioned this in the revised text.

Moreover, the large statistics used here, is reducing biased and quality differences. Similar number of actin filaments were used to reconstruct and back-mapping of the actin cytoskeleton. Therefore, the resolution in all samples is sufficient for the determination of actin polarity using the actin polarity toolbox (ATP, Martins et al. 2021) that was utilized here.

3. CrYOLO-based tracing is central to the analysis. However, performance data for the 3D filament tracing mode of crYOLO is not available in the literature. An analysis of error rates and potential picking bias using manual validation would strengthen confidence in the data.

We apologize that this central aspect of the analysis was insufficiently described in the original submission. In the revised version, we now provide a more detailed explanation of how the CrYOLO model for tracing actin filaments in tomograms was trained and iteratively improved (see revised Materials and Methods, lines 691-703).

We would also like to note that the APT method is relatively robust to false-positive actin detections, as these can be efficiently removed during subsequent 2D classification, where false-positive actin segments and low-quality particles are filtered out (lines 709-715). In this context, the primary challenge for the initial filament detection using CrYOLO is to minimize false-negative actin detections. As now described in the revised Materials and Methods, this was achieved through extensive visual inspection of the detection results and iterative retraining of the neural network by incorporating additional training data from tomograms with lower prediction accuracy.

4. The shift in polarity between WT (71%) and VASP-/- (68%) is modest. The authors attribute this to compensation by other Ena/VASP proteins, but this possibility is not directly tested. Additional experimental evidence is needed to support this interpretation.

We thank the reviewer for this comment. The compensation by other Ena/VASP proteins was demonstrated by two independent studies (Damiano-Guercio et al., 2020; Puleo et al., 2019). We are now citing these references when claiming the compensation.

5. The subtomogram averages used for polarity assignment are at ~12–13 Å. Although the authors claim reliable polarity determination at this resolution, the manuscript does not include adequate controls or validation. Given that polarity of individual filaments can be ambiguous in the tomograms at this resolution, the robustness of these assignments needs further support.

We thank the reviewer for this important comment. The determination of polarity in the actin subtomogram average, as well as its validation, is now described in detail in the revised manuscript (new Supplementary Fig. S10; lines 740-754).

Furthermore, as demonstrated in the original APT methodology paper (Martins et al., 2021), the a priori assumption that all actin segments belonging to the same filament share a common polarity direction allows the construction of a confidence score to assess the reliability of the polarity assignment for individual filaments.

In the revised manuscript, we now clearly describe how this actin polarity confidence analysis was performed (lines 755-773) and additionally stratified this analysis by cellular condition. As shown in the APT paper, the confidence of the polarity assignment depends primarily on the signal-to-noise ratio of the filaments. Since the fractions of retained filaments are similar across cellular conditions, this analysis further suggests comparable data quality among the datasets (lines 766-773; new Supplementary Fig. S11).

6. Although the knockout lines are well-validated, rescue experiments (e.g., re-expression of VASP or zyxin) are essential to establish causality. Without rescue, the conclusions remain correlative and cannot fully exclude secondary compensatory effects.

We thank the reviewer for this important suggestion. We performed rescue experiments by transiently re-expressing VASP and/or zyxin in the corresponding knockout cell lines. Re-expression of the respective proteins restored cell spreading, the organization of the actin cytoskeleton, and vinculin recruitment to adhesion complexes. In the rescued cells, VASP-mCherry and/or zyxin-mCherry localized to adhesion complexes, whereas neighbouring non-transfected knockout cells retained the characteristic phenotype of reduced vinculin recruitment and a disorganized actin cytoskeleton. These rescue experiments confirmed that the observed phenotypes are a direct consequence of VASP and/or zyxin loss rather than secondary effects associated with the knockout cell lines.

7. The interpretation that altered polarity contributes to defects in adhesion maturation is primarily correlative. The authors should provide direct measurements of FA dynamics or cytoskeletal behavior (e.g., retrograde flow) to substantiate the proposed mechanism.

We thank the reviewer for this valuable comment and appreciate the editor's assessment that a quantitative analysis of retrograde actin flow is beyond the scope of the present study. Nevertheless, to further substantiate our proposed mechanism, we have included live-cell imaging of cells confined on both circular and crossbow micropatterns, allowing direct visualisation of actin and focal adhesion dynamics. These experiments reveal the differential behaviour of adhesion-associated actin in wild-type and knockout cells, including the peeling and disengagement of stress fibres from high force-bearing adhesions in VASP-deficient cells, thereby providing dynamic support for our structural and quantitative analyses.

8. Tropomyosin decoration is presented as isoform-independent. However, different isoforms have distinct biochemical and regulatory roles at FAs. Without isoform-level resolution, the functional conclusions are limited.

We agree with the reviewer and therefore we do not wish to comment on isoform distribution but rather present the data that show where tropomyosins are situated and that we cannot detect differences within the mutants. Maybe due to the limited resolution. In light of the reviewer comment, we mentioned this in the revised manuscript line 234.

Minor Comments

1. The manuscript states that 186 tomograms were collected, but the number used for final polarity analysis varies across regions and genotypes. A clear flowchart or table of inclusion/exclusion criteria would improve transparency.

In the revised manuscript, the actin polarity analysis was stratified by cellular condition (new Supplementary Fig. S11; lines 766-773).

2. Only ~6% of annotated filaments show tropomyosin decoration. Additional discussion of whether this fraction is expected, and how detection sensitivity was evaluated, would be helpful.

We thank the reviewer for this comment. The 6% filaments that show tropomyosin decoration were simply calculated by dividing the number of decorated filaments by the overall filament in a tomogram. We are now mentioning the idea that the low percentage represent the requirement for highly dynamic actin network and the rich number of actin binding proteins to found in FAs (line 237-238).

