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Dishevelled coordinates phosphoinositide kinases PI4KIIIα and PIP5KIγ for efficient PtdInsP_2 synthesis

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MS TITLE: Dishevelled coordinates phosphoinositide kinases PI4KIIIα and PIP5KIγ for efficient PtdInsP2 synthesis

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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, while the reviewers agree that the manuscript examines an important question, they also raise a number of 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. In particular, both reviewers indicate that more direct evidence is required to link Dvl to PIP2 regulation (MS experiments). Addressing the reviews will strengthen the manuscript conclusions. 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.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

In this report, the authors investigated the role of Dvl in the regulation of PtdInsP2 production by PI4K and PIP5KI. PtdInsP2 plays important roles in a wide range of cellular processes including signal transduction and endocytosis.
While the regulation of its production has been extensively investigated, the subject still remains incompletely understood. The authors observed that PI4K expression in cells resulted in a reduction, rather an increase, in its immediate product PtdInsP. This observation, together with the previously published observation that Dvl forms a complex with PI4K and PIP5KI, leads to the hypothesis that Dvl may regulate cellular PtdIns2P contents via coordinating complex formation of PI4K and PIP5KI, in which PI4K may stimulate the activity of PIP5KI. This hypothesis, if well substantiated, provides new mechanistic insights into PIP2 production regulation.

Comments for the author

The authors used overexpression and siRNA-mediated knockdown approaches to provide evidence for this hypothesis. While the study contains some intriguing observations, there are a number of significant issues and gaps that should be addressed and filled.
1) A hypothesis that an interaction with PI4KIIIα increased the enzymatic activity of PIP5K was proposed based on the initial observation that PI4K expression in cells resulted in a reduction in PtdInsP, but this hypothesis was not really tested in the study. As this hypothesis is a key point of the manuscript, more experiments including the examination of PtdInsP contents by MS with Dvl knockdown need to be done to provide direct evidence.
2) The description of the effects of Dvl3 expression in Fig 3 should be based on the means shown in Fig. 3 G and H rather than the magnitudes of individual experiments shown in Fig 3B and E.
3) Dvl3 and Ror2 effects on PtdInsP2 were entirely inferred from the use of two indirect assays. Direct measurement of PtdInsP2 is needed to confirm the findings.
4) It is unclear what information the authors tried to convey with Fig. 4G.
5) The potential off-target effects of a single Dvl3 siRNA need to be considered. A second siRNA or rescue experiment should be done at the least.
6) It stated " We asked if Ror2 receptor expression can alter phosphoinositide levels and whether that change depends on Dvl (Fig. 6A)," but not evidence for the dependency of Dvl was offered.
7) Results in Fig. 6E and F only shows that Wnt5A increase plasma membrane localization, it does not allow concluding that Wnt5a acts through Ror2. The same is true for PIP5KIg.

Reviewer 2

Advance summary and potential significance to field

PIP2 is a key signaling molecule that has been implicated in diverse cellular functions. The current manuscript examines a model in which disheveled scaffolds PI4K and PIP5K to enable synthesis of PIP2 directly from PI, rather than a sequential and physically separated reaction. Providing convincing data supporting this mechanism is an important goal.

Comments for the author

The data presented primarily employ overexpression system to examine the effects of Dvl, PI4KIIIa, and PIP5KIg. The use of several different assays (mass spec electrophys, PIP2 fluorescence sensor) is a positive aspect of the manuscript.
However, some conclusions are preliminary require additional evidence and some controls are missing.
Comment 1: A major conclusion from this manuscript is that Dishevelled is necessary to organize the activities of PI4K and PIP5K. A key piece of data is that overexpression of Dishevelled

phenocopies the co-overexpression of PI4K and PIP5K. This correlation is suggestive of the relationship between Dishevelled and PI4K and PIP5K. However, to confirm a causative relationship, it should be tested whether depletion of Dishevelled impacts the effects of co-expression of PI4K and PIP5K on PIP2.
Comment 2: For the experiments in Fig 1, are the western blots in Fig 2 the same cells showing the levels of overexpression? If so, this should be made clear. If not, western blots showing the overexpression of cell lines used in Fig 1 should be provided.
Comment 3: To support the overall conclusions, it is important to include mass spectrometry experimenst examining the impact of Dishevelled PI, PIP and PIP2.
The other assays employed in the manuscript, while beneficial, are indirect reflections of PIP2.
Comment 4: What is the evidence that the puncta observed in Fig 4 are normal vesicular structures and not some type of protein aggregate from overexpression?
Comment 5: In Figure 5A, a loading control is necessary for this western blot.
In addition, to avoid off targets, a second siRNA targeting a distinct sequence should be included. KO and/or rescue are other options.

