nature portfolio

Peer Review File

Structural basis of the VANGL-PRICKLE interaction

Corresponding Author: Dr Zhe Zhang

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 Song et al (Zhe Zhang lab) addresses the protein structure of the human Van Gogh-like/Vangl1 and 2 proteins, alone and together with Pk1. The structural analyses are based on the cryo-EM technology and provide both novel and expected insight. Vang/Vangl proteins are at the center of one of the key asymmetric complexes, required during planar cell polarity establishment and associated tissue patterning. The presented data is of good quality and extends our understanding of the Vangl-Pk complex. It should serve as the basis for functional follow up studies.

Overall, I would recommend publication, once the paper is adequately revised. Please see my specific comments below, which I trust will help to further improve the paper.

There are two major points made in this paper: (i) demonstration that Vangl1/2 form trimers and hexamers (and I think it is fair to say by extension that all Vang/Vangl protein members share the same features) and (ii) evidence that Pk1 promotes formation of the hexamer of Vangl/vang via its interaction with the HBD region of the Vangl proteins.

Major points:
(1) Generally, the assumed structure of the Vangl1 or 2 monomer, as it is seen as part of the trimer, very much looks like what was predicted by the AlphaFold software, including the split of the JMD region by the HBD domain. This should be acknowledged in the text/results/discussion and properly referenced. The authors do reference AlphaFold when it come to the Pk structure, so I trust they are aware of the predicted Vang/vangl structure. It does not take anything away from their own work.

(2) Vang/vangl proteins have been shown to competitively bind to both, Dsh/Dvl and Pk proteins during the formation of the PCP complexes. This should be mentioned in the Introduction and revisited in the Discussion. The most relevant references for this are from Drosophila:
- Bastock et al (2003) Development. doi:10.1242/dev.00526
- Humphries et al (2023) PLoS Genetics. doi.org/10.1371/journal.pgen.1010849

(3) The N-terminal Vangl/Vang cytoplasmic tail/domain has been shown to be critical for complex formation and stability, and importantly it has been demonstrated that this is regulated by phosphorylation. As the N-terminal cytoplasmic tail is not detected in the structure as analyzed, this has to be put into context as a limitation of the study. And it has to be discussed accordingly. The appropriate references should include:
- Gao et al (2011) Dev Cell. doi:10.1016/j.devcel.2011.01.001
- Kelly et al (2016) Cell Reports. doi.org/10.1016/j.celrep.2016.06.010
- Strutt et al (2019) eLife. doi.org/10.7554/eLife.45107.001

(4) the structures as presented in Figure 2 are difficult to see. Likely some larger zoom-ins would be helpful. It is also confusing as presented and it needs to be integrated with published data. Based on the presented, I assume that there is one Pk molecule per 6 vangl proteins, or one Pk per each Vangl hexamer. This does make sense with previously published in vivo stoichiometric studies from the Strutt lab, yet none of these papers is cited/referenced. The published work makes actually the data presented here believable. Please integrate the presented observation with the papers:
- Strutt, Gamage, and Strutt (2016) Cell Reports, doi.org/10.1016/j.celrep.2016.11.021
- and also Strutt, Gamage and Strutt (2019) elife. doi.org/10.7554/eLife.45107.001

(5) Figure 3: This figure needs a much more detailed and comprehensive figure legend and better integration and explanation in the text. As it is, it is not explained at a sufficient level for the reader to understand what is shown. For example, which residues are mutated in Vangl1-10mut, this is not clear. Panels “b-d” are not explained. What is the reader looking at? The binding panels lack quantifications (panels “b-g”).
Importantly, a recent paper has defined a small region in Vang/Vangl2 to be critical for both binding of Pk to Vang/Vangl, and also for the selection of competitive binding between Dsh/Dvl and Pk to Vang/Vangl (a combination of mouse and Drosophila work). These authors have defined the critical residues in vang/vangl for binding.
- Humphries et al (2023) PLoS Genetics. doi.org/10.1371/journal.pgen.1010849
Strikingly this publication defines a small region, nearby but upstream of the proposed binding as suggested in your work. This difference needs to be addressed. It is worth noting that the Humphries et al (2023) paper shows both binding and functional in vivo significance for the respective residues.
As your data tries to suggest that there is no specific residues required for the Vangl-Pk interaction, but that it is rather just mediated by electrostatic charge interactions, this needs to be addressed and integrated, at least in the discussion. The authors here state “..specific recognition…. simply mediated by electrostatic attraction, rather than a structural stable complex.” (lines 190-192) This stands in contrast to what was published and confirmed in vivo, and it needs to be carefully discussed, as the authors here state “…Pk1 densities insufficient to..” (line 187-189).
Also in the same section: on line 161, it is stated “consistent with previous results…” with no reference or citation. What are the authors referring to?

(6) The region of Pk1 suggested to interact with vangl1/2 is 745-790. Previous work, Jenny et al (2003) EMBO J. (ref #40 in current draft), has also mapped the interaction between Pk and Vang/Stbm (both in Xenopus and flies) in vitro and confirmed in vivo, to a small region slightly N-terminal to what is proposed here. Please integrate previous data.
Along these lines, I am curious how a large protein, human Pk1/2 and Drosophila Pk are all >800 amino acids, do fit in the small cavity within the Vangl hexamer. Please try to propose a model.

(7) Figures 4 and 5 are largely artificial due to overexpression in non-polarized cells (PCP can only form properly in polarized cells). There is no physiological evidence for the data shown in these figures. This should be toned down.

(8) Figure 6 is useful, but it should be stated that it is mere correlation, as it is not addressed how these mutations might affect the structure. Also, while the mutations were found in human NTD patients, there is no proof that they are actually causing neural tube closure defects. The only way to do this is in model organisms, like mouse or Drosophila. For example, see:
- Humphries et al (2020) eLife. doi.org/10.7554/eLife.53532
for such studies demonstrating mutant behavior and causality.

(9) The title should be changed, maybe:
“Structural basis for the Vangl-Pk complex”?

Minor textual points:
- line 37: to reference the ciliary positioning mentioned here, the citation
Carvajal-Gonzalez et al (2016) BioEssays doi.org/10.1002/bies.201600154, should be added.

- line 54: the citations referring to Wnt-mediated PCP orientation must also include Wu et al. (2013) Nature Cell Biol. doi.org/10.1038/ncb2806

- line 58: It should be defined here that Vang is the genetic abbreviation for “Van Gogh” with the relevant citation: Taylor et al (1998), ref #25 in current draft.

- line 65: There are 4 Pk paralogs in vertebrates, not 2 as stated. Please see review Radaszkiewicz et al 2023 (Ref #34 in current draft) summarizing this.

