

Dact1 induces Dishevelled oligomerization to facilitate binding partner switch and signalosome formation during convergent extension

Corresponding Author: Dr Jianbo Wang

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study is a continuous effort of Dr. Wang's group to dissect the mechanisms of how Vang/Pk and Fz/Dsh can functionally antagonize each other during planar cell polarity, and they mainly used xenopus as a model organism in the context of convergence and extension (CE). In this manuscript, the authors proposed an attractive model in which Dact1 acts as a crosslinker to disengage Dvl from Vangl by inducing Dvl oligomerization and the subsequent formation of Fz-Dvl signalosome, triggered by non-canonical Wnt signals. This is a potentially interesting finding with clear novelty (if proved to be true), which may provide new insights into the mechanism of how Vang/Pk and Fz/Dsh complexes are separated. However, many experimental caveats must be addressed in order to support conclusions made in the manuscript. Specific comments follow:

Major concerns:

1. This study heavily relies on immunofluorescence of exogenously expressed proteins. To preclude the possible artefact due to overexpression, endogenous pattern should be provided at least in some instances. For example, does endogenous Vangl2 co-localize with Dvl2 and Dact1 (but not Fzd7) at any stage of CE or in the embryo without Wnt11? And then endogenous Dvl2 and Dact1 co-localize with Fzd7 (but not Vangl2) once PCP is established? This is particularly important to support the proposed model.

In discussion (line 419-422), they pointed out an important distinction between their data and the previous report is that in their system exogenous Dvl2 does not cause autonomous puncta formation and they thought it is because the moderate dosage they used is closer to the physiological level. They need to provide evidence that different levels of expression would cause different patterns of Dvl2 and the pattern of their exogenously expressed Dvl2 is same as endogenous one. If this is true, the expression pattern of Dvl2 needs to be treated with caution to ensure that the change of Dvl2 pattern is not caused by the change of expression level in each experiment.

2. Genetic interaction (synergy or antagonism) and functional test of a mutant form should also be demonstrated by loss of function experiments (and rescue experiments if applicable). A phenotypic enhancement by two exogenously expressed proteins does not necessarily mean they function in the same pathway.

3. The co-IP result in Figure 3 is not convincing. The change of Dvl2 is subtle and the subtle change could be explained by reduced Vangl2C in the pulldown complex. Is it possible that Dvl2 and Dact1 always strongly bind to each other and Dvl2 never reaches Vangl2 in the presence of Dact1? The recruitment of Dvl2 by Vangl2 might be an overexpression artifact? Does Vangl2 recruit endogenous Dvl in mammalian cells? In this regard, endogenous localization of Vangl2 and Dvl2 is important to verify the proposed disengagement of Dvl2 from Vangl2 to Fzd does occur. How about co-incubating Dvl2 and Dact1 and then use Vangl2C to compete? A relevant question is how Wnt induces Dvl2/Dact1 oligomerization? Is there biochemical evidence to support the oligomer formation of Dvl2 and Dact1 under the influence of Wnt and that oligomer formation disengaged Dvl2 from Vangl2? In general, more biochemical evidence is required to support the proposed model.

Other comments:

1. In Figure 1B, B', 0.2ng hDact1 induced phenotype, whereas 0.3ng hDact1 had no effect in Figure 1C, C'. Why?
2. In Figure 2, compared to WT (B-D), Δ SR mutant Dact1 (E-G) induces a better membrane localization of Vangl2, whereas in the presence of expressed Dvl2 (I-K), Δ SR mutant Dact1 partially abolishes the membrane localization of Vangl2 (P-S). Can authors explain these changes?
3. In many places, appropriate control group needs to be included. For example, Fig 2 lacks three control groups: Dvl2 and Dact1 single group, and Dvl2+Dact1 co-expression group. What is the localization pattern of Dvl2 and Dact1 in the absence of endogenous Vangl2? Similar control issues are also for Figure 4-6.
4. In supplementary Figure 4, the localization patterns of different forms of Dact1.L and Dvl2 look so different. Are the results in D-I are true signals or background signals?
5. In Figure 5, what happens if Vangl2 is expressed.
6. Does Dact1- Δ LZ or Dact1 Δ PDZb mutant have effects on Dvl/Fzd cluster formation under Wnt induction.
7. Authors proposed an important role of DIX domain in their model, which is different from the previous report (they also discussed this point). As this is critical for the conclusion, more stringent loss of function plus rescue experiments should be performed.

Minor points:

1. Line 176, (Fig. 2B-D) should be (Fig. 2E-G).
2. Molecular weight of western blot should be labelled. Figure 3Q and 3R should be run on the same gel.

Reviewer #2

(Remarks to the Author)

See attachment.

Reviewer #3

(Remarks to the Author)

After carefully reading the manuscript authored by A. Angermeier and colleagues, I am rather positive about the study. The research demonstrates a solid foundation and contributes meaningfully to the existing body of knowledge. However, I have identified a few key areas that require attention for improvement.

Positive Aspects: The study seem to be rigorous, well-structured argumentation, and clear presentation of results. The authors successfully address the role of Dact1 and provide an interesting novel mechanism how Dact1 regulates Dvl2/Vangl interaction in the convergent extension movements in *Xenopus*. In general, the conclusions are well supported by the presented data.

Major Concerns:

1. Figure 3: There is a crucial issue concerning the use of "DIX domain deletions." It is recommended that the authors revise this part using "DIX domain mutations" that are deficient in polymerization. This correction is essential to maintain the precision of the study's findings.
2. Figure 3P-R: The weakest aspect of the manuscript lies in the biochemical part - Figure 3P-R, particularly in the methodology employed. I believe this needs to be improved to support the general conclusions. I strongly recommend the authors to redo/supplement this section using conventional co-immunoprecipitation methods and mammalian cells. The authors do not have the direct control over the amount of proteins and their post-translational modification in the pulldown. This adjustment will enhance the reliability and robustness of the presented data.
3. Supplementary Figures 8-9: The immunocytochemistry data in Supplementary Figures 8-9 should be quantified (similarly to Figs. 2-4) and integrated into the main figures. This incorporation will strengthen the main findings and provide a more comprehensive overview of the results.

Minor Points:

1. Panel Labeling: The panels in the figures are not referenced in the text in the correct sequence/order.
2. Figure 4a Typo: A typographical error is identified in Figure 4a, where "bingding" should be corrected to "binding".

