nature portfolio

Peer Review File

Cryo-EM Structure and Oligomerization of the Human Planar Cell Polarity Core Protein Vangl1

Corresponding Author: Dr Shanshuang Chen

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)
This paper by Zhang et al (Wu and Chen labs) addresses the structure of the core planar cell polarity (PCP factor Vangl (named after the Drosophila gene Vang Gogh/Vang) via cryo-EM analyses. The work provides in principle useful insight into the structure and architecture of the Vang/Vangl associated PCP complex. While the data shown are interesting the paper lacks integration within the field and its context. There is one major conclusion made in this paper: demonstration that Vangl1 forms trimers as basic structural subunit and hexamers upon interaction with Pk (and I think it is fair to say by extension that all Vang/Vangl protein members share the same features). The Pk effect is. Not shown here but inferred. While this is relevant, the text is difficult to follow and interpret, as the data presented are not integrated and/or compared to previous existing publications in the PC field and the context of PCP establishment in general. It will require major revisions to achieve the quality the reader is expecting from a Nature Communications paper. Please see specific comments below.

The main issue with the current manuscript/data set is that is does not at all address how the obtained cryo-EM data fits in with the existing knowledge of the Vang/Vangl protein and its interaction partners, also including at least the mention of the Frizzled/Dsh-Dvl complexes. As such it is poorly presented and written, when thinking about the general impact and big picture.

Main points:
(1) The structure as shown in Figure 2 resembles very much the predicted structure as it comes from AlphaFold. This includes the split domain referred to here as the “Hand’ and similarly the N-terminal domain is not structured in this paper, same as in AlphaFold. I think there needs to be some referene to the predicted AlphaFold structure in the text, Results and Discussion.

(2) Figure 2: it is not explained how one goes from the pictures shown in panel 1a to the graphs shown in panels b, c and d. This needs to be explained in adequate detail.

(3) It seems that the Vangl protein forms a trimer as its basic unit. Why is it assumed that the hexamer with its predicted tail-to-tail arrangement of two trimers is really the physiological multimer. Could this be an artifact of the methodology? The Strutt lab has demonstrated – via careful and physiological in vivo studies – that there are 6 Vang/Vangl molecules for each Pk molecule so a hexamer makes sense, but they had no evidence for the arrangement as shown here, which would imply a juxtaposition of a plasma-membrane trimer with a vesicle-associated primer on the cytoplasmic side of the plasma-membrane trimer. At the minimum, the authors should show that such a scenario is possible in cells. I think it is unlikely the ‘arrangement’ in a stable membrane associated complex, but that might be just my opinion.
The reference for the Strutt lab data showing in vivo a 6:1 ration is:
Strutt, Gamage and Strutt (2016) Cell Reports, PMID 27926869. This paper needs to be discussed in the context of this work.
I recommend the authors read the relevant papers by the Strutt lab to allow them to better understand, interpret, and explain the Vangl structure.

(4) The formation of stable Vang/Vangl complexes depends on the phosphorylation of the N-terminal region. References: #25 and
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Kelly et al (2016) Cell Reports. PMID 27346358
Strutt et al (2019) Elife. PMID 31090542
How do the authors envision that fact that Pk is promoting stable complex formation/clustering by inhibiting N-terminal phosphorylation? As the authors propose that Pk serves as a bridge between the two trimers, this is highly relevant. Also, Pk1 and Pk2 (and Drosophila Pk) are a large proteins with >800 amino acids – significantly larger than a single Vang/Vangl molecula, and so it should be discussed how the model shown in Figure 5 is working. Here Pk is wrongly drawn as a much smaller unit than a single Vang/Vangl molecule.

(5) Figure 3: the CTL is not resolved (as stated on line 136). How is inserted into groove of the Hand domain?

(6) Stated on line 216 "… Pk binding domain…" lacking a link to published data. The most detailed analysis of the Pk binding region in Vang/Vangl is by Humphries et al (2023), PLoS Genetics, PMID 37463168 (which should be cited!). This defined phosphorylation of Tyr312 in human Vangl1 (and surrounding residues) as critical for Vang/Vangl-Pk binding. Where are these residues in the structure and how could the predicted Pk biding there promote the formation of a hexamer? Similarly, the authors suggest that binding of Dvl will "require additional biochemical investigations" as stated on line 321: the respective mapping of the Dvl/Dsh protein to Vang/Vangl is published in detail (with physiological in vivo confirmation) in the same above citation: Humphries et al (2023), PLoS Genetics, PMID 37463168

(7) Figure 4 (and lines 235-280 in the text) describes the mapping of Vangl1 and Vangl2 mutations found in human patients of NTDs on the structure. The presence of such mutations in humans is not proof of them causing neural tube defects, it's only a correlation. Functional studies in model organism are needed to document causality of such mutations for the disease phenotype. Recent work has demonstrated such causality for some of these mutations and should be cited: Humphries et al (2020) Elife, PMID 32234212.

(8) on line 328-330, the authors state: "However, Vangl lacks prominent extracellular domains, thus a structural basis for how it interacts with the distal PCP complex remains elusive." With distal complex I assume the authors mean the Frizzled-based complex, and the interaction between the Frizzled and Vang/Vangl complex has been published in detail, it is inter-cellular as it would need to be via the extracellular loop of Vangl. Please see and cite: Wu and Mlodzik (2008) Dev Cell, PMID 18804440.

A few specific textual points that need to be addressed:

- line 36: the authors should cite reviews that are relevant, up-to-date, and readable (by non-experts) from the leaders in the field. As it is now the only useful reviews cited are Adler 2012 (who is retired) and two reviews from the Strutt lab (at line 38). Additional reviews from Axelrod, Mlodzik, Devenport, and Wallingford should be cited. Refs #1 and #3 are not useful as they're too specialized on the mouse inner ear.

- line 38: same comment as above. Additional comprehensive reviews from Axelrod, Mlodzik, Devenport, and Wallingford should be cited.

- line 40: Ref #7 is not relevant and unreadable to non-experts… I am sure the authors did NOT read that paper. Comprehensive reviews from Axelrod, Mlodzik, Devenport, and Wallingford should be cited.

- line 41: ref #10 should also include the original reference describing the factor: Uemura et al (1999). Cell 98. Pg 585-595. PMID: 10490098

- line 42: ref #13… all the relevant Drosophila references are missing, for example:
Vinson et al (1989) Nature. PMID 2493583
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- line 47: at end of sentence there have to be references. I suggest to use the same reviews that should be added above from Axelrod, Mlodzik, Devenport, and Wallingford.

- line 50: references are missing: for example…
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- line 55: refs #15-20, the following citations should be added:
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- line 62: refs #23, 24. The dominant negative behavior has not been formally demonstrated except in: Humphries et al (2020) Elife. PMID 32234212

- lines 64-66: while ref #25 was the first to show a link from Fz-Ror to Vang phosphorylation, it did not address “localization and strict abundance control of Vang/Vangl”. These would be the following citations:
Strutt et al (2016) Cell Reports. PMID 27926869
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Strutt et al (2019) Elife. PMID 31090542
Strutt et al (2023) Current Biology. PMID 37995695

- line 72: the statement “biochemical” should be removed, structural is the point.

