# Exploring the mechanistic and temporal regulation of LRP6 endocytosis in canonical Wnt signaling

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DOI: 10.1242/jcs.243675

**Editor**: John Heath

**Review timeline**
Original submission: 14 January 2020
Editorial decision: 13 February 2020
First revision received: 10 June 2020
Accepted: 3 July 2020

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**Original submission**

First decision letter

MS ID#: JOCES/2020/243675

MS TITLE: Exploring mechanistic and temporal regulation of LRP6 endocytosis in canonical Wnt Signaling

AUTHORS: Fiete Haack, Kai Budde, and Adelinde M Uhrmacher
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. In particular, they draw attention to the clarity of writing and typographical errors. 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*

In this manuscript, Haack et al modelled and simulated the LRP6 internalisation, a key step for Wnt signalling. They comparatively simulated existing (and contradictory) models to assess which

scenario can account for the experimental findings. They found out that (i) LRP6 is likely to be endocytosed via clathrin-independent endocytosis, (2) membrane compartmentalisation is an important parameter (3) cytoplasmic assemblies should be taken into account for the signalling output.
Overall, despite involving a few simplifications and assumptions, I think this manuscript sheds light on an important question on Wnt signalling and it will help the experimental researchers to reconsider how to design new experiments and which parameters to control. Moreover, since Wnt signalling is one of the most important signalling pathways and LRP6 internalisation is the key step these findings will be important for broad audience.

*Comments for the author*

I have only minor comments:
- Authors state that "We were able to reproduce the experimental results only under the assumption that internalization of WNT LRP6 complexes occurs concurrently inside and outside of lipid rafts and is in both cases most likely caveolin-mediated, hence clathrin-independent". I understand how in both cases the endocytosis can be clathrin independent, but in several manuscripts, Cav is shown to localise exclusively in microdomains together with canonical Wnt (see one recent example here: PMID: 31803740). How could caveloin-dependent endocytosis take place in non-rafts if caveolin is only in rafts?
- Authors could reproduce the experimental results when they included the membrane compartmentalisation into their model. However, according to their best fitting model, endocytosis happens in both ordered and disordered membranes. It is somehow not clear that actually the endocytosis in the ordered domain is signalling active, and endocytosis outside the ordered domains does not lead to signalling? If this is the case, authors should clearly state this in Discussion.
- On page 7, there are missing information "…or inside (SUPPLEMENTAL xx)
of rafts. In the latter case Finally, we…"

Reviewer 2

*Comments for the author*

Up to now, the manuscript appears incomplete and too complicated in order to be reviewed properly. A few suggestions as remedies for the missing parts and in order to increase accessibility:

The work lacks a presentation and discussion of how the biological mechanisms were translated into the models and a systematic evaluation of their outcomes in comparison to measurements. What is model M? I would expect an overview of the models (with M, M1, M2, M3, M4, ...) and which mechanisms they contain. Which considerations were applied when combining mechanisms and how did they turn out in the models? If there were impossible combinations, this should be mentioned. Which challenges were encountered during the translation and how were these worked out? Is is infeasible to get even a rudimentary understanding of the models given two tables of parameter values without a single rule or model specification. Furthermore, as the computational approach is aiming at finding a minimal set of mechanisms, how was that minimality assessed? The use of the provenance graph in Fig. 4 is actually exacerbating an understanding of the modeling process, as it refers to publications by numbers that are not assigned in the references.

Regroup the figure panels in Figs. 1 to 3 according to their types:
1) Reactions within and across the involved compartments (Figs. 1(a), 2(a), 3(a)). This allows to present the discussed basic mechanisms (receptor/ligand binding CME vs CIE vs no endocytosis, raft-dependence vs raft-independence, signalosome formation, ...) with their respective biology. By the way, what is the difference between Fig. 2(a) and Fig. 3(a)?
2) Combinations of these basic mechanisms investigated in the models of the manuscript (Figs 2(b), 3(b), upper parts of the other panels). The implementations should be exemplified by source codes with explanations.
3) Comparison of combination models dynamics to measurements (lower parts of other panels) that can serve as a visual evaluation of the proposed signaling mechanisms. Furthermore, a more thorough evalutation will require not only a visual, but also a quantitative comparison of models (deviation of dynamics to measurements, number of parameters, ...).