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript has satisfactorily addressed most of my concerns and offers significant new insights into the Wnt/PCP field. I have two minor suggestions. 1. Supple Fig.12, check the protein levels of the injected Dvl2-EGFP at different doses (or at least lowest dose at 30pg) and compare them to the endogenous Dvl2 levels by immunoblotting. This comparison will provide a reference for the amount of injected Dvl2-EGFP. If the levels are still higher than endogenous Dvl2, authors should describe this in the manuscript. 2. Figure 3R and 3S, it appears that Dact1 promotes the phosphorylation of Dvl2? Authors should mention this and provide an explanation or speculation in either the Results or Discussion section.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

This revised manuscript by Angermeier et al. addresses the role of the protein Dact1 in regulating non-canonical/planar cell polarity Wnt signaling using the *Xenopus* model. Herein the authors show that Dact1 regulates non-canonical/planar polarity signaling via inducing oligomerization of the protein Dishevelled which then biases its interaction between either the Wnt receptor Frizzled (Fz) or the transmembrane planar cell polarity protein Vangl. The authors have responded well to my prior concerns and I firmly believe that these studies will significantly advance our mechanistic understanding of the role of Dact1 in Wnt signaling and convergent extension (CE) in general. I fully support its acceptance and I do have some minor concerns/suggestions for the authors.

1. It will be helpful to add the new reference by Carroll et al in eLife that shows a genetic knockout of Dact1/2 in zebrafish does indeed produce CE defects,
2. I am unsure if the studies in supplemental Figure 2 shows a synergistic or additive effect, can the authors clarify this?
3. In the western blot experiments with endogenous Vangl, can the authors indicate which is the Vangl band? Also, I am assuming that this antibody was validated using approaches as co-localized tagged versus endogenous Vangl localization studies etc?
4. Why does the Dvl-M2M4 construct migrate so differently than the wild type protein?
5. In trying to rap my head around the model presented by the authors I am left somewhat confused. In the absence of non-canonical Wnt, Vangl binds to both Dact1 and Dvl2. Would this be independent clusters of Vangl/Dvl and Vangl/Dact to keep the pathway off? But here I note Dvl-membrane localization is induced by non-canonical Wnt and Fz leaving the question where is the Dvl in the Vangl clusters coming from? With a non-canonical Wnt, the signal somehow "activates" Dact to disassociate from Vangl to Dvl to form 'signaling clusters' with Fz for CE. What could this signal possibly be, phosphorylation? And for CE, the cells must become polarized, so does this mean there should be observable enrichment of particular clusters in the 'front or leading-edge region' versus "back or lagging-edge region" of the cell but this is not observed? It will be helpful for the reader if the authors can speculate on what model is favored.
6. Lastly, I will respectfully disagree with the authors on the 'signalosome' issue but this is just one person's view. No one has yet shown that the clusters of Dvl etc. have any signaling role because there is no kinetic biochemical approaches deployed. It has been oddly assumed that because the clusters are observed, they are necessary for signaling with no evidence to substantiate this. It is always best to remember that useful old adage; "correlation does dictate causation".

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The authors have adequately addressed all my comments.

(Remarks on code availability)

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RESPONSE to REVIEWERS' COMMENTS

We thank the reviewers for their careful reading of our manuscript and their constructive critiques. We have performed additional experiments based on their suggestions, and thoroughly revised the manuscript. The editorial summary also emphasized the point of providing “*stronger data demonstrating localization, interaction, and expression levels of relevant proteins as indicated by the reviewers*”. To address these critiques, we have acquired an anti-Vangl2 antibody from our collaborators (Drs. Jean-Paul Borg and Sylvie Marchetto) to examine endogenous *Xenopus* Vangl2 location (Supplementary Fig. 3), analyzed a broad range of Dishevelled2 dose to determine the impact of expression levels on its localization and autonomous oligomerization (Supplementary Fig. 12), and carried out additional Western blot experiments to demonstrate biochemical interactions (Fig. 3). We made modifications to Figures 1, 2, 4, and 6, completely changed Fig. 3, added Fig. 7 and 8 by modifying the previous Suppl. Fig. 8 and 9, and included many additional data in 12 supplementary figures (expanded from previous 9). We believe that these new data and analyses have adequately addressed the concerns of the reviewers. The blue texts below are a point-by-point responses to all the comments.

Reviewer #1 (Remarks to the Author):

This study is a continuous effort of Dr. Wang's group to dissect the mechanisms of how Vang/Pk and Fz/Dsh can functionally antagonize each other during planar cell polarity, and they mainly used *xenopus* as a model organism in the context of convergence and extension (CE). In this manuscript, the authors proposed an attractive model in which Dact1 acts as a crosslinker to disengage Dvl from Vangl by inducing Dvl oligomerization and the subsequent formation of Fz-Dvl signalosome, triggered by non-canonical Wnt signals. This is a potentially interesting finding with clear novelty (if proved to be true), which may provide new insights into the mechanism of how Vang/Pk and Fz/Dsh complexes are separated. However, many experimental caveats must be addressed in order to support conclusions made in the manuscript. Specific comments follow:

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1. This study heavily relies on immunofluorescence of exogenously expressed proteins. To preclude the possible artefact due to overexpression, endogenous pattern should be provided at least in some instances. For example, does endogenous Vangl2 co-localize with Dvl2 and Dact1 (but not Fzd7) at any stage of CE or in the embryo without Wnt11? And then endogenous Dvl2 and Dact1 co-localize with Fzd7 (but not Vangl2) once PCP is established? This is particularly important to support the proposed model. In discussion (line 419-422), they pointed out an important distinction between their data and the previous report is that in their system exogenous Dvl2 does not cause autonomous puncta formation and they thought it is because the moderate dosage they used is closer to the physiological level. They need to provide evidence that different levels of expression would cause different patterns of Dvl2 and the pattern of their exogenously expressed Dvl2 is same as endogenous one. If this is true, the expression pattern of Dvl2 needs to be treated with caution to ensure that the change of Dvl2 pattern is not caused by the change of expression level in each experiment.

Response:

1.1 “To preclude the possible artefact due to overexpression, endogenous pattern should be provided at least in some instances. For example, does endogenous Vangl2 co-localize with Dvl2 and Dact1 (but not Fzd7) at any stage of CE or in the embryo without Wnt11? And then endogenous Dvl2 and Dact1 co-localize with Fzd7 (but not Vangl2) once PCP is established?”

We agree with the reviewer that examining endogenous patterns of Vangl2/Dvl2 should provide more definitive evidence to our model. Therefore we used a new anti-Vangl2 antibody developed by Dr. Jean-Paul Borg to examine endogenous *Xenopus* Vangl2 (XVangl2) localization and expression levels (Suppl. Fig. 3). For Dvl2, we attempted custom antibody and tried various commercially available anti-Dvl antibodies, but none worked to detect endogenous *Xenopus* Dvl. We therefore had to rely on injecting small amounts of fluorescently tagged Dvl2. In our hands, 30 pg mRNA of Dvl2-EGFP was the lowest dose that could provide detectable signal for Dvl2. We note that this dose is 2-3 folds lower than that reported in the literature to examine Dvl2 localization at physiological level, and over 15-30 times lower than that needed to induced convergent extension defects (Wallingford et. al., *Nature*. 2000;405(6782):81-5).

