Ubiquitin-assisted phase separation of dishevelled-2 promotes Wnt signalling

Vaishna Vamadevan, Neelam Chaudhary and Subbareddy Maddika
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First decision letter

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MS TITLE: Ubiquitin assisted phase separation of Dishevelled-2 promotes Wnt signaling

AUTHORS: Vaishna Vamadevan, Neelam Chaudhary, and Subbareddy Maddika
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Vamadevan et al. study the formation of Dvl2 signalosomes (but unfortunately call it phase separation-driven condensates). They find the WWP2, previously reported to interact with and negatively regulate Dvl2 signaling, here is a positive regulation of signalosome formation. They

propose that poly-ubiquitylation of Dvl2 via a K63 linkage facilitates signalosome formation by promoting Dvl2 dynamic polymerization. The role of the ubiquitin ligase WWP2 in signalosome formation is novel and and will be of interest to the field. The writing is clear and the experiments are generally clear. A missed opportunity is comparing WWP2 with the closely related NEDD4L with apparently an opposite role in Wnt/β-catenin signaling.

Comments for the author

Major issues: The limitations of the manuscript are several.

It relies very heavily on over-expression, usually without reference to endogenous concentration. This is a significant limitation that plagues the field.

Point 1: The authors would do well to include blots of endogenous protein in all over-expression experiments to let the reader decide the relevance of the findings.

All the studies are performed in a single cell line, HEK293T. HEK293T cells are fine for initial discovery, but they are weird cells with many mutations and several genes from adenovirus and SV40 not found in normal tissues.

Point 2: Repetition of key experiments in a few other cell lines would substantially strengthen the work.

Point 3: Key experiment that needs to be done: Does WWP2 KO impair Wnt-stimulated signalosome formation in HEK293 and other cells with endogenous levels of Dvl2?

Discussion point (or even experimentation) The conclusions on the role of WWP2 in Wnt/β-catenin signaling differ from prior studies (esp Mund et al.) and this ought to be addressed.

The role of ubiquitylation in phase separation of other proteins appears to be well-established. Please be careful to acknowledge prior work by others in the field. The interaction of HECT proteins including NEDD4L and WWP2 with Dvl2 was established previously by the Chen and Bienz labs. The Chen paper (Ding et al., JBC 288:8289, 2013) examining NEDD4L (with more hits in your Mass Spect data) needs to be cited and its contrary conclusions discussed.

Why is it necessary to use the trendy phrase ‘phase separation’ when the Wnt field already has studied this phenomenon and just called them signalosomes regulated by dynamic polymerization? I object to the use of a new jargon phrase when prior terminology works just fine.

The authors would do well to temper their enthusiasm and claims. Their work adds interesting information to the field, but is not as ground-breaking as comments like “Our study provides compelling evidence to suggest that the phase separated molecular condensates of Dvl2 are critical for activation of Wnt signaling” imply.

1,6 hexanediol is widely used but problematic. See PMID: 33814344, that shows “Already at 1% volume concentration, 1,6-hexanediol strongly impaired kinases and phosphatases…” This experiment should be removed, or the discussion qualified.

Extended data fig. 1d shows an excellent tool, stable expression of a tagged SFB-Dvl2 in HEK293T cells. I’d like to see this type of blot done for ALL transfection experiments (e.g. Fig 1a, 1b, 1d, 1e…where the reader has no idea what the fold over-expression is.

Fig 1f and fig 1a have two critical differences – Fig 1A shows ‘condensates’ in the absence of Wnt, and in fig 1A, Dvl2 is over-expressed. When Dvl2 is at near-endogenous levels, it does not form spontaneous condensates, but requires Wnt ligand (a well-established result). So, is it reasonable to extrapolate from behavior in over-expression to what happens at low concentrations? I think this over-expression approach can produce misleading and sometimes just irrelevant results.

What is a triple SFB tag (page 6)? Three copies of SFB, or one copy each of three tags?

Please clarify in the methods or results sections -was the tandem affinity purification done with the HEK293T cells with stable expression of SFB-Dvl2, or transient expression? If stable, were the cells treated with Wnt, or not treated with Wnt prior to lysis? If transient, what was the fold-overexpression of Dvl2?

