molecular systems biology

# Cell-Cycle Regulation of NOTCH Signaling during C. elegans Vulval Development

Stefanie Nusser-Stein, Antje Beyer, Ivo Rimann, Magdalene Adamczy, Nir Piterman, Alex Hajnal, and Jasmin Fisher

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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 12 April 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three 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. Thus, reviewer #1 refers to the need of a more precise time course to resolve potential conflicting evidence, and reviewer #3 raises the possibility that simpler mechanisms could lead to the required heterogeneity.

We would also ask you to provide in supplementary information (as 'dataset' file) a machine-readable version of your model.

*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 reporter gene measurements. Formatting guidelines for 'source data' are available at <http://www.nature.com/msb/authors/source-data.pdf>

As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology will

 

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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.

Yours sincerely,

Editor
Molecular Systems Biology

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Referee comments:

Reviewer #1 (Remarks to the Author):

The manuscript by Nusser-Stein et al. provides new insights into Notch signaling in C. elegans vulval development by showing that the degradation of Notch is linked to cell cycle progression. The authors have sought to computationally model the specification of vulval precursor cell (VPC) fates. The most recently published computational model by this group had invoked an artificial delay in Notch degradation in the primary cell precursor P6.p in order to correctly model VPC fates. In this manuscript, they demonstrate that the delay in Notch degradation is biologically grounded in cell cycle progression. The authors show that arresting P6.p in G1 phase (by overexpressing the CDK-inhibitor CKI-1) blocks the degradation of the Notch Intracellular domain (NICD). They further link Notch degradation to the cell cycle by showing that partial loss of the G1 cyclin D (CYD-1), which does not arrest the cell cycle, reduces expression of Notch on the apical membrane, while loss of the G1/S cyclin E (CYE-1) reduces expression of NICD. These results imply that the G1 cyclins promote Notch stability. In contrast, loss of the mitotic cyclin B3 (CYB-3), which also does not arrest the cell cycle, increases the levels of NICD, implying that CYB-3 normally acts to negatively regulate NICD levels. Analysis of synchronized larvae suggests that NICD is degraded in G2 phase prior to P6.p division. Inactivation of CDK-1, which induces a G2 phase arrest, stabilized NICD. The authors suggest that these results indicate that NICD is degraded in G2 phase or at the time of entry into M phase. The authors also show that the ANK and PEST domains of NICD are required for its degradation.

The authors' previously published model included "bounded asynchrony" in which the VPCs can progress independently but for only a limited number of steps. The authors show here that consistent with the concept of bounded asynchrony, the VPCs have independent cell cycle timing, wherein P5.p, P6.p, and P7.p divide at slightly different rates, and each of the cells enter mitosis before the others at roughly equal rates. The authors test their new computational model that incorporates cell cycle timing by comparing the readout under different mutant conditions that have known or predicted results.

Overall, the data is convincing, and the manuscript is well written. This work provides a significant advance in understanding the logic of VPC cell fate specification. There are a few points that should be addressed.

Points:
1. Fig. 3A: The time course of NICD degradation presented in Figure 3 appears to occur over multiple hours. This is not fully consistent with a late-G2 phase event (see below). It is possible that a higher degree of synchrony among animals in the experiment would reveal that the degradation of NICD is a more sudden event. The authors synchronize larvae by starvation. However, the

 

synchronization method of L1 starvation does not generally provide a high degree of synchrony, as the L1 larvae can sometimes develop to different extents during the starvation period and generally do not initiate development with a high degree of synchrony. A much more exact method is to transfer cohorts of newly hatched L1 onto food in short time 10-15 minute intervals. The newly-hatched L1 larvae can be obtained by mouth-pipeting newly-hatched larvae from collected pretzel-stage embryos onto bacterial plates at 10-15 min intervals. The timing of cell division should also be followed in the experiment, as this would provide a more accurate determination of when NICD degradation occurs relative to the cell cycle. Using the published timing of cell division is not a substitute for this analysis, as the culture conditions may be different and the published timing is based on following newly-hatched animals (not L1 starved animals placed on food).