Using the low dose of injected Dvl2-EGFP together with immunostaining of endogenous XVangl2, we demonstrated in *Xenopus* animal cap explants (Suppl. Fig. 3) that 1) XVangl2 is required to recruit Dvl2 to the

plasma membrane; 2) prior to the onset of convergent extension, Dvl2 co-localizes with XVangl2 and both proteins display even distribution on the plasma membrane: 3) during active convergent extension, Dvl2 (and a small amount of injected Fz7) forms small puncta on the plasma membrane, whereas XVangl2 remains evenly distributed on the plasma membrane and does not co-cluster with Dvl2 or Fz. These data are consistent with our later studies showing that Wnt11 and Dact1 induced Dvl2 and Fz to co-cluster (Fig. 5 and 6; Suppl. Fig. 9) on the plasma membrane, and support our model that as convergent extension progresses, Dvl2 disengages from Vangl2 and forms signalosome-like clusters with Fz (Fig. 9).

1.2 *"They need to provide evidence that different levels of expression would cause different patterns of Dvl2...., the expression pattern of Dvl2 needs to be treated with caution to ensure that the change of Dvl2 pattern is not caused by the change of expression level in each experiment."*

In Suppl. Fig. 12, we provided data of animal cap explant injected with different doses of Dvl2-EGFP, ranging from 30 to 1200 pg. Our results indicate that Dvl2-EGFP only autonomously forms puncta when injected at 1200 pg, whereas the doses we used for various experiments in the rest of the manuscript were between 30-600 pg and do not cause autonomous puncta formation.

2. Genetic interaction (synergy or antagonism) and functional test of a mutant form should also be demonstrated by loss of function experiments (and rescue experiments if applicable). A phenotypic enhancement by two exogenously expressed proteins does not necessarily mean they function in the same pathway.

Response: We thank the reviewer for the advice. Because three paralogous Dvl genes exist in *Xenopus laevis*, it is challenging to knockdown all three simultaneously. Therefore, we utilized a well-studied dominant negative Dvl mutant, Xdd1, to blocks endogenous Dvl function. Using Xdd1 and *dact1* morpholinos as loss of function approaches, we demonstrated synergy between Dvl and *dact1* during CE (Suppl. Fig. 2A, A'). Additionally, we performed a rescue experiment by co-injecting wild-type Dvl2 or Dvl2-M2M4 mutant with Xdd1 to demonstrate that DIX-mediated oligomerization is required for Dvl function in CE (Suppl. Fig 6C, C').

3. The co-IP result in Figure 3 is not convincing. The change of Dvl2 is subtle and the subtle change could be explained by reduced Vangl2C in the pulldown complex. Is it possible that Dvl2 and Dact1 always strongly bind to each other and Dvl2 never reaches Vangl2 in the presence of Dact1? The recruitment of Dvl2 by Vangl2 might be an overexpression artifact? Does Vangl2 recruit endogenous Dvl in mammalian cells? In this regard, endogenous localization of Vangl2 and Dvl2 is important to verify the proposed disengagement of Dvl2 from Vangl2 to Fzd does occur. How about co-incubating Dvl2 and Dact1 and then use Vangl2C to compete? A relevant question is how Wnt induces Dvl2/Dact1 oligomerization? Is there biochemical evidence to support the oligomer formation of Dvl2 and Dact1 under the influence of Wnt and that oligomer formation disengaged Dvl2 from Vangl2? In general, more biochemical evidence is required to support the proposed model.

Response:

3.1 *"The co-IP result in Figure 3 is not convincing...."*

We have completely revised Figure 3 with additional co-IP experiments to show that in addition to *in vitro* interaction with translated proteins (current Suppl. Fig. 5), Dact1 also reduced Dvl2-Vangl2 interaction *in vivo* in both *Xenopus* embryos (Fig. 3S, S') and mammalian cells (Fig. 3R, R'). In mammalian cells, we detected interaction between endogenous Vangl2 and Dvl that is reduced in the presence of hDACT1 (Fig. 3R, R'). We note that all our co-IP experiments were repeated three times or more, and quantified after normalizing the pulled down amount of Vangl2 (the bait protein in the co-IP experiments). We clarified the method used for quantifying the Co-IP results in the Materials and Methods section. Our quantification shows that the decrease of Dvl2-Vangl2 co-IP is statistically significant upon hDACT1 over-expression.

3.2 *"Dvl2 and Dact1 always strongly bind to each other and Dvl2 never reaches Vangl2 in the presence of Dact1"*. The reviewer raised an excellent point as we have pondered the same question. We note, however, that in both *Xenopus* animal cap and DMZ cells, XDact1 is known to be expressed, but endogenous XVangl2 is sufficient and necessary to bring low level of injected Dvl2-EGFP to the plasma membrane based on our current (Suppl. Fig. 3E-P) and previous studies (Fig. 3H-J, Seo *et. al.*, *Hum. Mol. Genet.* 2017; 26: 2053-2061). These data support that Dvl2 can reach Vangl2 in the presence of endogenously expressed Dact1. In the Discussion (second paragraph from last), we acknowledge that one remaining question is how Dact1-Dvl2 interaction could be regulated; we postulated that one mechanism could be local concentration change of Dvl and/or Dact1 triggered by non-canonical Wnt. Addressing this question will certainly be our future direction; we

feel that the most important contribution from the current manuscript is that Dact1-induced Dvl2 oligomerization can bias Dvl binding from Vangl2 to Fz.

3.3 "The recruitment of Dvl2 by Vangl2 might be an overexpression artifact? Does Vangl2 recruit endogenous Dvl in mammalian cells?"

As stated in Response 1.1, the data in Suppl. Fig. 3 demonstrated that endogenous XVangl2 is both necessary and sufficient to recruit low level of injected Dvl2 to the plasma membrane. Also in the new Fig. 3R, we used co-IP to demonstrate that in cultured SH-SY5Y cells, endogenous Vangl2 is able to co-IP endogenous Dvl2. These data support the notion that the recruitment of Dvl2 by Vangl2 is not an overexpression artifact. Lastly we note that a recent paper from Marek Mlodzik's group (*Humphries et. al., PLoS Genet. 2023; PMID: PMC10381084*) also demonstrated the importance of direct interaction between Vang and Dvl during PCP signaling in flies.

3.4 "How about co-incubating Dvl2 and Dact1 and then use Vangl2C to compete?"