First revision

Author response to reviewers' comments

We thank the reviewers for their helpful critiques. We consider the manuscript substantially improved by the additional experiments and revisions their comments inspired. We will detail our responses to their comments point by point (responses in red). The new version of the manuscript also shows changes or additions to the original text in red. This response has also been uploaded as a PDF “Response to Reviews” in the Supplementary Information.

Reviewer 1 Comments for the Author:
The authors used overexpression and siRNA-mediated knockdown approaches to provide evidence for this hypothesis. While the study contains some intriguing observations, there are a number of significant issues and gaps that should be addressed and filled.
1) A hypothesis that an interaction with PI4KIIIα increased the enzymatic activity of PIP5K was proposed based on the initial observation that PI4K expression in cells resulted in a reduction in PtdInsP, but this hypothesis was not really tested in the study. As this hypothesis is a key point of the manuscript, more experiments including the examination of PtdInsP contents by MS with Dvl knockdown need to be done to provide direct evidence.

Thank you, excellent point. As suggested, we attempted mass spectrometry analysis of PtdIns*P*, PtdInsP_2, and PtdInsP_3 in cells with knockdown of Dishevelled 3. Unfortunately, the percentage of cells positive for siDvl3 was very low, producing a signal-to-noise ratio too small to detect changes in phospholipid species. Not only did the protocol to transfect siRNA have a low transfection efficiency, but cells where Dvl3 had been knocked down also exhibited decreased viability, most likely due to disruption of cell division (Kikuchi et al., 2010). Given these constraints, we were unable to measure population effects of knocking down Dvl3 via mass spectrometry. In our other experiments using microscopy and electrophysiology, we overcame the challenges of knocking down Dvl by transfecting siDvl3 for a short duration (~12 hours) and selecting healthy individual cells labeled with the dye Tye563, a positive control for siRNA transfection.

As an alternative experiment, we measured PtdIns*P*, PtdInsP_2, and PtdInsP_3 in cells with overexpression of Dvl3. Results are shown in Figure 6.

2) The description of the effects of Dvl3 expression in Fig 3 should be based on the means shown in Fig. 3 G and H rather than the magnitudes of individual experiments shown in Fig 3B and E.

[Note that figure numbering is changed in this revision. The mass spectrometry data has been moved from Fig. 1 to Fig. 6 and the other figure numbers have been advanced accordingly (e.g. former Fig. 3 is now Fig. 2)].

We agree that the kinetic measures in Fig. 2G and H provide a more quantitative description of PH-PLCδ1 kinetics than Fig. 2B and E. We've edited the results section to draw conclusions primarily using Fig. 2G and H. To clarify a possible misunderstanding, the time courses in Fig. 2B and E are averaged responses from several cells (sample sizes are noted in the figure legend) and derive from the same data set used in Fig. 2G and H.

3) Dvl3 and Ror2 effects on PtdInsP2 were entirely inferred from the use of two indirect assays. Direct measurement of PtdInsP2 is needed to confirm the findings.

The indirect assays used for PtdInsP_2 (kinetics of activation/deactivation of KCNQ channels and localization of fluorescent PtdInsP_2 probe PH-PLCδ1) have been validated by mass spectrometry in several studies, confirming they faithfully report PtdInsP_2 availability (Dai et al., 2016; Horowitz et al., 2005; Traynor-Kaplan et al., 2017). We believe these two independent assays confer several advantages: they are highly sensitive, measurable within intact cells, and measurable from individual cells with known protein overexpression, providing an excellent signal-to-noise ratio.