2. Page 12, 3rd paragraph: The authors show that NICD is not degraded in cdk-1(RNAi) G2-phase-arrested P6.p cells. The authors also show a time course in Fig. 3A that implies that NICD degradation occurs over multiple hours. There is a potential conflict between the data. Inactivation of CDK-1 should block cells in late G2 (just before entry into mitosis), and if degradation occurs over multiple hours of G2 phase then an arrest in late G2 should allow all of the NICD to be degraded (as degradation is active over multiple hours of G2 phase). Further, if cyclin B3/CDK1 is involved in the degradation, then one would expect that the degradation would occur during mitosis, when the cyclin-CDK complex is presumably activated rather than over multiple hours of G2 phase. Potentially, a more careful time course (described above) will remove the conflict; but if not, this potential conflict should be addressed (at least in the text) by the authors.

3. Page 8, second paragraph: "The VPCs are born during the first larval stage and remain in the G1 phase until the transition ... (Sulston and Horvitz, 1977)." The reference cited does not justify the statement that the cells are in G1 phase, as the Sulston and Horvitz paper does not include cell cycle phase analysis.

4. Page 17, 2nd paragraph: "... CKI-1 specifically inhibits the CDK-2/CYE-1 complex (Fujita et al., 2007)." The reviewer does not think that this reference shows that CKI-1 inhibits CDK-2/CYE-1 specifically (implying that it does not inhibit other CDK/cyclins such as CYD-1). Further, another reference, Boxem and van den Heuvel, 2001 (which is not cited), also does not indicate that CKI-1 does not inhibit CYD-1. The latter paper presents biochemical evidence that CKI-1 binds to both CYE-1 and CYD-1, and while it shows genetic evidence that CKI-1 inhibits CYE-1, it does not present evidence that CKI-1 does not inhibit CYD-1. The authors should double-check their references, and either argue their position or change their statement.

5. Suppl. Fig. S3 shows that expression of NICD::GFP produces ectopic 2{degree sign} cells. It would be helpful if the authors clarified what percentage of animals have this phenotype. Also, the authors should indicate whether inactivation of CYD-1 and CYE-1 affect the level of ectopic 2{degree sign} cells in these mutants.

6. Fig. 5D: The graph indicates that the NICD::GFP signal in P5.p and P7.p for cyb-3 RNAi and vector RNAi are comparable, yet the image provided for vector RNAi does not show staining in P5.p and P7.p. A more representative image should be provided.

Reviewer #2 (Remarks to the Author):

Nusser-Stein et al. propose a conceptual approach to modeling the long-standing problem of cell fate specification among equipotential groups of cells and a molecular model that links cell cycle and cell fate specification. The findings are interesting, and the proposed biological model is provocative.

In previous work the authors proposed a concept, "bounded asynchrony", the implementation of which enabled them to more accurately model VPC specification. The idea was that cells could be neither strictly synchronous nor isolated from one another with respect to cell fate decisions. The fact that these cells must communicate during fate acquisition is well known from the previous work of Greenwald and colleagues. Further, Ambros and colleagues tested the connection between Notch and cell cycle over a decade ago, concluding that the activity of Notch in prior to and after S phase differentially influenced two possible cell fate decisions, and proposing that a cell-cycle link to Notch down-regulation may contribute. Data are presented here that support a model in which the

 

localization and stability of Notch is differentially influenced by G1 and G2 cyclins, and that Notch degradation is regulated not only by the PEST domain but by the ANK domain as well.

If I understood correctly, the claim is that cell cycle is the biological mechanism that corresponds to an "asynchronous" influence on cell fate acquisition that is nevertheless "bounded" by cell-cell communication via the influence of cell-cycle control on intercellular Notch signaling. However, as written, it is not clear what asynchrony refers to (asynchrony in fate specification? or cell cycle progression? or is it cell-cell communication?). Moreover, the results start with the modeling concept of "bounded asynchrony" and "search for" biology to match it. To me, the manuscript would be far more compelling if it started with the biological reality and went to the modeling concept rather than starting from a modeling concept and trying to fit reality to it.

