Single-minded 2 is required for left-right asymmetric stomach morphogenesis

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MS TITLE:*Single-minded 2* is required for left-right asymmetric stomach morphogenesis

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I have now received two 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, the referees express considerable interest in your work, but have some criticisms and suggestions for improvement. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

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This study by Wyatt et al. investigates the role of the transcription factor Sim2 during stomach morphogenesis. They find that Sim2 is asymmetrically expressed during stomach development downstream of left-right patterning cues.
With morpholino knockdown and CRISPR/Cas9 F0 mutagenesis they author show that Sim2 is required for normal gut development and LR patterning – although the mechanisms are not really addressed. The data are high quality and supporting the authors' conclusions. In principle this could be an important advance in our understanding a novel mechanism downstream of the core LR patterning pathway. While appreciating that this is a brief report, there is none the less, not much mechanistic insight into how sim2 regulates cell behaviour during stomach development. It would be important to provide some insight into the primary defect of the sim2 loss of function phenotypes.

Comments for the author

Major points
1.In its current form the study is very descriptive. The specific phenotype of epithelial cell disorganization in sim2 morphant and CRISPR mutant stomachs is unclear as the cells generally seem very large, rounded, and disorganized (Figure 3). The authors observe changes in cell-cell adhesion markers, cell shape, and epithelial organization. However, it is unclear if any of this is the primary defect. In some ways the cells just look like they are sick and dying. Can more insight be provided about when this defect first appears and what the primary defect is? Have changes in proliferation and apoptosis rates been ruled out as a mechanism? Can the cellular phenotype be rescued by adding back sim2 RNA? Appreciating that this is a brief report it is not expected that they completely work out the mechanism, but if they can point to a more specific direct function this would increase enthusiasm.

2.The validation of the Sim2-MO and CRISPR/Cas9 could be improved. There are many commercial antibodies to Sim2. Do any of these work in Xenopus to show loss of the endogenous protein – IF or western - this really is the gold standard. The Sim2 morpholino has been validated using tagged-sim2 morpholino resistant RNA. Does this also rescue the cellular phenotype of the MO and the sim2 mutant? Show and/or report that right-sided injection of the MO and CRISPR reagents has no phenotype. These additional controls would all strengthen the evidence of specific loss of function.

Minor points
1.When is Sim2 first expressed in the gut epithelium? A developmental profile would be helpful. Some comments on when and where Sim2 is expressed in mammals would also be helpful.

2.Insight into its potential transcriptional targets that may be related to cell polarity and cell-cell adhesion would provide mechanistic insight into what Sim2 is directly regulating and how its disruption leads to the stomach epithelial phenotype.

3.Figure 1G: the text reads (lines 98-102) that sim2 is bilaterally expressed in organs like the lungs, pronephros, etc. however the in situ hybridization sections in Figure 1G show expression in the lungs and pronephros on only one side of the embryo.

Reviewer 2

Advance summary and potential significance to field

A still unresolved issue in the field of left-right (LR) asymmetric development is what happens at the tissue level after the cascade initiated by Nodal and Pitx2 establishes laterally distinct signaling. In this paper, the researchers are able to identify one gene, Sim2, that acts at the organ level during stomach development in amphibians. They use a suite of manipulations to inhibit and mis-express this candidate, and demonstrate that it has a role in LR asymmetric development with a measurable phenotype. They also convincingly demonstrate that it is a member of the classical LR cascade downstream of Pitx2. Figures are clear and well-annotated, making them easy to understand and the conclusions evident. This work significantly advances the field and will be of interest to vertebrate developmental biologists and those interested in morphogenesis more broadly. It is appropriate for Development. However, there are a number of issues that should be addressed prior to publication.

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Comments for the author

1) Lines 162–164 describe the change in cellular morphology between the sim2 CRISPants and the controls. Is this contrasting phenotype consistent with what is seen in the Pitx2 knockouts? Does only removing sim2 recreate the phenotype at the cellular morphology level as the loss of a member at the beginning the whole cascade? This is to say, does the single gene convey all of the asymmetric information, or are there likely other players yet to be described?

2) Lines 201–202 state that "Interestingly, sim2 is insufficient to drive stomach curvature itself." However, the experiment cited—overexpression of Sim2 on the right side of the stomach—is insufficient to make this claim. An additional experiment in which Sim2 is overexpressed on the right and inhibited on the left (using one of the techniques in the paper that allows for spatial specificity in expression manipulation) could yield informative results.

