Robustness and timing of cellular differentiation through population-based symmetry breaking

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First decision letter

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I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Despite being experts in the field, both referees found the manuscript very difficult to read. I agree with this criticism and I would emphasise that for a study to be published in Development it must be accessible to a broad range of developmental biologists, many of whom will not be familiar with dynamical systems theory. The referees also highlight the need to better contextualise your work by citing and discussing relevant prior studies. In addition, the referees have specific suggestions and questions. I would particularly draw your attention to comments concerning the structural instability of pitchfork bifurcations and their relationship to twinned saddle-node bifurcations; the lack of clarity on the spatial simulations; the bifurcation analysis using \alpha_u; and the time scale of noise relative to other timescales in the system.

Because the changes necessary to adequately address these issues will be substantial and go beyond what we would consider a standard revision, I am afraid I have little choice other than to reject the paper at this stage. However, I do recognise the potential importance of this work. If you are able to address these criticisms, I would encourage you to submit a substantially revised manuscript. I would be happy to discuss the revisions with you, if that would be helpful.

Reviewer 1

Advance summary and potential significance to field

In the manuscript the authors propose a mechanism by which robust symmetry breaking can be achieve in a growing cell population without the necessity of any spatial cue. This mechanism, relying on a subcritical pitchfork bifurcations, is very interesting and the results from the simplified model are very appealing. The impact of the paper lies on the ability to show how a simple (but scalable) mechanism can achieve robust cell type proportions autonomously. My main criticism of the paper would be that it is quite hard to read. I have been working in dynamical systems theory applied to developmental biology for some years and I struggled to understand some of the plots and sentences in the paper. In addition, I found that the paper lacks contextualization with previous works, there is lots of work in the stability analysis of coupled switches (including subcritical pitchforks), for instance in the notch-delta patterning. Similarly, there is lots of work done in how a growing population of cells undergoes a bifurcation when changing its density (e.g. quorum sensing). I like that this paper brings together these two ideas, but some of the statements on its novelty are too strong.

Comments for the author

- All the spatial output of the model is omitted in the manuscript, and is hard to evaluate the validity of the model without studying the spatial cell onfiguration. Are there specific spatial patterns of cell fates obsered in the lattice? Do the patterns emerging change with cell number? Do these patterns correspond with what one observes in an biological scenario? Do the population numbers depend on how the division (sequential horizontal and vertial doubling) is implemented?

- Some of the centrals results of the paper rely on the details of cell-cell coupling. Nevertheless, information of how cells are arranged and how are they

coupled is scattered around the text. Additionally, in some points there are 3 different couplings and in some others there are 4. It would have helped me if this was made clearer earlier in the text. Perhaps moving the panels of Fig S2 to the main text? Also plots of the lattices from my previous point would help to understand the simulations.

- Some of the statements of the paper are overcomplicated and I had to reread them several times to understand their meaning. I personally think that the paper would benefit by being read by other biologists, it will just improve the future impact of the paper. Some examples, in no particular order:

line 105: "... resulting in the formation of an inhomogeneous steady state (IHSS) that reflects the cooperatively occupied differentiated cell fates**" line 614: "In a symmetry breaking mechanism that relies on the features of a population-based dynamical solution, cell fates are thus conjugate to one another."

line 438: "... Thus, reliable proportioning of cell types is an inherent property of the IHSS solution. Which proportion will be manifested for a specific system only relies on the **organization of that system** in parameter space."

- The main problem of pitchfork bifurcations from a biomathematical point of view is its structural unstability, meaning that small changes in the parameters will destroy the pitchfork bifurcation into two saddle-node bifurcations. Is this happening in the model? If it is not it would be really interseting to udnerstand why. On the other hand, if this is happening, then the pitchfork bifurcation is not required at all to observe the mechanism proposed, and should be pointed out. On the contrary, it looks that it is the accummulation of saddle-node bifurcations (at a population level) before losing the stability of the mlp state what allows to reach particular heterogeneous states. Is this related to the absence of pitchfork bifurcation in Figure 3c?

- I found really interesting the transition of states by changing the population size. Nevertheless, I am not convinced that the resulting bifurcation diagram (using a discrete parameter) is a direct exclusive consequence of the pitchfork you observed for the unrelated

parameter alpha_u. I agree that the subcritical organization is in charge of the available states. But then this behaviour is controlled by the position of the saddle nodes at the mlp multistable region. If the pitchfork is broken (due to its structural unstability) the pitchfork will disappear, but wouldn't the stable nodes still be there?

- In line 494, when discussing Figure 3b bottom, the authors write. "This reduces the necessity for fine-tuning parameter values, in particular in locally and short-range coupled systems". I do not fully understand this statement. In this plot we can see that a 10% change in the parameter alpha_u can change the critical value of the population one order of magnitude. Am I interpreting something wrong?

- I found the noise in the initial conditions a bit misleading. I expect the noise in the initial conditions to be important only when the timescale of the GRN is comparable to the cell cycle. Nevertheless, the timescale of the GRN is lambda = 50, which is 50 times faster than the cell cycle duration. Therefore, one would expect that the GRN relaxes to the steady state before the first cell division. Understanding these effects is paramount to understand where the "dynamical" effects of the model come from.

- The noise is introduced as a multiplicative noise proportional to the concentration of molecules. Is there any biological reason for this choice? I do not expect that more sofisticated choices will change much the results due to the nature of the discrete bifurcation, but this should be indicated.

Additional minor comments:

- Top and bottom parts of panels of Figure 4a,b,c where difficult to understand that they were related. Could the be shown closer together?
- Figure 4c, What are the units in time and u?
- Line 676: "and a fixed alpha_u organization before the symmetry breaking bifurcation". I think there is a typo in this sentence. Why is alpha_u organized?
- What is the "p-value" in the legend of figure 5b?
- line 699: "rate instances". Do the authors mean "rare instances"?

Reviewer 2

Advance summary and potential significance to field

In this paper, the authors combine bifurcation analysis and in silico experiments to show that symmetry breaking in a population of cells can be driven by the growth of the population itself. They then go on to characterise the dynamical mechanisms giving rise to these observed population fate dynamics as a pitchfork bifurcation from a homogeneous steady state (HSS) to an inhomogeneous steady state (IHSS) and show that the proportions of differentiated cells are insensitive to the initial conditions and expression noise of the cells for a given combination of parameter values. They also show that the proportions are recovered when the cell populations are separated according to fate in silico. The paper finishes off by showing that the proposed mechanism still holds if the cells start off oscillating synchronously.

Comments for the author

Overall impressions:

This paper tackles a very important question, namely whether the growth of a population of cells can be driving the dynamics of cell fate commitment, to report that indeed in silico experiments suggest that that is the case, and that furthermore, the growth of the population is also responsible for the proportions of the different cell types obtained and, to a certain extent, their dynamics. Unfortunately, in its current form, the paper is very hard to understand even for someone who is familiar with the methods being used.