As the reviewer pointed out, the interaction between Dvl2 and Dact1 is likely to be strong so we have not attempted to use Vangl2C to compete. Instead, we preincubated Dvl2 and Vangl2C, and then tested whether adding Dact1 could disrupt their binding (Suppl. Fig. 5, P-R').

3.5 "...how Wnt induces Dvl2/Dact1 oligomerization? Is there biochemical evidence to support the oligomer formation of Dvl2 and Dact1 under the influence of Wnt and that oligomer formation disengaged Dvl2 from Vangl2?" Our imaging data revealed that non-canonical Wnt can induce co-clustering of Fz/Dvl2/Dact1 at the plasma membrane through oligomerization (Fig. 6; Suppl. Fig. 9, 10, 11), but the detailed mechanism is not yet known. In a reviewed preprint on *eLife* (<https://elifesciences.org/reviewed-preprints/91199>), we reported that other components are also required for forming the non-canonical Wnt induced Fz/Dvl clusters, including the co-receptor Ror2. Elucidating how non-canonical Wnt may trigger Dvl2/Dact1 oligomerization will be the focus of our future studies.

Since the current manuscript is focused on Dact1, we did not present biochemical evidence for Wnt-induced dissociation of Dvl2 from Vangl2. But in our previous publication (Fig. 4D, *Seo et. al., Hum. Mol. Genet. 2017; 26: 2053-2061*) and the reviewed *eLife* preprint (Suppl. Fig. 7 and Suppl. Fig. 12a, <https://elifesciences.org/reviewed-preprints/91199>), we did show extensive biochemical data supporting dissociation of Dvl2 from Vangl2 by non-canonical Wnt. Testing biochemically whether Wnt induces oligomer formation of Dvl2 and Dact1 is a good idea but currently not possible since we do not have antibodies for endogenous *Xenopus* Dvl2 or Dact1; and when we co-injected epitope tagged Dvl2 and Dact1, they autonomously form oligomers even without Wnt induction. To circumvent this technical hurdle, we will need to directly tag endogenous Dvl2 and Dact1 in *Xenopus* through genome editing in the future.

Other comments:

1. In Figure 1B, B', 0.2ng hDact1 induced phenotype, whereas 0.3ng hDact1 had no effect in Figure 1C, C'. Why?

Response: We noticed that the 0.3 ng hDACT1 injected embryos in the previous Fig. 1C and C' had more variable CE phenotype and length-to-width ratio (LWR), which might have impacted the statistical analyses. We therefore repeated all the injections in Fig. 1B and C, and used a FIJI macro software to automatically measure the LWR of the embryos. The new data confirmed that 0.2 ng hDACT1 injection did induce a mild but statistically significant CE defect. The new data and analyses are included in Fig. 1B, B', B'', C and C'.

2. In Figure 2, compared to WT (B-D), Δ SR mutant Dact1 (E-G) induces a better membrane localization of Vangl2, whereas in the presence of expressed Dvl2 (I-K), Δ SR mutant Dact1 partially abolishes the membrane localization of Vangl2 (P-S). Can authors explain these changes?

Response: We thank the reviewer for pointing out the difference of Vangl2 localization in the original Fig. 2B-D vs. E-G. We checked other figures in the manuscript and noticed that in the original Suppl. Fig. 2J-L where wild-type hDACT1 was also co-injected with Vangl2, Vangl2 displayed much more robust plasma membrane localization. We therefore suspect that the poor Vangl2 membrane localization in original Fig. 2B-D was due to suboptimal imaging acquisition. We have since repeated the experiments multiple times and confirmed that Vangl2 displayed similarly strong plasma membrane localization when co-injected with either wild-type hDACT1 or Δ SR mutant. The new images are in Fig. 2C-E and Suppl. Fig. 4J-L and P-R.

3. In many places, appropriate control group needs to be included. For example, Fig 2 lacks three control

groups: Dvl2 and Dact1 single group, and Dvl2+Dact1 co-expression group. What is the localization pattern of Dvl2 and Dact1 in the absence of endogenous Vangl2? Similar control issues are also for Figure 4-6.

Response: In the previously submitted manuscript, we included most of the controls mentioned by the reviewer in supplementary figures (such as Suppl. Fig. 2) in order to reduce the number of the panels in the regular figures. In response to the reviewer's critique, we have incorporated these controls into Fig. 2, 4, 5 and 6. The Dvl2 and hDACT1 single injection controls were added to Fig. 2 (B, F, and K), the new Suppl. Fig. 4 (A-C, D-F, M-O), Fig. 5 (A-C), and Fig. 6 (A, E). In the revised Fig. 4B-M, we also included co-injection of Dvl2 or Dact1 (wild-type and mutants) with Vangl2 (B-M) as controls for their respective triple injections (Fig. 4N-Y).

In regard to Dvl2/Dact1 localization in the absence of endogenous Vangl2, we added a new Suppl. Fig. 3 as mentioned above in Response 1.1. Briefly, in naïve *Xenopus* animal cap explants where endogenous XVangl2 level is low, small amount of injected Dvl2-EGFP (30 pg) is distributed diffusely in the cytoplasm; in activin treated animal cap explants, Dvl2-EGFP is recruited to the plasma membrane as endogenous XVangl2 level is increased; morpholino knockdown of endogenous XVangl2 abolishes Dvl2-EGFP membrane localization, but the defect in the morphants can be rescued by small amounts of injected mouse Vangl2.

4. In supplementary Figure 4, the localization patterns of different forms of Dact1.L and Dvl2 look so different. Are the results in D-I are true signals or background signals?

Response: The reviewer raised a valid and critical point. Because Dvl2 and Dact1 often display diffuse cytoplasmic distribution when injected alone, it is sometimes difficult to distinguish true signals from the background in *Xenopus* embryos where yolk pellets tend to produce high auto-fluorescence. To rigorously address this criticism, we performed injection with Dvl2 or Dact1 in one blastomere and a plasma membrane marker (mem-iRFP670) in the adjacent blastomere. By imaging the mosaic region of the resulting animal cap explant, we were able to rule out the possibility that the observed fluorescent signal was from background noise. Additionally, we added single injections of wild-type or mutant Dact1 as controls to more clearly compare the distribution pattern changes before and after co-expression with Dvl2. These new data are in Supplementary Fig. 7, which replaced the previous Suppl. Fig. 4.

5. In Figure 5, what happens if Vangl2 is expressed.

Response: In our reviewed preprint on *eLife* (<https://elifesciences.org/reviewed-preprints/91199>), we performed the experiments similar to what the reviewer asked. In Figure 3 of the *eLife* preprint, we found that when non-canonical Wnt induced Dvl2 and Fz7 to co-cluster into distinct patches on the plasma membrane, moderately expressed Vangl2 localized broadly along the plasma membrane and displayed enrichment at the edge of Dvl2/Fz7 patches, but was diminished at the center of the patches where Dvl2 and Fz7 most strongly co-localized. In contrast, when Vangl2 is overexpressed at high level, it diminished Dvl2/Fz7 patch formation, induced Fz7 to undergo endocytosis and retained Dvl2 at the plasma membrane (Fig. 3, Fig. 4 and Suppl. Fig. 9 of the *eLife* preprint).