Minor point: Fig 2g – We need to know the expression levels of the Ub constructs, especially UbK63R, to interpret this figure. An alternative explanation of the results is, there was no expression of K63R.

Fig 2h/i: what protein concentrations were used in the various assays described, and how pure was the purified GST-Dvl2?

Fig 4a is not particularly compelling nor well described. What was the concentration of the His-Dvl2 CT? Does the input represent 100% of the material loaded in the pulldown lanes, or 10%. The lanes of the GST Dvl2 do not line up with the His lanes so it's hard to see what is happening.

Fig 4b is a nice result – endogenous WWP2 is needed for the Dvl2 interaction with Dvl2 CT. Two limitations of this figure that need to be stated are a) the test of the open-closed confirmation model is based on over-expression and is not demonstrated at endogenous levels and b) it is not demonstrated to be a Wnt-regulated event.

Prior papers (Mund, 2015; Ding, 2013) have found that WWP2 destabilized Dvl2 and inhibits Wnt/β-catenin and Wnt5a signaling. Your results are different. What happens to endogenous Dvl2 in WWP2 knockout cells?
Please test and discuss.

Fig 5 is titled: Dvl2 phase separation is essential for Wnt signaling, but I think the data are more correlative than proving causality.

Major problem: Fig 5c – without knowing the protein expression of the WT and delta-IDR1, this comparison is meaningless.

Extended data figure 5 is referred to in the text but is not included in the submission.

Reviewer 2

Advance summary and potential significance to field

This study focuses on the macromolecular complexes of Dishevelled, which have been a subject of a debate in the field for almost 30 years, because of their unclear physiological significance in Wnt signaling. The authors offer several novel observations. They demonstrate that Dvl2 undergoes phase separation to generate large puncta in HEK293T cells. This process is regulated via K63 ubiquitination by the HECT E3 ubiquitin ligase Wwp2 and appears to be involved in Wnt/beta-catenin signaling. These findings are consistent with a specific mechanism, in which the newly added ubiquitin chains cause a conformational change in Dvl2 and expose an intrinsically disordered region (IDR) triggering phase separation. The authors' data are well controlled and consistent with the involvement of Dvl2 phase separation in canonical Wnt signaling pathway however, their conclusions are somewhat overstated. Although the use of HEK 293T cells as the only experimental model and overreliance on overexpression are weaknesses, the findings would definitely be of interest to the readers.

Comments for the author

This study focuses on the macromolecular complexes of Dishevelled, which have been a subject of a debate in the field for almost 30 years, because of their unclear physiological significance in Wnt signaling. The authors offer several novel observations. They demonstrate that Dvl2 undergoes phase separation to generate large puncta in HEK293T cells. This process is regulated via K63 ubiquitination by the HECT E3 ubiquitin ligase Wwp2 and appears to be involved in Wnt/beta-catenin signaling. These findings are consistent with a specific mechanism, in which the newly

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Minor points:

1. Since WWP2 promotes Dvl2 phase separation in vitro (Figure 2i), I suggest that this important result can be added to the abstract.

2. In Figure 5, β-catenin localization looks unusual as compared to figure 5f. Is that reproducible? It is also hard to tell if β-catenin staining is in the same cell as the one expressing FLAG-Dvl2. Showing a different image may be advantageous.

3. Page 11, several figures are not referred to correctly, e. g. Extended data fig 5f is missing (should be 4f?), Extended data 5d (should be 5d).

4. "Required" or "critical for relaying the Wnt signal" As loss of puncta seem to only reduces TOP-Flash signal by about half, it is unlikely to be critical for Wnt signaling and most likely beneficial or enhances Wnt signaling.

5. Specify the Frizzled gene used for the experiments, because there are 13 Frizzled in vertebrates.

6. "The fluorescence signal of GFP tagged-Dvl2 was quickly recovered after photobleaching (FRAP) (Fig. 1b & 1c), suggesting mobile nature of the droplets." Please explain why "quickly" is justified? What is a 'normal' turnover time?

7. Better explain in discussion why the K63 linkage is interesting.

8. Some phrases are too strong and need to be softened to allow alternative interpretations. Examples: "rule out the possibility" (p5 line 22-24); "this data fully supports" (page 8) ; "data clearly demonstrate" (page 12). "Dvl2 condensates can accommodate all the components of destruction complex" All the components were not tested. Check the text for typos.