Generally, there is not enough information in the main text regarding the simulations. This makes it

very difficult to understand the results that are being reported, needing constant trips to the Methods section for basic information that should be in the text.

The choice of bifurcation parameter in section 2 of the results really confused me (more details below). It wasn't until after reading 2 more sections that it started making sense. Basically, you choose ▫u because later on you find that increasing the population size has the same effect as reducing this parameter, hence inducing differentiation. I realise that it's a difficult finding to explain but it might help the reader to summarise this finding at the very beginning of the results section 2. Like this the reader will know that there is nothing a priori special about ▫u and that it will remain constant throughout the simulations, but that it is worth looking at the bifurcation diagram with respect to this parameters because you have found that increasing the population size has effectively the same effect as reducing it.

Section headings are in general difficult to understand

Detailed feedback:

Results Section 1, starting on line 125

L 168: From the text, it is not entirely clear how the sensed extracellular concentrations of the signalling molecule are calculated and implemented in the simulations. You consider that the "communication between cells was short-range (nearest and second nearest neighbour)". Does this mean that every cell only senses the signal from 2 neighbours, or does it mean that it senses the signal from all neighbours that are closest and from those that are second closest to them? Figure 1b (right) seems to suggest the latter while the text suggests the former. Please clarify which is it. It is also not clear how the total signal sensed is computed and implemented. I assume that it is added and updates at every time point? Is the amount of signal secreted by cell i included in the calculation of the sensed signal from the neighbours? It would be good if this could be clarified in the results and then the reader can go to the methods for more details.

Figure 1 B: Where are the daughter cells positioned on the grid after a division? Is there a re-arrangement of the cells of the grid? I assume that this is important for the subsequent states since the daughter cells inherit their initial conditions from their mother, hence affecting the sensed signal of their neighbours at the next time point. In theory, their spatial distribution might be able to bias the dynamics.

Figure 1 C: Mention that this is a stochastic simulation in the caption. Does it start with one cell or is only one cell shown?

In this first section of the results, the authors claim that the timing of differentiation and the resulting proportions of the different fates are determined by the growth of the population. Whilst this might indeed be the case, I think that one single stochastic simulation (presented in Figure 1C) is not enough evidence to make such a claim. The authors need to show more than one simulation to establish whether the timing of differentiation is indeed conserved. In terms of claiming robustness, the authors will first have to be explicit about what is specifically that the proportions are robust to. Are they robust to the cell cycle length, to certain network parameters, to the initial population size? Once they have established what it is that they think the proportions are robust to, they will need to test this explicitly by simulating perturbations and showing that the proportions indeed don't change.

Results Section 2, starting on line 260

I am very confused by the choice of bifurcation parameter in this section. ▫u in the equations, is a parameter that indirectly determines the maximum concentration of u when there is no v, that is, it goes on to determine the maximum level of u with together with ▫u,s and ▫. As far as I can see, there is no obvious reason to study the bifurcations with respect to this particular parameter instead of any other, and the authors never provide one. In addition, this parameter does not itself change in previous and subsequent simulations, preventing the results of this section to be extrapolated further.

On the other hand, what does change and what is being studied on this paper is the size of the population of cells. I understand that one cannot really do a bifurcation analysis with respect to population size, but performing it with respect to ▫u, or any other network parameter for that matter is not a good proxy.

I also find the terminology used in the paper very confusing. Unless I have misunderstood, what the authors call a homogeneous steady state (HSS) is a point attractor in a monostable regime, and an inhomogeneous steady state (IHSS) is a multi-stable system. I think that the motivation behind the terminology is probably to highlight that different cells achieve different steady states, but I don't think that the chosen terminology aides the readers understanding. I'd urge the authors to reconsider their chosen terms to make them as accessible and in line with similar works as possible. If they decide to go forward with this terminology it would help if they clearly stated how it differs from mono and multistability.

L 319: It is well known that the same bifurcations can appear in many different systems. I would not state this as surprising.

L 324: The authors show that, with respect to parameter ▫u, mutistability requires more than one cell. That is not the same as showing that multistability "emerges on a population level via cell interactions". The effect of cell interactions has not been explicitly tested here. It would have been if $\alpha_{u,s}$ had been used as the bifurcation parameter, for example.

L 326: The idea of differentiation via bifurcations is old and well established, introduced at least as long ago as Waddington's "The Strategy of the Genes" in 1957.

Results Section 3, starting on line 384

The results presented in this section are really interesting, in particular the finding that cell population growth can in affect act as a bifurcation parameter controlling the differentiation of the system. I think that, although the previous section was probably introduced as a steping stone, it confuses and distracts the reader. For this reason, I would suggest merging the two and streamlining the argument

Figure 3c is difficult to understand from the information given in the text. The reader is again directed to the Methods in order to be able to understand what is going on. This disturbs the reading flow of the results, which should be generally understandable without recurrent trips to the Methods. I would like to encourage the readers to add a few lines or potentially an inset figure to illustrate how they are implementing heterogeneity within cells.

Results Section 4, starting on line 610

Please consider re-wording the title of this sub-section. As a stand-alone sentence, it is not understandable at the moment.

L 613: What do you mean by "cell fates are thus conjugate to one another"? And how does this suggest robust proportions?

L 690: Are you using realization to mean one given stochastic simulation? If so please clarify.

I love the in-silico fate separation experiment and I think that this is one of the coolest findings in the paper!

Results Section 5, starting on line 735

Figure 5a: Please mention explicitly what the parameter ▫p represents. Is the green line representing the unstable steady state at the centre of the limit cycle, rather than the actual limit cycle? If so, please clarify, if not please explain.

I think that showing that IHSS might happen with synchronously oscillating cells is not showing that

the mechanism is general, and that therefore the section title is an overstatement.

Resubmission

Author response to reviewers' comments

Response to the referees

We thank both referees for the constructive criticism and valuable comments that has significantly helped us to improve the manuscript, both in the presentation of our main findings, but importantly, also in addressing several open issues which we strongly believe strengthen our main hypothesis. We have thereby made substantial changes in the description and presentation both of the model/simulations scenarios used, as well as our main results in order to provide a more clear and simplified explanation. We have also revised previous Figs.1-5 to a large extent, yielding current Fig.1-4 and the supplementary figures 1-5 in order to incorporate the suggestion of the referees regarding the clarity of the presented concept. We have also included new Fig. 5, Figs. S7-9 and Movies 1-4 to address an important issue raised by the referees on the realization and characteristics of spatial patterns. We have also revised the introduction and the discussion in order to better incorporate our results with respect to previous/recent findings in the field. We strongly hope that the revised version of the manuscript is more clear and will be of significant interest to the broad readership of Development.

We next address point-by-point the comments from the referees.