- lines 100-101: This sentence does not make sense, syntax error?

- line 105: sentence does not make sense, syntax error?

- line 110: “earlier peak”? Do the authors mean “higher volume” peak?

- line 306: missing references of sentence ending on 306. Besides ref #32, the authors should list:
Jenny et al (2003) EMBO J, PMID 12941693.

This is not an exhaustive attempt at correcting and/or improving the text.
There are many more writing issues like this, which should be corrected for better integration of this work in the current context and to allow for better understanding of their data by the reader.

Reviewer #2

(Remarks to the Author)
The manuscript by Zhang et al, report structure of VANGL1, a membrane protein crucial for Planar Cell Polarity signaling in vertebrates. Overall, the manuscript is well written, and figure clearly illustrate the findings. The structure provides new insight on hexameric nature of VANGL1. The structure is very novel and opens new avenues for research. The major caveat in the manuscript is that author fails to provide clear insights the potential implication of oligomeric assembly for VANGL1 functions. To further strengthen the manuscript and to merit its publication in Nature Communication, authors should address some of the issues mentioned below.

Major
Authors shows that two distinct SEC fractions of VANGL1 correspond to the trimeric and hexameric population. Why cryo-EM analysis of hexameric species was pursued and trimeric state of VANGL1 is not? The structure of trimeric complex is interesting and could provide new insight for its VANGL1 function. Authors claim that PK1 preferentially interact with hexamer vs trimer. Author should provide additional binding experiments with purified VANGL1 hexamer and trimer peaks to support this claim. Further, since the binding site of PK1 is proposed to be at the interface of two VANGL trimers, the stoichiometry of VANGL:PK interaction should be clarified. Whether it is 1:0.5 not 1:1. Can in-vitro binding experiments resolve this? hill coefficient of binding curves? In either case, author should also consider reanalyzing the cryo-EM datasets using C3 or C1 symmetry, perform symmetry expansion (from C3 and D3 refined maps), no-align 3D classification, 3D variability analysis for improving the density features of PK1. The results should be included in supplementary files.
The FSEC profiles of VANGL1 wildtype and mutants cannot be used for comparison as the amount of protein injected for each protein is different (Supplementary Figure 8B). Does equilibrium of hexamer and trimer is concentration dependent? These experiments should be repeated by injecting similar amount of purified protein for each case. Clarify whether these purification / SEC profiles are reproducible and how many replicates were used before drawing the conclusion. Same goes to native PAGE experiments analysis.
Authors should provide the details of electrophysiology experiments and results in manuscript. Were electrophysiology experiments performed on whole cells or liposomes? Did author test the activity of hexamer and trimeric population? Again, the structure of trimer might be interesting for describing the channel properties. Hexamer assembly might be more restrained conformation

Minor
• Author should also clarify the expression and purification protocols described in material and methods. From current protocols, it’s not clear whether purification method is stated for VANGL1 or VANGL1-PK1 complex or both? Is expression-purification protocol of VANGL1 and VANGL-PK1 complex same? Why 500mM NaCl was used during solubilization? I guess high salt can potentially the destabilize VANGL1-PK1 complex and may be cause of poorly resolved density of VANGL1-PK1 complex?
• Figure 4 is hard to follow. Author should make the boxes/color more prominent or label the boxes in main figure.
• Page 26, line 470: What was final concentration of biotin? 150mM?
• Supplementary Figure 8; Sigma threshold levels of cryo-EM maps used for preparing figures should be provided in figure

legends.

Reviewer #3

(Remarks to the Author)
Zhang et al. reports the structure on VANGL1, which is a key factor for the establishment of plana cell polarity (PCP). The manuscript includes several important findings: 1) the first high resolution structure of VANGL1, 2) the interaction of two membrane-bound VANGL1 trimers, which is necessary for PK1-binding 3) Mapping of disease-causing mutations on the VANGL structures with the interpretation of disease-causing mechanisms. These findings significantly impoves our understanding of the complicated mechanism of PCP establishment. However, the proposed model of dynamic oligomerization equilibrium of VANGL1 (presented in Fig. 5) is not supported by sufficient experimental evidence, and thus needs to be revised before publication.

1. The equilibrium model and Fig. 5 need to be revised unless it is proved by more structural evidences.
1-(1) Although partially dissociated complexes of Vangl1 and PK1 have been identified in cryo-EM images, this does not necessarily represent an intermediate state of the equilibrium. The partial interaction of two Vangl1 trimers in the kinked complex should not be proposed without high-resolution evidence.
1-(2) Given that the PK1-binding was observed in 2d classes, at least partial density of the bound PK1 should be observed in the refined structure. PK1-bound and PK1-free Vangl1 hexamers might have been sorted out in 3D classification, and the final structure might have been reconstructed mainly with PK1-free hexamers. The authors need to thoroughly inspect all 3D classes.
1-(3) The kinked oligomers could be further analyzed to obtain high-resolution information. For example, trimer-focused image processing and 3d classification without symmetry imposition may produce a 3d class containing PK1-bound trimer substructures, which could be identified by comparison of the densities around three stick domains.

2. In the gel filtration fractions, the oligomerization states should be re-equilibrated. Therefore, the Native PAGE result for peak fractions needs to be reinterpreted. In the native gel result, high and low ratios of hexamer/trimer for Peak1 and 2 seem to be due to high and low protein concentration in Peak1 and 2 fractions. To assess the oligomerization equilibrium, concentration-dependent change in the oligomeric states needs to be examined by native gel electroporesis experiments with increasing concentrations of purified Vangl proteins. This may also allow the authors to roughly estimate the dimerization affinity.

3. If the dynamic dimerization equilibrium is true, the dimerization of Vangl trimers could be strengthened by PK1-binding, shifting the equilibrium. A similar native PAGE could readily address this hypothesis.

line 114, Unclear description. Does it mean "two-fold symmetry in dumbbell-shaped side views?" Please also indicate exactly which figure and panel.
line 116, Unclear description needs to be revised.
line 121, Error correction: "with each" to "and each"
line 125, "the opposite side" is clearer than "the other side"
line 140, Not layers but protein domains in each layer are related by three-fold symmetry.
line 159, "kinks into" seems grammartically wrong.
line 201, "Hexamer to trimer" is correct.
line 234, Electrophysiological data along with detailed methods need to be presented.
line 246, "extracellular halves" than “upper half" is clearer.
line 247, Does L242V belong to vangl1 or 2?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised version of this paper is much improved. The authors have carefully addressed all the comments from my side (I do not want to speak for the other reviewers). From my side the paper is now sufficiently well written and anchored/embedded in the field to make it a worthwhile contribution to Nature Communications.