First revision

Author response to reviewers' comments

Point-by-point response to reviewer's comments:

Reviewer 1:

Vamadevan et al. study the formation of Dvl2 signalosomes (but unfortunately call it phase separation-driven condensates). They find the WWP2, previously reported to interact with and negatively regulate Dvl2 signaling, here is a positive regulation of signalosome formation. They propose that poly-ubiquitylation of Dvl2 via a K63 linkage facilitates signalosome formation by promoting Dvl2 dynamic polymerization. The role of the ubiquitin ligase WWP2 in signalosome formation is novel and will be of interest to the field. The writing is clear and the experiments are generally clear.

Response: We thank the reviewer for appreciating our study.

A missed opportunity is comparing WWP2 with the closely related NEDD4L with apparently an opposite role in Wnt/β-catenin signaling.

Response: As indicated by the reviewer, NEDD4L along with other HECT ligases such as ITCH and SMURF1 are known to associate with DVL2 and negatively regulate Wnt signalling. Here, we focussed exclusively on E3 ligases that regulate phase separation of Dvl2 and possibly contributing to Wnt activation and therefore E3 ligases regulating Dvl2 stability seemed to be out of relevance for the current study.

Major issues: The limitations of the manuscript are several.

It relies very heavily on over-expression, usually without reference to endogenous concentration. This is a significant limitation that plagues the field.
Point 1: The authors would do well to include blots of endogenous protein in all over- expression experiments to let the reader decide the relevance of the findings.

Response: We agree with the reviewer that overexpression of proteins is a valid concern. To demonstrate phase separation of endogenous Dvl2, the real challenge was the non-availability of a good Dvl2 antibody that works specifically in immunofluorescence experiments. We screened several Dvl2 antibodies from various sources for this purpose but unfortunately none of the antibodies worked in our IF experiments. Therefore, we had to rely on overexpression experiments. As suggested by the reviewer, we now have included the western blot showing the relative expression of SFB Dvl2 and GFP Dvl2 (which have been widely used in the study) with respect to endogenous dvl2 in the revised manuscript (revised extended figure 1a and 1d).

All the studies are performed in a single cell line, HEK293T. HEK293T cells are fine for initial discovery, but they are weird cells with many mutations and several genes from adenovirus and SV40 not found in normal tissues. Repetition of key experiments in a few other cell lines would substantially strengthen the work.

Response: As suggested by the reviewer we have tested the formation of Dvl2 condensates in three other cell lines such as U2OS (Osteosarcoma epithelial cells), HepG2 (Hepatocellular carcinoma cells), RPE1 (retinal pigment epithelial cells). As seen in the revised manuscript, Dvl2 is able to form condensates in different cell lines (revised extended data figure 1b) and could efficiently sequester GSK3β in all tested cell lines (revised extended data figure 5d). Additionally, we have shown that WWP2 depletion in all these cell lines significantly reduced the Dvl2 condensates.

Point 3: Key experiment that needs to be done: Does WWP2 KO impair Wnt-stimulated signalosome formation in HEK293 and other cells with endogenous levels of Dvl2?

Reponse: As we have mentioned in our manuscript earlier, there are no good IF grade antibodies for Dvl2 to test the endogenous condensate formation. We screened several Dvl2 antibodies from various sources for this purpose but unfortunately none of the antibodies worked in our IF experiments. Therefore, we could not test Wnt dependent endogenous Dvl2 condensate formation in WWP2 KO cells. Nonetheless, we have clearly demonstrated that Dvl2 fails to form condensates in WWP2 KO 293T cells, as seen in figure 2b. To further test if loss of WWP2 impairs Dvl2 condensate formation in other cell types, we depleted WWP2 in U2OS, HepG2 and RPE1 cells and tested the condensate formation. In agreement with our earlier data from 293T cells, loss of WWP2 in all other tested cell lines led to defective Dvl2 condensates (shown in extended data figure 2).

Discussion point (or even experimentation) The conclusions on the role of WWP2 in Wnt/β-catenin signaling differ from prior studies (esp Mund et al.) and this ought to be addressed.

