VASP localization to lipid bilayers induces polymerization driven actin bundle formation

Timon Nast-Kolb, Philip Bleicher, Marco Payr, and Andreas Bausch

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RE: Manuscript #E21-11-0577
TITLE: VASP localization to lipid bilayers induces polymerization driven actin bundle formation

Dear Andreas,

Your manuscript has been reviewed by two experts in the field. Overall, they find your study interesting but have questions about the biochemical characterization of your experimental system and the broad impact of your conclusion according to other actin systems capable of coupling nucleation/elongation and cross-linking. These are important comments that need to be addressed before publication in MBoC.

Best regards,
Laurent

Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Bausch,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The submitted manuscript 'VASP localization to lipid bilayers induces polymerization driven actin bundle formation' by Nast-Kob et al, addresses the role of the actin binding protein VASP, a barbed elongator as well as a protein capable of filament bundling in solution, to induce the formation of bundles on fluid lipid surfaces. The authors of the study argue that VASP-induced polymerization is the main driving force of filament alignment on fluid lipid surfaces in in vitro assays with consequences on the actual filament organization in cells.
While I don't have any major problems with the conclusions reached by the authors, there are multiple issues with the way the experiments are performed and interpreted.
Major changes would thus be needed to get a substantial improvement in the quality of the article to be valuable to the scientific community of actin.
One aspect that has not been discussed by the authors of this study is whether thick bundles could be formed by having the processive elongation and filament-side binding activities being performed by two distinct proteins (eg formins & ERM proteins). I would really appreciate if the authors could elaborate on this question.

Major points:
- page 5: The authors report that after a minute bundles can be as large as 2.6µm, which would correspond roughly to 325 filaments being stacked horizontally onto the lipid bilayer by VASP. Did the authors double-check this reported size by measuring the actin fluorescence intensity in bundles ?
- page 5: the fact that bigger bundles are observed with increasing density of VASP anchored to the lipid surface probably correlates with the more filaments being nucleated by VASP, therefore with the overall filament density on the surface. Did the authors vary the actin concentration while maintaining VASP density and quantified bundle size ?
The authors specify that at low VASP concentrations, their bundle width measurement is limited by light diffraction. Still they report bundle width of ~ 0.5 µm. If I understood correctly, this value is obtained using the 'local thickness' FIJI plugin which is probably not adapted to quantify diffraction limited objects. Can the authors explain how accurate bundle width measurements are for small bundle sizes ?
- page 6, top: 'This effect is slightly enhanced by the presence of CP' Can the authors elaborate on this mechanism. It is probably not so straightforward...
- page 6, Still on the impact of CP on bundle formation by VASP, the authors claim that 'there is an increase of small filaments polymerizing away from the thick bundles ' (figure 1F) : how significantly different are the 2 distributions of bundle width in the presence or absence of CP ?
- At intermediate VASP (0.25 to 0.5 µM VASP) the authors point out that there is barely any increase in bundle width, while the density of total actin has increased by ~ 15%. They explain this by :'This is most likely due to the extent of VASP diffusion to the sides of the bundles, as well as the elongation proceeding at the sides of bundles.' This is a very cryptic explanation. Can the author try to explain this more clearly ? How could these two arguments explain their observations ?
- supp figure 2H should show error bars to be correctly appreciated and the author should indicate that these are normalized values.
I would integrate the measurement at 1µM VASP (shown in supp figure 2G) in figure 2D.
- page 6, the sentence 'The secondary actin incorporates preferentially at the sides of existing thick bundles and not equally distributed along the bundle' could be rewritten to be much clearer. For example :'Filaments elongating from Alexa647-actin preferentially grow and align with the external border of pre-existing bundles, further highlighting that actin polymerization is limited to two dimensions'.
- page 6: 'Even though the average bundle width of 0.832 µm is slightly smaller than for tetrameric VASP (1.024 µm), the resulting bundle thickness is more heterogeneous, ...' : quantification to say how these reported values are obtained (from a fit, from an average), but also indicating confidence intervals, standard deviations, sample sizes and number of 'biological' repeats are expected to appreciate how significant those differences are.
- page 7: the paragraph with 'The formation of thicker bundles than tetrameric VASP can be explained by the behavior of filaments growing at bundles upon filaments diverging from the large bundles.' This sentence should be rewritten to more clearly convey the author message.
- page 8, 'The reduced diffusion suffices to hinder processive barbed end elongation by VASP.' There are no data corresponding to this statement in the manuscript. Moreover, can the authors elaborate more on the amplitude of the friction force that would prevent barbed end processive elongation by VASP ?
- page 9: 'This negative colocalization to actin proceeds directly opposed to the accumulation of VASP at filaments during polymerization (Figure 4B,E, Video S4).' Once bundling has been achieved (by VASP polymerization), does VASP accumulation beneath actin filaments disappear ? if not, then can the author rephrase their statement by removing '...during polymerization' ?
- Related to the previous point is the sentence 'After the initial displacement coupled to actin polymerization observed under a bundle, the lipid intensity relaxes to around half of initial intensity over 90 min (Figure 4F).' This relaxation is correlated to the decrease in actin intensity in the bundle. An assay measuring the relaxation of VASP accumulation together with actin (or bundle width) would be more informative here.
- In the discussion, 'The thicker bundles have a higher propensity to bind VASP from the fluid bilayer than a single actin filament.' It would be more appropriate to say 'larger' than 'thicker' bundles.
- In the discussion, 'As a consequence, larger bundles with a heterogeneous width distribution are formed.' Can the authors explain why this should lead to a wider distribution of bundle sizes ?