Reviewer #2

(Remarks to the Author)
I am satisfied with the revisions done by the authors, and in principle, the manuscript is now suitable for publication.

Basavraj Khanppnavar

Reviewer #3

(Remarks to the Author)
The authors addressed all my questions. I have no more concern.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This paper by Zhang et al (Wu and Chen labs) addresses the structure of the core planar cell polarity (PCP factor Vangl (named after the Drosophila gene Vang Gogh/Vang) via cryo-EM analyses. The work provides in principle useful insight into the structure and architecture of the Vang/Vangl associated PCP complex. While the data shown are interesting the paper lacks integration within the field and its context. There is one major conclusion made in this paper: demonstration that Vangl1 forms trimers as basic structural subunit and hexamers upon interaction with Pk (and I think it is fair to say by extension that all Vang/Vangl protein members share the same features). The Pk effect is. Not shown here but inferred. While this is relevant, the text is difficult to follow and interpret, as the data presented are not integrated and/or compared to previous existing publications in the PC field and the context of PCP establishment in general. It will require major revisions to achieve the quality the reader is expecting from a Nature Communications paper. Please see specific comments below.

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We thank the reviewer for pointing out major issues with the current version of the manuscript, and we agree that the manuscript should be improved by making connections between our in vitro data and the frontier knowledge on PCP in existing publications. We have therefore revised our manuscript to address the major and minor points raised here, and a point-by-point response is provided below:

Main points:

(1) The structure as shown in Figure 2 resembles very much the predicted structure as it comes from AlphaFold. This includes the split domain referred to here as the “Hand’ and similarly the N-terminal domain is not structured in this paper, same as in AlphaFold. I think there needs to be some referene to the predicted AlphaFold structure in the text, Results and Discussion.

We appreciate the reviewer's suggestion. The structural model predicted by AlphaFold2 is indeed very similar and was used as a starting model to facilitate our cryo-EM structural modeling. We further compared each structural domain, including the split hand domain, with the predicted structure and further addressed whether the trimeric and hexameric assemblies can be accurately predicted by AlphaFold3. While the predicted trimer agrees with our experimental structure, AlphaFold3 failed to predict the dimeric interface formed between the trimeric stick domains in the hexamer, regardless of whether full-length Vangl1 or the isolated stick domain was used. The comparison between our experimental structure and the structure predicted by AlphaFold is now included in the second paragraph of the Discussion.

(2) Figure 2: it is not explained how one goes from the pictures shown in panel 1a to the graphs shown in panels b, c and d. This needs to be explained in adequate detail.

We believe the confusion is due to the inconsistent color code. We have therefore revised Figure 2 to clarify the confusion.

(3) It seems that the Vangl protein forms a trimer as its basic unit. Why is it assumed that the hexamer with its predicted tail-to-tail arrangement of two trimers is really the physiological multimer. Could this be an artifact of the methodology? The Strutt lab has demonstrated – via careful and physiological in vivo studies – that there are 6 Vang/Vangl molecules for each Pk molecule so a hexamer makes sense, but they had no evidence for the arrangement as shown here, which would imply a juxtaposition of a plasma-membrane trimer with a vesicle-associated primer on the cytoplasmic side of the plasma-membrane trimer. At the minimum, the authors should show that such a scenario is possible in cells. I think it is unlikely the 'arrangement' in a stable membrane associated complex, but that might be just my opinion.

The reference for the Strutt lab data showing in vivo a 6:1 ratio is:

Strutt, Gamage and Strutt (2016) Cell Reports, PMID 27926869. This paper needs to be discussed in the context of this work.

I recommend the authors read the relevant papers by the Strutt lab to allow them to better understand, interpret, and explain the Vangl structure.

We agree with the reviewer and believe that the trimer is likely to be a basic functional assembly in vivo, and it is not our intention to assume that the hexamer is the physiological assembly. Because our biochemical and structural analysis was limited to in vitro systems as we clearly stated throughout the manuscript, we have no evidence to directly support the physiological relevance of the hexamer. We referred to the 6:1 in vivo stoichiometry reported by the Strutt lab, which is consistent with our hexamer observed in vitro, and speculated on a few circumstances where adjacent membranes could accommodate the hexamer, including the plasma membrane-vesicle membrane in close proximity as you mentioned, as well as other organelle membranes involved in Vangl sorting and trafficking. Meanwhile, we do not exclude the possibility that Vangl only forms trimers only at the plasma membrane, and that there is only one molecule of Pk for every two independent trimers of Vangl.

We have revised the corresponding paragraph to discuss the physiological relevance of the hexamer in light of the Strutt lab paper.

(4) The formation of stable Vang/Vangl complexes depends on the phosphorylation of the N-terminal region. References:

#25 and

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How do the authors envision the fact that Pk is promoting stable complex formation/clustering by inhibiting N-terminal phosphorylation? As the authors propose that Pk serves as a bridge between the two trimers, this is highly relevant. Also, Pk1 and Pk2 (and Drosophila Pk) are a large proteins with >800 amino acids – significantly larger than a single Vang/Vangl molecula, and so it should be discussed how the model shown in Figure 5 is working. Here Pk is wrongly drawn as a much smaller unit than a single Vang/Vangl molecule.

We thank the reviewer for raising this interesting question and for pointing out an error in Figure 5. The three papers mentioned by the reviewer have provided strong evidence that the cell membrane localization of Vang/Vangl is dependent on Dco/CK1e mediated N-terminal serine/threonine phosphorylation. The Elife paper also showed that Pk negatively regulates the phosphorylation of Vang. Although our in vitro data indicated that Pk promotes the hexamerization of Vangl, the relationship between the oligomerization of Vangl and its localization to the cell membrane was not established. Given the flexible and thus disordered N-terminal region in our structure, the poorly resolved density that presumably represents some of the bound Pk (Figure S6b), and the absence of Dco/CK1ε in our in vitro system, we do not currently have a convincing explanation for how Pk inhibits the N-terminal phosphorylation of Vangl. The graphical error regarding the relative size of Pk has been corrected.

(5) Figure 3: the CTL is not resolved (as stated on line 136). How is inserted into groove of the Hand domain?

We apologize for the confusing statement caused by a typo. CTL stands for “carboxyl-terminal loop” instead of “carboxyl-terminal tail”. It is the C-terminal end, further beyond the resolved CTL, that is not resolved. The CTL is wedged between the hand domain and the intracellular loop of an adjacent protomer.