Reviewer 1

Advance Summary and Potential Significance to Field:
In the manuscript the authors propose a mechanism by which robust symmetry breaking can be achieve in a growing cell population without the necessity of any spatial cue. This mechanism, relying on a subcritical pitchfork bifurcations, is very interesting and the results from the simplified model are very appealing. The impact of the paper lies on the ability to show how a simple (but scalable) mechanism can achieve robust cell type proportions autonomously. My main criticism of the paper would be that it is quite hard to read. I have been working in dynamical systems theory applied to developmental biology for some years and I struggled to understand some of the plots and sentences in the paper. In addition, I found that the paper lacks contextualization with previous works, there is lots of work in the stability analysis of coupled switches (including subcritical pitchforks), for instance in the notch-delta patterning. Similarly, there is lots of work done in how a growing population of cells undergoes a bifurcation when changing its density (e.g. quorum sensing). I like that this paper brings together these two ideas, but some of the statements on its novelty are too strong.

We thank the referee for his/hers detailed assessment of our work, and the constructive criticism, which we have addressed in great detail. We have given special care into re-writing our manuscript, especially the explanations of the main findings and the descriptions of the dynamical systems terminology in order to provide better clarity. In the amended version of the manuscript, we also include discussion on previously described symmetry breaking concepts, and note in light of those findings, where our results bring a contribution. We also relate our work to previous descriptions, especially of pitchfork bifurcations in the Delta- Notch system, in the introduction and discussion.

Reviewer 1 Comments for the Author:
- All the spatial output of the model is omitted in the manuscript, and is hard to evaluate the validity of the model without studying the spatial cell configuration. Are there specific spatial patterns of cell fates obsered in the lattice? Do the patterns emerging change with cell number? Do these patterns correspond with what one observes in an biological scenario? Do the population numbers depend on how the division (sequential horizontal and vertial doubling) is implemented?

Following the importance of this issue raised by the referee, we have performed a systematic analysis of the spatial patterns emergence in growing populations, but also for cell populations with fixed size. In addition to the 4 distinct coupling types we considered before, for completeness, we now enlarged this set by varying the communication range both, for the deterministic as well as in the probabilistic, distance-based coupling scenarios. The results are summarized in the new Fig.5, Figs. S7-9 and Movies 1-4.

In short, we have demonstrated that regular spatial patterns are indeed formed, corresponding to fixed cell type proportions emerging from the IHSS, and their spatial frequency remains stable across the cell cycles in growing populations, when deterministic coupling of different range is considered. Even more, these patterns could be reproduced also for cell populations with a fixed size. They are reliably formed from diverse initial conditions, as demonstrated for 100 independent realizations in both instances. The type of patterns formed, however is directly related to the stable cell type proportion, as well as the coupling range. We found that the patterns formed under global and probabilistic coupling types however are mainly regulated by population growth, and are thus sensitive to initial conditions. The pattern emergence was independent from how the lattice duplication was implemented. We have also included a paragraph in the discussion placing our results in context of previous and recent findings on patterning at early differentiation stages, especially for the differentiation into PrE and Epi cells.

- Some of the centrals results of the paper rely on the details of cell-cell coupling. Nevertheless, information of how cells are arranged and how are they coupled is scattered around the text. Additionally, in some points there are 3 different couplings and in some others there are 4. It would have helped me if this was made clearer earlier in the text. Perhaps moving the panels of Fig S2 to the main text? Also plots of the lattices from my previous point would help to understand the simulations.

Following the referee's suggestion, in the amended version of the manuscript we describe in the main text how the simulations were performed, we included in the current Fig.1c the schematic representation of the 4 main coupling types used, and additionally added in Fig. S1 a schematic representation of the division process on the lattice. Throughout the manuscript, we compare all 4 coupling scenarios, which is now also included in the current Figure 2c. All of these coupling types, including an enlarged set, is then systematically considered in the analysis of the spatial patterns (Fig. 5 and the Figs. S8-9).

- Some of the statements of the paper are overcomplicated and I had to reread them several times to understand their meaning. I personally think that the paper would benefit by being read by other biologists, it will just improve the future impact of the paper. Some examples, in no particular order:

line 105: "... resulting in the formation of an inhomogeneous steady state (IHSS) that reflects the **cooperatively occupied differentiated cell fates**"
line 614: "In a symmetry breaking mechanism that relies on the features of a population-based dynamical solution, cell fates are thus conjugate to one another."
line 438: "... Thus, reliable proportioning of cell types is an inherent property of the IHSS solution. Which proportion will be manifested for a specific system only relies on the **organization of that system** in parameter space."

We thank the reviewer for pointing out problematic statements in the paper, which we have taken a great care to amend. We hope that the manuscript is significantly clearer and the readability is improved.

- The main problem of pitchfork bifurcations from a biomathematical point of view is its structural unstability, meaning that small changes in the parameters will destroy the pitchfork bifurcation into two saddle-node bifurcations. Is this happening in the model? If it is not it would be really interseting to udnerstand why. On the other hand, if this is happening, then the pitchfork bifurcation is not required at all to observe the mechanism proposed, and should be pointed out. On the contrary, it looks that it is the accummulation of saddle-node bifurcations (at a population level) before losing the stability of the mlp state what allows to reach particular heterogeneous

states. Is this related to the absence of pitchfork bifurcation in Figure 3c?

We thank the referee for this comment. Due to the scope of the current version of the manuscript, we chose not to elaborate this problem in details in the manuscript, however we would like to address it here. As previous work (of M. Golubitsky and others) have demonstrated, indeed, the unfolding of a pitchfork bifurcation results into a SN and another bifurcation. This work has been generally restricted to the normal form of the PB usually described for one variable (with exception to limited work on N-dim from S. Smale). The properties of the dynamical solution arising from a PB in an N-coupled system however are different from the 1D case. We therefore use the N-coupled system to point to the equivalence of the solutions in the perfect and imperfect bifurcation scenarios.

For example, for two coupled cells, the two IHSS branches denote two different manifestations, u1<u2 and u1>u2, indicating that when the first cell acquires the v+ fate the second will acquire the u+ fate, and vice versa. Thus each branch represents a symmetry broken solution. These branches are R-conjugate and related to each other through reflection. This renders the PB different than a SN bifurcation where formally, symmetry breaking does not occur. In general, for an N-globally-coupled system, N-1 stable IHSS solutions reflecting different distributions of the coupled subsystems into the two stable branches are always generated (Koseska et al., EPL 2009). We have demonstrated this for example for N=4 identical cells, where the distributions 1u+/3v+, 2u+/2v+ and 3u+/1v+ are possible (current Figure 3a). Thus, such sets of conjugate states are typical and unique for a pitchfork bifurcation, and what distinguishes it from the other symmetry-breaking bifurcation discussed in the literature, the transcritical bifurcation.