- Regarding FRAP experiments, have the authors been able to observe any correlation between the immobile fraction and the VASP-induced filament bundle size ? This would highlight the fact that part of the VASP proteins are actually 'trapped' by bundles ?
- In the discussion, 'In these actin structures, knock-down of VASP leads to a decrease in size as well (Damiano-Guercio et al. 2020; Tojkander et al. 2015). ' Please, specify what object has its size decreased in this sentence.
- In the discussion, 'The polymerization driven actin alignment proceeds on a second time scale, which is noticeably faster than the observed minute timescale alignment by linking F-actin filaments together (Falzone et al. 2012; Kelley et al. 2020). ' I am doubtful that fascin or alpha-actinin operate at a slower rate. For example, Breitsprecher et al, JCS 2008 reports fasicn-induced bundling zippering speed of hundreds of subunits per seconds.

Minor points:
- Supp Fig. 1E : the authors say there is no bundling in this case (VASP on ML, no CP). What would be the quantification if using the local thickness plugin here ?
in the methods section: 'For fluorescent actin, monomers were labelled at the N-terminus with Atto-488 (Jena Bioscience, FP-201-488) or Atto-647N NHS-ester (Jena Bioscience, FP-201-647N)'. I believe actin is not labeled at the N-terminus using NHS-ester but more probably on surface lysines.
- There is no mention of the mDia1 and alpha-actinin constructs used and how they were produced and purified.
the pH of the KMEI buffer is not specified.
- figure 2F: how 0.1µM atto-actin can be polymerized by VASP in the presence of 10µM profilin ?
- At 10% NTA lipids, there is apparently no saturation of NTA sites by His-VASP as incubating with more VASP results in more actin filaments and bigger bundles. Can you confirm this using fluo labeled VASP ?

Reviewer #2 (Remarks to the Author):

This work aims to describe how VASP coordinates its actin filament elongation and bundle formation activities. By targeting VASP to the surface of synthetic lipid bilayers, the authors are able to reconstitute the formation of massive actin bundles in the presence of actin, profilin and capping protein. This result leads them to the conclusion that the nature of VASP's localization, particularly its binding to a fluid membrane, is key to its function.

This experimental system is well designed, but some weak points need to be worked on in this study. I am convinced that VASP is forming these nice bundles and the model makes sense, but I am not convinced by the authors' arguments that elongation mediated bundling is unique to VASP. I detail below why some of the controls performed are not very convincing. Ideally, the authors should have studied different mutants of VASP to separate its bundling and elongation properties, rather than relying on very different proteins such as alpha-actinin or mDia1.

1/ I don't think the authors can build a strong argumentation based on negative results using alpha-actinin and mDia1. These experiments do not rule out the possibility for these proteins to form thick bundles from elongating actin filaments. Many arguments could be made that these experiments are not comparable. Are surface densities of proteins equivalent? Are these proteins diffusing at the surface of lipid bilayers similarly? Are their affinities for actin comparable? How does the fact that alpha-actinin pulls down F-actin from the bulk solution to the membrane excludes the possibility that this protein could, under other conditions, bundle actin filaments freely polymerizing at the surface of lipid bilayers?

2/ The idea that a factor combining nucleation/elongation and bundling activities can efficiently "amplify" actin bundle formation is not new in the field. Ironically, it is Laurent's team (who is editing this paper) who first put forward this idea so he will be a better judge than me on this matter. At the very least, I think his paper (Michelot and coauthors, Current Biology 2006) should be cited and discussed here.

3/ If bundling and filament elongation activities must be coordinated to promote the formation of actin bundles, would an experiment using simultaneously mDia1 and alpha-actinin (with alpha-actinin bound to lipid bilayers or in solution) lead to bundle formation similar to the VASP condition? If not, why?

Additional comments on data presentation and analysis:

4/ I have to admit that I do not understand how the authors see the elongation of filaments in Figures 1B-D. It is therefore impossible for me to properly evaluate this part of the work. The authors need to provide more obvious data or analysis to support their claims.

5/ Estimating bundle size from their diameter is not at all precise with fluorescence microscopy. I recommend presenting only the fluorescence intensity data, which is certainly much more accurate.

We thank the referees for their insightful comments and suggestions. Below, our point by point response is listed. According to the advice of the referees we performed additional experiments and added several new analyses, discussions and clarifications into the manuscript. We feel that the manuscript thus has significantly improved.