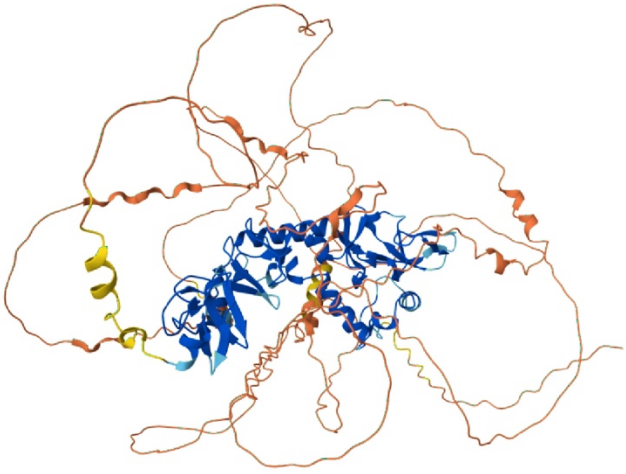
(6) Stated on line 216 “… Pk binding domain…” lacking a link to published data. The most detailed analysis of the Pk binding region in Vang/Vangl is by Humphries et al (2023), PLoS Genetics, PMID 37463168 (which should be cited!). This defined phosphorylation of Tyr312 in human Vangl1 (and surrounding residues) as critical for Vang/Vangl-Pk binding. Where are these residues in the structure and how could the predicted Pk biding there promote the formation of a hexamer?

Similarly, the authors suggest that binding of Dvl will “require additional biochemical investigations” as stated on line 321: the respective mapping of the Dvl/Dsh protein to Vang/Vangl is published in detail (with physiological in vivo confirmation) in the same above citation: Humphries et al (2023), PLoS Genetics, PMID 37463168

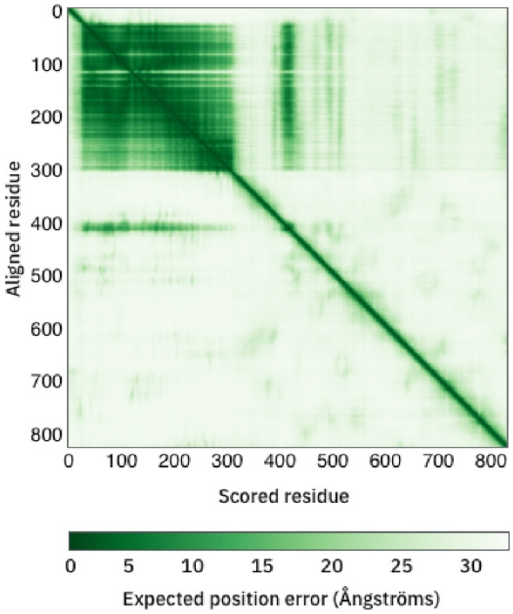
We thank the reviewer for highlighting this important work. This paper provides strong evidence that Stbm/Vang amino acids 364-375 are required for Pk binding using a series of C-terminally truncated Stbm/Vang. This region corresponds to a disordered loop in the stick domain between Helix α_2 and α_{3a}, suggesting that Pk recognizes a flexible linear region independent of the overall folding/oligomerization of Vang, as C-terminal truncations presumably disrupt the folding of the split hand domain and probably trimerization. The Dvl binding site was shown to be adjacent to the Pk binding sites with a partial overlap, but still is largely in the disordered loop.

Phosphorylation of Tyr312, located in the aforementioned unstructured loop, was elegantly shown to be critical for Pk binding. Since our Vangl1 sample was obtained in vitro, Tyr312 may not be phosphorylated, resulting in compromised Pk binding affinity, consistent with the observed low occupancy and thus poorly resolved Pk. Structural prediction of Pk suggests a structured N-terminal half and a poorly folded C-terminal half (see figure below). We therefore speculate that one possible pathway by which Pk stabilizes the hexamer is that the structured N-terminal half of Pk is responsible for recognizing of the Tyr312-containing loop, while the C-terminal half interacts non-specifically with the stick domain at the trimer-trimer interface, consistent with the observed density, which is substantially stronger than the background noise (Figure S6b).

We have discussed the above context in detail in the third paragraph of the Discussion.



Structure of Pk1 predicted by AlphaFold



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We thank the reviewer for pointing this out. We realized that many of the mutants do not necessarily cause neural tube defects due to the lack of phenotyping of model animals carrying disease-related mutations. To make this clearer, we have revised Figure 4 and colored the mutation sites based on whether there are documented animal PCP defects in animals or found in human patients. The accompanying text has also been revised accordingly.

(8) on line 328-330, the authors state: "However, Vangl lacks prominent extracellular domains, thus a structural basis for how it interacts with the distal PCP complex remains elusive." With distal complex I assume the authors mean the Frizzled-based complex, and the interaction between the Frizzled and Vang/Vangl complex has been published in detail, it is inter-cellular as it would need to be via the extracellular loop of Vangl. Please see and cite: Wu and Mlodzik (2008) Dev Cell, PMID 18804440.

It was indeed our intention to refer to the Fz/Fzd – Vang/Vangl interaction. We revised this sentence, which now reads "In particular, it is interesting to understand how direct interactions between Stbm/Vang and Fz in the intercellular signaling complex are achieved, given that Vangl lacks prominent extracellular domains. It has been reported that the extracellular domain of Fz can pull down wild-type but not mutant Stbm/Vang in Drosophila". How Fzd might interact with Vangl is also discussed in light of this paper.

A few specific textual points that need to be addressed:

- line 36: the authors should cite reviews that are relevant, up-to-date, and readable (by non-experts) from the leaders in the field. As it is now the only useful reviews cited are Adler 2012 (who is retired) and two reviews from the Strutt lab (at line 38). Additional reviews from Axelrod, Mlodzik, Devenport, and Wallingford should be cited. Refs #1 and #3 are not useful as they're too specialized on the mouse inner ear.

As suggested by the reviewer, we have revised our citation.

- line 38: same comment as above. Additional comprehensive reviews from Axelrod, Mlodzik, Devenport, and Wallingford should be cited.

Cited.

- line 40: Ref #7 is not relevant and unreadable to non-experts… I am sure the authors did NOT read that paper. Comprehensive reviews from Axelrod, Mlodzik, Devenport, and Wallingford should be cited.

Ref #7 was indeed cited by mistake. The correct references are now cited.

- line 41: ref #10 should also include the original reference describing the factor: Uemura et al (1999). Cell 98. Pg 585-595. PMID: 10490098

The reference is added.

- line 42: ref #13… all the relevant Drosophila references are missing, for example:

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The references are added.

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- line 62: refs #23, 24. The dominant negative behavior has not been formally demonstrated except in: Humphries et al (2020) Elife. PMID 32234212

The sentence has been rephrased with the Humphries paper cited.

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The sentence has been reworded with the above cited references.

- line 72: the statement "biochemical" should be removed, structural is the point.

The word "biochemical" has been removed from the sentence.

- lines 100-101: This sentence does not make sense, syntax error?

We rephrased the sentence, which now reads "We then confirmed direct interactions between Vangl1 and Pk1 by pull-down experiments".

- line 105: sentence does not make sense, syntax error?