In an N-coupled system therefore, although a small heterogeneity will effectively destroy the PB, this will not destroy the presence of the symmetry broken solution – N-1 distributions that reflect the symmetry broken states will still be present (shown in current Figure S4a, where a small difference in the α_V between the 4 cells is present). These conjugate states are not a consequence of the SNs through which they are stabilized and therefore cannot be equated to a generic bistable/multistable system, but rather are emerging from the remnant of the pitchfork bifurcation that disappeared through the unfolding. We are currently working in providing a formal proof of this using equivariant bifurcation theory, and thereby we hope to be able to generalize the role of conjugate states emerging from a PB, for biological systems with symmetries.

- I found really interesting the transition of states by changing the population size. Nevertheless, I am not convinced that the resulting bifurcation diagram (using a discrete parameter) is a direct exclusive consequence of the pitchfork you observed for the unrelated parameter alpha_u. I agree that the subcritical organization is in charge of the available states. But then this behaviour is controlled by the position of the saddle nodes at the mlp multistable region. If the pitchfork is broken (due to its structural unstability) the pitchfork will disappear, but wouldn't the stable nodes still be there?

In response to this comment, we would like to refer back to the answer of the previous comment from the referee. The symmetry-broken solutions will remain even after the unfolding of the PB. We would like to point again, that these symmetry-broken solutions are a hallmark and a unique characteristic of the PB that remain even after the unfolding.

- In line 494, when discussing Figure 3b bottom, the authors write. "This reduces the necessity for fine-tuning parameter values, in particular in locally and short-range coupled systems". I do not fully understand this statement. In this plot we can see that a 10% change in the parameter alpha_u can change the critical value of the population one order of magnitude. Am I interpreting something wrong?

We thank the reviewer for pointing out this miscommunication on our side that we have corrected. Our goal was to convey that the differentiation timing as well as stable proportions between the two cell types can be achieved, irrespective of initial conditions that commonly need to be fine-tuned.

I found the noise in the initial conditions a bit misleading. I expect the noise in the initial conditions to be important only when the timescale of the GRN is comparable to the cell cycle.

Nevertheless, the timescale of the GRN is lambda = 50, which is 50 times faster than the cell cycle duration. Therefore, one would expect that the GRN relaxes to the steady state before the first cell division. Understanding these effects is paramount to understand where the "dynamical" effects of the model come from.

We presume that the comment refers to Figure 4a,b and Figure S5a,b,d,e,g,h (Figure 4a,b and Figure S3a,b,d,e,g,h in the initial version of the manuscript), where the mean or standard deviation of the initial conditions are varied. There are no cell divisions in these results, and the population size is fixed to 32 cells (on a 4x8 grid). We aimed with these results to demonstrate that similar proportions will be achieved in steady state (indeed, we allow sufficient time for the system to reach the steady state), even when starting from markedly different initial conditions. Equivalently, we have also considered a case when the noise intensity was increased (stochastic system with multiplicative noise term was considered in this case). These results contrast the concept of multistability in single cells, where each cell will reach a given steady state depending on the distribution of initial conditions. In such case, all cells could potentially manifest only one cell type, without the other being realized.

In the IHSS case presented here, as there are multiple stable IHSS branches corresponding to distinct cell type proportions that coexist in the system, varying the initial conditions (or the noise in gene expression) will in principle result in different cell type proportions. However, due to positioning of the IHSS branches with similar proportions in sequential order in parameter space (as shown in Figure 3a for example), similar proportions are achieved (Figure 3b and 4a-c),even when the magnitude of the perturbation is increased.

- The noise is introduced as a multiplicative noise proportional to the concentration of molecules. Is there any biological reason for this choice? I do not expect that more sofisticated choices will change much the results due to the nature of the discrete bifurcation, but this should be indicated.

To mimic the effect of noise on the dynamics of the gene regulatory network considered, we chose a multiplicative noise term, which enables simpler numerical treatment, especially in the low molecule number ranges. The attained dynamics resembles a Gillespie treatment of the problem (similarity between both approaches for models of GRN was previously shown by one of the authors, Koseska et al., PRE 2007). An additive noise term would not influence the results from the stochastic simulations significantly, as all of the findings can be directly correlated to the deterministic results using the bifurcation analysis approach.

Additional minor comments:

- Top and bottom parts of panels of Figure 4a,b,c where difficult to understand that they were related. Could the be shown closer together?
- Figure 4c, What are the units in time and u?
- Line 676: "and a fixed alpha_u organization before the symmetry breaking bifurcation". I think there is a typo in this sentence. Why is alpha_u organized?
- What is the "p-value" in the legend of figure 5b?
- line 699: "rate instances". Do the authors mean "rare instances"?

We thank the reviewer for these comments; all of the points have been amended in the manuscript.

Reviewer 2 Comments for the Author:

Summary:

In this paper, the authors combine bifurcation analysis and in silico experiments to show that symmetry breaking in a population of cells can be driven by the growth of the population itself. They then go on to characterise the dynamical mechanisms giving rise to these observed population fate dynamics as a pitchfork bifurcation from a homogeneous steady state (HSS) to an inhomogeneous steady state (IHSS) and show that the proportions of differentiated cells are insensitive to the initial conditions and expression noise of the cells for a given combination of parameter values. They also show that the proportions are recovered when the cell populations are

separated according to fate in silico. The paper finishes off by showing that the proposed mechanism still holds if the cells start off oscillating synchronously.

Overall impressions:

This paper tackles a very important question, namely whether the growth of a population of cells can be driving the dynamics of cell fate commitment, to report that indeed in silico experiments suggest that that is the case, and that furthermore, the growth of the population is also responsible for the proportions of the different cell types obtained and, to a certain extent, their dynamics. Unfortunately, in its current form, the paper is very hard to understand even for someone who is familiar with the methods being used.

Generally, there is not enough information in the main text regarding the simulations. This makes it very difficult to understand the results that are being reported, needing constant trips to the Methods section for basic information that should be in the text.

We thank the referee for his in-depth assessment and the provided comments, which we have used as a guideline to significantly modify both the structure as well as the description of the main findings. In that regard, we have now included the crucial information on how simulations were performed / the used models throughout the text.

The choice of bifurcation parameter in section 2 of the results really confused me (more details below). It wasn't until after reading 2 more sections that it started making sense. Basically, you choose alpha_u because later on you find that increasing the population size has the same effect as reducing this parameter, hence inducing differentiation. I realise that it's a difficult finding to explain but it might help the reader to summarise this finding at the very beginning of the results section 2. Like this the reader will know that there is nothing a priori special about alpha_u and that it will remain constant throughout the simulations, but that it is worth looking at the bifurcation diagram with respect to this parameters because you have found that increasing the population size has effectively the same effect as reducing it.