Reviewer #1 (Remarks to the Author):

The submitted manuscript 'VASP localization to lipid bilayers induces polymerization driven actin bundle formation' by Nast-Kolb et al, addresses the role of the actin binding protein VASP, a barbed elongator as well as a protein capable of filament bundling in solution, to induce the formation of bundles on fluid lipid surfaces. The authors of the study argue that VASP-induced polymerization is the main driving force of filament alignment on fluid lipid surfaces in in vitro assays with consequences on the actual filament organization in cells.
While I don't have any major problems with the conclusions reached by the authors, there are multiple issues with the way the experiments are performed and interpreted.
Major changes would thus be needed to get a substantial improvement in the quality of the article to be valuable to the scientific community of actin.
One aspect that has not been discussed by the authors of this study is whether thick bundles could be formed by having the processive elongation and filament-side binding activities being performed by two distinct proteins (eg formins & ERM proteins). I would really appreciate if the authors could elaborate on this question.
We thank both referees for suggesting this experiment, which further clarifies the way VASP bundles filaments. We looked at mDia1 and α-actinin1 both being bound to membrane and added the experiment to Figure 3. The filaments are in this case bound and elongated on the membrane separately. As no direct barbed end capture at filament sides is happening, the growing filaments tend to get trapped within nematic defects formed by the filaments instead of being elongated in an aligned fashion. This leads to a high density nematic actin field, instead of large aligned bundles. This exemplifies the importance of VASP molecules being bound to the sides of filaments, capturing colliding barbed ends and elongating the filaments guided by the previous filament.

Major points:
- page 5: The authors report that after a minute bundles can be as large as 2.6µm, which would correspond roughly to 325 filaments being stacked horizontally onto the lipid bilayer by VASP. Did the authors double-check this reported size by measuring the actin fluorescence intensity in bundles?
We agree in that this is a valuable comparison. With actin fluorescence as well as the bundle width we get a number of filaments in the same order of magnitude.
For the actin intensity we have integrated the average intensity over the bundle width for bundles with 1-4 filaments to extrapolate the filament number from intensity. For the estimation of filament number from the width we assumed the diameter between VASP domains as a spacer between the stacked filaments. For VASP a diameter of 28-70 nm could be observed. We then compared the resulting number for 1 µm large bundles. The number of filaments calculated from intensity is 45±12, whereas calculated from the width depending on the estimated actin spacing a number of 14-40 would be expected. In TIRF the field of view is not evenly illuminated and small changes in Z direction have a large effect on the excitation intensity. Therefore, from the uncertainty of the intensity and the resulting high deviation we expect the number calculated from the intensities to be a slight overestimation, but both

methods still give a number of filaments in the same order of magnitude. This control was added to the methods section.

- page 5: the fact that bigger bundles are observed with increasing density of VASP anchored to the lipid surface probably correlates with the more filaments being nucleated by VASP, therefore with the overall filament density on the surface. Did the authors vary the actin concentration while maintaining VASP density and quantified bundle size?
This is an excellent suggestion made by the reviewer. We performed more systematical analysis and quantification of these experiments and added the results in Supplemental Figure 3 and to the main text.
The here presented bundling mechanism is dependent on VASP-mediated polymerization. With lower actin concentrations there are less monomers available and therefore less bundling present. At high actin concentrations, while keeping VASP, CP and profilin concentration constant, the increased polymerization speed of actin leads to non-VASP mediated polymerization and thus more polymerization away from the big bundles. The bundling mechanism is dependent on a stoichiometry regime were non-VASP mediated polymerization is still inhibited by CP and profilin and the VASP mediated polymerization is as high as possible.

The authors specify that at low VASP concentrations, their bundle width measurement is limited by light diffraction. Still they report bundle width of ~ 0.5 µm. If I understood correctly, this value is obtained using the 'local thickness' FIJI plugin which is probably not adapted to quantify diffraction limited objects. Can the authors explain how accurate bundle width measurements are for small bundle sizes?
We hope we can clarify this point. The lower limit for the bundle width does not come from the local thickness plugin directly, but from the segmentation of the filaments by ilastik. We have added a sentence stating that the ‘local thickness’ was determined for the segmented images in the methods. Ilastik classifies each pixel to either filament or background based on intensity, gradient and texture of the object. The limit in size is then dependent on its ability to identify the features and classify a pixel towards being in a filament. The local thickness plugin then is operating on the available segmentation.
The reviewer is correct in observing that differences in the bundles at small width are not as reliably resolved. For the bundle size at small concentrations a different method for analyzing would be better suited, but the main focus of that figure is the increase of width at higher VASP concentrations, which can be nicely quantified using the ‘local thickness’ plugin. The determination of the local thickness additionally enables comparison between the width distributions of the different conditions or constructs.

- page 6, top: 'This effect is slightly enhanced by the presence of CP' Can the authors elaborate on this mechanism. It is probably not so straightforward...
This effect was indeed not explained enough in the manuscript. We changed the text in the manuscript to clarify how CP effects the bundle formation. The important aspect leading to the alignment on the actin filament is the elongation and capture of barbed ends by VASP. As VASP is a potent actin polymerase most of the actin is polymerized by VASP and thus in a way leading to the observed bundles. Any free barbed ends polymerizing without VASP are capped. Without capping, these barbed ends can polymerize in a VASP-independent fashion and thus do not contribute to the bundling mechanism.