We rephrased the sentence to read "After tandem affinity purification, the Vangl1-Pk1 sample was subjected to preparative size-exclusion chromatography (SEC), …".

- line 110: "earlier peak"? Do the authors mean "higher volume" peak?

We have replaced "earlier peak" with "Peak 1" to be consistent with previous statements.

- line 306: missing references of sentence ending on 306. Besides ref #32, the authors should list:

Jenny et al (2003) EMBO J, PMID 12941693.

The missing reference is cited.

This is not an exhaustive attempt at correcting and/or improving the text.

There are many more writing issues like this, which should be corrected for better integration of this work in the current context and to allow for better understanding of their data by the reader.

We are grateful to the reviewer for pointing out many of our writing problems. We have made extensive attempts to improve the text by linking our findings to the existing literature, and believe that the revised manuscript is now a better contribution to the field.

Reviewer #2 (Remarks to the Author):

The manuscript by Zhang et al, report structure of VANGL1, a membrane protein crucial for Planar Cell Polarity signaling in vertebrates. Overall, the manuscript is well written, and figure

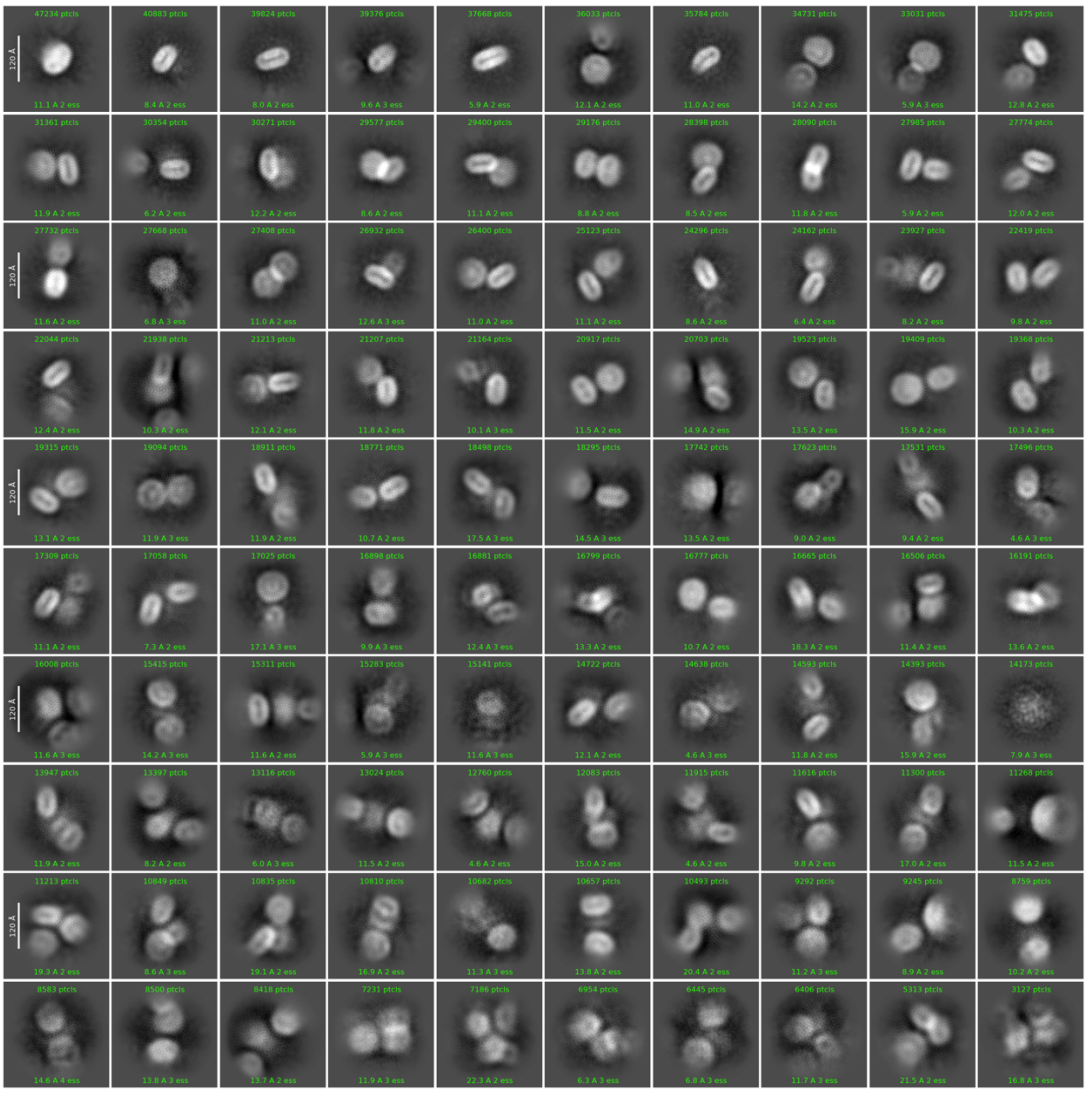
clearly illustrate the findings. The structure provides new insight on hexameric nature of VANGL1. The structure is very novel and opens new avenues for research. The major caveat in the manuscript is that author fails to provide clear insights the potential implication of oligomeric assembly for VANGL1 functions. To further strengthen the manuscript and to merit its publication in Nature Communication, authors should address some of the issues mentioned below.

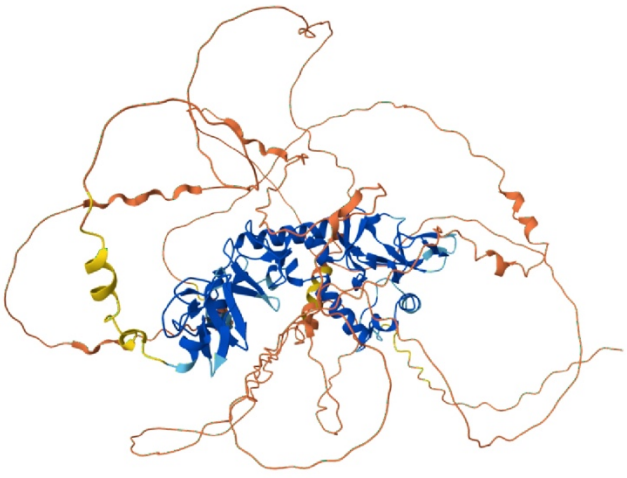
Major

Authors shows that two distinct SEC fractions of VANGL1 correspond to the trimeric and hexameric population. Why cryo-EM analysis of hexameric species was pursued and trimeric state of VANGL1 is not? The structure of trimeric complex is interesting and could provide new insight for its VANGL1 function. Authors claim that PK1 preferentially interact with hexamer vs trimer. Author should provide additional binding experiments with purified VANGL1 hexamer and trimer peaks to support this claim. Further, since the binding site of PK1 is proposed to be at the interface of two VANGL trimers, the stoichiometry of VANGL:PK interaction should be clarified. Whether it is 1:0.5 not 1:1. Can in-vitro binding experiments resolve this? hill coefficient of binding curves? In either case, author should also consider reanalyzing the cryo-EM datasets using C3 or C1 symmetry, perform symmetry expansion (from C3 and D3 refined maps), no-align 3D classification, 3D variability analysis for improving the density features of PK1. The results should be included in supplementary files.