We sincerely thank the referee for this suggestion, since we have struggled with the order for presentation of this concept in the initial version of the manuscript. We have now combined the most crucial features from Figures 2 and 3 from the previous version of the manuscript into a new Figure 2. In section 2, we now first describe the advantage of identifying the transition with respect to alpha_u, and then discuss the transition using N as bifurcation parameter, as the referee has suggested. We have also included additional simulations to demonstrate that the PB indeed shifts is position with increased N triggering the transition from the homogenous precursor to the heterogeneous differentiated state (Figure 3b) and estimated this shift using bifurcation analysis. We hope that the amended version has increased the clarity of the presented findings.

Section headings are in general difficult to understand

We have amended several section headings in order to better convey the presented results.

Detailed feedback:

Results Section 1, starting on line 125

L 168: From the text, it is not entirely clear how the sensed extracellular concentrations of the signalling molecule are calculated and implemented in the simulations. You consider that the "communication between cells was short-range (nearest and second nearest neighbour)". Does this mean that every cell only senses the signal from 2 neighbours, or does it mean that it senses the signal from all neighbours that are closest and from those that are second closest to them? Figure 1b (right) seems to suggest the latter while the text suggests the former. Please clarify which is it. It is also not clear how the total signal sensed is computed and implemented. I assume that it is added and updates at every time point? Is the amount of signal secreted by cell i included in the calculation of the sensed signal from the neighbours? It would be good if this could be clarified in the results and then the reader can go to the methods for more details.

It is the latter, as we schematically show in Figure 1c (former Fig.1b). We have also clarified this better in the amended version of the manuscript. In general, the sensed signal by cell *i* is calculated at each time point by averaging the current secreted amounts of all of its communicating cells, including itself, assuming immediate complete mixing (relative to the time scale of the GRN) of the secreted signals surrounding cell *i*.

Figure 1 B: Where are the daughter cells positioned on the grid after a division? Is there a rearrangement of the cells of the grid? I assume that this is important for the subsequent states since the daughter cells inherit their initial conditions from their mother, hence affecting the sensed signal of their neighbours at the next time point. In theory, their spatial distribution might be able to bias the dynamics.

At every cell division, each mother cell is divided along the same axis (horizontally or vertically, interchangeably throughout subsequent cell cycle events) and substituted by two daughter cells. After the first cell division, the single cell / grid point is horizontally 'expanded' and divided to two cells (1x2 placement), each of those two are subsequently vertically-divided and substituted by two daughter cells (2x2 placement), and so on. Therefore, the grid doubles in size at every cell cycle event. We have included a schematic representation of this process in Fig. S1, and introduced clarification in relation to current Fig. 1d. Sufficient time is ensured between cell cycle events for the system to reach a steady state, and we show that the resulting proportions are consistent (Figure 1d, and additionally we included estimation from 100 independent realisations in Fig.3c). In addition, in the amended version of the manuscript we have included a systematic analysis (new Figure 5 and Figs. S8-9), where we have also characterized the established regular spatial patterns, and demonstrated that under diverse realizations of deterministic coupling, the established spatial patterns are preserved across the cell cycle events, while maintaining the robust cell type proportions. We show that this is conserved for multiple (n=100) independent simulations realizations. The evolution of the patterns along with the cell divisions though the lineage tree can be also observed in Movies 1-4.

Figure 1 C: Mention that this is a stochastic simulation in the caption. Does it start with one cell or is only one cell shown?

The simulations start with one single cell. We have amended the figure caption to indicate stochastic simulations where necessary.

In this first section of the results, the authors claim that the timing of differentiation and the resulting proportions of the different fates are determined by the growth of the population. Whilst this might indeed be the case, I think that one single stochastic simulation (presented in Figure 1C) is not enough evidence to make such a claim. The authors need to show more than one simulation to establish whether the timing of differentiation is indeed conserved. In terms of claiming robustness, the authors will first have to be explicit about what is specifically that the proportions are robust to. Are they robust to the cell cycle length, to certain network parameters, to the initial population size? Once they have established what it is that they think the proportions are robust to, they will need to test this explicitly by simulating perturbations and showing that the proportions indeed don't change.

According to the referee's suggestion, we have performed 100 independent stochastic simulations and quantified the timing of the differentiation event (current Fig. 3c), demonstrating that this transition is manifested in 94% of the cases, in line with the results shown in Fig. 2b. The mechanism behind the reliable proportioning is also elaborated in grater extent and demonstrated with additional results in the current Fig.3b. Additionally, we have preformed the same analysis also for different coupling types in relation to the quantification of the spatial patterns, and the results are presented in Fig. S8.

Also, we have re-worded our conclusions in the description of Fig.1d, which shows that steady proportions of differentiated cell types are generated and maintained across the cell cycle events. Later on however, in Fig. 4, we demonstrated that the established cell type proportions are robust with respect to variance in the initial conditions and their mean value, as well as noise intensity.

Results Section 2, starting on line 260

I am very confused by the choice of bifurcation parameter in this section. Alpha_u in the equations, is a parameter that indirectly determines the maximum concentration of u when there is no v, that is, it goes on to determine the maximum level of u with together with alpha_u,s and l. As far as I can see, there is no obvious reason to study the bifurcations with respect to this particular parameter instead of any other, and the authors never provide one. In addition, this parameter does not itself change in previous and subsequent simulations, preventing the results of this section to be extrapolated further.

As noted to the related comment above, we have amended the respective description in the text. In brief, Fig. 1d indicates that the population growth and thereby the size of the population N acts as a bifurcation parameter. However, as N is not an explicit parameter of the model, bifurcation analysis cannot formally be carried out. We have therefore chosen alpha_u, as u is used throughout the manuscript as the readout variable through which the dynamics of the system is studied. The dynamical mechanism (subcritical pitchfork bifurcation) is revealed with respect to this parameter and all of the characteristics of the IHSS unravelled through the bifurcation analysis directly correspond to the characteristics of the systems identified through stochastic search numerical simulations performed for different N (compare Fig.2a/3a to Fig.2b/3b). We additionally show how both parameters are related: for increasing N, the alpha_u at which the PB occurs is shifted to lower values, thereby enabling the transition to the differentiated state. We demonstrated this with numerical simulations (new Fig.3b) and additionally the value of alpha_u for the PB at N=8 cells when the transition occurs in the lineage tree that is obtained from the bifurcation analysis is given (text).

On the other hand, what does change and what is being studied on this paper is the size of the population of cells. I understand that one cannot really do a bifurcation analysis with respect to population size, but performing it with respect to alpha_u, or any other network parameter for that matter is not a good proxy.

As mentioned above, we performed an extensive stochastic search simulations to identify the dynamical characteristics of the symmetry-breaking transition for increased N (in the amended version of the manuscript, Fig. 2b (compare to Fig. 2a), but also simulations for fixed alpha_u and fixed N from a broad range of initial conditions that directly reflect the findings from the bifurcation analysis (Fig. 3b, compared to Fig. 3a). We thereby demonstrated that all of the features of the system obtained for increased N correspond and can be directly explained by the IHSS features uncovered with respect to the bifurcation analysis performed for varying alpha_u. Even more, we revised the explanation of the two-parameter bifurcation-like diagram between alpha_u and N, demonstrating that for fixed alpha_n a transition towards a symmetry-broken state can be triggered at a crticial N.