3. The dorsal localization (~110 nm above substrate) is interesting, but the manuscript could benefit from clearer figures or schematic illustrations to highlight this spatial compartmentalization.

We agree with the reviewer that the dorsal localization is interesting and consistent with previous work from Clare Waterman, Peter Gunning, and colleagues. However, we feel that including a schematic illustration of the proposed spatial organization may be misleading, as the cryo-EM data do not directly resolve the localization of zyxin or VASP. A schematic focusing only on tropomyosin while omitting these key adhesion components may imply a level of certainty that is not supported by our data. For this reason, we prefer to use the rendered image in figure 7a.

4. Some comparisons (e.g., density differences) lack explicit p-values or effect-size reporting. Adding these would improve scientific rigor.

5. Terms such as “forward,” “barbed-end-toward-membrane,” and “oriented toward the plasma membrane” should be standardized for clarity.

We thank the reviewer for points 4 and 5. This has now been corrected.

Response to reviewer #4 (Remarks to the Author):

The authors invested significant effort in revising the manuscript. The revised version is stronger, with the addition of rescue experiments, expanded methodological descriptions, complementary imaging, live-cell analyses, and improved statistical presentation. These revisions address several of the original concerns and improve the overall quality of the manuscript.

We thank the reviewer for these comments and the time invested.

The remaining concerns are as follows:

1. Validation of actin polarity assignments remains incomplete. While the authors expanded the description of the APT workflow and introduced confidence metrics, these constitute internal validation rather than independent confirmation of polarity assignments. The confidence metrics increase confidence in the proposed assignments but do not establish whether the polarity assignments are correct. Moreover, the biological application of the APT workflow remains relatively limited outside the work of its developers, with comparatively little independent validation across other biological systems. Because filament polarity underpins the principal biological conclusions of this study, the absence of orthogonal validation remains a significant limitation.

We thank the reviewer for the comment. Here we used a variant of the conventional STA procedures and therefore the method for polarity assignment is limited to internal validation and depends finally on the resolution of the sub-tomogram average and the S/N ratio of the data. We agree that orthogonal validation approach for *in situ* structural determination would increase the confidence of polarity assignment of actin filaments but orthogonal validation approaches for *in situ* STA are yet to be developed.

2. The proposed compensation by other Ena/VASP family members remains speculative. The modest polarity difference between WT and VASP-null cells is attributed to compensation by Mena/EVL; however, this hypothesis is not experimentally tested. The literature on

redundancy makes this explanation biologically plausible but does not demonstrate that such compensation occurs in the cells examined in this study.

We agree with the reviewer that the compensation by Mena and/or EVL could explain the limited effect we observed on actin polarity in the context of VASP loss remains speculative. We have now explicitly stated in the manuscript that compensation by other Ena/VASP family members is a possible explanation for the limited effect of VASP loss on actin polarity, but that this interpretation remains speculative (line 252-254).

3. Sampling bias and statistical independence remain insufficiently addressed. Although the authors clarify their sampling strategy and substantially increase the number of analyzed filaments, larger datasets increase statistical precision but do not address potential sampling bias or pseudoreplication. The manuscript still does not quantify variability across cells, regions, or grids, nor does it clearly distinguish biological from technical replication. Consequently, the underlying concerns regarding statistical independence remain unresolved.

We agree with the reviewer that this study focuses exclusively on the focal adhesions located at the cell periphery, which may introduce a potential bias in our analysis. However, for each cell line, cells were grown on several grids across three independent experiments. Thus, by analyzing at least two grids per cell culture experiment, we ensured that our data accurately represent the peripheral focal adhesions for each cell line. This information has been added to the Material and Methods section (line 674-676). In light of the reviewer's comment, we have clearly stated that a limitation of our study is that it analyzes the actin architecture of focal adhesions located exclusively at the cell periphery (line 203-204), and we have added a statement to the "Limitations of the Study" section acknowledging this limitation and clarifying the scope of our analysis (line 438-445).

4. The proposed mechanism remains primarily correlative. The additional live-cell imaging strengthens the manuscript, but direct quantitative evidence linking altered actin polarity to focal adhesion maturation is still lacking.

We agree with the reviewer that the proposed mechanism may not be exclusive, however, very much relevant and plausible. We believe that correlating actin polarity to the maturation

of FAs is beyond the scope of this current study. We have clearly stated in the discussion section that this potential relationship remains to be investigated (line 359-361).

Collectively, these revisions improve the manuscript and make the proposed model more consistent and biologically plausible. However, they do not resolve the principal methodological limitations or provide the independent experimental validation and quantitative evidence needed to substantiate the central conclusions.

The limitation of the study is now clearly stated.

Finally, the comparison with a major cytoskeletal intermediate filament protein such as vimentin is not particularly informative in this context. A more relevant comparison is the well-established functional redundancy within the zyxin/LIM-domain focal adhesion family. While such redundancy provides a biologically plausible explanation for the relatively mild phenotype, it does not constitute evidence that compensatory mechanisms are responsible for the observations reported here.

We agree with the reviewer that functional redundancy within the zyxin/LIM-domain focal adhesion family provides a biologically plausible explanation for the relatively mild phenotype observed in mice. However, we emphasize that this possibility remains speculative and does not constitute evidence that such compensatory mechanisms are involved in the alterations we report. We have clarified this point in the Results section (line 254-257).

Overall, the manuscript represents a valuable technical contribution and has been substantially improved through revision. However, the principal mechanistic conclusions continue to rely, in part, on inference rather than direct experimental validation, and key methodological uncertainties remain unresolved. As a result, the study does not yet provide the level of mechanistic rigor and broad biological insight expected for publication in Communications Biology.