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Deconvolving the roles of Wnt ligands and receptors in sensing and amplification

Rui Zhen Tan, Ni Ji, Remco A. Mentink, Hendrik C. Korswagen and Alexander van Oudenaarden

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   11 April 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now finally heard back from the referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. However, they raise several concerns on your work, which should be convincingly addressed in a major revision of the present study. The recommendations provided by the reviewers are very clear in this regard.

NOTE: Molecular Systems Biology strongly encourages authors to upload the 'source data'-for example, tables of individual numerical values and measurements-that were used to generate figures. These files are separate from the traditional supplementary information files and are submitted using the "figure source data" option in the tracking system. Source data are directly linked to specific figure panels so that interested readers can directly download the associated 'source data' (see, for example, http://tinyurl.com/365zpej), for the purpose of alternative visualization, re-analysis or integration with other data. In the context of your study, we would encourage you to supply source data files for figures showing quantitative mRNA counts. Formatting guidelines for 'source data' are available at <http://www.nature.com/msb/authors/source-data.pdf>

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If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favourable.

Best wishes,

Editor
Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

Review of the manuscript "Deconvolving the roles of Wnt ligands and receptors in sensing and amplification"

The manuscript by Tan et al. investigates the polarization of the P-cells by Wnt signaling in the C. elegans larva. The authors show that Wnt ligands secreted by posterior cells polarize the P-cells (P3 to P10), which undergo an asymmetric cell division generating an anterior Pn.a neuroblast and a posterior epidermal Pn.p cell. Using quantitative single molecule FISH analysis, they identify lag-2 and lin-31 as specific markers for the Pn.a and Pn.p fates, allowing them to observe and reliably quantify P-cell polarity. The key experimental observation in this work is that mutations in Wnt ligands and Wnt receptors cause different phenotypes. While mutations in Wnt ligands result in a polarity reversal, mutations in the Wnt receptors cause a depolarization and symmetric division of the P cells. This is a very interesting observation, suggesting that Wnt receptors are involved in a positive feedback loop, such that small stochastic differences in receptor activity are amplified to induce spontaneous P-cell polarization in the absence of Wnt ligands.
To further investigate this phenomenon, they build a phenomenological computational model. After including a signal amplification loop into the model, they are able to fit the parameters to the experimental data.
Both the experimental work and computational model are well done and explained and the general model is plausible. I was hoping the computational model would suggest a possible mechanism for the signal amplification allowing ligand independent polarization or exclude alternative explanations for the data. However, the molecular mechanism underlying the experimental observations remains open. What would be necessary to test the model is a condition which disrupts signal amplification without affecting signal sensing.
My main concern with respect to the experimental data is the problem of functional redundancy. Both Wnt ligands and receptors act in a highly redundant manner and it is therefore very difficult to exclude that the data obtained in the double or triple mutants are not confounded by the activity of yet another ligand or receptor. Because of this, I think it is necessary to build the ligand & receptor quadruple mutant (cwn-1, egl-20, mom-5, lin-17) to exclude that the presence of additional Wnt ligands or receptors may confound the interpretation. Moreover, since the Notch ligand lag-2 is highly expressed in Pn.a cells, could an interaction between Notch and Wnt be involved in signal amplification?
With respect to the model, I am am bit surprised that the authors treated all P cells equally. Since the anterior P cell must experience a very different dose and shape of the Wnt gradient than the posterior P cells, it seems unlikely that the same values for the g and rf parameters would fit all P cells. This point is shown in the experimental data in Fig. 5C and 6, hence the model parameters should be fitted individually for each P cell.
In summary, this is a technically well-executed work investigating a relevant issue, though the insights into the underlying mechanism are limited.

Specific comments
p.3, second para .."excluding cells immediately after division." How was this done?
fig. 1b. Why do some Pn.p cells not express lin-31?

fig 2d. Is this a real lineage analysis or just inferred from 2b? If the latter is true, then the authors have not shown sufficient data to support an exact duplication of the Pn.a lineage.

fig 3. As mentioned above, the data should be split up according to the individual P cells to reveal differences between the anterior and posterior P cells in the mutant backgrounds. Maybe, even in the wild-type the ratios of lag-2 and lin-31 expression counts vary among the Pn.a and Pn.p cells?

p.6 end of first para and. What are the predictions made by the model, which are tested in the following paragraph, e.g. what can be concluded by the fitting of the parameters g and rf?

p.6, 3d para. has the contribution of sys-1 to P cell polarity been tested experimentally?

p.7, end of second para. The finding that a further reduction of ligands in the mom-5; cwn-1 background causes more symmetric divisions in P6 and P5 sounds trivial. This could have been predicted without any modeling.

Typos
p.4 line 6: "... upon inhibiting the Wnt..."
p.6, 3d para "...., we used this mutant to efficiently..."

Reviewer #2 (Remarks to the Author):

Tan et al describe their interesting and informative analysis of how the ligands and receptors required for Wnt signaling differ with respect to their roles in sensing versus amplification of the initial signal. They used single cell quantitative analysis of mRNA transcript levels, focusing on two mRNAs expressed in complementary Ant-Post patterns, as a readout for signal response in wild-type and mutant backgrounds. A mathematical analysis (phenomenological model) of the results suggests that (i) the Wnt ligands predominantly influence sensing, and (ii) the Wnt receptors influence both sensing and amplification. While the authors do not offer insight into how receptors might do both and ligands only one, the findings are very intriguing. Moreover, the application of this method to the mechanisms of Wnt signaling is appealing; these findings should promote efforts to use this approach in other settings. If the authors can first address the two following comments, the manuscript should be suitable for publication in Molecular Systems Biology.