I therefore suggest an alternate organization starting with the general question of the relationship between cell fate specification and cell cycle and its amenability to combined experimental and modeling approaches. An introduction of the VPC system and Notch could follow. Here, a clear historical context of previous findings of Ambros and Greenwald work is essential. Results could begin with laboratory findings from the experiments that simultaneously interfere with cell cycle and monitor cell fate. These results could then be related back to the previous modeling (perhaps starting with the problem elaborated in the section starting on p. 9), the concept of "bounded asynchrony" (making clear what "asynchrony" refers to), and the computational means used to model the concept. Finally, the additional in silico and laboratory experiments could be presented, leading to the model of cell cycle-regulated degradation of Notch by different mechanisms.

In summary, my two main concerns are that the modeling concepts are presented as de facto constructs rather than as concepts that facilitate implementation of the computational model to understand the biology, and that the current work is not put into sufficiently clear context given the prior findings in the field.

Additional comments:
The following terms should be reconsidered: "diverging" and "drifting apart" evoke a physical distance rather than distinct cell cycle progression. "Bonds" evokes chemistry.

Make clear in the abstract and throughout the manuscript where the word "model" refers to the biological system and where it refers to the computational model.

Typo, p. 3: "Fbw7" or "Fbxw7", not "Fbv7"

Reviewer #3 (Remarks to the Author):

This manuscript contains a model of NOTCH signaling that is based on an earlier published model. The addition is an external regulator that introduces heterogeneity into the differentiation process by affecting the timing of the cell cycle. The concept of an external regulator is interesting and the resulting model agrees very well with the authors' experimental data. However, no actual mechanism is proposed that could accomplish that.

It is not clear that the desired heterogeneity cannot also be accomplished much more simply by introducing some level of noise in the cell cycle progression of the individual cells. Both mechanisms are consistent with the experimental evidence presented. In this way, only a minor modification would be required to the earlier model.

Another concern of this reviewer is about the appropriateness of the modeling methodology. Time discrete models might not be the best tool to use when studying processes that include fairly subtle temporal changes, since this requires time to be explicit. Also, for state-discrete models data need to be categorical, which introduces its own problems with dynamic artifacts. These issues definitely need to be addressed in the paper.

1st Revision - authors' response 13 July 2012

 

**Reviewer #1:**

**A more precise time-course analysis of NICD::GFP expression**

*Fig. 3A: The time course of NICD degradation presented in Figure 3 appears to occur over multiple hours. This is not fully consistent with a late-G2 phase event (see below). It is possible that a higher degree of synchrony among animals in the experiment would reveal that the degradation of NICD is a more sudden event. The authors synchronize larvae by starvation. However, the synchronization method of L1 starvation does not generally provide a high degree of synchrony, as the L1 larvae can sometimes develop to different extents during the starvation period and generally do not initiate development with a high degree of synchrony. A much more exact method is to transfer cohorts of newly hatched L1 onto food in short time 10-15 minute intervals. The newly-hatched L1 larvae can be obtained by mouth-pipeting newly-hatched larvae from collected pretzel-stage embryos onto bacterial plates at 10-15 min intervals. The timing of cell division should also be followed in the experiment, as this would provide a more accurate determination of when NICD degradation occurs relative to the cell cycle. Using the published timing of cell division is not a substitute for this analysis, as the culture conditions may be different and the published timing is based on following newly-hatched animals (not L1 starved animals placed on food).*

We have repeated the time course analysis of NICD degradation using the synchronization methods suggested by reviewer #1 (p.11, 2nd paragraph & Fig. 3A, B). This new analysis yielded a higher resolution and indicates that NICD expression reaches a peak during the late L2 stage and then gradually declines. Before the first VPC division NICD expression in P6.p is almost undetectable. By comparing NICD expression to P6.p division we find that NICD levels in P6.p are significantly lower already before the first division (see % division in Fig 3A & Fig3B +33 hrs time point). The fact that we did not observe a sharp decline at a particular cell-cycle stage (e.g. G2) is probably due to the fact that NICD is both stabilized by G1 cyclins and destabilized by G2 cyclins. Thus, the observed kinetics is a combination of lack of stabilization plus active destabilization.