- page 6, Still on the impact of CP on bundle formation by VASP, the authors claim that 'there is an increase of small filaments polymerizing away from the thick bundles ' (figure 1F) : how significantly different are the 2 distributions of bundle width in the presence or absence of CP ?

Even though the median bundle width between the measurements is very similar, without CP the network has around 12 % more bundles in the range form 0.6-1 µm width and due to diverging filaments almost no bundle larger than 2 µm is observable.

- At intermediate VASP (0.25 to 0.5 µM VASP) the authors point out that there is barely any increase in bundle width, while the density of total actin has increased by ~ 15%. They explain this by: 'This is most likely due to the extent of VASP diffusion to the sides of the bundles, as well as the elongation proceeding at the sides of bundles.' This is a very cryptic explanation. Can the author try to explain this more clearly? How could these two arguments explain their observations?
We thank the reviewer for pointing this out. As this was not necessarily important for the general conclusions of the concentration dependent bundle size increase and formulated in a very unclear way, we have decided to take this explanation out of the manuscript.

- supp figure 2H should show error bars to be correctly appreciated and the author should indicate that these are normalized values.
We are thankful for the reviewer for pointing this out. The decoration was measured in triplicates and we have added the error bars of the standard deviation. This measurement shows the segmented area divided by total area, therefore it shows the ratio of decoration. The updated graph was moved to Supplemental Figure 2G.

I would integrate the measurement at 1µM VASP (shown in supp figure 2G) in figure 2D.
This measurement was kept out, as it would expand the X-axis and is not very meaningful, as at 1 µM VASP the bilayer is almost completely decorated and therefore apparent bundle width is the result of the distance between holes in the decoration and less comparable to the measurements of individual bundle widths at lower VASP concentrations. To clarify this we have added a sentence to the results.

- page 6, the sentence 'The secondary actin incorporates preferentially at the sides of existing thick bundles and not equally distributed along the bundle' could be rewritten to be much clearer. For example: 'Filaments elongating from Alexa647-actin preferentially grow and align with the external border of pre-existing bundles, further highlighting that actin polymerization is limited to two dimensions'.
We appreciate this suggestion and reworded this section in the manuscript.

- page 6: 'Even though the average bundle width of 0.832 µm is slightly smaller than for tetrameric VASP (1.024 µm), the resulting bundle thickness is more heterogeneous, ...' : quantification to say how these reported values are obtained (from a fit, from an average), but also indicating confidence intervals, standard deviations, sample sizes and number of 'biological' repeats are expected to appreciate how significant those differences are.
Again we appreciate the reviewer pointing out the insufficient documentation of these values. Upon this suggestion we have changed all the graphs comparing the median bundle widths from an average to a box plot, so that the comparison of median and distribution of bundle width is more apparent. The amount of repeats of the experiments was added to method section.

- page 7: the paragraph with 'The formation of thicker bundles than tetrameric VASP can be explained by the behavior of filaments growing at bundles upon filaments diverging from the large bundles.' This sentence should be rewritten to more clearly convey the author message.
We agree that this explanation was not clear enough. We reworded the explanation in the revised manuscript.

- page 8, 'The reduced diffusion suffices to hinder processive barbed end elongation by VASP.' There are no data corresponding to this statement in the manuscript. Moreover, can the authors elaborate more on the amplitude of the friction force that would prevent barbed end processive elongation by VASP?
Indeed, we expect there to be a more complicated interaction between diffusivity and processive elongation. For VASP mediated polymerization the low diffusion within in the lipid monolayer acts as a barrier the polymerization needs to act against. This leads to a slowdown of polymerization depending of the diffusion. The slower VASP mediated polymerization on the monolayer leads to free actin polymerization being faster and more favored and thus dissociation of the barbed end from VASP. The free barbed end then immediately gets capped. The slower diffusion thus decreases the likelihood for barbed ends to be polymerized by VASP, more so than accumulation of VASP to existing filaments. To emphasize this, we added an experiment with prepolymerized actin on a monolayer in Supplemental Figure 4H.

- page 9: 'This negative colocalization to actin proceeds directly opposed to the accumulation of VASP at filaments during polymerization (Figure 4B,E, Video S4).' Once bundling has been achieved (by VASP polymerization), does VASP accumulation beneath actin filaments disappear? if not, then can the author rephrase their statement by removing '...during polymerization'?
“During polymerization” here should have described the time at which point the negative correlation starts to appear. Indeed though, VASP accumulation starts to slowly disappear after polymerization as well as shown now in Figure 4D and 5C. We have reworded the part to make this clearer.

- Related to the previous point is the sentence 'After the initial displacement coupled to actin polymerization observed under a bundle, the lipid intensity relaxes to around half of initial intensity over 90 min (Figure 4F).' This relaxation is correlated to the decrease in actin intensity in the bundle. An assay measuring the relaxation of VASP accumulation together with actin (or bundle width) would be more informative here.
We thank the reviewer for this helpful remark. We have measured the relaxation of VASP as well and changed the plot to the average of 5 bundles in Figure 5C, so there is less confusion about the bleaching observed in the actin intensity of an individual bundle. Indeed we feel the relaxation of the lipid intensity is now explained in a more clear way.