6. Does Dact1-ΔLZ or Dact1-ΔPDZb mutant have effects on Dvl/Fzd cluster formation under Wnt induction.

Response: This is an excellent question and we addressed in two ways. First, we performed a straightforward co-expression experiment, and found that irrespective of co-injection with wild-type or mutant Dact1, Dvl2 could still form patches under Wnt11 induction. Quantification using the "Mean to Sigma ratio", however, revealed a moderate but statistically significant decrease of Dvl2 enrichment within the patches when co-expressed with the two Dact1 mutants vs. wild-type Dact1. Second, we performed a rescue experiment to test how wild-type or mutant Dact1 could restore diminished Dvl2 patch formation when endogenous Dact1 was knocked down by morpholino. In this case we observed more pronounced difference: whereas wild-type Dact1 significantly restored Dvl2 patch formation in the *Dact1* morphant, the mutants had minimal or no rescue effect. These results, which are in the new Suppl. Fig. 11, provided strong cellular and molecular evidence that both the LZ and PDZb domains of Dact1 are crucial for its function in promoting Dvl cluster formation under Wnt induction.

7. Authors proposed an important role of DIX domain in their model, which is different from the previous report (they also discussed this point). As this is critical for the conclusion, more stringent loss of function plus rescue experiments should be performed.

Response: The function of DIX domain in PCP/ non-canonical Wnt signaling indeed was controversial in the literature. Since it is difficult to knockdown all three Dvl paralogs simultaneously in *Xenopus*, we used a well-

studied dominant negative mutant Xdd1 to block endogenous Dvl function during CE, and then tested rescuing Xdd1-induced CE defect with wild-type or DIX mutant form of Dvl2. Furthermore, to demonstrate more definitively that the oligomerization ability of the DIX domain is responsible for its involvement in CE/ non-canonical Wnt signaling, we added to our study the Dvl2-M2M4 mutant. M2M4 harbors three point mutations (Y27D/ V67A/ K68A) in the DIX domain, and prior work from Mariann Bienz's group showed that the three point mutations compromise the ability of DIX domain to undergo head-to-tail assembly into oligomers but preserve its overall structure (Schwarz-Romond *et. al.*, *Nat Struct Mol Biol.* 2007;14(6):484-92). In Suppl. Fig. 6C, we demonstrated that Dvl2-M2M4 mutant could no longer rescue Xdd1-induced CE defects like wild-type Dvl2. We also used the M2M4 mutant to repeat all the studies previously done with the delta-DIX deletion mutant. These data are in Fig. 3, Fig. 5, Suppl. Fig. 6B and Suppl. Fig. 10.

Minor points:

1.Line 176, (Fig. 2B-D) should be (Fig. 2E-G).

2.Molecular weight of western blot should be labelled. Figure 3Q and 3R should be run on the same gel.

Response: We have made the suggested changes.

Reviewer 2:

The manuscript by Angermeier *et al.* seeks to address the role of the protein Dact1 in regulating non-canonical/planar cell polarity Wnt signaling using the *Xenopus* model. The role of Dact1 frankly remains perplexing as it was previously shown to be a regulator of canonical and/or non-canonical signaling and this controversy remains unclarified. Herein the authors show that Dact1 regulates non-canonical/planar polarity signaling via inducing oligomerization of the protein Dishevelled which then biases its interaction between either the Wnt receptor Frizzled (Fz) or the transmembrane planar cell polarity protein Vangl. The manuscript is very well written and clear and the studies uses cutting edge imaging and subcellular localization studies coupled with using convergent extension in the frog as a physiological readout. I believe these studies can significantly advance our understanding of the role of Dact1, Wnt signaling and gastrulation in general but I have some concerns that are listed below:

1. The authors use a morpholino to knockdown Dact1 and find a role in convergent extension. However, there is no examination about a potential role in maternal Wnt signaling which when disrupted can also produce defects in convergent extension. I think this is a critical point that must be addressed.

Response: The reviewer correctly pointed out that in *Xenopus*, Dact1 was initially identified “in a maternal cDNA library and is expressed maternally by RT-PCR” (Cheyette *et. al.*, *Developmental Cell*, 2002, Vol. 2, 449–461). On the other hand, additional studies also reported zygotic expression of Dact1 during embryogenesis (Gloy *et. al.*, *Nat. Cell Bio.* 2002, Vol 4, 351-357; Hikasa and Sokol, *Development*, 2004, Vol. 131, 4725-4734). In the 2002 *Developmental Cell* paper, Cheyette *et. al.* specifically addressed the function of maternal Dact1 using the host-transfer technique and *Xenopus* oocytes injected with *dact1* morpholino; the phenotypes they observed includes “acephaly/ microcephaly, severely reduced and/or displaced notochords, reduced/malformed neural tubes, and overspecification of the lateral mesoderm”. By contrast, in the 2004 *Development* paper, Hikasa and Sokol performed dorsal injection of *dact1* morpholino into four-cell stage *Xenopus* embryos; in this case the embryos displayed shortened A-P axis and dorsal bending, phenotypes characteristic of convergent extension defect, in addition to reduced head structures. The distinct phenotypes in these two reports suggest that for convergent extension, maternal Dact1 may not be indispensable whereas zygotic Dact1 is likely to play, at least in part, a critical role. In our manuscript we further confirmed a requirement of endogenous Dact1 in convergent extension by performing MO knockdown in the DMZ as well as in the animal caps induced by activin for elongation assays (Suppl. Fig. 1A and B). Although these experiments do not determine the contribution of maternal vs. zygotic pool of *dact1* mRNA, the results suggest that knockdown of zygotic Dact1 protein alone is sufficient to induce CE defects, and that maternal Dact1 protein is not able to compensate for the loss of zygotic Dact1. Our studies agree with the current literature which did not implicate a strong contribution of maternal Dact1 for convergent extension. We therefore did not further examine a potential role of maternal Wnt signaling in convergent extension. Instead, we used functional interaction, cell biological and biochemical assays to elucidate how Dact1 interacts with Vangl2 and Dvl2, two components of the non-canonical Wnt signaling pathway, to regulate convergent extension.

2. One can argue that most of the studies rely on non-physiological exogenous expression of proteins and I would suggest that the authors include some biochemical (physical interaction) studies to show concurrence with their imaging conclusions. This is especially important for the studies examining Wnt-dependence of the observed effects.