**Role of CDK-1 in NICD degradation**

*Page 12, 3rd paragraph: The authors show that NICD is not degraded in cdk-1(RNAi) G2-phase-arrested P6.p cells. The authors also show a time course in Fig. 3A that implies that NICD degradation occurs over multiple hours. There is a potential conflict between the data. Inactivation of CDK-1 should block cells in late G2 (just before entry into mitosis), and if degradation occurs over multiple hours of G2 phase then an arrest in late G2 should allow all of the NICD to be degraded (as degradation is active over multiple hours of G2 phase). Further, if cyclin B3/CDK1 is involved in the degradation, then one would expect that the degradation would occur during mitosis, when the cyclin-CDK complex is presumably activated rather than over multiple hours of G2 phase. Potentially, a more careful time course (described above) will remove the conflict; but if not, this potential conflict should be addressed (at least in the text) by the authors.*

The continuous degradation over several of NICD is not necessarily a contradiction to our model. As explained above, NICD is both stabilized and destabilized during the cell-cycle. The reviewer is absolutely right with his remark concerning the role of *cdk-1*/*cyb*-3. The fact that NICD levels persist in *cdk-1* arrested cells can be interpreted in two ways. NICD may be degraded shortly before or upon entry into M-phase, or alternatively (our favoured model) cdk-1/cyb-3 may be directly involved in NICD degradation (p.13, end of 1st paragraph & p.18, 1st paragraph).

**The Sulston and Horvitz, 1977 reference**

*Page 8, second paragraph: "The VPCs are born during the first larval stage and remain in the G1 phase until the transition ... (Sulston and Horvitz, 1977)." The reference cited does not justify the statement that the cells are in G1 phase, as the Sulston and Horvitz paper does not include cell cycle phase analysis.*

Has been replaced with the Euling and Ambros (1996) reference, which refers to the cell-cycle arrest (p.8, 2nd paragraph).

**Inhibition of CDK-2/CYE-1 complex by CKI-1**

*Page 17, 2nd paragraph: "... CKI-1 specifically inhibits the CDK-2/CYE-1 complex (Fujita et al., 2007)." The reviewer does not think that this reference shows that CKI-1 inhibits CDK-2/CYE-1 specifically (implying that it does not inhibit other CDK/cyclins such as CYD-1). Further, another reference, Boxem and van den Heuvel, 2001 (which is not cited), also does not indicate that CKI-1 does not inhibit CYD-1. The latter paper presents biochemical evidence that CKI-1 binds to both CYE-1 and CYD-1, and while it shows genetic evidence that CKI-1 inhibits CYE-1, it does not present evidence that CKI-1 does not inhibit CYD-1. The authors should double-check their references, and either argue their position or change their statement.*

We have rewritten this part in the discussion and added the relevant references (p.17, bottom to p.18). There exists no direct evidence in C. elegans indicating that CKI-1 does not inhibit CDK-4(CYD-1). However, data from mammals and *Drosophila* indicate that Dacapo, p21 & p27 act primarily on Cyclin E complexes and that p21 family members positively regulate Cyclin D activity.

**A phenotypic effect of *cyd-1* and *cye-1* mutants on NICD signaling**

*Suppl. Fig. S3 shows that expression of NICD::GFP produces ectopic $2^0$ cells. It would be helpful if the authors clarified what percentage of animals have this phenotype. Also, the authors should indicate whether inactivation of CYD-1 and CYE-1 affect the level of ectopic $2^0$ cells in these mutants.*

In our opinion, this is one of most important new results included in the revised manuscript: We have quantified the phenotype caused by NICD::GFP and show that both cyd-1 and cye-1 mutants weakly suppress the NICD phenotype (Tab. 1 and p.13, last paragraph, p.14 1st

paragraph). This is the first indication that the stabilizing effect of the G1 cyclins also modulates the strength of the NOTCH signal, which can be detected in a modification of the phenotype. One should also take into account the positive autoregulation of LIN-12 (i.e. NOTCH signaling induces LIN-12 expression, first observed by Levitan et al 1998). Hence, the suppression of the NICD phenotype by *cyd-1* (in the absence of an effect on NICD stability, as observed by *cyd-1i*) could also be due to a block in this feedback loop from NICD to endogenous LIN-12 (end of 1st paragraph on p.14)

**Vector RNAi in Fig. 5D**

*Fig. 5D: The graph indicates that the NICD::GFP signal in P5.p and P7.p for cyb-3 RNAi and vector RNAi are comparable, yet the image provided for vector RNAi does not show staining in P5.p and P7.p. A more representative image should be provided.*

The image has been replaced with a clearer picture showing the 2° specific NICD expression.