Furthermore, the omission of FZD from the models should be discussed in detail both justified by biology and describing the influence on the models, since it forms an integral part of the Wnt receptor complex. The manuscript also should emphasize that rule-based modeling approaches are currently studied, contrasting the results with similar methods especially for those dealing with (Wnt) signaling. Occasionally, sentences contain redundancies, e.g. "[...] a simplistic model [...] serves as a simplistic, but general description [...]". Last but not least, I would strongly encourage to use a spellchecker as I saw some errors in that regard.

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**First revision**

Author response to reviewers' comments

First of all, we would like to thank the reviewers for the detailed and critical review of our manuscript. The comments are insightful and have brought out several points that were not clearly stated in the first version of the manuscript. We have thoroughly restructured the manuscript and revised the line of discussion, as well as important details following the suggestions of the reviewers. A manuscript version with all changes highlighted was submitted as a supplementary file.
Point-to-Point answers to the reviewer's comments are given below:

Reviewer 1 Comments for the author
I have only minor comments:
-Authors state that “We were able to reproduce the experimental results only under the assumption that internalization of WNT LRP6 complexes occurs concurrently inside and outside of lipid rafts and is in both cases most likely caveolin-mediated, hence clathrin-independent”. I understand how in both cases, the endocytosis can be clathrin independent, but in several manuscripts, Cav is shown to localise exclusively in microdomains together with canonical Wnt (see one recent example here: PMID: 31803740). How could caveloin-dependent endocytosis take place in non-rafts if caveolin is only in rafts?

>response:
Many thanks for this comment. For us this result was puzzling as well. We are aware that caveolin is exclusively localized in raft domains (often times caveolin is used as marker for lipid rafts fractions) and that caveolin-dependent endocytosis should be restricted to microdomains. In fact, some statements in the manuscript were not sufficiently precise to express that the internalization of LRP6 outside of rafts domains is clathrin-independent (CIE) and has (only) similar temporal dynamics as the caveolin-mediated endocytosis.
We have corrected the misleading statements and added a short paragraph to the discussion in which we shortly review potential alternative internalization pathways:

'This means, our simulation results indicate a raft- and clathrin-independent endocytosis pathway with similar internalization dynamics as raft-dependent caveolin-mediated internalization. However, Caveolin is raft-associated, which makes it highly unlikely, that caveolin is also mediating the internalization of non-raft associated LRP6. Recently a flotillin-dependent internalization mechanism of LRP6 has been discussed in Yamamoto et al. (2017) Also, other potential clathrin-independent processes capable of building endocytotic pits have been discussed Johannes et al. (2015), such as the clathrin-independent carrier (CLIC)-GPI-anchored protein-enriched early endosomal compartment (GEEC) pathway (Sabharanjak et al. (2002); Kirkham et al. (2005)) or the formation of clathrin-independent pits induced by receptor-clusting and mediated by short Actin structures (Rao and Mayor (2014)). Clearly, this interesting aspect requires further investigations.'

-Authors could reproduce the experimental results when they included the membrane compartmentalisation into their model. However, according to their best fitting model, endocytosis happens in both ordered and disordered membranes. It is somehow not clear that actually the

endocytosis in the ordered domain is signalling active, and endocytosis outside the ordered domains does not lead to signalling? If this is the case, authors should clearly state this in Discussion.

>response:
Yes, this is indeed the case; thereby we are following the large number of studies showing that LRP6 phosphorylation, hence pathway activation is exclusively occurring in raft domains. We have added a corresponding statement to the discussion. However, please note, that the activation of beta-catenin pathway was not the primary focus of our study.

-On page 7, there are missing information "…or inside (SUPPLEMENTAL xx)
of rafts. In the latter case Finally, we…"

>response:
Thank you - we have fixed the missing link.