Reviewer #2

(Remarks to the Author)
The manuscript by Song et al, report structure of VANGL1 and VANGL2, a key membrane proteins involved vertebrate planar cell polarity signaling. The resolution of VANGl1 & VANGL2 cryo-EM maps is very good and structures are very novel, and interesting on its own. Subsequently authors also determine structures of VANGL1-PK1 complexes. The author also performs cellular experiments and based on which propose a potential role for VANGL1 oligomerization, and VANGL-PK1 interaction may mediate asymmetric recruitment of VANGL1 and PK1 to plasma membrane. Overall, the study is very extensive with multiple cryo-EM structures and biochemical/cellular experiments and is breakthrough for its field. However, I have one major and a few minor concerns, which need to be addressed prior to its publication:

Major
• The major issue in manuscript is limited resolution for PK1 in cryo-maps. The author should also not rule out the possibility of additional density seen in presence of PK1 or PK1 peptide, may be also as part of VANGL1 that is not resolved in cryo-EM map. Although this seems to be less likely but needs to be discussed in manuscript at least, especially in absence of clear experimental evidence.
• The stoichiometry of VANGL and PK1 binding is not clear. Authors use D3 symmetry for cryo-EM processing without any

clear evidence for 1:1 binding. The poor density of PK1 was concluded with lower affinity of interaction between VANGL1-PK1. I suspect this may not be the real situation. If the PK1 was co-purified with VANGL1, it likely the affinity of interaction is not low. Considering, the binding of PK1 at the interface of two VANGL trimers, it possible the stoichiometry is 1:0.5 (VANGL:PK). I recommend author to consider analyzing the cryo-EM datasets using C3 or C1 symmetry. Also perform symmetry expansion (from C3 and D3 refined maps) and 3D classification of each protomers to improve the density features of PK1. The results of these analysis including angular distribution after applying C1, C3 symmetry should be included in supplementary information's. A figure depicting the density features of PK1 and PK1 peptides (close views) at different contour is needed. Authors should also do side-side by comparison of any density features in proposed PK1 binding site in apo state, and PK1 bound state, and compare density features in C1 C3, and D3 symmetry maps.
• It would be useful to depict the VANGL1 binding regions of PK1 in AlphaFold model. Are these regions in unstructured regions or core of protein fold. Can it explain or provide more insights? Did author try to predict the interaction of VANGL-PK1 using AlphaFold multimer? Does the program predict something like proposed interaction sites? Will the binding site can accommodate two PK1 at the same time without creating steric clash.

Minor
• The structural feature of PK1 binding site is not clearly illustrated. Does PK1 binding site is at the interface of two protomers of VANGL trimer. Author should color the each protomer / chains in different color or shades to depict this.
• The line 95-102 is not clear.
• The author does not provide sigma threshold levels of maps used for preparing figures. This is important information that should be included, in figure legends.
• The statement on page 13, line 271-273 is not accurate "This study showed that VANGL and PK did not form a structurally stable complex. Indeed, their weak electrostatic interaction makes them more ….". A author should not careful before claiming the weak interaction of VANGL1-PK1 without any experimental evidence. Inability to structurally resolve complex may be due to several factors, which may not necessarily link with lower affinity. If
• Extended figure 1,2, 4 & 7; The scale bar for micrographs is missing.

Reviewer #3

(Remarks to the Author)
Song et al. reports structural and biochemical studies on the VANGL-PRIKLE interaction, which is essential for the establishment of plana cell polarity (PCP). The manuscript includes several important findings: 1) the first high resolution structures of VANGL1 and 2, 2) the oligomer states of VANGL proteins, 3) identification of the PK1-binding site by structural and biochemical studies, 4) mapping of disease-causing mutations on the VANGL structures with the interpretation of disease-causing mechanisms. This study significantly impoves our understanding of the complicated mechanism of PCP establishment, and thus is suitable for publication in Nature Communications. I have only minor comments.

1. Because the PK1-binding site is close to the dimeric interface, the mutant VANGL1 (10mut) may have a defect in dimerization. If this is true, the cell experiment results need to be reinterpreted. To exclude the possibility, the authors need to provide the gel filtration or AUC data of the purified mutant VANGL1, which is expected to show the major peak at the position of the hexameric size.

2. Fig 4f. Why is VANGL1(1-495) colocalized with PK1(WT)? Does monomeric VANGL1 also interact with PK1?

3. A schematic diagram showing the domain composition in the primary structure in Fig 1 would be helpful to understand the manuscript.

4. Did the side chain conformations in the PK1 binding site of VANGL1 change upon the PK1 binding? Then, the structural comparison needs to be provided.

5. How strong would be the dimerization of the VANGL trimers? It seems quite strong in the purified sample. With this high affinty, the trimers in the plasma membrane could recruit VANGL-rich vesicles without PK1, but do not appear to in this study.

line 81. The peak profile show a large void peak and a sholder, and AUC also showed trimer population. Protein samples are not homogenous.
line 105. A typo (DILI) needs to be corrected.
lines 395-397. Not only the ratio but also protein concentrations need to be provided.
line 126. The PK1/VANGL ratio would be difficult to be judged by SDS-PAGE. High molecular weight protein bands are sharp and not stained well, so the amounts cannot be compared with those of low molecular weight bands.
line 129. No need to be assumed.
line 135. "Shapes/sizes/strengths of densities are distinct" would be a clearer expression.
line 137. Did the authors mean "the bound PK1 was flexible" or "the binding was weak?"
line 137. Did the authors mean "our findings in protein purification?"
line 148. "may originate" would be proper.
line 178. The authors need to provide molar concentrations rather than molar ratios, which are necessary to estimate the binding affinity.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript provides significant improvement vis-a-vis the original submission. The authors have addressed almost all comments raised by me and the other two referees. The paper is improved with additional data sets and also easier to read with the additional explanations and referencing. I recommend the revised, current manuscript for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
The authors have addressed all of my concerns, and the revised manuscript has significantly improved in clarity and overall quality. Therefore, I believe it is now suitable for publication.

Basavraj Khanppnavar

Reviewer #3

(Remarks to the Author)
The authors addressed all my previous concerns, and I recommend publication of this paper.