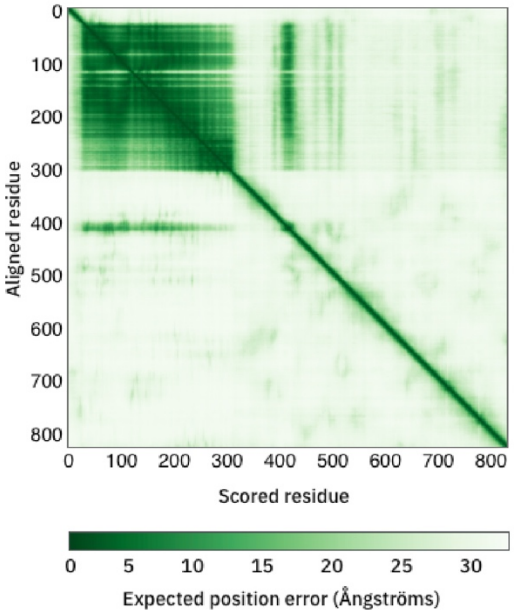
We thank the reviewer for the comments. While our cryo-EM analysis of Peak 1 in SEC revealed a hexameric assembly of Vangl1, we do not have direct evidence to support that Peak 2 is composed of Vangl1 trimers. In fact, we did perform similar cryo-EM analysis of Peak 2 in replicates, but did not see any homogeneous species (representative 2D classes are shown below). Without direct evidence, we revise our interpretation and argue that hexamerization of Vangl1 is important for Pk1 binding. Binding experiments in vitro are challenging, because it is difficult to purify stable Pk1, consistent with the structural prediction (see prediction results below). However, the binding sites and stoichiometry have been reported in vivo in Drosophila. The Strutt Lab showed a stoichiometry of 6:1 (Vang : Pk) in Drosophila pupal wings using quantitative fluorescence imaging (ref #39). The Mlodzik Lab showed that phosphorylation of Tyr312 in the stick domain is important for Pk binding (ref #47). The binding sites and stoichiometry are discussed in detail in our revised Discussion.

We followed the reviewer's suggestion and re-analyzed the cryo-EM data. Despite extensive attempts at 3D classification by imposing C3 or C1 symmetry using either the original or symmetry-extended particles, the density feature putatively for Pk1 was not improved to unambiguousness. Nevertheless, the best results obtained by removing the imposed symmetry are shown in Figure S6B.





Structure of Pk1 predicted by AlphaFold



The FSEC profiles of VANGL1 wildtype and mutants cannot be used for comparison as the amount of protein injected for each protein is different (Supplementary Figure 8B). Does equilibrium of hexamer and trimer is concentration dependent? These experiments should be repeated by injecting similar amount of purified protein for each case. Clarify whether these purification / SEC profiles are reproducible and how many replicates were used before drawing the conclusion. Same goes to native PAGE experiments analysis.

We appreciate the reviewer's comments. The difference in the amount of protein injected was likely due to a different batch of baculovirus production. We repeated all experiments, including FSEC, native PAGE, and pull-down assays with mutant Vang1. With improved baculovirus production from the same batch, wild-type and mutant Vangl1 yielded similar expression levels as shown in updated Figure S8B. Overexpression and FSEC analysis of Vangl1 were performed in 6 replicates, and the preparative SEC and native PAGE were performed in 3 replicates, yielding consistent results (now described in the figure legend for Fig. S8).

Authors should provide the details of electrophysiology experiments and results in manuscript. Were electrophysiology experiments performed on whole cells or liposomes? Did author test the activity of hexamer and trimeric population? Again, the structure of trimer might be interesting for describing the channel properties. Hexamer assembly might be more restrained conformation.

We appreciate the reviewer's suggestion. The electrophysiological experiments were performed in a whole-cell patch clamp configuration using HEK293T cells. Although the structural analysis revealed a hexameric assembly of Vangl1, it remains unclear whether Vangl1 on the cell membrane adopts the same oligomerization or becomes trimeric. Although it is not as easy as it seems to stabilize and capture the structure of trimeric Vangl1, the structural prediction of trimeric Vangl1 by AlphaFold3 indicated that the trimer is similar to half of the experimentally determined hexamer (Figure S6B). However, we do agree with the reviewer and will strive to test a similar hypothesis that the hexamer may represent an autoinhibited state by structural determination of the trimeric Vangl, and other cell-based functional assays.

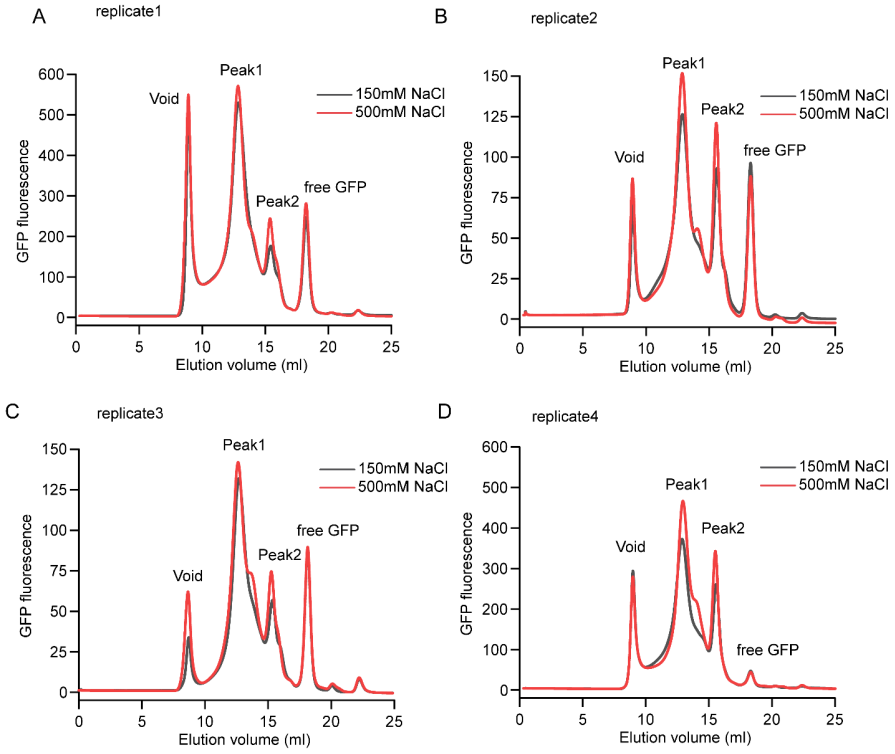
Minor

• Author should also clarify the expression and purification protocols described in material and methods. From current protocols, it’s not clear whether purification method is stated for VANGL1 or VANGL1-PK1 complex or both? Is expression-purification protocol of VANGL1 and VANGL-PK1 complex same? Why 500mM NaCl was used during solubilization? I guess high salt can potentially the destabilize VANGL1-PK1 complex and may be cause of poorly resolved density of VANGL1-PK1 complex?