Nevertheless, we agree with the reviewer that direct quantification of PtdInsP_2 levels is important to confirm our findings with these indirect assays. Therefore, as suggested, we performed mass spectrometry on cells overexpressing Dvl3. Note that in this case the signal-to-noise ratio is not optimal as it depends on cell populations with moderate transfection efficiency.

The new mass spectrometry results with overexpression of Dvl3 provide direct evidence that Dvl3 decreases PtdIns$P$ and increases PtdInsP_2 and PtdInsP_3 (Fig. 6D-F). These results corroborate our observations with two well-established indirect assays of PtdInsP_2.

4) It is unclear what information the authors tried to convey with Fig. 4G.

We agree with the reviewer that this figure was confusing and conveyed little information. We replaced former Figure 4G with new analysis probing the colocalization of PI4KIIIα and PIP5Kγ in the context of Dvl3 overexpression and Dvl3 knockdown (new Fig. 3G). From confocal micrographs of cells overexpressing PI4KIIIα and PIP5Kγ, we measured the Pearson coefficient of colocalization of PI4KIIIα and PIP5Kγ within the plasma membrane. We found that colocalization was increased by Dvl3 overexpression and decreased by knocking down Dvl3.

5) The potential off-target effects of a single Dvl3 siRNA need to be considered. A second siRNA or rescue experiment should be done at the least.

The reviewer is right in pointing this out. The siRNA for Dvl3 we obtained commercially from Santa Cruz Biotechnology is a cocktail of three different duplexed siRNAs directed against Dvl3 and rescue plasmids are not made available. As a result, it was not feasible for us to perform a rescue experiment. To control for a non-specific effect of siDvl3 on expression of PI4KIIIα and PIP5Kγ, we measured kinase expression with knockdown of Dvl3 and found that endogenous protein levels were unaffected by Dvl3 siRNA (Fig. 4B). The new mass spectrometry results with Dvl3 overexpression provide additional evidence that Dvl3 is acting on PI4K and PIP5K to increase phosphoinositides (Fig. 6D-F).

6) It stated " We asked if Ror2 receptor expression can alter phosphoinositide levels and whether that change depends on Dvl (Fig. 6A)," but not evidence for the dependency of Dvl was offered.

Good point. We have not tested whether Dvl is required for the change of phosphoinositides induced by Ror2 agonist Wnt5a and have adjusted the text accordingly. However, we performed additional experiments to test whether Wnt5a induces phosphorylation of Dvl3, a step thought to be necessary for this scaffolding protein to start assembling larger signaling complexes (Nishita et al., 2010; Witte et al., 2010). The new data presented in Fig. 5E shows that Wnt5a does phosphorylate Dvl3, and that the effect is specific for this non-canonical ligand. Wnt7a, a canonical Wnt ligand that does not activate Ror2 receptors (Cerpa et al., 2011; Cerpa et al., 2015; McQuate et al., 2017), fails to increase Dvl3 phosphorylation.

7) Results in Fig. 6E and F only shows that Wnt5A increase plasma membrane localization, it does not allow concluding that Wnt5a acts through Ror2. The same is true for PIP5KIg.

The reviewer is right pointing this out. Wnt5a can activate multiple receptor types. We have adjusted the results and discussion sections of the manuscript to better reflect our data.

Reviewer 2 Comments for the Author:
The data presented primarily employ overexpression system to examine the effects of Dvl, PI4KIIIa, and PIP5KIg. The use of several different assays (mass spec, electrophys, PIP2 fluorescence sensor) is a positive aspect of the manuscript.
However, some conclusions are preliminary require additional evidence and some controls are missing.

Comment 1: A major conclusion from this manuscript is that Dishevelled is necessary to organize the activities of PI4K and PIP5K. A key piece of data is that overexpression of Dishevelled phenocopies the co-overexpression of PI4K and PIP5K. This correlation is suggestive of the relationship between Dishevelled and PI4K and PIP5K. However, to confirm a causative relationship, it should be tested whether depletion of Dishevelled impacts the effects of co-expression of PI4K and PIP5K on PIP2.