Point-by-point responses

We sincerely thank the editor and all reviewers for their valuable feedback. These comments are very helpful for revising and improving our article. According to the Reviewers' comment, we have made extensive revisions to our previous draft. The detailed point-by-point responses are listed below. We believe all the questions or concerns have been appropriately addressed. The key parts of the revision include:

1) We have thoroughly correlated our results and findings with previous research, as requested by Reviewer #1.
2) We have utilized AlphaFold3 to model the VANGL oligomer, VANGL-PK1, and VANGL-PK1$^{745\text{-}790}$ complexes, as requested by Reviewers #1 and #2. The modelled complexes revealed that PK1 indeed binds to the positively charged surface of VANGL, employing the same set of negatively charged residues we suggested (Extended Data Fig. 4).
3) We have conducted the refinement of the cryo-EM datasets for VANGL-PK1 complexes using both C1 and C3 symmetry, and have included more detailed density features of PK1 and PK1 peptides in Extended Data Fig. 6, in response to Reviewer #2's request.

Referee's remarks are shown in ***black*** and our responses are in **blue**.

Reviewer #1 *(Comments to the Author):*

This paper by Song et al (Zhe Zhang lab) addresses the protein structure of the human Van Gogh-like/Vangl1 and 2 proteins, alone and together with Pk1. The structural analyses are based on the cryo-EM technology and provide both novel and expected insight. Vang/Vangl proteins are at the center of one of the key asymmetric complexes, required during planar cell polarity establishment and associated tissue patterning. The presented data is of good quality and extends our understanding of the Vangl-Pk complex. It should serve as the basis for functional follow up studies.

Overall, I would recommend publication, once the paper is adequately revised. Please see my specific comments below, which I trust will help to further improve the paper.

There are two major points made in this paper: (i) demonstration that Vangl1/2 form trimers and hexamers (and I think it is fair to say by extension that all Vang/Vangl protein members share the same features) and (ii) evidence that Pk1 promotes formation of the hexamer of Vangl/vang via its interaction with the HBD region of the Vangl proteins.

Response: We thank the reviewer for the positive comments.

Major points:

(1) Generally, the assumed structure of the Vangl1 or 2 monomer, as it is seen as part of the trimer, very much looks like what was predicted by the AlphaFold software, including the split of the JMD region by the HBD domain. This should be acknowledged in the text/results/discussion and properly referenced. The authors do reference AlphaFold when it come to the Pk structure, so I trust they are aware of the predicted Vang/vangl structure. It does not take anything away from their own work.

Response: We sincerely appreciate the Reviewer's comment. We have incorporated the AlphaFold3-predicted VANGL structure into the revised manuscript. The predicted VANGL1 and VANGL2 trimer structures align well with our cryo-EM structures, with a root-mean-square deviation (RMSD) of 1.0 Å and 0.9 Å, respectively (Extended Data Fig. 4a, b). However, the hexamer structure, which was modelled as two adjacent trimers, did not match our experimental findings (Extended Data Fig. 4c).

Furthermore, we have also utilized AlphaFold3 to model the VANGL-PK1 and VANGL-PK1$^{745\text{-}790}$ complexes. The modelled complexes revealed that PK1 indeed binds to the positively charged surface of VANGL, employing the same set of negatively charged residues we suggested (Extended Data Fig. 4d-g). Collectively, our structural evidence, pull-down results, and AlphaFold3 modelling substantiated the proposed VANGL-PK recognition mechanism. We have revised the manuscript accordingly (Pages 6-7 and 10).

(2) Vang/vangl proteins have been shown to competitively bind to both, Dsh/Dvl and Pk proteins during the formation of the PCP complexes. This should be mentioned in

the Introduction and revisited in the Discussion. The most relevant references for this are from Drosophila:

- Bastock et al (2003) Development. doi:10.1242/dev.00526

- Humphries et al (2023) PLoS Genetics. doi.org/10.1371/journal.pgen.1010849

Response: We appreciate the critical feedback from the Reviewer and acknowledge our oversight in discussing the VANGL-DVL complex. To address this, we have now incorporated this topic in both the Introduction and the Discussion sections. Specifically, we have added the following points: (1) It is noteworthy that DVL can also interact with VANGL in a competitive manner with PK, a process that is regulated by the post-translational phosphorylation of a specific tyrosine residue within VANGL during the establishment of PCP[10,11] (Page 3). (2) A recent study has pinpointed a specific region within the Vang/Vangl proteins–residues 302-324 of human VANGL1, residues 298-322 of human VANGL2, and residues 364-387 of Drosophila Vangl–as critical for binding both PK and DVL[11]. Interestingly, this region was almost invisible in our structural models and is situated upstream of the binding sites we identified. These findings imply that multiple areas of VANGL may be involved in PK1 binding, highlighting the intricate nature of the VANGL-PK interaction (Page 15). The relevant references have also been included.

(3) The N-terminal Vangl/Vang cytoplasmic tail/domain has been shown to be critical for complex formation and stability, and importantly it has been demonstrated that this is regulated by phosphorylation. As the N-terminal cytoplasmic tail is not detected in the structure as analyzed, this has to be put into context as a limitation of the study. And it has to be discussed accordingly. The appropriate references should include:

- Gao et al (2011) Dev Cell. doi:10.1016/j.devcel.2011.01.001

- Kelly et al (2016) Cell Reports. doi.org/10.1016/j.celrep.2016.06.010

- Strutt et al (2019) eLife. doi.org/10.7554/eLife.45107.001

Response: We appreciate the Reviewer's valuable comment. We acknowledge that the absence of the VANGL N-terminal domain in our structures represents a limitation in our work. To address this, we performed pull-down experiments to examine the impact

of the N-terminal domain on the interaction between VANGL1 and PK1. The gel filtration result showed that the N-terminal truncated VANGL1 (VANGL1$^{115-524}$) maintained its hexameric assembly, similar to the full-length protein (Fig. R1a). Moreover, the pull-down assays demonstrated that the N-terminal truncation of VANGL1 did not affect its interaction with PK1 (Fig. R1b). Collectively, these results confirm that the N-terminus of VANGL1 might not be involved in VANGL1 oligomerization or the VANGL-PK interaction. Given the high conservation between VANGL1 and VANGL2, it is reasonable to infer that this conclusion extends to VANGL2 as well.

Although phosphorylation of the N-terminal regions has been established as crucial for the polarized membrane localization of Vangl and other PCP proteins, which is even potentially regulated by Pk, our data indicate that the N-terminus of VANGL1 is not directly involved in binding to PK1. The detailed investigation of the N-terminal phosphorylation's role in PCP complex localization and the downstream signaling is not the focus and beyond the scope of this study, hence we have chosen not to include it in the revised manuscript.