We thank the reviewer for raising this point. In fact, we used the same expression and purification method to purify Vangl1 and the Vangl1-Pk1 complex. Cells overexpressing Vangl1

alone were only used for FSEC analysis. The relevant statements in the Methods have been revised to clarify the confusion.

We agree with the reviewer's concern regarding the use of high concentrations of NaCl during solubilization. We thus performed FSEC analysis on the same batch of cells overexpressing Vangl1-Pk1, but solubilized separately in 500 mM and 150 mM NaCl, in 4 replicates shown below. We did not observe any shift in the hexamer peak, indicating that Pk1 did not dissociate from Vangl1.



• Figure 4 is hard to follow. Author should make the boxes/color more prominent or label the boxes in main figure.

We apologize for the confusion and have revised Figure 4 accordingly.

• Page 26, line 470: What was final concentration of biotin? 150mM?

We have revised the description to read “Wash Buffer A supplemented with biotin at a final concentration of 150 mM”.

• Supplementary Figure 8; Sigma threshold levels of cryo-EM maps used for preparing figures should be provided in figure legends.

We appreciate the reviewer's suggestion. The contour level varied in the range of 5-12 σ and are now labeled on each figure panel.

Reviewer #3 (Remarks to the Author):

Zhang et al. reports the structure on VANGL1, which is a key factor for the establishment of plana cell polarity (PCP). The manuscript includes several important findings: 1) the first high resolution structure of VANGL1, 2) the interaction of two membrane-bound VANGL1 trimers, which is necessary for PK1-binding 3) Mapping of disease-causing mutations on the VANGL structures with the interpretation of disease-causing mechanisms. These findings significantly impoves our understanding of the complicated mechanism of PCP establishment. However, the proposed model of dynamic oligomerization equilibrium of VANGL1 (presented in Fig. 5) is not supported by sufficient experimental evidence, and thus needs to be revised before publication.

We agree with the reviewer and have revised our dynamic oligomerization equilibrium model, due to the lack of direct evidence for the trimer. We have carefully revised the relevant figure and context, which is summarized in the point-by-point response below.

1. The equilibrium model and Fig. 5 need to be revised unless it is proved by more structural evidences.

1-(1) Although partially dissociated complexes of Vangl1 and PK1 have been identified in cryo-EM images, this does not necessarily represent an intermediate state of the equilibrium. The partial interaction of two Vangl1 trimers in the kinked complex should not be proposed without high-resolution evidence.

We agree with the reviewer and have revised Figure 5 and related content, to reflect that "assembly of Vangl1 as a hexamer promotes Pk1 binding" instead of "Vangl1 is in an oligomerization equilibrium between hexamers and trimers".

1-(2) Given that the PK1-binding was observed in 2d classes, at least partial density of the bound PK1 should be observed in the refined structure. PK1-bound and PK1-free Vangl1 hexamers might have been sorted out in 3D classification, and the final structure might have been reconstructed mainly with PK1-free hexamers. The authors need to thoroughly inspect all 3D classes.

We thank the reviewer for this point. We did indeed observe a partial density in the 3D reconstructions that would have appeared if the threshold had been lowered, indicating that the particle set contains Pk1-bound particles. We also performed extensive 3D classification on the same particle set, but were unable to separate Pk1-bound and Pk1-free classes. We believe that the poorly resolved Pk1 is due to its high content of intrinsic disorder, which is consistent with the predicted structure. We further suspected low abundance of Pk1 and therefore performed 3D classification imposing C3 or C1 symmetry using either the original or symmetry-extended particles, which improved the density feature putatively for Pk1 but not to unambiguity. Nevertheless, the best results obtained by removing the imposed symmetry are shown in Figure S6B.

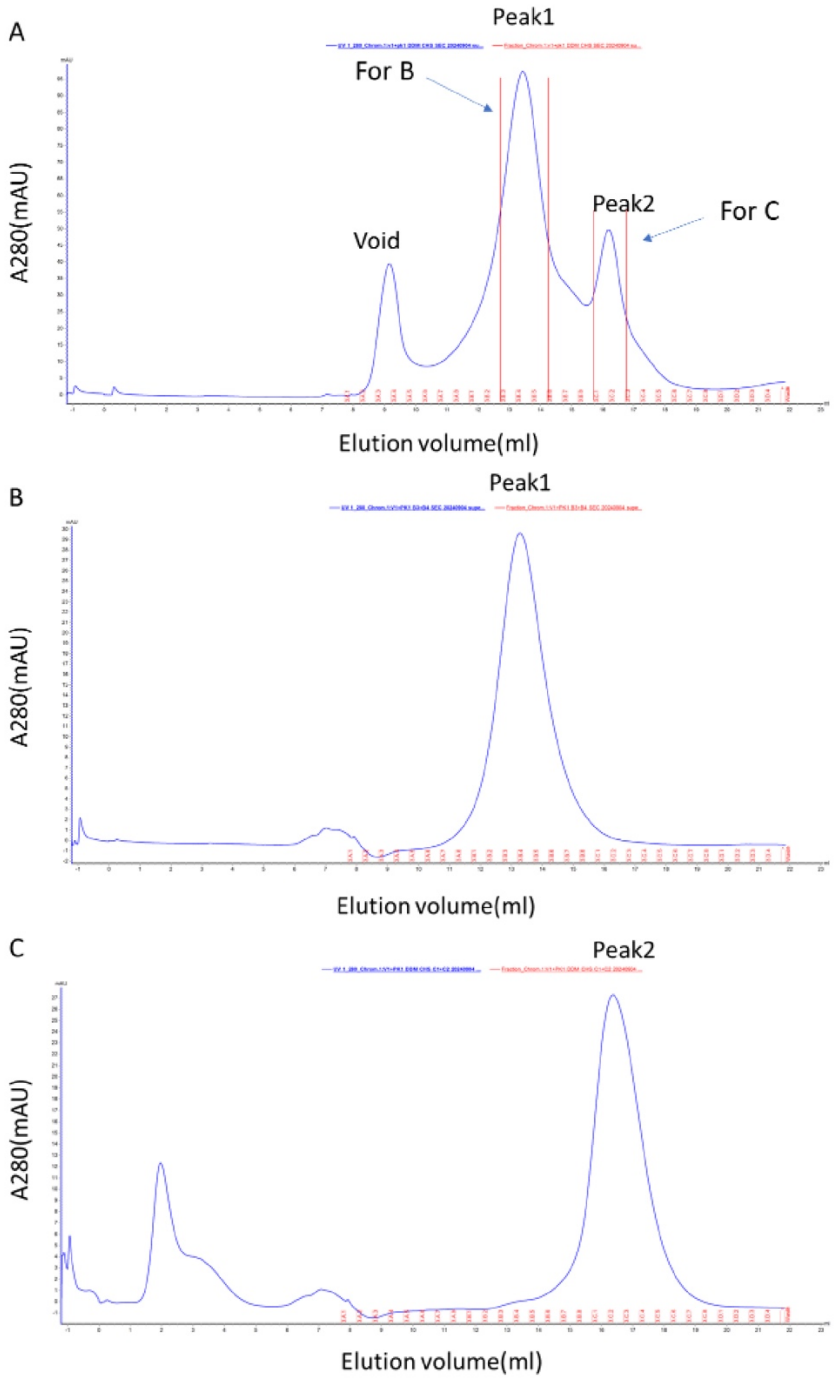
1-(3) The kinked oligomers could be further analyzed to obtain high-resolution information. For example, trimer-focused image processing and 3d classification without symmetry imposition may