Response: We agree with the reviewer and performed a number of experiments to address this criticism. Firstly, in the new Fig. 3, we performed a co-IP experiment using cultured SH-SY5Y cells to show interaction between endogenous Dvl2 and Vangl2, which could be reduced by transfected hDACT1 (Fig. 3R, R'). Secondly, in *Xenopus* embryos, hDACT1 reduced Vangl2 interaction with wild-type Dvl2 but not Dvl2-M2M4 mutant that could not oligomerize (Fig. 3S, S'). Thirdly, in the new Suppl. Fig. 3, we used low dose of injected Dvl2-EGFP (30 pg mRNA) together with immunostaining of endogenous XVangl2 to demonstrate that, in *Xenopus* animal cap, 1) XVangl2 is required to recruit Dvl2 to the plasma membrane; 2) prior to the onset of convergent extension, Dvl2 co-localizes with XVangl2 and both proteins display even distribution on the plasma membrane; and 3) during active convergent extension, Dvl2 (and small amount of injected Fz7) forms small puncta on the plasma membrane, whereas XVangl2 remains evenly distributed on the plasma membrane and does not co-cluster with Dvl2 or Fz. These Dvl2/Fz7 puncta are reminiscent of Wnt11 and Dact1 induced Dvl2 and Fz cluster (Fig. 5 and 6; Suppl. Fig. 9) on the plasma membrane, supporting our model that as convergent extension progresses, Dvl2 disengages from Vangl2 and forms clusters with Fz (Fig. 9).

Since the current manuscript is focused on Dact1, we did not present biochemical evidence for Wnt-induced dissociation of Dvl2 from Vangl2. But in our previous publication (Fig. 4D, *Seo et al., Hum. Mol. Genet.* 2017; 26: 2053-2061) and our reviewed *eLife* preprint (Suppl. Fig. 7 and Suppl. Fig. 12a, <https://elifesciences.org/reviewed-preprints/91199>), we did show extensive biochemical data supporting dissociation of Dvl2 from Vangl2 by non-canonical Wnt.

3. In delineating the role(s) of the individual domains (DIX, PDZ and DEP) domains of Dishevelled, the authors use internal deletions within the context of the full-length protein. I think here it is critical for the authors to examine the role(s) of expression of the individual domains by themselves to show coherence with the observed/proposed effects.

Response: To prove our model more definitively, we have now added to our study the Dvl2-M2M4 mutant in which three point mutations (Y27D/ V67A/ K68A) in the DIX domain compromise the ability of DIX domain to self-oligomerize but preserve its overall structure (*Schwarz-Romond et al., Nat Struct Mol Biol.* 2007;14(6):484-92). In Suppl. Fig. 6C, we demonstrated that unlike wild-type Dvl2, Dvl2-M2M4 mutant could no longer rescue Xdd1-induced CE defects. We also used the M2M4 mutant to repeat all the studies previously done with the delta-DIX deletion mutant. These data are in Fig. 3, Fig. 5, Suppl. Fig. 6B and Suppl. Fig. 10. The studies using point mutants in both DIX and DEP domains support our model in Fig. 9.

Regarding to individual domains, many previous publications have already examined the function of these (DIX, PDZ and DEP) domains of Dvl, which are largely consistent with our data using internal deletions or point mutations of these domains. For instance, prior work from Mariann Bienz/ Melissa Gammons has delineated how the DIX and DEP domains contribute to Dvl oligomerization, and their conclusions fully agree with our data using DIX and DEP mutants. The new model we proposed, based on their prior work and our new data, is that Dact1 reinforces “both DIX-oligomerization and DEP-dimerization to help Dvl to cluster, and it does so by binding to the PDZ of Dvl with the PDZ-b domain and homodimerize via the LZ domain”. Therefore, in our model Dact1’s action on Dvl requires not only direct binding to PDZ, but also relies indirectly on the self-oligomerization ability of DIX and DEP. Because of the mutual dependent nature of the three domains in our model, it is difficult to use individual domains of Dvl to further validate our model.

4. Why do the authors think that Fz needs to be co-clustered for a signaling function? Biochemical studies from the Weis group would argue against this. Along this line, I am unsure why the authors use the term ‘signalosome’? This term which appears to have been created de-novo implies that higher-ordered protein oligomerization is critical for a signaling function, however no signaling role is demonstrated anywhere in the presented studies.

Response: In a 2016 paper from David Strutt’s group, the term “signalosome” was first used to describe the action of core proteins during PCP signaling (Dvl, Vang, etc.), as they seem to aggregate into non-stoichiometrically polymerized complexes that “bear striking resemblance to those seen for higher-order assemblies of signaling molecules known as signalosomes” (*Strutt et al., Cell Reports* 2016; (17) 2660-2671; <http://dx.doi.org/10.1016/j.celrep.2016.11.021>). The authors further stated: “We therefore propose that core

proteins assemble into signalosome-like structures, but importantly, unlike in the conventional view of signalosomes, core protein assembly is not a transient response to ligand binding but part of the process of robust establishment of stable cell polarity". Their work and model were instrumental for our current study of Dvl/Dact1 oligomers, which can be induced to cluster with Fz on the plasma membrane by non-canonical Wnt ligands (Fig. 6, Suppl. Fig. 9, 10, 11). We therefore feel that it is appropriate to use "signalosome" or "signalosome-like clusters" to describe the Dvl/Dact1 oligomers in our study. We do, however, understand the concern raised by the reviewer, and changed our title to "Dact1-induced Dishevelled oligomerization facilitates ... and signalosome-like cluster formation ...". Similar modifications were made throughout the text by changing "signalosome" to "signalosome-like cluster (or complex)". We hope that the reviewer will find these modifications reasonable.

We think that Fz and Dvl may need to be co-clustered for non-canonical Wnt signaling because not only clustering of Fz/Dvl complex is an inherent feature of PCP signaling, but also Fz-dimerization appears to be sufficient to activate non-canonical Wnt signaling based on recent work from Henry Ho's group (*Griffiths et al. eLife 2024;13:e71980. DOI: <https://doi.org/10.7554/eLife.71980>*). Whereas Wnt5a-induced non-canonical Wnt signaling normally requires both Fz receptor and co-receptors Ror1/2, the authors reported in this publication that in *Ror1/2* null cells and without the addition of Wnt5a, a bivalent anti-Fz7 antibody can strongly activate non-canonical Wnt signaling, arguing that Wnt5a and Ror may function by inducing Fz dimerization or co-clustering. In support of this idea, our reviewed preprint on *eLife* showed that in non-canonical Wnt induced Fz/Dvl clusters, Ror2 is not only a component but also required for their formation (Fig. 7, <https://elifesciences.org/reviewed-preprints/91199>). For these reasons, we think that that inducing Fz co-clustering is likely an important mechanism for non-canonical Wnt signaling.