We thank the reviewer for this observation. As suggested, we directly tested whether knockdown of Dvl counteracts the effect of overexpressing either PI4KIIIα or PIP5KIγ.
First, we probed the colocalization of PI4KIIIα and PIP5KIγ in the context of Dvl3 overexpression and Dvl3 knockdown (new Fig. 3G). From confocal micrographs of cells overexpressing PI4KIIIα and PIP5KIγ, we measured the Pearson coefficient of colocalization of PI4KIIIα and PIP5KIγ within the plasma membrane. We found that colocalization was increased by Dvl3 overexpression and decreased by knocking down Dvl3.

Second, we attempted mass spectrometry analysis of PtdIns*P*, PtdInsP_2, and PtdInsP_3 in cells with overexpression of either PI4KIIIα and PIP5KIγ and concurrent knockdown of Dishevelled 3. Unfortunately, as mentioned in our response to Reviewer 1, constraints with expression of siDvl3 prevented us from collecting useful population data with those transfection conditions.

Comment 2: For the experiments in Fig 1, are the western blots in Fig 2 the same cells showing the levels of overexpression? If so, this should be made clear. If not, western blots showing the overexpression of cell lines used in Fig 1 should be provided.

The experiments in both figures (now Figures 1 and 6) were performed on populations of transiently transfected cells where the same transfection conditions were used, therefore there is no reason to expect different levels of overexpression. This is now clearly stated in the text of the manuscript.

Comment 3: To support the overall conclusions, it is important to include mass spectrometry experiments examining the impact of Dishevelled PI, PIP and PIP2.
The other assays employed in the manuscript, while beneficial, are indirect reflections of PIP2.

We agree with the reviewer that the assays used to determine relative levels of PtdInsP_2 are indirect. Nevertheless, these indirect assays used for PtdInsP_2 (kinetics of activation/deactivation of KCNQ channels and localization of fluorescent PtdInsP_2 probe PH-PLCδ1) have been validated by mass spectrometry in several studies, confirming they faithfully report PtdInsP_2 availability (Dai et al., 2016; Horowitz et al., 2005; Traynor-Kaplan et al., 2017). We believe these two independent assays confer several advantages: they are highly sensitive, measurable within intact cells, and measurable from individual cells with known protein overexpression, providing an excellent signal-to-noise ratio.

Nevertheless, we agree with the reviewer that direct quantification of PtdInsP_2 levels is important to confirm our findings with these indirect assays. Therefore, as suggested, we performed mass spectrometry on cells overexpressing Dvl3. Note that in this case the signal-to-noise ratio is not optimal as it depends on cell populations with moderate transfection efficiency.

The new mass spectrometry results with overexpression of Dvl3 provide direct evidence that Dvl3 decreases PtdIns*P* and increases PtdInsP_2 and PtdInsP_3 (Fig. 6D-F). These results corroborate our observations with two well-established indirect assays of PtdInsP_2.

Comment 4: What is the evidence that the puncta observed in Fig 4 are normal vesicular structures and not some type of protein aggregate from overexpression?

We don't have evidence to distinguish between vesicular structures and protein aggregation. While we would like to better understand the nature of these structures, we believe it is outside of the scope of the present study. Therefore, we focused our experimental attention and discussion on the effect of Dvl3 expression on colocalization of PI4KIIIα and PIP5Kγ (Fig. 3G).

Comment 5: In Figure 5A, a loading control is necessary for this western blot.
In addition, to avoid off targets, a second siRNA targeting a distinct sequence should be included. KO and/or rescue are other options.

As suggested by the reviewer, loading controls (GAPDH) have been added to the Western blot (Fig. 4A). The siRNA for Dvl3 we obtained commercially from Santa Cruz Biotechnology is a cocktail of three different duplexed siRNAs directed against Dvl3 and rescue plasmids are not made available. As a result, it was not feasible for us to perform a rescue experiment. However, mass spectrometry results with Dvl3 overexpression provide additional evidence that Dvl3 is acting on phosphoinositides.

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Second decision letter

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MS TITLE: Dishevelled coordinates phosphoinositide kinases PI4KIIIα and PIP5KIγ for efficient PtdInsP2 synthesis

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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. Thank you for your thoughtful responses to the referee comments and for submitting this interesting work to J Cell Science!