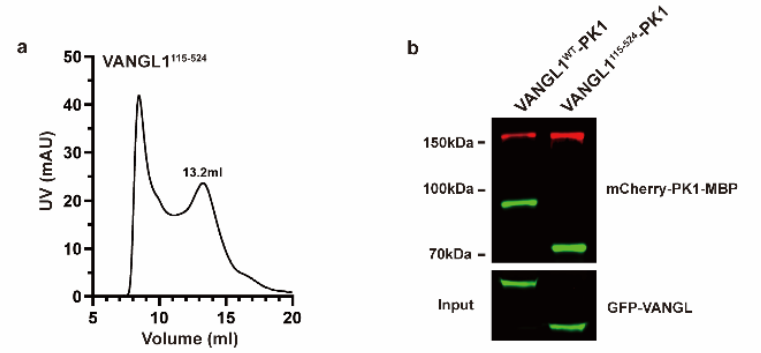

Fig. R1 Investigation of the role of the N-terminal region of VANGL1 in the interaction with PK1.

a, Gel filtration profile of N-terminal truncated VANGL1 (VANGL1$^{115-524}$). **b,** Pull-down assay to verify the interaction between VANGL1$^{115-524}$ and PK1.

(4) the structures as presented in Figure 2 are difficult to see. Likely some larger zoom-ins would be helpful. It is also confusing as presented and it needs to be integrated with published data. Based on the presented, I assume that there is one Pk molecule per 6

vangl proteins, or one Pk per each Vangl hexamer. This does make sense with previously published in vivo stoichiometric studies from the Strutt lab, yet none of these papers is cited/referenced. The published work makes actually the data presented here believable. Please integrate the presented observation with the papers:

- Strutt, Gamage, and Strutt (2016) Cell Reports, doi.org/10.1016/j.celrep.2016.11.021

- and also Strutt, Gamage and Strutt (2019) elife. doi.org/10.7554/eLife.45107.001

Response: We greatly appreciate the Reviewer's comments. As suggested, we have included a new supplementary figure (Extended Data Fig. 6) to further illustrate the density features of PK1 and PK1 peptides at various contour levels. This figure also provided a side-by-side comparison of the PK1 binding site of VANGL1 in both the apo and PK1-bound states, as well as a comparison of the PK1 density features across C1, C3, and D3 symmetry maps.

In terms of the stoichiometry of the VANGL-PK1 interaction, our structural data does not allow us to determine an exact binding ratio due to the ambiguous PK densities. Given that VANGL forms a symmetric hexamer, with PK1-binding sites that are also symmetrical, we speculate that a single VANGL hexamer could potentially bind either three or six PK1 molecules. However, considering the size of the positively-charged surface at the VANGL trimer-trimer interface, there is a substantial risk of steric clash if six PK1 molecules were to bind simultaneously in a 1:1 ratio (please refer to Fig. R2 in the response to Reviewer #2). Furthermore, our SDS-PAGE result indicates that the proportion of PK1 in the VANGL1-PK1 complex is lower than that of VANGL1 (Extended Data Fig. 5a). This suggests that PK1-VANGL1 stoichiometric ratio is likely less than 1:1. Therefore, we hypothesize that the most probable stoichiometric ratio is three PK molecules per VANGL hexamer, which corresponds to a 1:2 ratio. It is also possible that the potential densities observed in the three symmetrical PK1 binding sites of VANGL could be misaligned when C1 symmetry was applied during refinement or could be enhanced due to the refinements conducted using C3 or D3 symmetries (Extended Data Fig. 6). This raises the alternative possibility that only one PK1 molecule binds to each VANGL hexamer, in a 1:6 ratio, as proposed by previous studies.

Nevertheless, further studies are necessary to clarify these possibilities. We have added a brief discussion in the revised manuscript (Page 15).

(5) Figure 3: This figure needs a much more detailed and comprehensive figure legend and better integration and explanation in the text. As it is, it is not explained at a sufficient level for the reader to understand what is shown. For example, which residues are mutated in Vangl1-10mut, this is not clear. Panels "b-d" are not explained. What is the reader looking at? The binding panels lack quantifications (panels "b-g").

Response: We sincerely apologize for any confusion that may have arisen due to the lack of a detailed and comprehensive figure legend, as well as for the insufficient integration and explanation within the text. We have made the following revisions to address these issues: (1) We have specified the mutation residues of VANGL1^{10mut} in the main text (Page 8). (2) We have expanded the legend of Fig. 3, ensuring that each panel is clearly described (Pages 35-36). (3) We have included the quantifications of the pull-down experiments of Fig. 3b-g in Extended Data Fig. 7.

Importantly, a recent paper has defined a small region in Vang/Vangl2 to be critical for both binding of Pk to Vang/Vangl, and also for the selection of competitive binding between Dsh/Dvl and Pk to Vang/Vangl (a combination of mouse and Drosophila work). These authors have defined the critical residues in vang/vangl for binding.

- Humphries et al (2023) PLoS Genetics. doi.org/10.1371/journal.pgen.1010849

Strikingly this publication defines a small region, nearby but upstream of the proposed binding as suggested in your work. This difference needs to be addressed. It is worth noting that the Humphries et al (2023) paper shows both binding and functional in vivo significance for the respective residues.

Response: We sincerely appreciate the Reviewer's comments and apologize for any lack of clarity in correlating our findings with previous research. As mentioned in our previous response, we have now included a paragraph in the Discussion section of the revised manuscript that highlights the distinct binding region of Vang/Vangl to Pk identified in previous study: "A recent study has pinpointed a specific region within the

Vang/Vangl proteins–residues 302-324 of human VANGL1, residues 298-322 of human VANGL2, and residues 364-387 of Drosophila Vangl–as critical for binding both PK and DVL[11]. Interestingly, this region was almost invisible in our structural models and is situated upstream of the binding sites we identified. These findings imply that multiple areas of VANGL may be involved in PK1 binding, highlighting the intricate nature of the VANGL-PK interaction." (Page 15). It is plausible that both of these regions contribute to the interaction between VANGL and PK, potentially in different experimental or physiological contexts.

As your data tries to suggest that there is no specific residues required for the Vangl-Pk interaction, but that it is rather just mediated by electrostatic charge interactions, this needs to be addressed and integrated, at least in the discussion. The authors here state "..specific recognition…. simply mediated by electrostatic attraction, rather than a structural stable complex." (lines 190-192) This stands in contrast to what was published and confirmed in vivo, and it needs to be carefully discussed, as the authors here state "…Pk1 densities insufficient to.." (line 187-189).

Response: We thank the Reviewer for pointing this out. Actually, our structural data, pull-down results, and AlphaFold3 modelling collectively support the proposed VANGL-PK recognition mechanism. It is probably that the electrostatic interaction between VANGL and PK1 is mediated by specific attractions between certain residues within their binding regions. However, due to the inability to resolve the high-resolution structure of the VANGL-PK complex, drawing a definitive conclusion remains challenging. To clarifying this point and avoid any potential misunderstanding, we have revised the manuscript as follows: "Based on the findings thus far, we inferred that the specific recognition between VANGL and PK1 is mediated by the electrostatic attraction within a defined region of these proteins." (Page 10).