1. In the introduction, the authors refer to ligands and receptors in C. elegans having the same requirements in genetic loss of function studies. This is roughly true, but in detail it is more interesting. In brief, ligand mutants (mom-2/Wnt) show the loss of function phenotype (excess mesoderm at the expense of endoderm) with fairly low penetrance, in around 60% of the mutant embryos (although all die from later defects). In the receptor mutant (mom-5/Fz), only 5% of mutant embryos lack endoderm and make excess mesoderm. Curiously, in Wnt/Fz double mutant embryos (mom-2; mom-5), only 5% of the embryos lack endoderm and make excess mesoderm. In other words, mom-5 acts as if it is downstream of mom-2 Wnt, but the requirements are not identical. I am not sure how this fits into the view of sensing vs amplification but the authors might want to consider modifying their text somehow to reflect this information. The above summary comes from: Bei et al Developmental Cell 3, 113-125 (2002), from Craig Mello laboratory.

2. The discussion is very brief. It might be interesting if the authors would add text that discusses: how at a molecular level do the ligands and receptors have the different requirements, with respect to sensing and amplification.

3. Can the authors could provide a review reference, or other literature citations, for background on "phenomenological modeling"?

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors study the polarization of P daughter cells by the Wnt asymmetry pathway in C. elegans. After division, the anterior daughter cells become neuronal blast cells, and the posterior daughters become epithelial cells. The authors use single molecule mRNA detection to follow the fate of the P daughter cells. They find that the normal polarity of the daughters of the Pn.p cells is altered differently in Wnt ligand vs. Wnt receptor mutants. In Wnt ligand mutants, the polarity of the daughter cells become reversed, while in Wnt receptor mutants, the polarity is lost. To explain this difference, the authors construct a phenomenological model, and they find that the difference in phenotype can be explained if Wnt receptors are not only involved in receiving the signal, but also in an amplification phase.

The experiments that are presented seem well executed, and certainly the single molecule fish approach has been demonstrated to work well in C. elegans. The modeling presents a solution to what seems a non-intuitive outcome, namely that ligand and receptor mutants have a very different effect on the polarity of the P-cell daughters.

The main problem I have with the manuscript is the limited data on the P cells as a polarity model. Little is known about how the cell fate of the P-cell daughters is established. The authors elaborate on the findings from the Sawa group and show conclusively that in a mom-4; lit-1 double mutant, the fate of the Pn.a and Pn.p cells is altered. The authors make a number of assumptions on the polarity of P cells for which no data is shown. The authors describe the Wnt asymmetry pathway on page 3, and conclude from the mom-4; lit-1 experiment that this pathway acts the same in the P cells as it does in e.g. the seam cells. However, this single experiment leaves several questions unanswered:
1. Is the P mother cell polarized before division? On page 4, the authors say that lag-2 expression is a good reflection of the polarity of the mother P cell before division. We do not know however if the mother cell is polarized: the fate of the daughters could be established after an initially symmetric division. Do Wnt components localize asymmetrically in the P cells before division, as in other Wnt asymmetry pathway cells?
2. Similarly, are POP-1 and SYS-1 levels asymmetric in the daughter cells? Why were these genes not included in the analysis in Table S1? If the P cells polarize in a similar fashion to seam and other cells, the anterior daughters should show high POP-1 and low SYS-1, and vice versa.
3. What is the role of Notch signaling in P cell polarity? The two markers used to show polarity are lag-2 and lin-31. lag-2 is a ligand for Notch receptors. Is a Notch signal involved in establishing Pn.a and Pn.p identity, similar to its role later in vulval development?

If these questions can be addressed and the authors can demonstrate better that the Wnt asymmetry pathway indeed functions similarly in P cells as in established cell types like the seam cells, the paper would be suitable for publication. It would also help to elaborate more on the details that are in fact known. For example, the modeling assumes a Wnt gradient. The authors own work showing the existence of a gradient is not mentioned until the discussion, leaving the reader uncertain that the assumptions going into the modeling are based on experimental findings. It is not entirely clear to me why the authors use a novel system, rather than performing their experiments using the much better established seam cell model. In these cells, loss of Wnt ligands also results in a reversal of polarity in half of the divisions.

Other remarks and typos:

The authors describe cell polarity as the result of the sensing of an external gradient, followed by amplification through intracellular signaling networks involving positive feedback regulation. Though this is the case for some types of polarity (migration along a chemotactic gradient for example), this is a much to narrow description of all polarity. For example, the one cell C. elegans embryo polarizes in the absence of an extracellular gradient, and this involves mostly negative interactions between anterior and posterior PAR proteins. The question phrased in the title is interesting enough, without claiming to offer insights into the much broader topic of cell polarity.

Abstract: Caenorhabditis elegans' P cells: remove apostrophe
Page 2: six pairs of P cells should be twelve.

Reviewer #4 (Remarks to the Author):

The article aims to disentangle two different roles of the Wnt signalling system, sensing and amplification in polarity establishment. To this purpose it uses a phenomenological model, describing cell polarization as a stochastic multistep process where the per step chances to adjust polarity in one or the other direction depend on gradient direction and steepness and the current polarity of the cell.