5. In a number of the presented studies, rescue is shown using a single dose and it becomes important to show dose-dependency of such a rescue to strengthen the argument.

Response: This is an excellent suggestion. We performed rescue of Vangl2 over-expression induced CE defects with different dose of hDACT1 ranging from 0.05 to 0.8 ng, and found that 0.1-0.2 ng injection provided the best rescue. The new data are shown in Suppl. Fig.2B.

6. The authors suggest a membrane localization of Dact1 is required for its functional role (or modulation of interaction between Dishevelled and Vangl), why no mis-localize Dact1 and test if it then loses this regulatory ability?

Response: This is an interesting idea. John Wallingford's group previously expressed a mitochondria-tethered Dvl to induce mis-localization of endogenous Dvl (via DIX-mediated dimerization), and provided evidence for Dvl plasma membrane localization as an important step in non-canonical Wnt signaling during CE (Park et. al., *Current Biology*, 2005, Vol. 15, 1039–1044). We considered a similar approach by expressing mitochondria-tether Dact1, but predict that it will not only induce mis-localization of endogenous Dact1 (via LZ-mediated dimerization), but also endogenous Dvl given Dact1's strong potency in inducing Dvl oligomerization. This complexity will make it difficult to interpret the result. Therefore, until we can directly mis-localize endogenous Dact1 by making a mutant via genome editing in *Xenopus*, we have not pursued this idea.

Reviewer #3 (Remarks to the Author):

After carefully reading the manuscript authored by A. Angermeier and colleagues, I am rather positive about the study. The research demonstrates a solid foundation and contributes meaningfully to the existing body of knowledge. However, I have identified a few key areas that require attention for improvement.

Positive Aspects: The study seem to be rigorous, well-structured argumentation, and clear presentation of results. The authors successfully address the role of Dact1 and provide an interesting novel mechanism how Dact1 regulates Dvl2/Vangl interaction in the convergent extension movements in *Xenopus*. In general, the conclusions are well supported by the presented data.

Major Concerns:

1. Figure 3: There is a crucial issue concerning the use of "DIX domain deletions." It is recommended that the authors revise this part using "DIX domain mutations" that are deficient in polymerization. This correction is essential to maintain the precision of the study's findings.

Response: We agree with and thank the reviewer for this excellent advice. Since there were reports for other possible functions of the DIX domain such as actin binding, it is indeed critical to validate our result using additional mutants that specifically abolish the polymerization activity but present the overall structural integrity of DIX. We therefore added to our study the Dvl2-M2M4 mutant in which three point mutations (Y27D/ V67A/ K68A) specifically disrupt the interface mediating DIX oligomerization (*Schwarz-Romond et. al., Nat Struct Mol Biol. 2007;14(6):484-92*). We repeated all prior studies using the delta-DIX mutants, and these data are in Fig. 3, Fig. 5, Suppl. Fig. 6B an C and Suppl. Fig. 10.

2. Figure 3P-R: The weakest aspect of the manuscript lies in the biochemical part - Figure 3P-R, particularly in the methodology employed. I believe this needs to be improved to support the general conclusions. I strongly recommend the authors to redo/supplement this section using conventional co-immunoprecipitation methods and mammalian cells. The authors do not have the direct control over the amount of proteins and their post-translational modification in the pulldown. This adjustment will enhance the reliability and robustness of the presented data.

Response: We followed the reviewer's advice and performed conventional co-IP using a mammalian cell line SH-SY5Y. We demonstrated binding between endogenous Vangl2 and Dvl2, which could be reduced by transfection of hDACT1 but not GFP (Fig. 3R, R'). We also performed co-IP using *Xenopus* embryos and showed that hDACT1 reduced Vangl2 interaction with wild-type Dvl2 but not Dvl2-M2M4 mutant that could not oligomerize (Fig. 3S, S'). We hope the reviewer will find that these data sufficiently addressed the weakness in the previous manuscript.

3. Supplementary Figures 8-9: The immunocytochemistry data in Supplementary Figures 8-9 should be quantified (similarly to Figs. 2-4) and integrated into the main figures. This incorporation will strengthen the main findings and provide a more comprehensive overview of the results.

Response: We performed quantification for the images in the previous Suppl. Fig. 8 and 9 and incorporated them into the main figures (now Fig. 7 and 8, respectively) as advised by the reviewer.

Minor Points:

1. Panel Labeling: The panels in the figures are not referenced in the text in the correct sequence/order.

2. Figure 4a Typo: A typographical error is identified in Figure 4a, where "bingding" should be corrected to "binding".

Response: We have made corrections to the figure numbers and the typo.

RESPONSE to REVIEWERS' COMMENTS

We thank the reviewers for their additional constructive critiques. We have made revisions to the manuscript according to their comments. The blue texts below are a point-by-point responses to all the comments.

Reviewer #1 (Remarks to the Author):

The revised manuscript has satisfactorily addressed most of my concerns and offers significant new insights into the Wnt/PCP field. I have two minor suggestions.

1. Supple Fig.12, check the protein levels of the injected Dvl2-EGFP at different doses (or at least lowest dose at 30pg) and compare them to the endogenous Dvl2 levels by immunoblotting. This comparison will provide a reference for the amount of injected Dvl2-EGFP. If the levels are still higher than endogenous Dvl2, authors should describe this in the manuscript.

Response: As explain in our previous response, we acquired an antibody for endogenous *Xenopus* Vangl2, but failed to identify an antibody capable of detecting endogenous *Xenopus* Dvl2 after trying many commercial antibodies and attempting custom antibody. We note that the injection dose of 30 pg used to validate our conclusion is 2-3 folds lower than that reported in the literature to examine Dvl2 localization at physiological level (*Wallingford et. al., Nature. 2000;405(6782):81-5*), and over 15-30 times lower than that needed to induced convergent extension defects (*Seo et. al., Hum. Mol. Genet. 2017; 26: 2053-61*).

2. Figure 3R and 3S, it appears that Dact1 promotes the phosphorylation of Dvl2? Authors should mention this and provide an explanation or speculation in either the Results or Discussion section.

Response: Yes, the data from mammalian cell culture (Fig. 3R) and *Xenopus* embryos (Fig. 3S) both support this conclusion; and Fig. 3S additionally suggests that the oligomerization ability of the DIX domain is required for Dvl2 to undergo Dact1 induced phosphorylation. We add description in the Results and speculation in the Discussion for the Dact1-induced Dvl2 phosphorylation.