**Reviewer #2**:

**Synchronous vs asynchronous cell-cycle progression and NOTCH signaling**

*Nusser-Stein et al. propose a conceptual approach to modeling the long-standing problem of cell fate specification among equipotential groups of cells and a molecular model that links cell cycle and cell fate specification. The findings are interesting, and the proposed biological model is provocative. In previous work the authors proposed a concept, "bounded asynchrony", the implementation of which enabled them to more accurately model VPC specification. The idea was that cells could be neither strictly synchronous nor isolated from one another with respect to cell fate decisions. The fact that these cells must communicate during fate acquisition is well known from the previous work of Greenwald and colleagues. Further, Ambros and colleagues tested the connection between Notch and cell cycle over a decade ago, concluding that the activity of Notch in prior to and after S phase differentially influenced two possible cell fate decisions, and proposing that a cell-cycle link to Notch down-regulation may contribute. Data are presented here that support a model in which the localization and stability of Notch is differentially influenced by G1 and G2 cyclins, and that Notch degradation is regulated not only by the PEST domain but by the ANK domain as well. If I understood correctly, the claim is that cell cycle is the biological mechanism that corresponds to an "asynchronous" influence on cell fate acquisition that is nevertheless "bounded" by cell-cell communication via the influence of cell-cycle control on intercellular Notch signaling. However, as written, it is not clear what asynchrony refers to (asynchrony in fate specification? or cell cycle progression? or is it cell-cell communication?). Moreover, the results start with the modeling concept of "bounded asynchrony" and "search for" biology to match it. To me, the manuscript would be far more compelling if it started with the biological reality and went to the modeling concept rather than starting from a modeling concept and trying to fit reality to it.*

All processes in the cell are processes that take time and for which timing could be important. Timing of events is extremely important when multiple cells are involved and need a coordinated decision to take place. Such processes require synchronization rather than asynchronization. However, we do not expect the entire worm to have a global clock. So the question is how cells can synchronize using an independent time-keeping mechanism. The conjecture raised in this paper is whether the cell cycle could be a time keeping mechanism that is kept by cells independently and used for synchronization purposes. It is well known that the cell cycle is a time keeping mechanism.

We answer two questions regarding the cell cycle:

1. Does it behave asynchronously enough to justify thinking about it as a keeper of bounded asynchronous behaviour? For that we checked that it is not synchronous and we show that the cell cycle has a great variability as well as that different cells progress through the different stages of the cell cycle in a semi-independent way (Fig. 1E).

2. Do processes in the cell rely on the cell cycle as a time keeping mechanism? In particular, processes that are related to the messaging between the cells should rely on this time keeping mechanism as it is particularly important to keep them synchronized. The answer to this question is also yes. In this work we show that the degradation of LIN-12, which is a manifest of the cell-cell communication, is tightly linked to the suggested synchronization mechanism.

To summarize, the progression through the cell cycle is asynchronous. However, it is used to coordinate important communication related mechanisms between the cells and thus used as a synchronizer between the different cells.

We have emphasized these points in the introduction.

**General organization of the manuscript**

*I therefore suggest an alternate organization starting with the general question of the relationship between cell fate specification and cell cycle and its amenability to combined experimental and modeling approaches. An introduction of the VPC system and Notch could follow. Here, a clear historical context of previous findings of Ambros and Greenwald work is essential. Results could begin with laboratory findings from the experiments that simultaneously interfere with cell cycle and monitor cell fate. These results could then be related back to the previous modeling (perhaps starting with the problem elaborated in the section starting on p. 9), the concept of "bounded asynchrony" (making clear what "asynchrony" refers to), and the computational means used to model the concept. Finally, the additional in silico and laboratory experiments could be presented, leading to the model of cell cycle-regulated degradation of Notch by different mechanisms.*

We have changed various parts of the manuscript. First, the last paragraph of the introduction (pages 4-5) was completely rewritten to stress that we are using the modelling

to complement experiments and to try and understand better the biological processes underlying cell-fate specification. Regarding the order in which we present the model and experimental data in the results section: We have tried several combinations when writing the manuscript and think the current structure of the manuscript starting with a model and its predictions followed by experimental data, a refined model and additional experiments to verify the new model is the best solution. This organization of the manuscript clearly represents the iterative approach of modelling and prediction followed by experimental verification we have taken in this work.