I have a few major issues that need to be solved before the paper can be considered for publication.

Major comments

1. Although the system at work in these C. elegans P cells is not exactly the same as the planar cell polarity system, it does resemble that system. Therefore it strikes me that the authors completely fail to discuss the extensive literature on planar cell polarity models and the ongoing dispute in this literature on the importance of gradients versus cell-cell signalling
in setting up polarity. Indeed this is a missed opportunity since the current work in a sense unites these two opposing directions. I suggest the authors repair this omission.

2. It appears that the authors simply assume that the Wnt ligands are present in a gradient. No literature is cited or own experiments are done to support this assumption. Their data would also be consistent with a system in which the presence of Wnt ligands is indeed needed for the polarity system to work, but in which Wnt ligands do not provide the directionality cue for the system. Other directional cues have been suggested for planar cell polarity like systems, such as the mechanical forces arising during growth. It is crucial that the authors clear up this issue.

3. The authors should spend more effort on explaining how their model works to non-modelers. Sentences such as "The chemical master equation is solved" or "deltat is the timestep of the numerical simulation" should be rephrased / extended. In the model section of the methods, also a more extensive explanation is needed to allow a non-modeler to have some idea of what is being done. Eg explain that due to the 0-90 degrees you get a 91 times 91 transition matrix, or that reflecting boundary conditions mean that you can not increase beyond 90 or decrease beyond 0 degrees.

4. How do their results depend on total simulation time T? Did they run untill a steady state distribution was achieved? These issues should be clarified.

5. Page 4: It seems a bit odd to use assymmetric classification rules <15 is reverse and >75 is ok, why not 25 and 75 or 15 and 85? This way it is harder to have revers than ok polarity, which seems to introduce a bias into the analysis.

6. The authors should explain the rationale behind the extended version of their model that incorporates the reinforcement. It should be discussed that a choice was made here, by assuming that gradient and prior polarization have a synergystic (multiplication in the equations) rather than additive
(alternative equations with sum of the two effects) effect on further polarization. What would happen if instead additive effects were assumed. Why did they deem a synergystic effect to be more likely? Again, this should be clarified.

Minor comments

1. In the abstract "The initiation" should be "The establishment"

2. Introduction, page 1: It is not correct that all polarity processes require sensing and amplification, this is only for systems in which the apolar rest state is a stable equilibrium. If this is not the case noise may already lead to spontaneous polarisation.

3. Page 3: Correct the following "from the nuclear into the cytoplasm"

4. There are a lot of other language errors that need to be repaired.

1st Revision - authors' response   17 October 2012

Response to Referee 1:

We thank Ref. 1 for his/her positive remarks regarding our manuscript and his/her valuable and constructive suggestions, which enable us to improve the manuscript. Remarks of Ref. 1 are denoted in *italics*.

> *The manuscript by Tan et al. investigates the polarization of the P-cells by Wnt signaling in the C. elegans larva. The authors show that Wnt ligands secreted by posterior cells polarize the P-cells (P3 to P10), which undergo an asymmetric cell division generating an anterior Pn.a neuroblast and a posterior epidermal Pn.p cell. Using quantitative single molecule FISH analysis, they identify lag-2 and lin-31 as specific markers for the Pn.a and Pn.p fates, allowing them to observe and reliably quantify P-cell polarity. The key experimental observation in this work is that mutations in Wnt ligands and Wnt receptors cause different phenotypes. While mutations in Wnt ligands result in a polarity reversal, mutations in the Wnt receptors cause a depolarization and symmetric division of the P cells. This is a very interesting observation, suggesting that Wnt receptors are involved in a positive feedback loop, such that small stochastic differences in receptor activity are amplified to induce spontaneous P-cell polarization in the absence of Wnt ligands.*

> *To further investigate this phenomenon, they build a phenomenological computational model. After including a signal amplification loop into the model, they are able to fit the parameters to the experimental data.*

> *Both the experimental work and computational model are well done and explained and the general model is plausible.*

We thank Ref. 1 for this positive assessment of our work.

> *I was hoping the computational model would suggest a possible mechanism for the signal amplification allowing ligand independent polarization or exclude alternative explanations for the data. However, the molecular mechanism underlying the experimental observations remains open. What would be necessary to test the model is a condition which disrupts signal amplification without affecting signal sensing.*

> *My main concern with respect to the experimental data is the problem of functional redundancy. Both Wnt ligands and receptors act in a highly redundant manner and it is therefore very difficult to exclude that the data obtained in the double or triple mutants are not confounded by the activity of yet another ligand or receptor. Because of this, I think it is necessary to build the ligand & receptor quadruple mutant (cwn-1, egl-20, mom-5, lin-17) to exclude that the presence of additional Wnt ligands or receptors may confound the interpretation.*