produce a 3d class containing PK1-bound trimer substructures, which could be identified by comparison of the densities around three stick domains.

We thank the reviewer for this comment. The kinked oligomers revealed by the 2D classification analysis were rare in the population. In addition, projections of these kinked particles along the "top-down" direction (approximately perpendicular to the membrane) are difficult to detect, without which high-resolution reconstructions were unlikely to be achieved. However, we were nontheless inspired by this suggestion and re-extracted two "trimer" particles from each hexamer using coordinates derived from the final hexameric reconstruction. After removal of duplicates (particles too close to each other), subsequent trimer-focused classifications were performed, aiming to separate particles with and without the extra density around the stick domain. Unfortunately, such attempt did not work out, presumably because of the heterogeneity of the densities.

2. In the gel filtration fractions, the oligomerization states should be re-equilibrated. Therefore, the Native PAGE result for peak fractions needs to be reinterpreted. In the native gel result, high and low ratios of hexamer/trimer for Peak1 and 2 seem to be due to high and low protein concentration in Peak1 and 2 fractions. To assess the oligomerization equilibrium, concentration-dependent change in the oligomeric states needs to be examined by native gel electrophoresis experiments with increasing concentrations of purified Vangl proteins. This may also allow the authors to roughly estimate the dimerization affinity.

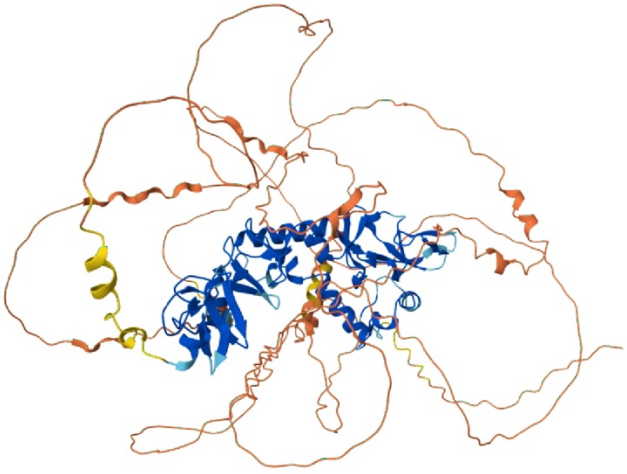
We appreciate the reviewer's comments. We tested for re-equilibration of the two peak fractions by injecting each fraction individually for a second SEC. The results showed little re-equilibration between the two peak positions (see figure below). We therefore revised our dynamic oligomerization equilibrium model, and reinterpreted the related data as supporting evidence for our revised hypothesis that hexamerization of Vangl1 promotes Pk1 binding.



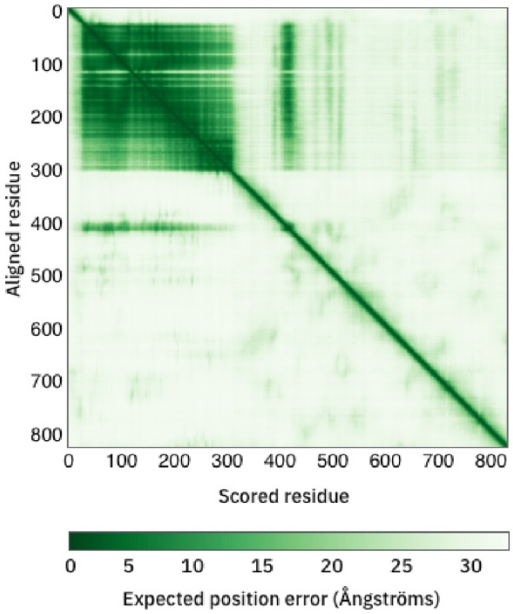
3. If the dynamic dimerization equilibrium is true, the dimerization of Vangl trimers could be strengthened by PK1-binding, shifting the equilibrium. A similar native PAGE could readily address this hypothesis.

We thank the reviewer for this point. Since we do not have direct evidence for the presence of the Vangl1 trimer, it remains unclear whether the dimerization equilibrium is true. However, we do believe that the dimerization of Vangl trimers may be enhanced by Pk1 binding, as mutations

at the dimer interface promoted the presence of a lower order of oligomer and reduced the amount of co-purified Pk1 (Figure S8C). Native PAGE analysis of Vangl in the presence or absence of Pk1 is a great suggestion; however, we are unable to overexpress and purify Pk1 in large quantities, consistent with the structural prediction that Pk1 may be poorly folded (see prediction results below).



Structure of Pk1 predicted by AlphaFold



line 114, Unclear description. Does it mean "two-fold symmetry in dumbbell-shaped side views?" Please also indicate exactly which figure and panel.

line 116, Unclear description needs to be revised.

The unclear description in lines 114-116 has been revised to read “Two-dimensional class averages revealed dumbbell-shaped “side views” with two-fold symmetry, where the weight of the dumbbell resembles the transmembrane domain surrounded by detergent micelles and the handle represents the soluble domain (Figure 1A, boxed in cyan). Besides the side views, three-fold related “top-down views” were also observed (Figure 1A, boxed in cyan).”

line 121, Error correction: "with each" to "and each"

Corrected.

line 125, "the opposite side" is clearer than "the other side"

Corrected.

line 140, Not layers but protein domains in each layer are related by three-fold symmetry.

Corrected.

line 159, "kinks into" seems grammartically wrong.

The grammatical error has been corrected.

line 201, "Hexamer to trimer" is correct.

Corrected.

line 234, Electrophysiological data along with detailed methods need to be presented.

We agree with the reviewer and have included the electrophysiological data in Figure S9 and Methods.

line 246, "extracellular halves" than “upper half" is clearer.

Corrected.

line 247, Does L242V belong to vangl1 or 2?

L242V belongs to Vangl1. We have clarified this in the revised text.