Also in the same section: on line 161, it is stated "consistent with previous results…" with no reference or citation. What are the authors referring to?

Response: We apologize for any confusion caused by this statement. We intended to convey that the pull-down results in Fig. 3d aligns with those in Fig. 3c, thereby confirming the essential role of the 46 residues of PK1 ($PK1^{745-790}$) in binding to VANGL1. This observation is based solely on our experimental data and does not reference any previous research. To avoid any misunderstanding, we have made the following revision to the manuscript: "To confirm that these 46 residues in PK1 ($PK1^{745-790}$) are essential for binding to VANGL1, we examined the VANGL1-PK1 interaction solely using PK1 C-terminal peptides. Indeed, these results further supported our conclusion (Fig. 3d and Extended Data Fig. 7c)." (Page 9).

(6) The region of Pk1 suggested to interact with vangl1/2 is 745-790. Previous work, Jenny et al (2003) EMBO J. (ref #40 in current draft), has also mapped the interaction between Pk and Vang/Stbm (both in Xenopus and flies) in vitro and confirmed in vivo, to a small region slightly N-terminal to what is proposed here. Please integrate previous data.

Along these lines, I am curious how a large protein, human Pk1/2 and Drosophila Pk are all >800 amino acids, do fit in the small cavity within the Vangl hexamer. Please try to propose a model.

Response: We appreciate the Reviewer's reminder. We have revised the manuscript to include this information: "Of note, a previous study identified a C-terminal fragment of Pk1, corresponding to residues 628-767 of human PK1, as being responsible for Vang binding in Drosophilia[44]. This region encompasses the first negatively charged patch ($^{757-}$EDDD^{-760}) and is partially consistent with our findings." (Page 9).

Regarding the interaction mode between VANGL and PK. It is important to note that despite PK being a large protein comprising over 800 residues, its C-terminal 500 residues are predicted to be intrinsically disordered, as indicated by AlphaFold. This includes the VANGL-binding regions. Consequently, only a small fragment of PK is required to engage with the VANGL trimer-trimer interface, such as the $PK1^{745-790}$ peptide. This segment could potentially adopt either a loop or short helical structure to perform its function, as shown in Extended Data Fig. 4d-g. The highly flexible structure

of PK might be essential for its functional promiscuity, such as interactions with multiple protein partners such as VANGL, DVL, INVERSIN, and RICTOR.

(7) Figures 4 and 5 are largely artificial due to overexpression in non-polarized cells (PCP can only form properly in polarized cells). There is no physiological evidence for the data shown in these figures. This should be toned down.

Response: We sincerely appreciate the Reviewer's comment. We acknowledge that the cell imaging experiments in Figs. 4 and 5 were conducted using non-polarized U2OS cells, and we recognize the need to validate the findings in polarized cells. As suggested, we have revised the manuscript accordingly: "However, further investigation in polarized cells is necessary to confirm the PK1-mediated membrane recruitment of VANGL-containing vesicles and to elucidate the underlying mechanism." (Page 13).

(8) Figure 6 is useful, but it should be stated that it is mere correlation, as it is not addressed how these mutations might affect the structure. Also, while the mutations were found in human NTD patients, there is no proof that they are actually causing neural tube closure defects. The only way to do this is in model organisms, like mouse or Drosophila. For example, see:

- Humphries et al (2020) eLife. doi.org/10.7554/eLife.53532

for such studies demonstrating mutant behavior and causality.

Response: We are grateful for the Reviewer's constructive comment. We concur that our current work has focused on structural analyses to anticipate the possible consequences of these loss-of-function or disease-related mutations in VANGL. We agree that further biochemical analyses and animal model studies are imperative to confirm the actual impact and clinical significance of these mutations. We have revised the manuscript to emphasize this point. (Page 14)

(9) The title should be changed, maybe:

"Structural basis for the Vangl-Pk complex"?

Response: We thank the Reviewer for this suggestion. We have changed the title to "Structural basis of the VANGL-PK interaction" to better reflect the content of our manuscript.

Minor textual points:

- line 37: to reference the ciliary positioning mentioned here, the citation Carvajal-Gonzalez et al (2016) BioEssays doi.org/10.1002/bies.201600154, should be added.

- line 54: the citations referring to Wnt-mediated PCP orientation must also include Wu et al. (2013) Nature Cell Biol. doi.org/10.1038/ncb2806

- line 58: It should be defined here that Vang is the genetic abbreviation for "Van Gogh" with the relevant citation: Taylor et al (1998), ref #25 in current draft.

- line 65: There are 4 Pk paralogs in vertebrates, not 2 as stated. Please see review Radaszkiewicz et al 2023 (Ref #34 in current draft) summarizing this.

Response: We thank the Reviewer's insightful comments. The manuscript has been revised accordingly.

***Reviewer #2** (Remarks to the Author):*

The manuscript by Song et al, report structure of VANGL1 and VANGL2, a key membrane proteins involved vertebrate planar cell polarity signaling. The resolution of VANGl1 & VANGL2 cryo-EM maps is very good and structures are very novel, and interesting on its own. Subsequently authors also determine structures of VANGL1-PK1 complexes. The author also performs cellular experiments and based on which propose a potential role for VANGL1 oligomerization, and VANGL-PK1 interaction may mediate asymmetric recruitment of VANGL1 and PK1 to plasma membrane. Overall, the study is very extensive with multiple cryo-EM structures and biochemical/cellular experiments and is breakthrough for its field. However, I have one major and a few minor concerns, which need to be addressed prior to its publication:

Response: We highly appreciate the positive feedback from the Reviewer and the constructive suggestions.

Major

• The major issue in manuscript is limited resolution for PK1 in cryo-maps. The author should also not rule out the possibility of additional density seen in presence of PK1 or PK1 peptide, may be also as part of VANGL1 that is not resolved in cryo-EM map. Although this seems to be less likely but needs to be discussed in manuscript at least, especially in absence of clear experimental evidence.