Reviewer 2 Comments for the author
Up to now, the manuscript appears incomplete and too complicated in order to be reviewed properly. A few suggestions as remedies for the missing parts and in order to increase accessibility:

The work lacks a presentation and discussion of how the biological mechanisms were translated into the models and a systematic evaluation of their outcomes in comparison to measurements. What is model M? I would expect an overview of the models (with M, M1, M2, M3, M4, ...) and which mechanisms they contain. Which considerations were applied when combining mechanisms and how did they turn out in the models? If there were impossible combinations, this should be mentioned. Which challenges were encountered during the translation and how were these worked out? Is is infeasible to get even a rudimentary understanding of the models given two tables of parameter values without a single rule or model specification. [..]

Regroup the figure panels in Figs. 1 to 3 according to their types:
1) Reactions within and across the involved compartments (Figs. 1(a), 2(a), 3(a)). This allows to present the discussed basic mechanisms (receptor/ligand binding, CME vs CIE vs no endocytosis, raft-dependence vs raft-independence, signalosome formation, ...) with their respective biology. By the way, what is the difference between Fig. 2(a) and Fig. 3(a)?
2) Combinations of these basic mechanisms investigated in the models of the manuscript (Figs 2(b), 3(b), upper parts of the other panels). The implementations should be exemplified by source codes with explanations.
3) Comparison of combination models dynamics to measurements (lower parts of other panels) that can serve as a visual evaluation of the proposed signaling mechanisms. Furthermore, a more thorough evalutation will require not only a visual, but also a quantitative comparison of models (deviation of dynamics to measurements, number of parameters, ...).

>response:

We are sorry, that our argumentation line was not sufficiently clear and we are grateful for the suggestions to improve the accessibility and understandability of our paper.
Following this advise, we restructured the manuscript and regrouped the figures accordingly.
All in all we tried to incorporate as many recommendations as possible, while keeping the main structure of the manuscript.
Most importantly, we now separated our considerations about the general models (we now term it abstract models), from the actual simulation model of LRP6 internalization.
This allows us to better transport the idea of transfering abstract, pathway independent concepts, such as membrane compartmentalization, localization-dependent endocytosis or signalosome formation and to the actual WNT/beta-catenin pathway. Thereby we can first give an overview of the models and describe the general mechanisms (as suggested); and only afterward how these have been incorporated in the pathway-specific simulation model of WNT/beta-catenin (including a description of the pathway-specific assumptions and simplifications we made during the translation); and lastly what model configurations were analyzed in terms of simulation and why.

Furthermore, as the computational approach is aiming at finding a minimal set of mechanisms, how was that minimality assessed?

>response:

We have not used a computational approach to identify a minimal set of mechanisms or to minimize an existing model.
Instead we have followed a bottom-up approach, starting out from a simple (mechanistic) model which considers ligand-induced receptor internalization.
This model was stepwise extended by mechanisms that we considered most natural in terms of WNT/LRP6 internalization.
The adjective minimal is therefore misleading, we removed it.
Instead, we emphasize the development process, i.e., starting with a simple model which was successively extended.

The use of the provenance graph in Fig. 4 is actually exacerbating an understanding of the modeling process, as it refers to publications by numbers that are not assigned in the references.

>response:

Thank you for pointing this out. Indeed, we did not notice this mistake, which was introduced during the submission process.
We are sorry about the confusion. We adapted the provenance graph accordingly.

Furthermore, the omission of FZD from the models should be discussed in detail both justified by biology and describing the influence on the models, since it forms
an integral part of the Wnt receptor complex.