- In the discussion, 'The thicker bundles have a higher propensity to bind VASP from the fluid bilayer than a single actin filament.' It would be more appropriate to say 'larger' than 'thicker' bundles.
We have adapted this suggestion for the text.

- In the discussion, 'As a consequence, larger bundles with a heterogeneous width distribution are formed.' Can the authors explain why this should lead to a wider distribution of bundle sizes?
The larger bundles have more binding sites for VASP and more VASP accumulation. In turn the higher VASP concentration leads to more nucleation or polymerization of actin at these bundles. Additionally, competition of barbed ends for actin monomers is expected. This results in some bundles growing larger, while small bundles or not favored to grow. We have expanded the explanation in the manuscript for this point.

- Regarding FRAP experiments, have the authors been able to observe any correlation between the immobile fraction and the VASP-induced filament bundle size? This would highlight the fact that part of the VASP proteins are actually 'trapped' by bundles?
Unfortunately, due to the small mesh size or small bundle size it is difficult to perform the FRAP on isolated structures using the same FRAP geometry. Within the FRAP ROI the VASP at bundles

seems to have a larger immobile fraction, but this is can be due to differences in recovery geometry as well.

- In the discussion, 'In these actin structures, knock-down of VASP leads to a decrease in size as well (Damiano-Guercio et al. 2020; Tojkander et al. 2015). ' Please, specify what object has its size decreased in this sentence.
This should have been in regard to the listed actin structures (focal adhesions, stress fibers) in the sentence before. To make this point clearer we have reworded the sentence and mentioned the focal adhesions and stress fibers again.

- In the discussion, 'The polymerization driven actin alignment proceeds on a second time scale, which is noticeably faster than the observed minute timescale alignment by linking F-actin filaments together (Falzone et al. 2012; Kelley et al. 2020). ' I am doubtful that fascin or alpha-actinin operate at a slower rate. For example, Breitsprecher et al, JCS 2008 reports fascin-induced bundling zippering speed of hundreds of subunits per seconds.
We agree that this was formulated in an unclear way. This was now changed in the manuscript to discuss the alignment processes in a more precise way. For zippering dependent bundling processes larger bundle formation is limited by the decreased likelihood of filaments meeting due to the decreased Brownian motion of larger bundles. Therefore, large bundle formation is limited.

Minor points:
- Supp Fig. 1E : the authors say there is no bundling in this case (VASP on ML, no CP). What would be the quantification if using the local thickness plugin here?
The quantification gives a median thickness of 0.577. The quantification for the measurement of VASP on ML, no CP as well as VASP on SLB no CP is now in Supplemental Figure 4C and we have added the box plots for the quantification in Supplemental Figure 4F as well.

in the methods section: 'For fluorescent actin, monomers were labelled at the N-terminus with Atto-488 (Jena Bioscience, FP-201-488) or Atto-647N NHS-ester (Jena Bioscience, FP-201-647N)'. I believe actin is not labeled at the N-terminus using NHS-ester but more probably on surface lysines.
The reviewer is absolutely correct. Structurally the N-terminus is exposed in actin filaments, but apparently lysine 328 is mostly targeted by Alexa488-NHS, whereas other reactive species preferentially target other lysines.
We changed this part in the methods.

- There is no mention of the mDia1 and alpha-actinin constructs used and how they were produced and purified. the pH of the KMEI buffer is not specified.
We apologize for forgetting to add this information. The protocols or references to the purification were added to the method section.

- figure 2F: how 0.1µM atto-actin can be polymerized by VASP in the presence of 10µM profilin ?
We thank the reviewer for pointing this out and apologize for this error. We actually added 0.5 µM Atto647-actin. This was corrected in the manuscript.

- At 10% NTA lipids, there is apparently no saturation of NTA sites by His-VASP as incubating with more VASP results in more actin filaments and bigger bundles. Can you confirm this using fluo labeled VASP?

Indeed, we confirmed this using labeled VASP. Above 1 µM of VASP, the VASP fluorescence the VASP fluorescence only slightly increases further with incubating higher concentrations. From 0.05-1 µM VASP a steady increase in fluorescence depending on the VASP concentration can be observed, whereas above 1 µM the bilayer starts to get more saturated.

Reviewer #2 (Remarks to the Author):

This work aims to describe how VASP coordinates its actin filament elongation and bundle formation activities. By targeting VASP to the surface of synthetic lipid bilayers, the authors are able to reconstitute the formation of massive actin bundles in the presence of actin, profilin and capping protein. This result leads them to the conclusion that the nature of VASP's localization, particularly its binding to a fluid membrane, is key to its function.