Response: As indicated by the reviewer, earlier studies (Mund et al (2015) Open Biol) have shown the association of WWP2 and Dvl2. However, the precise role of WWP2 in Wnt/β-catenin signalling is not studied. In fact, Mund et al focussed on regulation of Notch and not Wnt signalling by WWP2 in their study. In our study we have extensively tested the effect of WWP2 in Wnt signaling at

various stages of the pathway starting from Dvl2- frizzled interaction till Wnt target gene transcript levels. As you may find in our manuscript WWP2 depletion did not alter interaction of Dvl2 with Frizzled or any of the destruction protein components that we tested. However, WWP2 depletion (both knock down and knock out) severely impaired Dvl2 condensate formation, activation of beta catenin and its translocation to the nucleus followed by defective Wnt target gene expression. Together these data clearly indicate that WWP2 plays a significant role in the Wnt signalling pathway.

The role of ubiquitylation in phase separation of other proteins appears to be well-established. Please be careful to acknowledge prior work by others in the field. The interaction of HECT proteins including NEDD4L and WWP2 with Dvl2 was established previously by the Chen and Bienz labs. The Chen paper (Ding et al., JBC 288:8289, 2013) examining NEDD4L (with more hits in your Mass Spect data) needs to be cited and its contrary conclusions discussed.

Response: Studies on role of ubiquitination during phase separation are emerging. Association of proteins with free ubiquitin chains was shown to affect phase separation of proteins such as UBQLN2, NEMO and p62, These studies are now included in the discussion of our revised version of manuscript. However it may be noted that a direct role of covalent linkage of ubiquitination to the substrate and the its role in substrate phase separation is not established. Our study demonstrated that substrate (Dvl2) ubiquitination with a specific linkage type is important for its phase separation.
Apologies for our oversight on missing out the inclusion of the references related to known HECT E3 ligases for Dvl2. We now have included these references along with the relevant discussion in the revised manuscript.

Why is it necessary to use the trendy phrase 'phase separation' when the Wnt field already has studied this phenomenon and just called them signalosomes regulated by dynamic polymerization? I object to the use of a new jargon phrase when prior terminology works just fine.

Response: We completely agree with the reviewer that the term signalosome was appropriate for DVL2 structures as it clearly conveys the function of those protein assemblies in the signalling pathway. It may be noted that we used the term 'phase separation' to clearly describe the nature of these protein assemblies rather than replacing the existing term of signalosomes. We want to clearly emphasize that signalosomes are formed by the phase separation of Dvl2 rather than mere aggregation of proteins and it is functionally relevant unlike many protein aggregates in the cell. Even though polymerisation is a pre-requisite for Dvl2 phase separation, dependency of Dvl2 on its IDR clearly demonstrates that polymerisation is not sufficient for Dvl2 phase separation to form signalosomes. Phase separation here defines the process by which the signalosomes are formed during Wnt signaling.

The authors would do well to temper their enthusiasm and claims. Their work adds interesting information to the field, but is not as ground-breaking as comments like "Our study provides compelling evidence to suggest that the phase separated molecular condensates of Dvl2 are critical for activation of Wnt signalling" imply.

Response: As suggested by the reviewer, we modified these statements in the revised manuscript

1,6 hexanediol is widely used but problematic. See PMID: 33814344, that shows "Already at 1% volume concentration, 1,6-hexanediol strongly impaired kinases and phosphatases…" This experiment should be removed, or the discussion qualified.

Response: We agree with the reviewer regarding the adverse effects of 1,6- hexanediol in cells and therefore as you may have noticed we haven't used 1,6 hexanediol for any functional experiments. However, given that it is a widely accepted reagent and routinely used for testing the phase separation, we utilized this to show the sensitiveness of Dvl2 condensates to 1,6- hexanediol as only a supporting evidence to other data shown in figure 1.

Extended data fig. 1d shows an excellent tool, stable expression of a tagged SFB-Dvl2 in HEK293T cells. I'd like to see this type of blot done for ALL transfection experiments (e.g., Fig 1a, 1b, 1d, 1e…where the reader has no idea what the fold over-expression is.

Response: As suggested by the reviewer, we now have included the western blot showing the relative expression of SFB Dvl2 and GFP Dvl2 with respect to endogenous dvl2 in the revised manuscript (revised extended figure 1a and 1d).