Following the suggestion of Ref. 1 we constructed the quadruple mutant *(cwn-1; egl-20; mom-5; lin-17)* and quantified the division of the P cells. We find that in this mutant about 70% of the P cells exhibit loss of polarity compared to 60% in the triple mutant *cwn-1 egl-20 mom-5* (Fig. R1). This shows that *cwn-1, egl-20, mom-5,* and *lin-17* are the main players in the determining cell polarity of the P cells and it is unlikely that other Wnt receptors or ligands play a major role in determining P cell polarity. It is very hard to obtain more statistics on the quadruple mutant because of a combination of low survival of the

homozygote quadruple mutants (identified by the absence of GFP) and the difficulty with synchronizing the worms all to the right stage.
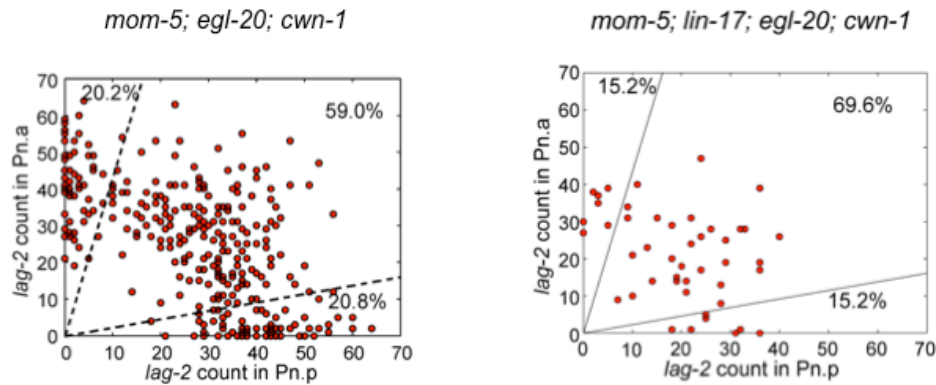

Figure R1. *lag-2* expression in the *mom-5; egl-20; cwn-1* mutant (left panel) compared to the quadruple mutant *mom-5; lin-17; egl-20; cwn-1*.

Moreover, since the Notch ligand lag-2 is highly expressed in Pn.a cells, could an interaction between Notch and Wnt be involved in signal amplification?

Indeed the high expression of *lag-2* in the P cells suggests a role of Notch signaling in P cell division. To explore the role of Notch signaling we examined a large panel of mutants that weaken Notch signaling including a *lag-1* temperature sensitive strain and a *lin-12* loss of function mutation *lin-12(n941)*. We also examined animals with the semi-dominant mutation, *lin-12(n137)* and did not observe any change in the polarity of P cell divisions in these mutants. Taken together this suggests that LIN-12/Notch does not play a major role in regulating P cells division. Of course we cannot rule out that in the loss of function mutants there is still some residual Notch activity left. Additionally GLP-1, the other Notch family member or the Delta family members (e.g. APX-1, DSL-1), might compensate for the reduced Notch activity. Because of these complexities we decided to focus on the role of Wnt signaling on P cell polarity, which resulted in well-defined phenotypes as described in our manuscript.

With respect to the model, I am a bit surprised that the authors treated all P cells equally. Since the anterior P cell must experience a very different dose and shape of the Wnt gradient than the posterior P cells, it seems unlikely that the same values for the g and rf parameters would fit all P cells. This point is shown in the experimental data in Fig. 5C and 6, hence the model parameters should be fitted individually for each P cell.

The reviewer is correct that each P cell is different. Therefore in our fit, we indeed fit the individual P cells separately. We regret that this was not clear for Ref. 1. We now further clarify this point in our manuscript. Although in Fig. 3 data from different P cells are combined in one scatter plot, the data in

Fig. 5c and Fig. 6a show lag-2 expression for specific P cell lineages (P3, P4, …, P10). Similarly in Fig. 5d, the parameters that yield the best fit for each individual P cell in the different mutants are shown.

In summary, this is a technically well-executed work investigating a relevant issue, though the insights into the underlying mechanism are limited.

The objective of the work is not to elucidate the underlying molecular mechanism; rather we hope to provide a general quantitative systems-level framework for accessing the contributions of gradient sensing and amplification to the establishment of polarity. The model proposed allows us to determine quantitative measures to describe the amount of gradient sensing and amplification that each of the P cell is experiencing. Furthermore since the model does not depend on knowledge of the exact molecular mechanism used in establishing polarity, it is general and can potentially be used to describe other polarity systems. We hope that future work more focused on the molecular and cell biology of the P cell will reveal a detailed molecular mechanism.

Specific comments

p.3, second para .."excluding cells immediately after division." How was this done?

This was done by rejecting Pn.a and Pn.p cells with similar sizes. Since the Pn.a cells continues to divide during the L1 stage and the Pn.p cells do not divide until the L3 stage, the Pn.a cells will continue to increase in size whereas the size of Pn.p cell remains relatively unchanged. As seen in Fig. 2c of the new manuscript, immediately after division, the Pn.a and Pn.p cells are similar in sizes. But after some time, the Pn.a cells continues to grow whereas the Pn.p cells do not, leading to the different sizes for the two cells observed in Fig. 2a.

fig. 1b. Why do some Pn.p cells not express lin-31?

We are looking at the Pn.p cells at the point of fixation. It is possible that some of these cells have not started to express *lin-31*. *lag-2* is always expressed at at least 20 mRNA copies per cell and we therefore use *lag-2,* instead of *lin-31,* as a quantitative marker of cell polarity.

fig 2d. Is this a real lineage analysis or just inferred from 2b? If the latter is true, then the authors have not shown sufficient data to support an exact duplication of the Pn.a lineage.

This is inferred from Fig. 2b in the previous manuscript and Fig. 2d in the new manuscript. We have decided to remove this lineage analysis as it also involved some data from the Pn.aa divisions that we have not included in this paper.