This experimental system is well designed, but some weak points need to be worked on in this study. I am convinced that VASP is forming these nice bundles and the model makes sense, but I am not convinced by the authors' arguments that elongation mediated bundling is unique to VASP. I detail below why some of the controls performed are not very convincing. Ideally, the authors should have studied different mutants of VASP to separate its bundling and elongation properties, rather than relying on very different proteins such as alpha-actinin or mDia1.
We agree with the reviewer, that this would be nice to see. We tried using these constructs as well. Unfortunately, The GAB or FAB binding domain of VASP can each compensate a part of the other domain's activity respectively. Therefore, it is not really possible to separate these functions in VASP mutants and get reliable information about the contributions of the respective domains.

1/ I don't think the authors can build a strong argumentation based on negative results using alpha-actinin and mDia1. These experiments do not rule out the possibility for these proteins to form thick bundles from elongating actin filaments. Many arguments could be made that these experiments are not comparable. Are surface densities of proteins equivalent? Are these proteins diffusing at the surface of lipid bilayers similarly?
We tried to approximate similar conditions in these regards. All proteins are bound via His-tag to the bilayer and as the monomeric concentrations were used for the incubation, the same amount of His-tagged protein can be expected. The proteins have a fairly similar size and it has been shown, that bound protein has comparably little effect on the diffusion on a bilayer compared to the diffusivity of the lipids.
Are their affinities for actin comparable?
We are aware, that mDia1 has longer processive run length and α-actinin binds F-actin stronger than VASP. These differences in binding mode illustrate how the specific properties VASP as protein has contribute to the presented actin bundling mechanism, in contrast to canonical crosslinkers or processive barbed end elongation. The ability to move on the actin filaments plays an important part in its abilities compared to static crosslinking or processive elongation without being dynamically bound to F-actin sides.
How does the fact that alpha-actinin pulls down F-actin from the bulk solution to the membrane excludes the possibility that this protein could, under other conditions, bundle actin filaments freely polymerizing at the surface of lipid bilayers?
The α-actinin experiments were performed with profilin and capping protein present as well as without these proteins. In the shown condition in Fig. 3B with polymerizing actin, very little alignment is visible. The alignment and hence bundling ability of VASP is precisely governed by

the fact that the filaments are being solely polymerized by VASP and hence bound to the surface where they can be captured by VASP decorating the existing filament. Non-VASP polymerization is not restricted to 2D and has little binary collisions with the previously formed filaments.

2/ The idea that a factor combining nucleation/elongation and bundling activities can efficiently "amplify" actin bundle formation is not new in the field. Ironically, it is Laurent's team (who is editing this paper) who first put forward this idea so he will be a better judge than me on this matter. At the very least, I think his paper (Michelot and coauthors, Current Biology 2006) should be cited and discussed here.
We thank the reviewer for finding this paper, which we unfortunately missed before. We added a comparison between the bundling mechanisms in the discussion. Interestingly, even though both VASP and AFH1 can nucleate, elongate and bind F-actin, the bundling mechanism is different. AFH1 is creating bundles by facilitating filament growth in the same direction, which then bundle through zippering and Brownian motion. VASP is creating the bundles by processively polymerizing filaments, while being dynamically bound to the existing bundles. This leads to larger bundles, as it is not limited by reduced Brownian motion of larger bundles and displays a positive feedback of larger bundles recruiting more VASP.

3/ If bundling and filament elongation activities must be coordinated to promote the formation of actin bundles, would an experiment using simultaneously mDia1 and alpha-actinin (with alpha-actinin bound to lipid bilayers or in solution) lead to bundle formation similar to the VASP condition? If not, why?
This is an excellent suggestion for an experiment and both reviewers requested a similar experiment in this regard. We performed the experiment and added the results to the manuscript in Figure 3. Both mDia1 and α-actinin lead to fast polymerization of actin and all filaments being anchored to the bilayer. Nevertheless, very little alignment of polymerizing filaments and no processive elongation guided to existing filaments was observed. Instead the formation of a dense nematic actin field can be seen. This is in line with the explanation that the ability of VASP to freely move bound to the filaments, capture barbed ends and continuously elongate guided to the bundles plays an important role in the bundle formation. These properties are not recapitulated by the separate anchoring and elongation abilities of mDia1 and α-actinin.

Additional comments on data presentation and analysis:

4/ I have to admit that I do not understand how the authors see the elongation of filaments in Figures 1B-D. It is therefore impossible for me to properly evaluate this part of the work. The authors need to provide more obvious data or analysis to support their claims.
We apologize for making this unclear in the figure. Over the time series (Figure 1B) it is visible, that the filament seeds are getting longer until they merge together. Subsequently an intensity increase on the previous filament is visible in line with were the elongation is happening at the same elongation rate as before the alignment. We have added more explanations concerning this part of the figure in the main text. Additionally, we made the kymograph more intuitive to understand by adding the axes.