Fig 1f and fig 1a have two critical differences – Fig 1A shows 'condensates' in the absence of Wnt, and in fig 1A, Dvl2 is over-expressed. When Dvl2 is at near-endogenous levels, it does not form spontaneous condensates, but requires Wnt ligand (a well-established result). So, is it reasonable to extrapolate from behavior in over-expression to what happens at low concentrations? I think this over-expression approach can produce misleading and sometimes just irrelevant results.

Response: It is very well established that phase separation is a concentration dependent process and most of the phase separating proteins have saturation concentration above which it undergoes phase separation. Dvl2 at near endogenous levels can form condensates during Wnt signaling even without an 'overexpression' implies that the phase separation of Dvl2 inside the cells is tightly regulated through post translational modification or conformational inhibition rather than protein expression levels. This is further supported by our findings in WWP2 depleted cells and delta IDR mutants, despite of over expression, condensate formation of Dvl2 was significantly reduced in WWP2 KO cells and cells over expressing delta IDR mutant of Dvl2. On the other hand, this data may also suggest that Wnt treatment may provide the signal to enhance the critical local concentration that is required for Dvl2 phase separation, which may be mimicked during overexpression in the absence of Wnt. While Dvl2 overexpression doesn't necessarily mimic complete physiological setting, it does phenocopy Wnt 'ON' conditions such as beta-catenin activation, gene expression etc, therefore we believe that it serves as decent alternative tool to study the phase separation behaviour of Dvl2.

What is a triple SFB tag (page 6)? Three copies of SFB, or one copy each of three tags?

Response: SFB is one copy of each of the three tags: S-protein binding protein, Streptavidin binding protein and Flag tag. This has been extensively used in our earlier manuscripts to describe this construct. We modified it to 'SFB-triple tag' rather than 'triple-SFB tag' in the revised version of the manuscript.

Please clarify in the methods or results sections -was the tandem affinity purification done with the HEK293T cells with stable expression of SFB-Dvl2, or transient expression? If stable, were the cells treated with Wnt, or not treated with Wnt prior to lysis? If transient, what was the fold-overexpression of Dvl2?

Response: Affinity purification was done using transient expression of SFB-Dvl2 under normal conditions. No Wnt treatment was done in this experiments. Representation of fold expression of Dvl2 was shown in extended data figure 1a.

Minor point: Fig 2g – We need to know the expression levels of the Ub constructs, especially UbK63R, to interpret this figure. An alternative explanation of the results is, there was no expression of K63R.

Response: We have included the blots showing the input levels of Ub mutants in the revised manuscript. As shown in revised figure 2g, although the Ub K63 mutant is readily expressed in cells, Ub Chain formation on Dvl2 is severely hampered.

Fig 2h/i: what protein concentrations were used in the various assays described, and how pure was the purified GST-Dvl2?

Response: The assay was performed on GST Dvl2 bound to glutathione Sepharose beads, which limits measuring the concentration of Dvl2, however we have used the same volume of beads containing GST Dvl2 across the samples. Due to the limited solubility of recombinant DVL2 FL it was challenging to detect protein via CBB staining. We always utilized western to detect Dvl2 specifically and thus purity could not be estimated. Since same DVL2 protein samples was utilized with WT and C/A in these assays, we do not anticipate the purity of GST- Dvl2 would have affected the assay results.

Fig 4a is not particularly compelling nor well described. What was the concentration of the His-Dvl2 CT? Does the input represent 100% of the material loaded in the pulldown lanes, or 10%. The lanes of the GST Dvl2 do not line up with the His lanes so it's hard to see what is happening.

Response: The concentration of His CT used was 7 µg. This information is now included in the revised figure legend. Input represents 100% of the material loaded in the pull-down lanes. GST Dvl2 lanes are in fact in line with His lanes. Four lanes of GST DVL2 proteins are in line with 4 lanes of His-DVL2 CT. Two additional lanes were seen in His-CT blot, one with marker and another showing His-CT input protein.

Fig 4b is a nice result – endogenous WWP2 is needed for the Dvl2 interaction with Dvl2 CT. Two limitations of this figure that need to be stated are a) the test of the open-closed confirmation model is based on over- expression and is not demonstrated at endogenous levels and b) it is not demonstrated to be a Wnt-regulated event.