Instead we now focus more on the Pn.a and Pn.p cells. In the revised manuscript, we included a new Fig. 2 to highlight the fact that *lag-2* is expressed in both the Pn.a and Pn.p cells in the *mom-4;lit-1* mutant grown at 25°C (Fig. 2b). Furthermore, we also include a new panel to show that both the Pn.a and Pn.p cells divide during the L1 stage (Fig. 2d) in this mutant. This provide compelling evidence that the Pn.p cell acts more Pn.a-like in the *mom-4;lit-1* mutant grown at 25°C.

fig 3. As mentioned above, the data should be split up according to the individual P cells to reveal differences between the anterior and posterior P cells in the mutant backgrounds. Maybe, even in the wild-type the ratios of lag-2 and lin-31 expression counts vary among the Pn.a and Pn.p cells?

3

We found that in wild-type animals, *lag-2* and *lin-31* expression does not vary significantly among the Pn.a and Pn.p cells (Supplementary Fig. S1). In different levels of lag-2 in the different P cells in different mutant background are presented in Fig. 5c and Fig. 6a.

p.6 end of first para and. What are the predictions made by the model, which are tested in the following paragraph, e.g. what can be concluded by the fitting of the parameters g and rf?

In the section "A phenomenological model for cell polarity", we explain the rationale and steps involved in constructing the model used to fit the empirical distribution. In the simplest model in which we did not include amplification, we were unable to observe the coexistence of correct and reverse polarity observed in the ligand mutants. After we included the r_f term to represent amplification, we obtain the model that leads to behavior that we observed in our experiments, namely correct divisions, symmetric divisions and also coexistence of correct and reverse polarity. The parameters *g* and r_f were used to characterize the strength of the gradient and amplification in the model.

p.6, 3d para. has the contribution of sys-1 to P cell polarity been tested experimentally?

The contribution of *sys-1* to P cell polarity has not been tested experimentally. Our model is independent of the exact molecular mechanism involved in setting up polarity hence whether *sys-1* is involved would not have any impact on our conclusions. Nonetheless, it would be interesting to test for the contribution of *sys-1* to P cell polarity in the future.

p.7, end of second para. The finding that a further reduction of ligands in the mom-5; cwn-1 background causes more symmetric divisions in P6 and P5 sounds trivial. This could have been predicted without any modeling.

In the earlier part of the paper, we found that reduction of ligands induces polarity reversals whereas reduction of receptors leads to symmetric divisions. Hence it is unclear what would be the phenotype observed in a receptor and ligand compound mutant. Since symmetric divisions are observed in P3 and P4 in the *mom-5* background, one might expect symmetric divisions to occur in the other P cells upon loss of other receptors or ligands. This inference may work for the P5 and P6 cells where symmetric divisions are observed but it certainly does not work for P9 and P10 where polarity reversals are observed. We feel that the phenomenological model helps to explain this non-intuitive behavior.

For most previous studies the fractions of correct, reverse and symmetric divisions have been used to characterize the mutant phenotypes. Here, we took a step further by fitting our experimental results to a phenomenological model describing divisions using two parameters, gradient sensing and amplification. This allows us to gain greater insight into the system. For example, instead of just observing that the anterior P cells undergo symmetric divisions whereas the posterior P cells undergo polarity reversals in the *mom-5; egl-20* background, we could obtain parameters for sensing and amplifications using our model. We found that the anterior P cells have lower amplification strength than the posterior P cells. We also found that the amplification strength is highly correlated with the differential expression of *lin-17* mRNA in individual P cells. These findings would not have been possible if we did not transform the observed fractions of correct, reverse and symmetric divisions into the quantitative parameters of sensing and amplification.

Typos
p.4 line 6: "... upon inhibiting the Wnt..."

p.6, 3d para "...., we used this mutant to efficiently..."

These errors have been corrected.

Response to Referee 2:

We thank Ref. 2 for his/her insightful suggestions, which enabled us to improve our manuscript. Remarks of Ref. 2 are denoted in *italics*.

Tan et al describe their interesting and informative analysis of how the ligands and receptors required for Wnt signaling differ with respect to their roles in sensing versus amplification of the initial signal. They used single cell quantitative analysis of mRNA transcript levels, focusing on two mRNAs expressed in complementary Ant-Post patterns, as a readout for signal response in wild-type and mutant backgrounds. A mathematical analysis (phenomenological model) of the results suggests that (i) the Wnt ligands predominantly influence sensing, and (ii) the Wnt receptors influence both sensing and amplification. While the authors do not offer insight into how receptors might do both and ligands only one, the findings are very intriguing. Moreover, the application of this method to the mechanisms of Wnt signaling is appealing; these findings should promote efforts to use this approach in other settings. If the authors can first address the two following comments, the manuscript should be suitable for publication in Molecular Systems Biology.

We thank Ref. 2 for this positive assessment of our work.

1. In the introduction, the authors refer to ligands and receptors in C. elegans having the same requirements in genetic loss of function studies. This is roughly true, but in detail it is more interesting. In brief, ligand mutants (mom-2/Wnt) show the loss of function phenotype (excess mesoderm at the expense of endoderm) with fairly low penetrance, in around 60% of the mutant embryos (although all die from later defects). In the receptor mutant (mom-5/Fz), only 5% of mutant embryos lack endoderm and make excess mesoderm. Curiously, in Wnt/Fz double mutant embryos (mom-2; mom-5), only 5% of the embryos lack endoderm and make excess mesoderm. In other words, mom-5 acts as if it is downstream of mom-2 Wnt, but the requirements are not identical. I am not sure how this fits into the view of sensing vs amplification but the authors might want to consider modifying their text somehow to reflect this information. The above summary comes from: Bei et al Developmental Cell 3, 113-125 (2002), from Craig Mello laboratory.