5/ Estimating bundle size from their diameter is not at all precise with fluorescence microscopy. I recommend presenting only the fluorescence intensity data, which is certainly much more accurate.
We agree that for very small bundles, the width is not very reliable to observe differences in size. The main point of the manuscript focusses on the formation of large bundles though, where differences

in size can be quantifiably assessed using the bundle diameter. In TIRF microscopy the field of view is not evenly illuminated leading to large intensity differences over the image. Additionally, minimal differences in scattering or Z direction lead to bigger differences in the excitation intensity. To get more quantitative TIRF intensities a TIRF setup with a spinning azimuth angle would be needed. This would decrease the frame rate, which would make observation of the polymerization more difficult. Unfortunately, our TIRF setup does not have this option, therefore the diameter of the bundles above the diffraction limit enables more accurate comparisons between the different constructs and conditions.

RE: Manuscript #E21-11-0577R
TITLE: "VASP localization to lipid bilayers induces polymerization driven actin bundle formation"

Dear Andreas,
You will see that the reviewer found your manuscript improved. But he/she still has some comments on your manuscript, and I would like to give you the opportunity to address them before accepting your manuscript for publication in MBoC.
All the best,
Laurent

Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Bausch,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Overall, I am satisfied by the responses given by the authors to reviewer comments.
Still, I think there is room for improvement to be made in the way the text is written to better express concepts, describe results and discuss them. This would really improve the readibility of the manuscript. Some sentences are sometimes very hard to connect to the previous ones, or are not clearly connected to the main idea of a paragraph.
That being said, I have still a few points that I would have liked to be clarified/improved.

Major:
- sentence "Double the actin concentration of 4 µM compared to 2 µM during polymerization actually leads to a decrease of bundle width, as the faster polymerization leads to some filaments are polymerizing in a VASP independent fashion and thus not bound to the bundles" does not seem to be correct according to the data shown in supp figure 3F. Besides, it provides explanation/interpretation of events that have not been verified. Was the amount of CP and profilin in the presence of larger amount of actin also been increased ?
- page 7, "On larger bundles VASPΔTetra accumulates and therefore is able to track and elongate actin barbed ends" : At a specific VASP concentration (whether monomeric or tetrameric), the binding affinity of VASP for filament side sets the density of VASP per micrometer of filaments. Apart from the immediate neighboring filament, the presence of other filaments for large bundles will not change the local density of VASP. Thus, I am very doubtful that large bundles will induce a faster barbed end elongation rate (which is not shown either), which will provoke a reinforcement mechanism leading to bundles to be even larger...
- figure 4D: it is a pity that we do not see the actin network overlaid with the VASP intensity.

Minor:
- figure 2D : please improve the quality of the graph (use connected points rather than single isolated data points).
- supp figure 3 A-C is very surprising as the images in A and B are very different.
- page 6 "This polymerization mediated bundling stands in contrast to bundling by traditional crosslinkers, which pull together filaments with multiple F-actin binding domains.", replace 'pull' by 'hold' or 'bind'.
- page 7: "For these experiments profilin and capping protein had to be emitted to observe actin polymerization (Figure 3E)." typo 'emitted' for 'omitted'. Please correct.
- page 7: there seems to be typo for the a-actinin concentration in figure 3E (0.2 and 0.5µM, whereas the main text specifies 0.05µM).
- Supplemental Figure 4G: a quantitative assessment of the VASP phase separation area as a function of VASP concentration would have been very much appreciated.

Reviewer #1 (Remarks to the Author):

Overall, I am satisfied by the responses given by the authors to reviewer comments. Still, I think there is room for improvement to be made in the way the text is written to better express concepts, describe results and discuss them. This would really improve the readibility of the manuscript. Some sentences are sometimes very hard to connect to the previous ones, or are not clearly connected to the main idea of a paragraph.
That being said, I have still a few points that I would have liked to be clarified/improved.

We thank the reviewer for the detailed comments and suggestions. In order to improve the readability, we had a lector service proof reading it, and hope now that the English is improved. As listed in the point by point response, we rewrote the parts specifically mentioned in the suggestions to be more precise and added more data to the figures. Therefore, we believe we can present an improved and finalized manuscript.

Major:
- sentence "Double the actin concentration of 4 µM compared to 2 µM during polymerization actually leads to a decrease of bundle width, as the faster polymerization leads to some filaments are polymerizing in a VASP independent fashion and thus not bound to the bundles" does not seem to be correct according to the data shown in supp figure 3F. Besides, it provides explanation/interpretation of events that have not been verified. Was the amount of CP and profilin in the presence of larger amount of actin also been increased?
We apologize for the confusing sentence and have reworded it to be more clear. The normal conditions are polymerization of 2 µM of actin. At 4 µM of actin, the bilayer is expectedly more decorated with actin filaments, but the median bundle size as well as the maximal bundle width is smaller. Consequently, no bundle displays a diameter larger than 1.8 µm. The concentration of CP and profilin was kept constant, which definitely plays a part in the enhanced polymerization at 4 µM. As the complete lack of CP only leads to a small decrease in bundle size and actin is already used at a high excess compared to CP, we expect the faster actin polymerization to still play a part in promoting VASP-independent polymerization, which is not bound to the bundles. In the text we did not discuss the contributions of the CP to actin ratio in this case. Therefore, we changed the text to incorporate this as well.