I also find the terminology used in the paper very confusing. Unless I have misunderstood, what the authors call a homogeneous steady state (HSS) is a point attractor in a monostable regime, and an inhomogeneous steady state (IHSS) is a multi-stable system. I think that the motivation behind the terminology is probably to highlight that different cells achieve different steady states, but I don't think that the chosen terminology aides the readers understanding. I'd urge the authors to reconsider their chosen terms to make them as accessible and in line with similar works as possible. If they decide to go forward with this terminology it would help if they clearly stated how it differs from mono and multistability.

In order to better clarify our findings, we have strongly revised the respective explanations in the text. In brief, both, the HSS and IHSS are point attractors in the joint 3N-dimensional space (N being the number of coupled cells in the system), and existence of either one of them does not directly imply mono- or multistability. While the HSS can exist as a sole attractor, it can also co-exist with the IHSS point attractors (as shown for different α values in Fig.2a, and with the shaded region in Fig.3a).

What is characteristic about the IHSS is that it describes a heterogeneous manifestation of the system in a single attractor (for two coupled cells, 6dim. attractor): high u-expression level (u+) in one cell and low u- expression level (v+) in the other cell. The IHSS is therefore a symmetry-broken state because it describes simultaneously both cell types with mutually exclusive gene expression

patterns. As there is no preference which cell will acquire the u+ or v+ cell type, both realizations (u1<u2 or u1>u2) are represented as two branches of the IHSS solution (upper and lower branch in Fig.2a). Thus, the same fixed point will be manifested as upper branch in the u+ cell, but as lower branch when plotting the equivalent bifurcation diagram for the v+ cell. Thus, these two branches do not correspond to a classical multistable system, but are R-conjugate and related to each other through reflection. We have also clarified the explanation in the text how this symmetry broken solution differs from a multistable system, where the steady states simply emerge from SN bifurcations.

L 319: It is well known that the same bifurcations can appear in many different systems. I would not state this as surprising.

The aim of our work was to provide a unified dynamical mechanism to describe how timing of cell differentiation can be regulated in self-organized manner, resulting in robust proportions of differentiated cell types that can recover upon perturbation. Thus, we have demonstrated that the proposed mechanism is not realized only for the specific network topology we mainly analyse, but rather for systems that have an effective negative feedback coupling (currently Fig. S3). We have however changed the wording in order to better reflect our aim.

L 324: The authors show that, with respect to parameter au, mutistability requires more than one cell. That is not the same as showing that multistability "emerges on a population level via cell interactions". The effect of cell interactions has not been explicitly tested here. It would have been if alpha_u,s had been used as the bifurcation parameter, for example.

In order to address this point, we have included in the amended version of the manuscript numerical simulations that mimic inhibition of the cell-cell communication (affecting alpha_s; Fig. S6) and show that in this case, not only the proportions, but also the two different cell types cannot be maintained. These results resemble previous experimental findings of cell-cell communication inhibition during early embryogenesis (Yamanaka et al., 2010).

We would also like to note that in our recent complementary study (briefly noted in the discussion, (Raina et al., 2020), we have tackled this problem in a mouse embryonic stem cell model, looking at the differentiation step from ICM into PrE and Epi-like cells. Generating a communication-deficient mutant cell line thereby resembling a bistable system has demonstrated that robust proportioning of differentiated cell types cannot be generated.

L 326: The idea of differentiation via bifurcations is old and well established, introduced at least as long ago as Waddington's "The Strategy of the Genes" in 1957.

We for sure agree with the referee. What we aimed to present in this manuscript is the concept that differentiation and acquiring two cell types with mutually exclusive gene expression patterns can be described through a single, symmetry-broken population-based steady state. We identify a distinction between a mechanism relying on single cell multistability and population-based inhomogeneous state, such that the latter conveys to the system robust proportioning of the differentiated cell types, reproducible spatial patterns as well as timing of the differentiation event.

Results Section 3, starting on line 384

The results presented in this section are really interesting, in particular the finding that cell population growth can in affect act as a bifurcation parameter controlling the differentiation of the system. I think that, although the previous section was probably introduced as a steping stone, it confuses and distracts the reader. For this reason, I would suggest merging the two and streamlining the argument

Following the referee's suggestion (as also noted above), we have now merged these two sections and showed the results in one figure (Figure 2). We hope that together with the amended version of the text, the results are clearer.

Figure 3c is difficult to understand from the information given in the text. The reader is again directed to the Methods in order to be able to understand what is going on. This disturbs the

reading flow of the results, which should be generally understandable without recurrent trips to the Methods. I would like to encourage the readers to add a few lines or potentially an inset figure to illustrate how they are implementing heterogeneity within cells.

We thank the referee for the suggestion, which is implemented, both in the text as well as the respective figure in the amended version of the manuscript.

Results Section 4, starting on line 610

Please consider re-wording the title of this sub-section. As a stand-alone sentence, it is not understandable at the moment.

We have modified majority of the section headings to state more clearly the respective findings.

L 613: What do you mean by “cell fates are thus conjugate to one another”? And how does this suggest robust proportions?

What we meant by this statement is that both cell fates are described through a single, joint solution, the IHSS, as we have discussed above. The conjugacy itself is a technical term that describes this type of dynamical solution. However, in order to achieve better clarity, we have revised the description of the IHSS related to Fig.2 and in general throughout the text.

L 690: Are you using realization to mean one given stochastic simulation? If so please clarify.

In l.690 (previous version of the manuscript) indeed one stochastic realization is meant to describe how the shift of the mean value of initial conditions is changed, however, at each mean value, 10 independent realizations are performed and the results are summarized in Fig. 4.

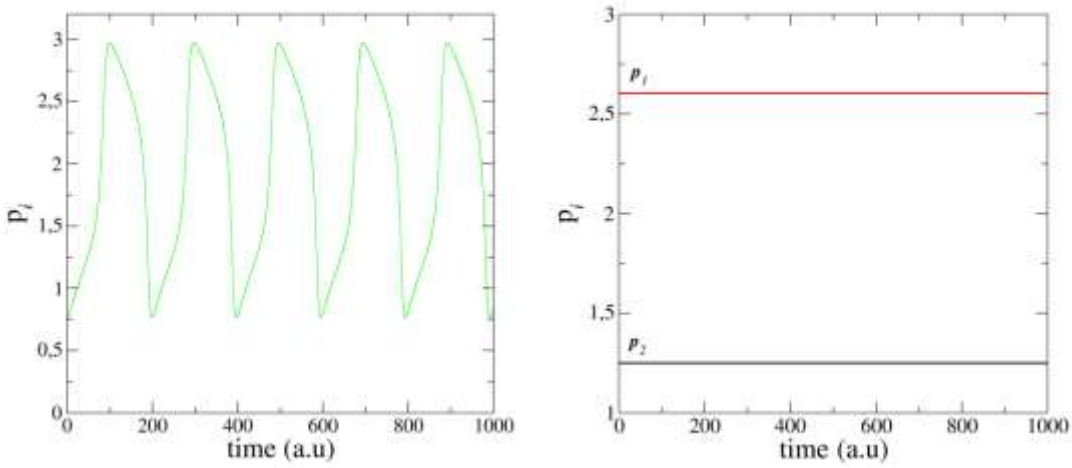
I love the in-silico fate separation experiment and I think that this is one of the coolest findings in the paper!