**Use of terms "diverging" and "drifting apart" & "Bonds"**

*The following terms should be reconsidered: "diverging" and "drifting apart" evoke a physical distance rather than distinct cell cycle progression. "Bonds" evokes chemistry.*

These have been changed with the appropriate terms to indicate a temporal de-synchronization. "Bonds" was replaced with "boundaries" which is commonly used when referring to cell-cycle checkpoints (p.8, 2nd paragraph).

**Models and systems**

*Make clear in the abstract and throughout the manuscript where the word "model" refers to the biological system and where it refers to the computational model.*

We have changed the text to reflect that the word "model" always refers to a computational model and "system" to the VPC specification.

**The typo**, *p. 3: "Fbw7" or "Fbxw7", not "Fbv7"*

The typo p. 3: "Fbw7" has been corrected.

**Reviewer #3:**

**Introducing noise into the model**

*This manuscript contains a model of NOTCH signaling that is based on an earlier published model. The addition is an external regulator that introduces heterogeneity into the differentiation process by affecting the timing of the cell cycle. The concept of an external regulator is interesting and the resulting model agrees very well with the authors' experimental data. However, no actual mechanism is proposed that could accomplish that.*

*It is not clear that the desired heterogeneity cannot also be accomplished much more simply by introducing some level of noise in the cell cycle progression of the individual cells. Both mechanisms are consistent with the experimental evidence presented. In this way, only a minor modification would be required to the earlier model.*

This is exactly what we do. The model introduces some level of noise in the cell cycle. However, this noise needs to be corrected in order to capture the fact that cells do not drift apart. Noise by itself leads to drifting apart as shown by the timed automata model. If we allow the cells to continue with noise alone long enough we expect their coordinated progression to be broken after a long enough time. This is exactly the point of the timed automata model (see middle of p. 6: "A completely asynchronous model of VPC differentiation…").

**Modelling methodology**

*Another concern of this reviewer is about the appropriateness of the modeling methodology. Time discrete models might not be the best tool to use when studying processes that include fairly subtle temporal changes, since this requires time to be explicit. Also, for state-discrete models data need to be categorical, which introduces its own problems with dynamic artifacts. These issues definitely need to be addressed in the paper.*

The duality between the timed automata model and the discrete model answers exactly this concern. We start with a model that includes real time and represents time explicitly. We then analyze this model and show that the schedules it gives rise to (up to global synchronization) are exactly the schedules that can be reproduced by a discrete mechanism. It is the discrete mechanism that is incorporated in the detailed model to make analysis of the model tractable.

The main results of this paper are experimental. Our computational model accompanies these results by stimulating experimentation and checking whether experimental results can be incorporated in models that reproduce these results. More elaborate models that are not state-discrete could be created to answer different questions but this is out of the scope of this paper.

**The source code of the computational model**

*We would also ask you to provide in supplementary information (as 'dataset' file) a machine-readable version of your model.*
Separate files with the code are now supplied as supplementary material.

2nd Editorial Decision 20 August 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who accepted to re-evaluate the revised study. As you will see, the reviewers are now supportive and I am pleased to inform you that we will be able to accept your paper for publication, pending the following minor points:

- the reviewers raise minor issues that we would kindly ask you to address by suitable amendements in the text.
- we would appreciate if you could provide text files rather than PDF files for the model scripts.
Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor
Molecular Systems Biology

http://www.nature.com/msb

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Referee reports:

Reviewer #1 (Remarks to the Author):

The authors have addressed my concerns and I think that the manuscript is acceptable for publication. The only minor point that the authors may consider addressing concerns the model in Fig. 7. The legend for Fig. 7 does not indicate for which cells the molecular pathway is applicable. While cyclin E and D affect P5.p, P6.p, and P7.p cells, cyclin B only appears to affect P6.p but not P5.p or P7.p. Additionally, it would be helpful if the authors addressed in the main text the extent to which their computer model reflects the different extent of cell cycle regulation on Notch stability in P6.p vs. P5.p and P7.p.