Response: We appreciate the Reviewer's insightful comments and acknowledge the potential limitation highlighted. Our observation of these additional densities only in the presence of PK1 or PK1 peptide leads us to believe that they most likely originate from PK1. To provide further clarity, we have introduced a new supplementary figure (Extended Data Fig. 6) that delineates the density features of PK1 and PK1 peptides at various contour levels. This figure also provides a comparison of the PK1 density features across C1, C3, and D3 symmetry maps. Moreover, we have utilized AlphaFold3 to model the VANGL-PK1 and VANGL-PK1$^{745\text{-}790}$ complexes. The modelled complexes confirmed that PK1 binds to the same position as the additional densities we observed in the VANGL-PK1 and VANGL-PK1$^{745\text{-}790}$ structures, employing the same set of negatively charged residues we suggested to interact with the positively charged surface of VANGL (Extended Data Fig. 4d-g). These findings collectively reinforce our conclusion. Nonetheless, to enhance the rigor of our study, we have revised the manuscript to include the possibility raised by the Reviewer: "Thus, we presumed that these densities **most likely** originated from PK1 and that PK1 binding influenced the structure of VANGL1. **Nevertheless, it remains possible that these densities could arise from other unresolved regions of VANGL1, such as the N-terminal domain.**" (Page 7)

• The stoichiometry of VANGL and PK1 binding is not clear. Authors use D3 symmetry for cryo-EM processing without any clear evidence for 1:1 binding. The poor density of PK1 was concluded with lower affinity of interaction between VANGL1-PK1. I suspect this may not be the real situation. If the PK1 was co-purified with VANGL1, it

likely the affinity of interaction is not low. Considering, the binding of PK1 at the interface of two VANGL trimers, it possible the stoichiometry is 1:0.5 (VANGL:PK). I recommend author to consider analyzing the cryo-EM datasets using C3 or C1 symmetry. Also perform symmetry expansion (from C3 and D3 refined maps) and 3D classification of each protomers to improve the density features of PK1. The results of these analysis including angular distribution after applying C1, C3 symmetry should be included in supplementary information's. A figure depicting the density features of PK1 and PK1 peptides (close views) at different contour is needed. Authors should also do side-side by comparison of any density features in proposed PK1 binding site in apo state, and PK1 bound state, and compare density features in C1 C3, and D3 symmetry maps.

Response: We sincerely appreciate the Reviewer's constructive suggestions. We have tried to perform symmetry expansion and 3D classification for each protomers. However, it did not improve the density features of PK1 in our case. To provide further clarity, we have introduced a new supplementary figure (Extended Data Fig. 6) that delineates the density features of PK1 and PK1 peptides at various contour levels. This figure also provided a side-by-side comparison of the PK1 binding site of VANGL1 in both the apo and PK1-bound states, as well as a comparison of the PK1 density features across C1, C3, and D3 symmetry maps. As per the Reviewer's suggestion, we have included the orientation distribution of the particles utilized in the final reconstruction when applying different symmetries in Extended Data Figs. 5, 9, and 10.

In terms of the stoichiometry of the VANGL-PK1 interaction, as mentioned in our response to Reviewer #1, our structural data does not allow us to determine an exact binding ratio due to the ambiguous PK densities. Given that VANGL forms a symmetric hexamer, with PK1-binding sites that are also symmetrical, we speculate that a single VANGL hexamer could potentially bind either three or six PK1 molecules. However, considering the size of the positively-charged surface at the VANGL trimer-trimer interface, there is a substantial risk of steric clash if six PK1 molecules were to bind simultaneously in a 1:1 ratio (please refer to Fig. R2 in our response to the next question). Furthermore, our SDS-PAGE result suggests that PK1-VANGL1

stoichiometric ratio is likely less than 1:1 (Extended Data Fig. 5a). Therefore, we hypothesize that the most probable stoichiometric ratio is three PK molecules per VANGL hexamer, which corresponds to a 1:2 ratio, just as proposed by the Reviewer. It is also possible that the potential densities observed in the three symmetrical PK1 binding sites of VANGL could be misaligned when C1 symmetry was applied during refinement or could be enhanced due to the refinements conducted using C3 or D3 symmetries (Extended Data Fig. 6). This raises the alternative possibility that only one PK1 molecule binds to each VANGL hexamer, in a 1:6 ratio, as proposed by previous studies (Strutt et al. ***Cell Reports***, 2016; Strutt et al. ***Elife***, 2019). Nevertheless, further studies are necessary to clarify these possibilities. We have added a brief discussion in the revised manuscript (Page 15).

• *It would be useful to depict the VANGL1 binding regions of PK1 in AlphaFold model. Are these regions in unstructured regions or core of protein fold. Can it explain or provide more insights? Did author try to predict the interaction of VANGL-PK1 using AlphaFold multimer? Does the program predict something like proposed interaction sites? Will the binding site can accommodate two PK1 at the same time without creating steric clash.*

Response: We appreciate the Reviewer's insightful suggestions. As mentioned above, we have utilized AlphaFold3 to model the VANGL-PK1 and VANGL-PK1$^{745\text{-}790}$ complexes (Extended Data Fig. 4). The predicted VANGL1 and VANGL2 trimer structures align well with our cryo-EM structures, with an RMSD of 1.0 Å and 0.9 Å, respectively (Extended Data Fig. 4a, b). However, the hexamer structure, which was modelled as two adjacent trimers, did not match our experimental findings (Extended Data Fig. 4c). Therefore, we have focused on the VANGL trimer for complex structure modeling in the present of three PK1 or PK1$^{745\text{-}790}$ molecules (Extended Data Fig. 4d-g). The modelled complexes indicate that PK1 binds to the same position as the additional densities we observed in the VANGL-PK1 and VANGL-PK1$^{745\text{-}790}$ structures, employing the same set of negatively charged residues (^{757}-EDDD-^{760} and

^{771}-DSEEE-^{775}) we suggested to interact with the positively charged surface of VANGL. These findings further substantiate our results.

Moreover, the C-terminal 500 residues of PK1 are predicted to be intrinsically disordered, which includes the VANGL-binding regions. Consequently, only a small fragment of PK is required to engage with the VANGL trimer-trimer interface, such as the PK1$^{745-790}$ peptide. This segment could potentially adopt either a loop or short helical structure to perform its function, as shown in Extended Data Fig. 4d-g. When we superimposed the predicted binding mode of PK1$^{745-790}$ onto our VANGL1 hexamer structure at a 1:1 ratio, it becomes evident that steric clashes would occur between the two PK1 peptides (Fig. R2). However, additional studies are necessary to clarify the binding ratio of VANGL to PK1.