We thank the referee for this comment.

Results Section 5, starting on line 735

Figure 5a: Please mention explicitly what the parameter alpha_p represents. Is the green line representing the unstable steady state at the centre of the limit cycle, rather than the actual limit cycle? If so, please clarify, if not please explain.

The solid green line represents the stable limit cycle solution, stabilized by a subcritical Hopf bifurcation as identified from the bifurcation analysis. We include here for the referee’s perusal the corresponding time- series. The are estimated for alpha_p=3.1, for which there is a coexistence of the limit cycle (both oscillators are synchronised, left) and the IHSS solution (right):



I think that showing that IHSS might happen with synchronously oscillating cells is not showing that the mechanism is general, and that therefore the section title is an overstatement.

In accordance with the referee's comment, we have re-distributed these results in a shorter version to the discussion for which network topologies IHSS can be observed (Fig. S3) and re-phrased the wording.

Second decision letter

MS ID#: DEVELOP/2020/197608

MS TITLE: Robustness and timing of cellular differentiation through population-based symmetry breaking

AUTHORS: Angel Stanoev, Christian Schroeter, and Aneta Koseska

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Stanoev et al present a theoretical study of the decision-making capabilities of populations of cells that contain a mutually inhibitory circuit motif, under different cell-cell coupling configurations. The study performed here of the connection between cellular decision making and the bifurcation structure of the underlying cellular circuits has value, in my opinion.

Comments for the author

The issues raised by previous reviewers have mostly been addressed satisfactorily, with the exception of a point made by reviewer 2 regarding the choice of control parameter made. The authors claim that the behavior they report is an instance of population-based symmetry breaking. However, since the intracellular mutual inhibition circuit they consider is capable of inducing cellular decision by itself (with no cell-cell coupling needed), I concur with reviewer 2 that it would be important to discuss what happens when alpha_u,s is used as control parameter in this system instead of alpha_u (irrespective of previous works in different systems published previously by the authors, cited in their response to reviewer 2). This would not be hard to do in this theoretical model, and therefore I see no reason for not doing it. At least a comparison with the case alpha_u,s=0 should be presented. This study would be complementary to that shown in Fig. S6, which already shows that cell-cell communication is needed for a well-balanced population outcome (although this conclusion is not emphasized enough, in my opinion, in the main text). In Figure S6 the authors consider the effect of a decrease in alpha_s, but the limit alpha_s=0 corresponds to an intrinsically asymmetric mutual inhibition switch (since for s=0, u would have a non-zero basal production level and v wouldn't). Thus for alpha_s=0 the cell-cell communication pathway would still play a role in the system. Therefore I'm left wondering what happens if alpha_u,s=0, which would correspond to a symmetric switch. In that case, there would still be bistability in a certain alpha_s window near the regime of a balanced switch, and that situation would lead to symmetry breaking not arising at the population level. Not robust, for sure, but I think it would be good to emphasize this point in the paper.

As an additional comment, although the authors seem to have made an effort to make the paper easier to follow in response to reviewer 1 (to what extent I can't tell, since I didn't have access to the first version of the manuscript), it would still be helpful to make their models less abstract by relating them to specific decision-making circuits such as those listed by the authors in their introduction and discussion sections. Can the authors name, when they introduce their model, which genes or pathways would their variables u, v, s, etc represent? This is specially important in the context of the new Fig. 5, which shows two-dimensional simulations that might have little biological realism depending on the system that they are trying to model (e.g. a developing mouse embryo).

Finally, I would suggest that these new results are discussed in the context of an earlier paper, published previously by the last author (Koseska et al 2009, PMID 1928306), in which she studied the role of stochasticity in a model closely related to the ones considered here. Since the authors perform stochastic simulations of their models in this manuscript, I wonder if noise effects similar to those published in the 2009 paper could arise in these models.

Reviewer 2

Advance summary and potential significance to field

Already provided the first time I reviewed this paper

Comments for the author

I'd like to thank the authors for exhaustively addressing my comments. This version of the manuscript reads well and is clearly structured. The methods of the paper are now presented early

on and should not be too difficult for the readers of Development understand. In addition, the extra simulations ground the author's claims.

In my opinion, the findings presented in this paper constitute an important contribution to the field by highlighting the effect that cell population size and growth might play in differentiation, offering a much needed link between morphogenesis and pattern formation. I would be happy to see this paper published in Development.

Second revision

Author response to reviewers' comments

Response to referees

We thank the referee for his detailed comments and suggestions on the manuscript that we address as follows:

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, Stanoev et al present a theoretical study of the decision-making capabilities of populations of cells that contain a mutually inhibitory circuit motif, under different cell-cell coupling configurations. The study performed here of the connection between cellular decision making and the bifurcation structure of the underlying cellular circuits has value, in my opinion.
Reviewer 1 Comments for the Author:
The issues raised by previous reviewers have mostly been addressed satisfactorily, with the exception of a point made by reviewer 2 regarding the choice of control parameter made.

The authors claim that the behavior they report is an instance of population-based symmetry breaking. However, since the intracellular mutual inhibition circuit they consider is capable of inducing cellular decision by itself (with no cell-cell coupling needed), I concur with reviewer 2 that it would be important to discuss what happens when alpha_u,s is used as control parameter in this system instead of alpha_u (irrespective of previous works in different systems published previously by the authors, cited in their response to reviewer 2). This would not be hard to do in this theoretical model, and therefore I see no reason for not doing it.
At least a comparison with the case alpha_u,s=0 should be presented. This study would be complementary to that shown in Fig. S6, which already shows that cell-cell communication is needed for a well-balanced population outcome (although this conclusion is not emphasized enough, in my opinion, in the main text).

We thank the referee for this suggestion. Indeed, in absence of communication (for alpha_u,s=0), the single cell dynamics is governed by the toggle switch. In this case, the system will be bistable when changing alpha_u (Fig. 1 in response). However, we would like to note that the parameters of the model in the manuscript are such that the system is organized in the monostable regime (alpha_u=2.3), therefore only low u-expressing cells will be present.
According to the referee’s suggestion, to systematically describe this effect of effective reduction in cell-cell communication, we performed additional simulations (complementary to the simulations with inhibition of alpha_s) and included in the amended version of the manuscript the summarized results as another panel in Fig. S6, demonstrating the effect of subsequent alpha_u,s decrease on robust cell type proportioning.