>response:
Many thanks for the remark. We completely agree that Frizzled is a crucial part of the Wnt receptor complex in both canonical and non-canonical Wnt signaling. We also agree with the reviewer, that the omission of Frizzled should be discussed in more detail. Accordingly we changed and extended this part in the manuscript as follows:

'Despite its crucial role in controlling canonical- and non-canonical WNT signaling, we do not explicitly consider the presence of FZ for LRP6-receptor internalization in the context of WNT signaling. While several studies indicate that LRP6 is internalized in form of a ternary complex comprising WNT, FZ, and LRP6, the binding affinity of FZ to WNT is similar to LRP6 and both receptors are homogeneously distributed throughout the membrane (Sezgin et al., 2017; Bourhis et al., 2010; Brennan et al., 2004; Semënov et al., 2001). Thus, the impact of FZ on LRP6 internalization dynamics can be neglected, as both receptors are activated through WNT3a and internalized in similar way and dynamics Yamamoto et al. (2006). Therefore, we assume that FZ is either implicitly part of the WNT/LRP6 complex, or the WNT-LRP6 interaction alone is sufficient for inducing LRP6 internalization'

The manuscript also should emphasize that rule-based modeling approaches are currently studied, contrasting the results with similar methods especially for those dealing with (Wnt) signaling.

>response:

We used for our simulation studies a rule-based modeling language (ML-Rules). We included a sentence to emphasize that rule-based approaches have been and are used for modeling and simulating signalling pathways. As suggested by the reviewer, we considered to include small snippets of the model code in the document, to explain how models are defined in ML-Rules and include the complete simulation models as supplemental material; however, the editorial office asked us to remove the code snippets as well as the model implementations from the supplemental material and instead refer to the GitHub repository. Therefore we included more references to the model code located in the github repository (e.g. in figure captions). Also, it should be noted that the models do not use the full set of features of ML-Rules and thus could have also been easily realized in other modeling and simulation approaches. Our motivation for using ML-Rules has been

that the models can more easily be extended to include e.g., the growth of lipid rafts, endosomal sorting or recycling, which will become relevant for upcoming studies. We included several sentences and added references to similar methods, such as Kappa (also applied in the context of WNT signaling) or BioNetGen, to clarify this in the paper. Also we emphazised the paper of Helms et al., in which the syntax and semantics of the modeling language ML-Rules is thoroughly described and also compared to similar approaches.

Occasionally, sentences contain redundancies, e.g. "[...] a simplistic model [...] serves as a simplistic, but general description [...]". Last but not least, I would strongly encourage to use a spellchecker as I saw some errors in that regard.

>response:
Many thanks for the remark, we have checked the spelling and, also revised the wording.

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Second decision letter

MS ID#: JOCES/2020/243675

MS TITLE: Exploring mechanistic and temporal regulation of LRP6 endocytosis in canonical Wnt Signaling

AUTHORS: Fiete Haack, Kai Budde, and Adelinde M Uhrmacher
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*

The authors addressed all my concerns in the revised manuscript.

*Comments for the author*

Therefore, I recommend the publication of the manuscript.
Erdinc Sezgin

Reviewer 2

*Advance summary and potential significance to field*

The manuscript has minor improvement since the last revision. However, only biological issues and figures rearrangements were addressed in the last revision and my previous mentioned comments on the models were only partially considered.
Thus, the manuscript still appears incomplete and the basis of the whole study including all drawn conclusion can’t be reviewed properly.

*Comments for the author*

The manuscript has minor improvement since the last revision. However, only biological issues and figures rearrangements were addressed in the last revision and my previous mentioned comments on the models were only partially considered.

Thus, the manuscript still appears incomplete and the basis of the whole study including all drawn conclusion can't be reviewed properly.

The manuscript should contain a rudimentary description of the established models. This includes a complete model representation including equations. Given tables of parameters in the manuscript is insufficient without equations that are currently only on GitHub.

Although model M is highlighted in figure 6. It is not mentioned in the whole manuscript or on GitHub. What is model M? Furthermore, the model setup is still not clear for me. Figure 6 seems to describe the development process without mentioning the newly introduced models A1-A3. Only by looking at the provenance graph, it seems that model M1 would be the most complex one.

Conclusions about the internalisation process were drawn on a visual comparison of data curves. The establishment of new mechanistic regulations should be based on more solid analyses and thus quantitative comparisons of the models
(deviation of dynamics to measurements number of parameters, …)

Moreover, a commented source code or at least an extensive ReadMe file would be desirable to get a rudimentary understanding of the crowded GitHub repository and the performed analyses.