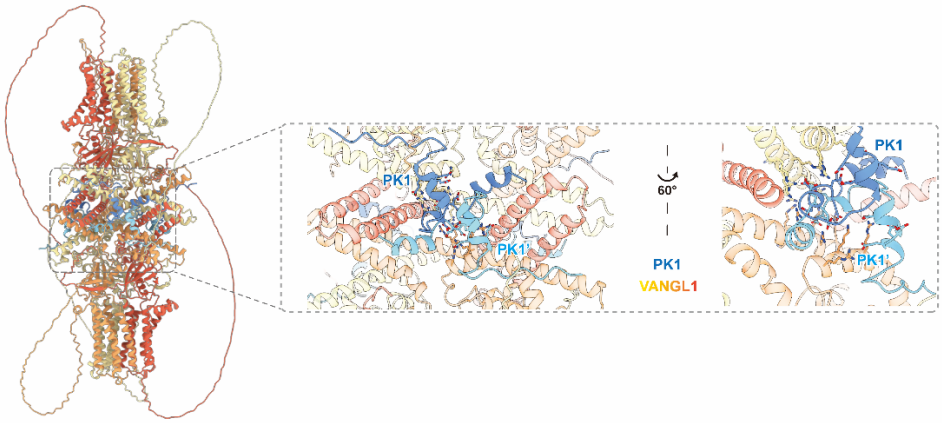

Fig R2. The artificial model of the VANGL1-PK1$^{745-790}$ hexamer structure. The VANGL1-PK1$^{745-790}$ trimer structure is predicted by AlphaFold3. VANGL1 is colored by chains and PK1 is in blue. The proposed interaction sites are shown with side chains.

Minor

• The structural feature of PK1 binding site is not clearly illustrated. Does PK1 binding site is at the interface of two protomers of VANGL trimer. Author should color the each protomer / chains in different color or shades to depict this.

Response: We apologize for any confusion. The PK1 binding site is primarily located on the surface of a single protomer within the VANGL trimer. We have made the requested adjustments in Fig. 2c to enhance clarity.

• *The line 95-102 is not clear.*

Response: We thank the Reviewer's valuable feedback. We have now introduced a schematic representation in Fig. 1c to delineate the domain composition within the primary structures of VANGL1 and VANGL2 to improve understanding.

• *The author does not provide sigma threshold levels of maps used for preparing figures. This is important information that should be included, in figure legends.*

Response: We apologize for the oversight regarding the absence of contour levels. We have now included them in all the relevant figure legends.

• *The statement on page 13, line 271-273 is not accurate "This study showed that VANGL and PK did not form a structurally stable complex. Indeed, their weak electrostatic interaction makes them more …". A author should not careful before claiming the weak interaction of VANGL1-PK1 without any experimental evidence. Inability to structurally resolve complex may be due to several factors, which may not necessarily link with lower affinity.*

Response: We appreciate the Reviewer's comment. We have now removed this statement from the revised manuscript (Page 14).

• *Extended figure 1,2, 4 & 7; The scale bar for micrographs is missing.*

Response: We appreciate the Reviewer's reminder. We have now included scale bars in the relevant figures.

Reviewer #3 *(Remarks to the Author):*

Song et al. reports structural and biochemical studies on the VANGL-PRIKLE interaction, which is essential for the establishment of plana cell polarity (PCP). The manuscript includes several important findings: 1) the first high resolution structures of VANGL1 and 2, 2) the oligomer states of VANGL proteins, 3) identification of the PK1-binding site by structural and biochemical studies, 4) mapping of disease-causing

mutations on the VANGL structures with the interpretation of disease-causing mechanisms. This study significantly impoves our understanding of the complicated mechanism of PCP establishment, and thus is suitable for publication in Nature Communications. I have only minor comments.

Response: We appreciate the Reviewer's summarization of our work and the positive comments.

1. Because the PK1-binding site is close to the dimeric interface, the mutant VANGL1 (10mut) may have a defect in dimerization. If this is true, the cell experiment results need to be reinterpreted. To exclude the possibility, the authors need to provide the gel filtration or AUC data of the purified mutant VANGL1, which is expected to show the major peak at the position of the hexameric size.

Response: We greatly appreciate the Reviewer's insightful comment. To confirm the oligomerization status of VANGL1^{10mut}, we have purified the VANGL1^{10mut} protein following the same protocol as for the wild-type VANGL1. We then performed AUC analysis. Notably, the results from gel filtration and AUC suggested that the VANGL1^{10mut} oligomers have a molecular weight that corresponds to the size of a trimer (Fig. R3). This indicates that VANGL1^{10mut} trimer indeed has a defect in dimerization, which hinders it from forming the hexameric structure as presumed by the Reviewer. In light of these findings, we have re-evaluated the cell experimental outcomes and adjusted our interpretations accordingly: "Notably, the VANGL1^{10mut} formed trimers instead of hexamers (Extended Data Fig. 11e, f), indicating that these mutations impede the dimerization of VANGL1 trimers, beyond just disrupting PK1 binding. These findings suggest that the positively charged residues in VANGL1 are essential for its hexameric assembly and vesicular localization, in addition to their role in attracting PK1." (Page 12).

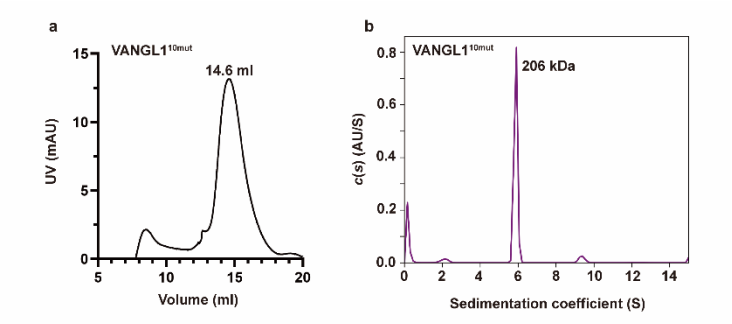

Fig. R3 Characterization of the oligomerization state of VANGL1^{10mut}.

a, Gel filtration profile of VANGL1^{10mut}. **b,** AUC analysis of VANGL1^{10mut}.

2. Fig 4f. Why is VANGL1(1-495) colocalized with PK1(WT)? Does monomeric VANGL1 also interact with PK1?

Response: We appreciate the Reviewer's incisive query. Our pull-down results have indeed validated that monomeric VANGL1$^{1-495}$ is capable of interacting with PK1 (Fig. R4). Consequently, we postulate that the interaction between VANGL and PK1 is likely mediated by electrostatic attractions between specific regions of these two proteins, rather than requiring the formation of a VANGL hexamer. Consistent with this hypothesis, our structural analysis shows that the PK1 binding site is primarily located on the surface of an individual VANGL protomer (Fig. 2c).

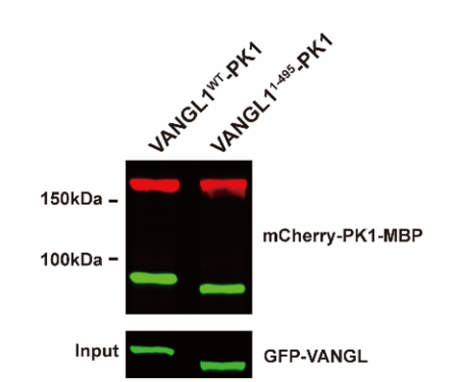

Fig. R4 Pull-down assay to verify the interaction between VANGL1$^{1-495}$ and PK1(WT).