We thank Ref. 2 for bringing up this interesting phenomenon. We now discuss this reference in the introduction of our revised manuscript.

2. The discussion is very brief. It might be interesting if the authors would add text that discusses: how at a molecular level do the ligands and receptors have the different requirements, with respect to sensing and amplification.

We expanded the discussion section of our manuscript and included a paragraph on how sensing and amplification might be implemented at the molecular level.

3. Can the authors could provide a review reference, or other literature citations, for background on "phenomenological modeling"?

We included several new references with other examples of phenomenological models.

Response to Referee 3:

We thank Ref. 3 for his/her insightful suggestions, which enabled us to improve our manuscript. Remarks of Ref. 3 are denoted in *italics*.

In this manuscript, the authors study the polarization of P daughter cells by the Wnt asymmetry pathway in C. elegans. After division, the anterior daughter cells become neuronal blast cells, and the posterior daughters become epithelial cells. The authors use single molecule mRNA detection to follow the fate of the P daughter cells. They find that the normal polarity of the daughters of the Pn.p cells is altered differently in Wnt ligand vs. Wnt receptor mutants. In Wnt ligand mutants, the polarity of the daughter cells become reversed, while in Wnt receptor mutants, the polarity is lost. To explain this difference, the authors construct a phenomenological model, and they find that the difference in phenotype can be explained if Wnt receptors are not only involved in receiving the signal, but also in an amplification phase.

The experiments that are presented seem well executed, and certainly the single molecule fish approach has been demonstrated to work well in C. elegans. The modeling presents a solution to what seems a non-intuitive outcome, namely that ligand and receptor mutants have a very different effect on the polarity of the P-cell daughters.

We thank Ref. 2 for this positive assessment of our work.

The main problem I have with the manuscript is the limited data on the P cells as a polarity model. Little is known about how the cell fate of the P-cell daughters is established. The authors elaborate on the findings from the Sawa group and show conclusively that in a mom-4; lit-1 double mutant, the fate of the Pn.a and Pn.p cells is altered. The authors make a number of assumptions on the polarity of P cells for which no data is shown. The authors describe the Wnt asymmetry pathway on page 3, and conclude from the mom-4; lit-1 experiment that this pathway acts the same in the P cells as it does in e.g. the seam cells. However, this single experiment leaves several questions unanswered:

1. Is the P mother cell polarized before division? On page 4, the authors say that lag-2 expression is a good reflection of the polarity of the mother P cell before division. We do not know however if the mother cell is polarized: the fate of the daughters could be established after an initially symmetric division. Do Wnt components localize asymmetrically in the P cells before division, as in other Wnt asymmetry pathway cells?

We agree with Ref. 3 that we do not have direct experimental evidence of polarization of the mother cell before division. We use this as an assumption to interpret the expression of *lag-2* in the daughter P cells. Using the assumption we can explain our full data-set with the proposed model.

2. Similarly, are POP-1 and SYS-1 levels asymmetric in the daughter cells? Why were these genes not included in the analysis in Table S1? If the P cells polarize in a similar fashion to seam and other cells, the anterior daughters should show high POP-1 and low SYS-1, and vice versa.

We thank Ref. 3 for the suggestion to explore POP-1 and SYS-1 asymmetry in the daughter P cells. Unfortunately we were not able to visualize SYS-1-YFP in the daughter P cells. The expression level of this fusion protein was too low to be resolved above background levels. We were able to visualize POP-1-

GFP and we found that nuclear levels of POP-1 are asymmetric in the daughter cells. Pn.a has a higher level of nuclear POP-1 than Pn.p. These data are presented in new figure in the revised manuscript (Fig. 2c). The POP-1 asymmetry in the P daughter cells is consistent with the asymmetry seen in the seam cells, whereby anterior daughters show higher levels of POP-1.

3. What is the role of Notch signaling in P cell polarity? The two markers used to show polarity are lag-2 and lin-31. lag-2 is a ligand for Notch receptors. Is a Notch signal involved in establishing Pn.a and Pn.p identity, similar to its role later in vulval development?

Indeed the high expression of *lag-2* in the P cells suggests a role of Notch signaling in P cell division. To explore the role of Notch signaling we examined a large panel of mutants that weaken Notch signaling including a *lag-1* temperature sensitive strain and a *lin-12* loss of function mutation *lin-12(n941)*. We also examined animals with the semi-dominant mutation, *lin-12(n137)* and did not observe any change in the polarity of P cell divisions in these mutants. Taken together this suggests that LIN-12/Notch does not play a major role in regulating P cells division. Of course we cannot rule out that in the loss of function mutants there is still some residual Notch activity left. Additionally GLP-1, the other Notch family member or the Delta family members (e.g. APX-1, DSL-1), might compensate for the reduced Notch activity. Because of these complexities we decided to focus on the role of Wnt signaling on P cell polarity, which resulted in well-defined phenotypes as described in our manuscript.