Response: As suggested, we tested the trans CT binding with endogenous Dvl2 in the presence of Wnt. As shown in revised figure 4c, treatment of cells with Wnt3a readily enhanced the interaction of CT fragment with endogenous Dvl2 (Fig. 4c), thus suggesting that Dvl2 conformational switching is a Wnt regulated event.

Prior papers (Mund, 2015; Ding, 2013) have found that WWP2 destabilized Dvl2 and inhibits Wnt/β-catenin and Wnt5a signaling. Your results are different. What happens to endogenous Dvl2 in WWP2 knockout cells?
Please test and discuss.

Response: It is not entirely true that prior indicated papers (Mund, 2015; Ding, 2013) have found that WWP2 destabilized Dvl2 and inhibits Wnt/β-catenin and Wnt5a signalling. No data was available in these studies that suggest WWP2 inhibits Wnt signalling. In fact, Mund et al 2015 studied the connection of Dvl2-WWP2 in regulation of NOTCH signalling rather than Wnt signalling. Also, no direct experimental evidence was provided in these studies to suggest Dvl2 as a degradative substrate of WWP2. Throughout our study, we could detect ubiquitination of Dvl2 without any proteosomal inhibitors suggesting that ubiquitinated Dvl2 is rather stable inside the cells (Fig 2f, 2g, 3h) which was further supported by our cycloheximide chase assay (Extended Fig 3h). We assume that the decrease in the intensity of Dvl2 band shown in earlier study (Mund et al 2015) may be due to the shift in the size of Dvl2 protein due to extensive ubiquitination by WWP2. In fact, Mund et 2015 has noted in their Fig 2a representing the ubiquitination and destabilization of Dvl2 by Wwp2 that 'the levels of ubiquitylation of Dvl2 do not strictly correlate with its destabilization.' Given that no data was available in earlier studies to clearly suggest that WWP2 destabilizes Dvl2, our data may not necessarily be seen contrary to published data.

Also, as suggested by the reviewer we have tested the levels of endogenous Dvl2 in WWP2 KO and KD cells. As shown in the revised extended data figure 3, endogenous levels of Dvl2 remain relatively unaltered in both WWP2 KO and KD cells.

Fig 5 is titled: Dvl2 phase separation is essential for Wnt signaling, but I think the data are more correlative than proving causality.

Response: The statements were modified in the revised manuscript.

Major problem: Fig 5c – without knowing the protein expression of the WT and delta-IDR1, this comparison is meaningless.

Response: We now have included the blots showing the expression levels of WT and delta IDR1 in the revised version. As shown in the extended data figure 5e, although the expression of delta-IDR1 in comparision to WT is not altered, delta IDR1 is defective in activating Wnt signalling.

Extended data figure 5 is referred to in the text but is not included in the submission.

Response: Apologies for the error. All the appropriate figures are now referred in the revised manuscript.

Reviewer 2:

This study focuses on the macromolecular complexes of Dishevelled, which have been a subject of a debate in the field for almost 30 years, because of their unclear physiological significance in Wnt signaling. The authors offer several novel observations. They demonstrate that Dvl2 undergoes phase separation to generate large puncta in HEK293T cells. This process is regulated via K63 ubiquitination by the HECT E3 ubiquitin ligase Wwp2 and appears to be involved in Wnt/beta-catenin signaling. These findings are consistent with a specific mechanism, in which the newly added ubiquitin chains cause a conformational change in Dvl2 and expose an intrinsically disordered region (IDR) triggering phase separation.

Response: We thank the reviewer for appreciating our study.

The authors' data are well controlled and consistent with the involvement of Dvl2 phase separation in canonical Wnt signaling pathway, however, their conclusions are somewhat overstated. Although the use of HEK 293T cells as the only experimental model and overreliance on overexpression are weaknesses, the findings would definitely be of interest to the readers. Acceptance is recommended after the manuscript is strengthened by editing.

Response: As suggested by both the reviewers, we included data from three additional cell lines in the revised manuscript to complement our original observations in 293T cells (see revised extended figure 1b and extended figure 2. We strengthened the manuscript with additional data along with the suggested edits.

Minor points:

1. Since WWP2 promotes Dvl2 phase separation in vitro (Figure 2i), I suggest that this important result can be added to the abstract.