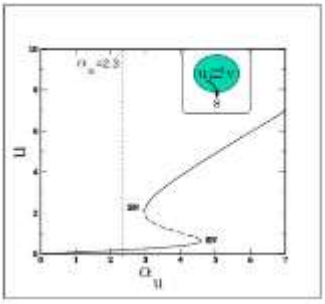


Fig.1. Bifurcation analysis of a single cell. Dashed line corresponds to alpha_u value used in the growing population simulations in the manuscript.

We would like to emphasize once more that the actual bifurcation parameter of the system is the number of cells (N), and thereby the organization before the PB is crucial, as described with respect to Fig. 2a in the manuscript. Due to such organization, within our model, the expression profiles of the multilineage primed, as well as the two differentiated cell types can be captured without any change in the parameters, and the transition between them is established at a given N, as the position of the PB shifts to lower alpha_u as N increases (Figure 3b in the manuscript). Thus, alpha_u is only chosen as a representative bifurcation parameter to identify the type of dynamical transition. Since the same transition can be observed when changing alpha_u,s (Fig. 2 in the response yielding equivalent transition as Fig. 2a in the manuscript), we would like to retain the analysis with respect to alpha_u, in order to remain consistent with the description throughout the text.

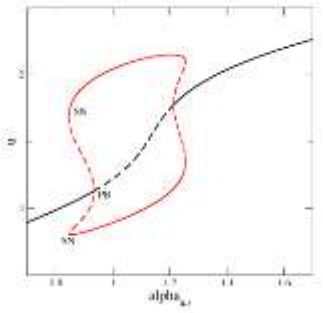


Fig.2. Bifurcation diagram for N=2 coupled cells using alpha_u,s as a bifurcation parameter. Solid lines denote stable homogeneous (black) and inhomogeneous (red) steady states, and dashed lines- unstable steady states. The diagram resembles Fig. 2a in the manuscript, indicating that alpha_u and alpha_u,s can be both used as exemplary bifurcation parameters to uncover the type of dynamical transition (sub-critical pitchfork bifurcation).

In Figure S6 the authors consider the effect of a decrease in alpha_s, but the limit alpha_s=0 corresponds to an intrinsically asymmetric mutual inhibition switch (since for s=0, u would have a non-zero basal production level and v wouldn't). Thus for alpha_s=0 the cell-cell communication pathway would still play a role in the system.
Therefore I'm left wondering what happens if alpha_u,s=0, which would correspond to a symmetric switch. In that case, there would still be bistability in a certain alpha_s window near the regime of a balanced switch, and that situation would lead to symmetry breaking not arising at the population level. Not robust, for sure, but I think it would be good to emphasize this point in the paper.

As briefly described in the response to the previous question, for alpha_u,s=0, indeed there is a bistability on the level of a single cell, however with respect to alpha_u (Fig. 1 in the response; we note that equivalent diagram will also be obtained using alpha_v as a bifurcation parameter). We would like to note that alpha_s only determines the production of s, and therefore the

properties of the toggle switch are independent of alpha_s.
In the manuscript however, alpha_u=2.3 rendering organization in the monostable regime on the level of single cells, such that all cells will have low u-expressing state. This is also reflected in the newly added Fig. S6b.

As an additional comment, although the authors seem to have made an effort to make the paper easier to follow in response to reviewer 1 (to what extent I can't tell, since I didn't have access to the first version of the manuscript), it would still be helpful to make their models less abstract by relating them to specific decision-making circuits such as those listed by the authors in their introduction and discussion sections. Can the authors name, when they introduce their model, which genes or pathways would their variables u, v, s, etc represent? This is specially important in the context of the new Fig. 5, which shows two-dimensional simulations that might have little biological realism depending on the system that they are trying to model (e.g. a developing mouse embryo).

We aimed at presenting a generic mechanism that describes how timing of differentiation into reliable cell type proportions, as well as their recovery upon perturbation could be explained through a single dynamical mechanism. We therefore choose to discuss the possible biological circuits in the introduction and the discussion. In that regard, Fig. 5 is also studying systematically different coupling scenarios, giving an overview on the possible spatial organization that can emerge from the proposed mechanism, and how establishing a specific patterns and population growth are related. As the diffusion range of the signaling molecules in different early development systems might be different, we therefore prefer to refrain from making a direct relation to a specific biological system. To the best of our knowledge, only the basic characteristics of a coupling range as well as the pattern features for example in early mouse development have been discussed in the literature (Saiz et al., 2020; Mathew et al., 2019; Fischer et al., 2020), and we therefore would like to limit this discussion in light of the available observations in the discussion section to avoid possible overinterpretation or speculation, but also to maintain a broader theoretical description demonstrating the possibilities of the proposed mechanism.

Finally, I would suggest that these new results are discussed in the context of an earlier paper, published previously by the last author (Koseska et al 2009, PMID 1928306), in which she studied the role of stochasticity in a model closely related to the ones considered here. Since the authors perform stochastic simulations of their models in this manuscript, I wonder if noise effects similar to those published in the 2009 paper could arise in these models.

The Koseska et al., 2009 manuscript describes how a noisy population of coupled switches can make reliable decisions as the population grows in size, as the noise scales with the size of the population as 1/sqrt(N).
One of the main differences of the current with the mechanism presented in the 2009 study is the sub-critical organization. This allows us to approach the observations in development systems more closely, by additionally explaining in a single mechanism the existence of a stable mlp state and the transition to reliable proportions of differentiated cell types, and how these can be recovered upon perturbation. As the mlp state is a homogenous steady state, there is no stochastic switching as in a bistable system, but in contrast, we demonstrate that the mlp state is stable even in the presence of noise.

Reviewer 2 Advance Summary and Potential Significance to Field: Already provided the first time I reviewed this paper
Reviewer 2 Comments for the Author:
I'd like to thank the authors for exhaustively addressing my comments. This version of the manuscript reads well and is clearly structured. The methods of the paper are now presented early on and should not be too difficult for the readers of Development understand. In addition, the extra simulations ground the author's claims.
In my opinion, the findings presented in this paper constitute an important contribution to the field by highlighting the effect that cell population size and growth might play in differentiation, offering a much needed link between morphogenesis and pattern formation. I would be happy to see this paper published in Development.

We thank the referee for his assessment and once again, for his detailed and insightful comments and suggestions that helped us to revise the manuscript to the current format.

Third decision letter

MS ID#: DEVELOP/2020/197608

MS TITLE: Robustness and timing of cellular differentiation through population-based symmetry breaking

AUTHORS: Angel Stanoev, Christian Schroeter, and Aneta Koseska
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed satisfactorily the reviewers' comments, in my opinion.

Comments for the author

No further comments.