If these questions can be addressed and the authors can demonstrate better that the Wnt asymmetry pathway indeed functions similarly in P cells as in established cell types like the seam cells, the paper would be suitable for publication.

It would also help to elaborate more on the details that are in fact known. For example, the modeling assumes a Wnt gradient. The authors own work showing the existence of a gradient is not mentioned until the discussion, leaving the reader uncertain that the assumptions going into the modeling are based on experimental findings.

The referee has raised a good point. We now describe Wnt gradients in the introduction of the manuscript.

It is not entirely clear to me why the authors use a novel system, rather than performing their experiments using the much better established seam cell model. In these cells, loss of Wnt ligands also results in a reversal of polarity in half of the divisions.

The reviewer makes a good point about the seam cell model being a much more established model. But we think the P cells are interesting for many reasons. First of all, unlike the seam cells, they are not found at their final position in hatchlings. Instead, they migrate from the left and right side of the body into the ventral cord before division. Secondly, they divide much more rapidly. In this work, we have focused our efforts on the first division of the P cells. In the future, we aim to examine the other divisions, for example divisions of Pn.a and Pn.aa. It will be interesting to determine whether there an erroneous division in the P cell will lead to a higher chance of erroneous division in its Pn.a daughter.

Other remarks and typos:

The authors describe cell polarity as the result of the sensing of an external gradient, followed by amplification through intracellular signaling networks involving positive feedback regulation. Though this is the case for some types of polarity (migration along a chemotactic gradient for example), this is a much to narrow description of all polarity. For example, the one cell C. elegans embryo polarizes in the absence of an extracellular gradient, and this involves mostly negative interactions between anterior and posterior PAR proteins. The question phrased in the title is interesting enough, without claiming to offer insights into the much broader topic of cell polarity.

Abstract: Caenorhabditis elegans' P cells: remove apostrophe

Page 2: six pairs of P cells should be twelve.

These errors have been corrected.

Response to Referee 4:

We thank Ref. 4 for his/her insightful suggestions, which enabled us to improve our manuscript. Remarks of Ref. 4 are denoted in *italics*.

The article aims to disentangle two different roles of the Wnt signalling system, sensing and amplification in polarity establishment. To this purpose it uses a phenomenological model, describing cell polarization as a stochastic multistep process where the per step chances to adjust polarity in one or the other direction depend on gradient direction and steepness and the current polarity of the cell.

I have a few major issues that need to be solved before the paper can be considered for publication.

Major comments

1. Although the system at work in these C. elegans P cells is not exactly the same as the planar cell polarity system, it does resemble that system. Therefore it strikes me that the authors completely fail to discuss the extensive literature on planar cell polarity models and the ongoing dispute in this literature on the importance of gradients versus cell-cell signaling in setting up polarity. Indeed this is a missed opportunity since the current work in a sense unites these two opposing directions. I suggest the authors repair this omission.

We thank Ref. 4 for pointing out this omission. We included a paragraph in the introduction where we cover the planar cell polarity system and discuss the ongoing dispute on the role gradient sensing versus cell-to-cell signaling in determining polarity.

2. It appears that the authors simply assume that the Wnt ligands are present in a gradient. No literature is cited or own experiments are done to support this assumption. Their data would also be consistent with a system in which the presence of Wnt ligands is indeed needed for the polarity system to work, but in which Wnt ligands do not provide the directionality cue for the system. Other directional cues have been suggested for planar cell polarity like systems, such as the mechanical forces arising during growth. It is crucial that the authors clear up this issue.

We now describe the recent literature demonstrating that Wnt ligands are expressed in a gradient in the introduction of the manuscript. Referee 4 is correct that the Wnt ligand could act instructively (and define directionality) or permissively (Wnt signal is needed but does not provide directionality). To determine if ligands act instructively or permissively, we expressed *cwn-2* posteriorly using an *egl-20* promoter or uniformly using a *myo-3* promoter in a *cwn-1; cwn-2; egl-20* triple ligand mutant (Supplementary Figure 6). We found that ubiquitous expression of *cwn-2* using both promoters are able to rescue the polarity reversals, suggesting that it is the ligand level rather than the gradient that is important for the sensing process at least for this Wnt ligand. However, the recent identification of Wnt inhibitor *sfrp-1* in the anterior region of the worm suggests that ligand profile is not shaped by ligand expression alone (Harterink et al., 2011). Anterior expression of *sfrp-1* could potentially convert uniform ligand expression into a ligand gradient. Future work identifying more Wnt components and quantitative measurement of ligand protein profiles will be necessary to resolve this issue.

3. The authors should spend more effort on explaining how their model works to non-modelers. Sentences such as "The chemical master equation is solved" or "deltat is the timestep of the numerical

simulation" should be rephrased / extended. In the model section of the methods, also a more extensive explanation is needed to allow a non-modeler to have some idea of what is being done. Eg explain that due to the 0-90 degrees you get a 91 times 91 transition matrix, or that reflecting boundary conditions mean that you can not increase beyond 90 or decrease beyond 0 degrees.

We significantly expanded the description of the in the methods section of the manuscript.

4. How do their results depend on total simulation time T? Did they run untill a steady state distribution was achieved? These issues should be clarified.

Since in the simulations time is measured in arbitrary units, the total simulation time T is underdetermined and is set to 1 for all the simulations and different mutants analyzed. In other words the rate r_f (which is one of the fit parameters) is measured in units of T. We expanded the modeling section to further explain this.