Response: As suggested by the reviewer, we now included this in the abstract of revised manuscript.

2. In Figure 5, β-catenin localization looks unusual as compared to figure 5f. Is that reproducible? It is also hard to tell if β-catenin staining is in the same cell as the one expressing FLAG-Dvl2. Showing a different image may be advantageous.

Response: In figure 5A, the β catenin staining in the cells expressing flag dvl2 represents Total beta catenin in figure 5 whereas Fig.5f is stained by active β-catenin antibody. The observed difference in staining in these panels is due to their relative abundance in the cellular locations.

3. Page 11, several figures are not referred to correctly, e. g. Extended data fig 5f is missing (should be 4f?), Extended data 5d (should be 5d).

Response: Apologies for the error. All the appropriate figures are now properly referred in the revised manuscript.

4. "Required" or "critical for relaying the Wnt signal" As loss of puncta seem to only reduces TOP-Flash signal by about half, it is unlikely to be critical for Wnt signaling and most likely beneficial or enhances Wnt signaling.

Response: As suggested the statement has been modified in the revised manuscript.

5. Specify the Frizzled gene used for the experiments, because there are 13 Frizzled in vertebrates.

Response: We have used Frizzled 5 for our experiments. This information has been included in the revised figure legend.

6. "The fluorescence signal of GFP tagged-Dvl2 was quickly recovered after photobleaching (FRAP) (Fig. 1b & 1c), suggesting mobile nature of the droplets." Please explain why "quickly" is justified? What is a 'normal' turnover time?

Response: We agree with the reviewer that we cannot make relative statement since we do not have a measurable time comparison. Thus, we modified the statement by removing 'quickly' in the revised manuscript.

7. Better explain in discussion why the K63 linkage is interesting.

Response: We thank the reviewer for the suggestion. We included relevant discussion in the revised manuscript.

8. Some phrases are too strong and need to be softened to allow alternative interpretations. Examples: "rule out the possibility" (p5 line 22-24); "this data fully supports" (page 8) ; "data clearly demonstrate" (page 12). "Dvl2 condensates can accommodate all the components of destruction complex" All the components were not tested. Check the text for typos.

Response: We thank the reviewer for the suggestions. As suggested we have made necessary corrections in the revised manuscript by toning down the statements.

Second decision letter

MS ID#: JOCES/2022/260284

MS TITLE: Ubiquitin assisted phase separation of Dishevelled-2 promotes Wnt signaling

AUTHORS: Vaishna Vamadevan, Neelam Chaudhary, and Subbareddy Maddika
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

Repeating my prior comments, Vamadevan et al. study the formation of Dvl2 signalosomes (but unfortunately call it phase separation-driven condensates). They find the WWP2, previously reported to interact with and negatively regulate Dvl2 signaling, here is a positive regulation of signalosome formation. They propose that poly-ubiquitylation of Dvl2 via a K63 linkage facilitates signalosome formation by promoting Dvl2 dynamic polymerization. The role of the ubiquitin ligase WWP2 in signalosome formation is novel and will be of interest to the field.

Comments for the author

The authors have largely addressed my concerns. I have two minor requests.

Response to Point 1: The concern about over-expression is supported by the marked over-expression illustrated in new Supplemental/Extended figure 1A. The image is over-exposed so relative quantitation is challenging, but it's easy to estimate there is >10-fold over endogenous.

Request 1: This needs to be stated in the results section.
Notably, the Boutros lab recently addressed this issue by knocking a fluorescent tag into endogenous Dvl2 (see Schubert et al., “Superresolution microscopy localizes endogenous Dvl2 to Wnt signaling-responsive biomolecular condensates”, pmid: 35867833).

Request 2: I recognize that this PNAS paper examining endogenous Dvl2 was published only slightly before the first manuscript was submitted, but with the revision, the findings of single centrosome-located condensates of the Schubert ought to be at least be discussed.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript has substantially improved with the addition of the new data and text modifications. I was already very enthusiastic after my read of the first version and the authors did a terrific job in responding to the critiques despite the existing limitations of their system. This work uncovers the nature and the regulation of Dvl ‘puncta’ which have been misinterpreted in the literature a number of times. The authors also demonstrate the important mechanistic role of Wwp2 in the Wnt pathway. I recommend acceptance.

Comments for the author

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