3. A schematic diagram showing the domain composition in the primary structure in Fig 1 would be helpful to understand the manuscript.

Response: We appreciate the Reviewer's suggestion. We have now included the domain compositions of VANGL1 and VANGL2 in their primary structures in Fig. 1c.

4. Did the side chain conformations in the PK1 binding site of VANGL1 change upon the PK1 binding? Then, the structural comparison needs to be provided.

Response: We are grateful to the Reviewer's comment. As suggested, we have compared the side chain conformations at the PK1 binding site of VANGL1 before and after PK1 binding (Fig. R5 and Extended Data Fig. 6b). Our observations indicate that there are no significant conformational changes upon PK1 binding.

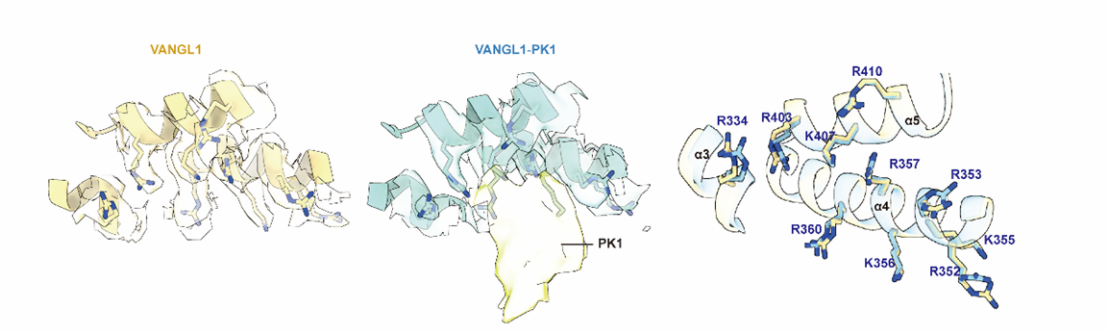

Fig R5 The structural comparison of the side chain conformations at the PK1 binding site of VANGL1.

5. How strong would be the dimerization of the VANGL trimers? It seems quite strong in the purified sample. With this high affinty, the trimers in the plasma membrane could recruit VANGL-rich vesicles without PK1, but do not appear to in this study.

Response: We appreciate the Reviewer's comment. In our biochemical and cryo-EM studies, we found that the dimerization of VANGL trimers is quite stable, with only a minor portion of trimers being present (Extended Data Fig. 1b). However, the cellular situation may be more complex. It is plausible that other proteins could interfere with the hexameric assembly of VANGL or that the dimerization strength of VANGL trimers might be less robust. As a result, VANGL alone might not be sufficient to form hexamers and target VANGL-rich vesicles to the plasma membrane. In this context, PK1 appears to be essential for the membrane localization of VANGL-containing vesicles as indicated in our experiments. Nevertheless, further investigation in polarized

cells is necessary to confirm the PK1-mediated membrane recruitment of VANGL-containing vesicles and to elucidate the underlying mechanism.

line 81. The peak profile show a large void peak and a sholder, and AUC also showed trimer population. Protein samples are not homogenous.

Response: We agree with Reviewer's observation regarding the non-homogeneity of protein samples. We have made the necessary revisions to the manuscript to address this point: "Their gel filtration peaks indicate that VANGL exists as an oligomer (the molecular weight of the monomer is ~60 kDa) (Extended Data Fig. 1a). To clarify the oligomerization status, we performed analytical ultracentrifugation (AUC) analyses; the results showed that the molecular weight of the VANGL oligomers approximately matched the hexamer size, with only a minor portion of trimers being present (Extended Data Fig. 1b)." (Page 5).

line 105. A typo (DILI) needs to be corrected.

Response: We have corrected it to "DALI server".

lines 395-397. Not only the ratio but also protein concentrations need to be provided.

line 178. The authors need to provide molar concentrations rather than molar ratios, which are necessary to estimate the binding affinity.

Response: We appreciate the Reviewer's reminder. We have now revised the Methods section to specify the molar concentrations of each protein: "The final molar concentrations were 21 μM for VANGL1 and 84 μM for PK1$^{745-790}$, respectively. Similarly, the concentrations for VANGL2 and PK1$^{745-790}$ were 24 μM and 96 μM, respectively." (Page 21).

line 126. The PK1/VANGL ratio would be difficult to be judged by SDS-PAGE. High molecular weight protein bands are sharp and not stained well, so the amounts cannot be compared with those of low molecular weight bands.

Response: We appreciate the Reviewer's comment. Generally, the intensities of the bands in SDS-PAGE are proportional to the molecular weights of the proteins. Hence, for a 1:1 complex, we would expect a more intense band for the protein with the greater molecular weight compared to its lighter counterpart. In the case of the VANGL1-PK1 complex, despite the MBP-tagged PK1 (~135 kDa) having a significant higher molecular weight than VANGL1 (~60 kDa), the band for PK1 appears relatively faint which is not caused by the staining (Extended Data Fig. 5a). This observation leads us to suggest that the PK1-VANGL1 interaction may be relatively weak or that the stoichiometric ratio may be less than 1:1. While this is not a definitive conclusion, it is a plausible inference. Furthermore, our structural analyses corroborate this hypothesis, as detailed in our responses to Reviewers #1 and #2. Therefore, we have decided to retain this statement in the revised manuscript.

line 129. No need to be assumed.

Response: We have made the following revision to the manuscript: "Upon binding to PK1, VANGL1 **maintained** its hexamer structure, similar to its unbound form, but the two trimers were compressed by approximately 3 Å along their long axis (Fig. 2b)." (Page 7).

line 135. "Shapes/sizes/strengths of densities are distinct" would be a clearer expression.

line 137. Did the authors mean "the bound PK1 was flexible" or "the binding was weak?"

line 137. Did the authors mean "our findings in protein purification?"

Response: We thank the Reviewer for the great suggestions. We have revised the manuscript accordingly: "When we conducted three-dimensional (3D) reconstruction with C1 symmetry (rather than C3 or D3 symmetry), we found that the **shapes, sizes, and strengths** of the additional densities associated with each VANGL1 subunit were distinct (Extended Data Fig. 6); these results suggested that PK1 could not bind in a comparable manner to each VANGL1 subunit and/or that **the bound PK1 was flexible**,

consistent with **our findings in protein purification** (Extended Data Fig. 5a).” (Pages 7-8).

line 148. "may originate" would be proper.

Response: We have corrected it in the revised manuscript (Page 8).