When the simulations are run until a steady state distribution is achieved (which would happen for large values of r_f), the fit to the experimental data is poor. The best fit obtained indicates that θ is approaching a steady-state but did not reach it yet. These issues are further clarified in the method section.

5. Page 4: It seems a bit odd to use asymmetric classification rules <15 is reverse and >75 is ok, why not 25 and 75 or 15 and 85? This way it is harder to have reverse than ok polarity, which seems to introduce a bias into the analysis.

We agree that the thresholds 15 and 75 are rather arbitrary. It was necessary to define thresholds in order to classify the polarity as correct, reversed, or absent. However note that the angle ranges from 0 to 90° so it makes sense to define symmetrically (15 = 0 +15; 75 = 90 – 15). For example using the thresholds 25 and 75 would results in a bias since a wider range for correct polarity (0-25) than for reversed polarity (75-90) would be chosen. Changing the thresholds symmetrically which change the classification but does not affect the main conclusions of our work.

6. The authors should explain the rationale behind the extended version of their model that incorporates the reinforcement. It should be discussed that a choice was made here, by assuming that gradient and prior polarization have a synergystic (multiplication in the equations) rather than additive (alternative equations with sum of the two effects) effect on further polarization. What would happen if instead additive effects were assumed? Why did they deem a synergystic effect to be more likely? Again, this should be clarified.

As shown in Fig. R2, additive effects of gradient sensing and amplification are unable to result in the coexistence of correct and reverse polarity. This shows that an additive relationship cannot be used to represent the relationship between sensing and amplification.
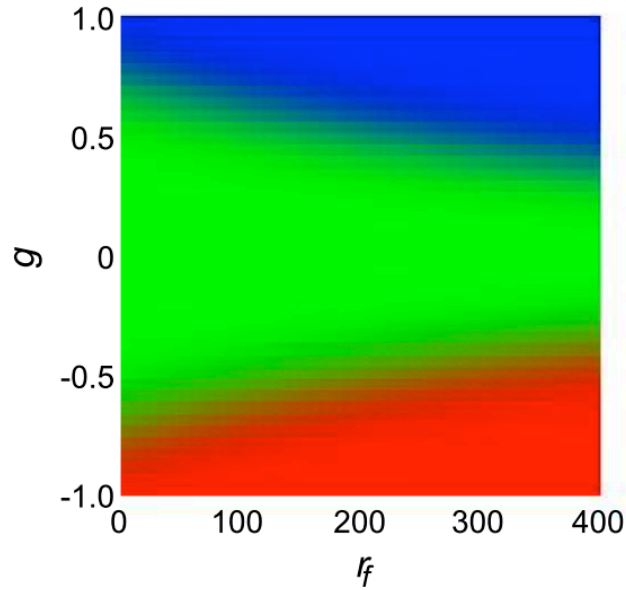
Figure R2.   Phase diagram showing the parameter space of g and r_f giving rise to correct polarity (blue), reverse polarity (red) and unpolarized divisions (green) for a model assuming additive effects of gradient sensing and amplification.

A way to view the polarization process represented by the synergistic relationship is that initially when the cell is unpolarized ($\theta = 45^\circ$), the contributions of r_f to the probabilities of taking a right or left step are equal. Hence the sensing process, represented by g, plays a more important role is setting up polarization. Once sensing has occurred and θ is no longer close to 45°, contributions of amplification represented by r_f will become important and amplify the polarization set up by the sensing process. This representation agrees with our current understanding of polarization.

Minor comments

1. In the abstract "The initiation" should be "The establishment"

2. Introduction, page 1: It is not correct that all polarity processes require sensing and amplification, this is only for systems in which the apolar rest state is a stable equilibrium. If this is not the case noise may already lead to spontaneous polarisation.

3. Page 3: Correct the following "from the nuclear into the cytoplasm"

4. There are a lot of other language errors that need to be repaired.

We corrected these mistakes.

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Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Proofs will be forwarded to you within the next 2-3 weeks.

Thank you very much for submitting your work to Molecular Systems Biology.

Best wishes,

Editor
Molecular Systems Biology

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript by Tan et al has improved significantly. I think, most the points raised by the reviewers have been addressed and some important changes/ clarifications were made to the manuscript. Even though the exact molecular mechanism of the postulated feedback loop remains unknown, this work is interesting, the experimental as well as the modeling data are of high quality. I therefore believe the current manuscript is suitable for publication in MSB.

Reviewer #3 (Remarks to the Author):

In their rebuttal and rewritten manuscript, the authors have addressed the two main points I raised:

1. The examination of POP-1 in the P daughter cells, and the observation that POP-1 is indeed asymmetric, strengthens the argument that P cell asymmetry is mediated by the Wnt pathway.

2. The authors have added a paragraph on the potential role for Notch signaling (or rather the lack thereof) in setting up P cell polarity. The finding that lag-2 and lin-12 are differentially expressed in the P daughters while Notch signaling appears to play no role is still an interesting observation.

These points, as well as the minor points I raised, have been adequately addressed.

The authors have also addressed the comments by the other reviewers, adding experimentation and figures, and making clarifications. It seems that the extra experiments address several important questions, and that the clarifications have improved the manuscript.

Overall, I am now happy to support publication of the manuscript by Tan et al. in Molecular Systems Biology.