Reviewer #2 (Remarks to the Author):

This revised manuscript by Angermeier et al. addresses the role of the protein Dact1 in regulating non-canonical/planar cell polarity Wnt signaling using the *Xenopus* model. Herein the authors show that Dact1 regulates non-canonical/planar polarity signaling via inducing oligomerization of the protein Dishevelled which then biases its interaction between either the Wnt receptor Frizzled (Fz) or the transmembrane planar cell polarity protein Vangl. The authors have responded well to my prior concerns and I firmly believe that these studies will significantly advance our mechanistic understanding of the role of Dact1 in Wnt signaling and convergent extension (CE) in general. I fully support its acceptance and I do have some minor concerns/suggestions for the authors.

1. It will be helpful to add the new reference by Carroll et al in eLife that shows a genetic knockout of Dact1/2 in zebrafish does indeed produce CE defects,

Response: We thank the reviewer for noting this new report and have added this article to the reference.

2. I am unsure if the studies in supplemental Figure 2 shows a synergistic or additive effect, can the authors clarify this?

Response: We interpret the data in Suppl. Fig. 2A and A' to mean synergy between Dact1 and Dvl, because partial blocking of Dvl (with 0.25 ng *Xdd1* mRNA) or Dact1 (with 15 ng *dact1.S* morpholino) induced only mild or no CE defect, respectively, whereas their combined injection caused severe and highly penetrant CE defects.

3. In the western blot experiments with endogenous Vangl, can the authors indicate which is the Vangl band? Also, I am assuming that this antibody was validated using approaches as co-localized tagged versus endogenous Vangl localization studies etc?

Response: In fact, both bands in Suppl. Fig. 3Q represents endogenous XVangl2. Based on ref. 46, vertebrate *Vangl2* has a conserved alternative translation start codon AUA in the 5' UTR region, which leads to production of a less abundant Vangl2-long isoform 50 aa longer than the major Vangl2 isoform translated from the canonical start codon AUG. The 36E3 anti-Vangl2 antibody we used can detect both isoforms with approximate size of 70 Kd (for Vangl2-Long isoform) and 62 Kd (for the major Vangl2 isoform). The *XVangl2* morpholino we used could efficiently block translation of the major Vangl2 isoform but less so of Vangl2-long, presumably because it was designed to target the canonical start codon. Suppl. Fig. 3R accessed the total amount of endogenous Vangl2 under each condition by measuring the intensity of both isoforms.

The 36E3 anti-Vangl2 antibody was first reported and validated in ref. 46; in the current manuscript we used morpholino knockdown and western blot to validate the specificity of this antibody (Suppl. Fig. 3Q).

4. Why does the Dvl-M2M4 construct migrate so differently than the wild type protein?

Response: As explained in the response to Reviewer 1's second comment, the different migration pattern of Dvl2-M2M4 on western blot (Fig. 3S) is most likely due to its failure to undergo Dact1-induced phosphorylation due to its deficiency in oligomerization.

5. In trying to rap my head around the model presented by the authors I am left somewhat confused. In the absence of non-canonical Wnt, Vangl binds to both Dact1 and Dvl2. Would this be independent clusters of Vangl/Dvl and Vangl/Dact to keep the pathway off? But here I note Dvl-membrane localization is induced by non-canonical Wnt and Fz leaving the question where is the Dvl in the Vangl clusters coming from? With a non-canonical Wnt, the signal somehow "activates" Dact to disassociate from Vangl to Dvl to form 'signaling clusters' with Fz for CE. What could this signal possibly be, phosphorylation? And for CE, the cells must become polarized, so does this mean there should be observable enrichment of particular clusters in the 'front or leading-edge region' versus "back or lagging-edge region" of the cell but this is not observed? It will be helpful for the reader if the authors can speculate on what model is favored.

Response: Firstly, our model predicts that Vangl2 can directly recruit Dvl (and Dact1) to the plasma membrane, independent of Fz and non-canonical Wnt. This is based on our previous publication (ref. 41) and the data in Suppl. Fig. 3. Fig. 8E further suggests that Vangl2 recruits Dvl2 to the plasma membrane through binding to the PDZ domain.

Secondly, we do not know whether Vangl binds to Dvl2 and Dact1 to form dimeric Vangl/Dvl and Vangl/Dact1 complexes, or Dvl/Vangl/Dact1 trimers. We do know that the PDZ domain mediates Dvl binding to both Vangl2 and Dact1, such that Vangl2-bound Dvl is probably inhibited from binding to Dact1 simultaneously. This may contribute to the inhibitory effect of Vangl2 on non-canonical Wnt signaling.

Thirdly, we do not know what signal triggered by non-canonical Wnt can activate Dact1 to dissociate Dvl from Vangl. It could be phosphorylation, or the local concentration of Dact1/Dvl as

we speculated in the Discussion. Additional reagents enabling tracking endogenous Dvl and Dact1 will be needed to address this question.

Finally, we have not done live imaging to correlate the distribution of Dvl clusters with the mediolateral or anteroposterior edge of the cells undergoing CE, so we cannot make any speculation in the current manuscript.

6. Lastly, I will respectfully disagree with the authors on the 'signalosome' issue but this is just one person's view. No one has yet shown that the clusters of Dvl etc. have any signaling role because there is no kinetic biochemical approaches deployed. It has been oddly assumed that because the clusters are observed, they are necessary for signaling with no evidence to substantiate this. It is always best to remember that useful old adage; "correlation does dictate causation".

Response: We definitely agree with the reviewer that correlation does not imply causation, but causation often occurs with correlation. Our current manuscript is to establish a correlation between Dact1-induced Dvl oligomerization and non-canonical Wnt signaling during CE. As explained in the previous response, we used the term "signalosome" because it has been used in the literature to describe PCP protein clusters or Dvl oligomers in canonical Wnt signaling (for example, ref ⁶⁹ and <https://doi.org/10.1016/j.molcel.2016.08.026>) We agree with the reviewer that the biochemical nature of the Dvl clusters will need to be studied in the future to substantiate their signaling roles during CE.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed all my comments.

The manuscript by Angermeier et al. seeks to address the role of the protein Dact1 in regulating non-canonical/planar cell polarity Wnt signaling using the *Xenopus* model. The role of Dact1 frankly remains perplexing as it was previously shown to be a regulator of canonical and/or non-canonical signaling and this controversy remains unclarified. Herein the authors show that Dact1 regulates non-canonical/planar polarity signaling via inducing oligomerization of the protein Dishevelled which then biases its interaction between either the Wnt receptor Frizzled (Fz) or the transmembrane planar cell polarity protein Vangl. The manuscript is very well written and clear and the studies uses cutting edge imaging and subcellular localization studies coupled with using convergent extension in the frog as a physiological readout. I believe these studies can significantly advance our understanding of the role of Dact1, Wnt signaling and gastrulation in general but I have some concerns that are listed below:

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