- page 7, "On larger bundles VASPΔTetra accumulates and therefore is able to track and elongate actin barbed ends" : At a specific VASP concentration (whether monomeric or tetrameric), the binding affinity of VASP for filament side sets the density of VASP per micrometer of filaments. Apart from the immediate neighboring filament, the presence of other filaments for large bundles will not change the local density of VASP. Thus, I am very doubtful that large bundles will induce a faster barbed end elongation rate (which is not shown either), which will provoke a reinforcement mechanism leading to bundles to be even larger...
We agree that it would be very interesting to see the barbed end elongation speed. Unfortunately, in our setup we cannot observe individual filaments on the bundles. Nevertheless, we expect a slight concentration dependent increase in polymerization

speed. We believe that our data indicate contributions to the local concentration of VASP of more than the outer most actin filaments, thus providing the necessary concentration to speed up actin polymerization.
The isotropic distribution of VASP under the bundles suggests VASP and actin are not excluding each other on the bilayer. The diameter of actin of 6 nm is very small compared to the average diameter between VASP ends of 35-40 nm. Additionally, the VASP domains have a high degree of freedom of movement due to the intrinsically disordered region. It seems unlikely that actin filaments would therefore be able to create a physical barrier for VASP. As the bond of VASP to F-actin is very transient, it should be able to freely move between filaments of the bundle. This can be seen by the FRAP data as well. There we do not see a clear directional recovery or movement blockade by the bundles, as would be the case if only side binding was possible. The movement by VASP over long time periods to the sides of bundles seems to be more indicative of the slow reformation of VASP clusters. For this the VASP movement does not seem to be impeded in a major way as well.
In contrast to an increase of polymerization speed leading to larger bundles would be an increase of the number of barbed ends polymerizing at the bundles. The high local VASP concentration should lead to continuous processive polymerization by multiple individual processive run lengths of VASP molecules. As very little elongating filaments are diverging and other filaments continuously converging on the bundles, a high number of polymerizing barbed ends is formed on the large bundles. Additionally, nucleation events on the bundles by the high VASP concentration might be possible.

- figure 4D: it is a pity that we do not see the actin network overlaid with the VASP intensity.
We agree that it would be nice to see the actin channel for these images as well. We have thus added the actin images there. We initially thought this would be redundant with the images in Figure 4A. The overlay of both channels was not shown as the VASP distribution is less visible due to the high degree of colocalization at actin.

Minor:
- figure 2D : please improve the quality of the graph (use connected points rather than single isolated data points).
This graph should definitely be clearer. We have changed the graph by adding a fit using log-normal distribution. We think the fit over the distribution is more appropriate than connected points, as the data represent histograms with binned width and therefore not a continuous graph. In line with this adjustment, we also changed the similar graphs in Supplemental Figure 2I and Supplemental Figure 4C.

- supp figure 3 A-C is very surprising as the images in A and B are very different.
There is definitely a noticeable change in bundle width observable. This is expected as the concentration of 0.5 µM of actin in SuppFig 3A is already fairly close to the critical concentration. But there are already large bundles visible with the diameter clearly bigger than the diffraction limit. This is striking compared to the width differences at varying

VASP concentrations, as these noticeable bundle widths like in SuppFig 3A,F are not observable below 0.25 µM VASP (Figure 2E).

- page 6 "This polymerization mediated bundling stands in contrast to bundling by traditional crosslinkers, which pull together filaments with multiple F-actin binding domains.", replace 'pull' by 'hold' or 'bind'.
We agree that this wording might be confusing and changed it to ‘bind’. ‘Pull’ was initially used in regard to the measureable force exerted by crosslinkers like anillin on filaments as described in Kučera et al. 2021.

- page 7: "For these experiments profilin and capping protein had to be emitted to observe actin polymerization (Figure 3E)." typo 'emitted' for 'omitted'. Please correct.
We apologize for this mistake and corrected the typo.

- page 7: there seems to be typo for the a-actinin concentration in figure 3E (0.2 and 0.5µM, whereas the main text specifies 0.05µM).
This was actually not a typo. Different concentrations of α-actinin were used to compare the actin networks with VASP. The high concentration of 0.5 µM was used in conjunction with CP and profilin, to create a stronger case that the polymerization is severely hindered. The intermediate concentration of 0.2 µM was used as a similar concentration regime to the shown polymerization kinetic with VASP. The low concentration of 0.05 µM had to be used for measurements were filaments or bundles should be distinguishable and thus for the shown image as well. We agree that this might be confusing to the reader and have therefore changed the shown actin intensity kinetic to the 0.05 µM α-actinin experiment. Additionally, we have changed the main text to emphasize the different concentrations used and the reasoning for these conditions.

- Supplemental Figure 4G: a quantitative assessment of the VASP phase separation area as a function of VASP concentration would have been very much appreciated.
This is an excellent suggestion and we now added a respective plot showing the area fraction of the VASP phase.

RE: Manuscript #E21-11-0577RR
TITLE: "VASP localization to lipid bilayers induces polymerization driven actin bundle formation"

Dear Andreas

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Best regards,

Laurent Blanchoin
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Bausch:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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