Reviewer #2 (Remarks to the Author):

The authors have sufficiently satisfied my previous concerns. Remaining suggestions are listed below.
Abstract: I still find the statement "While searching for a biological manifestation of bounded asynchrony..." off-putting and unnecessary to justify this work. However, the authors can choose to retain it.

Section entitled "VPCs enter M phase...": in the second sentence, delete "...differentiate in a random order...". Cell fate markers do not appear in a random order. Nor is terminal differentiation ultimately "random", as shown. Here the question is the order in which VPCs enter M, and the surprising result is that this order is not fixed. Also later in the section, consider rewording "could not adopt a defined fate" so as to better reflect the observation that they express a combination of 1 and 2 markers. The manifestation of the fate is classically defined as the cell division pattern, not only marker expression. Again, in the final sentence, clarify "pattern" as a gene expression pattern not a fate (cell division) pattern.

 

Thank you for considering our manuscript entitled "Cell-Cycle Regulation of NOTCH Signaling during C. elegans Vulval Development". We were delighted to read that you have accepted our work for publication. We have made the following minor changes to the manuscript to address the remaining points raised by the reviewers:

**Reviewer #1**

The authors have addressed my concerns and I think that the manuscript is acceptable for publication. The only minor point that the authors may consider addressing concerns the model in Fig. 7. The legend for Fig. 7 does not indicate for which cells the molecular pathway is applicable. While cyclin E and D affect P5.p, P6.p, and P7.p cells, cyclin B only appears to affect P6.p but not P5.p or P7.p.

*We indicate in the legend to figure 7 which of the cells are affected by the different cyclins (see highlight below).*

*"The activity of the G1-specific CDK-4/CYD-1 and CDK-2/CYE-1 complexes positively regulate LIN-12 NOTCH signaling in P5.p, P6.p and P7.p by stabilizing full-length NOTCH at the apical plasma membrane and NICD in the nucleus, respectively. Degradation of NICD in P6.p occurs during the G2 phase, when activation of the G2-specific CDK-1/CYB-3 complex terminates NOTCH signaling by inducing ubiquitin-mediated proteasomal degradation of NICD."*

Additionally, it would be helpful if the authors addressed in the main text the extent to which their computer model reflects the different extent of cell cycle regulation on Notch stability in P6.p vs. P5.p and P7.p.

*We added the following sentences on page 12 in the section entitled: A computational model of VPC fate specification based on the cell-cycle: "Furthermore, the coupling between cell-cycle progression and LIN-12 NOTCH degradation was implemented in all VPCs in an identical manner (see Fig. 13 in the supplementary material). However, when executing the model we found that LIN-12 levels in VPCs progressing towards a 1° fate stabilized at lower levels than in VPCs adopting a 2° fate."*

**Reviewer #2**

The authors have sufficiently satisfied my previous concerns. Remaining suggestions are listed below.

Abstract: I still find the statement "While searching for a biological manifestation of bounded asynchrony..." off-putting and unnecessary to justify this work. However, the authors can choose to retain it.

*We have changed this sentence as follows to better explain our approach:*

*"While searching for a molecular mechanism underlying bounded asynchrony, we discovered...."*

Section entitled "VPCs enter M phase...": in the second sentence, delete "...differentiate in a random order...". Cell fate markers do not appear in a random order. Nor is terminal differentiation ultimately "random", as shown. Here the question is the order in which VPCs enter M, and the surprising result is that this order is not fixed.

*To address this point, we deleted "...differentiate in a random order..."*

Also later in the section, consider rewording "could not adopt a defined fate" so as to better reflect the observation that they express a combination of 1 and 2 markers. The manifestation of the fate is classically defined as the cell division pattern, not only marker expression. Again, in the final sentence, clarify "pattern" as a gene expression pattern not a fate (cell division) pattern.

*We have rewritten these sentences as follows to make clear hat we infer the cell fates from the expression pattern of the fate markers (changes highlighted below):*

*"However, our results also indicate that G1-arrested P6.p cells could not establish a fate-specific gene expression pattern, as they simultaneously expressed 1° and 2° cell fate markers. We conclude that a coordinated cell-cycle progression of the VPCs is necessary for the specification of a stable cell fate as judged by the expression of cell-fate markers."*

We hope you will agree with these changes and are looking forward to see our work published in *Molecular Systems Biology*.

 