3) This paper is eager to draw parallels across animals and claim that the role of sim2 in directing laterally asymmetric development is "deeply conserved." While the paper explores the role of this gene in frogs and cites evidence that its homolog is necessary for asymmetric midgut development in flies, the connections they draw to humans require more evidence. They state on lines 239-240 that "Sim2 is also expressed in the human embryo stomach (although whether it is left-sided is unknown; Rachidi et al., 2005)". The conservation argument would be aided with the presence of expression data (e.g. in situ hybridizations in mouse) showing whether or not the mammalian SIM2 shows LR asymmetric expression in the gut or elsewhere. In addition, as sim2 was found to be symmetrically expressed in frog lungs, it would be interesting to see if this tissue (symmetric in frog but asymmetric in mouse) shows changes in gene expression in the mouse. Conversely, the role of sim2 as a LR asymmetry gene may be conserved in its specificity to the gut.

4) Even if additional data were provided regarding human/mammalian function, to call the parallel observations in flies and vertebrates "conserved" is not warranted. "Deep homology" is term applied to homologous genetic circuitry in analogous – but not strictly homologous – settings. When it is not a pathway used to a similar end, but rather the activity of a single gene, it is much more appropriate to frame it as convergent cooption of sim2/singleminded in the two settings. (If the fly utilized a pathway including Nodal -> Pitx2 -> Sim, the suggestion of deep conservation would be more justified).

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

Major points

1. In its current form the study is very descriptive. The specific phenotype of epithelial cell disorganization in sim2 morphant and CRISPR mutant stomachs is unclear as the cells generally seem very large, rounded, and disorganized (Figure 3). The authors observe changes in cell-cell adhesion markers, cell shape, and epithelial organization. However, it is unclear if any of this is the primary defect. In some ways the cells just look like they are sick and dying. Can more insight be provided about when this defect first appears and what the primary defect is? Have changes in proliferation and apoptosis rates been ruled out as a mechanism? Can the cellular phenotype be rescued by adding back sim2 RNA? Appreciating that this is a brief report it is not expected that they completely work out the mechanism, but if they can point to a more specific direct function this would increase enthusiasm.

We now include NEW data addressing these salient points:

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(1) First, we show that *sim2*-MO injected cells remain proliferative through curvature (see NEW Figure S4A-B), confirming the cells are not dying early, and suggesting *sim2*-deficient curvature phenotypes are initiated independently of any decrease in cell number. We now mention these results in the text (lines 183-5).

(2) We show that, even *prior to obvious morphological curvature* (NF37), *sim2*-MO injected cells display round shapes and irregularly distributed adhesion markers, coinciding with aberrant MT orientation (see NEW Figure S4C-J). These alterations in cell properties suggest that *sim2*-deficient cells become more mesenchymal, a phenotype consistent with the known role of Sim2 in regulating epithelial-mesenchymal plasticity in other contexts. Indeed, in some *sim2*- MO injected cells, □-catenin appears to become localized to the nucleus (e.g., cells with dotted outlines in NEW Figure S4H-I)—a state associated with epithelial to mesenchymal transition (e.g., *Sci Rep* 9, 18440, 2019)—in contrast to control cells, where □-catenin is predominantly localized to the membrane (Figure S4D-E). We now mention these results in the text (lines 193-7).

(3) We show that the entire suite of cellular phenotypes caused by the *sim2*-MO are rescuable by exogenous, morpholino-resistant *sim2* mRNA (NEW Figure S4K-V), suggesting that Sim2 affects multiple inter-related cell properties and behaviors involved in epithelial morphogenesis/plasticity. We now mention these results in the text (lines 200-202).

2. The validation of the Sim2-MO and CRISPR/Cas9 could be improved. There are many commercial antibodies to Sim2. Do any of these work in Xenopus to show loss of the endogenous protein – IF or western - this really is the gold standard. The Sim2 morpholino has been validated using tagged-sim2 morpholino resistant RNA. Does this also rescue the cellular phenotype of the MO and the sim2 mutant? Show and/or report that right-sided injection of the MO and CRISPR reagents has no phenotype. These additional controls would all strengthen the evidence of specific loss of function.

We are certainly aware that IF/Western is the gold standard for MO validation, and we did try multiple commercial antibodies (in Western and IF, plus and minus antigen retrieval) predicted to detect the *Xenopus* epitope, but were unable to find a suitable reagent that didn't cause impracticable artifacts. Therefore, in lieu of Sim2 antibody-based methods, we validated our MO results with three complementary strategies:

1) we confirmed that 2 different *sim2*-MOs, targeting different regions of the *sim2* mRNA, elicit similar phenotypes (Figure S3A);
2) we showed that the level of translation (fluorescence) of a GFP transcript fused with the *sim2*-MO target site is reduced *in vivo* (both visibly and by Western with a GFP antibody) upon co-injection with the *sim2*-MO (Figure S3B); and
3) we showed that we can rescue the *sim2*-MO phenotype at both the gross anatomical (Figure S3C) and cellular (NEW Figure S4K-V) level by co-injection of MO-resistant *sim2* mRNA. [We now mention the cellular rescue in the text (lines 200-202).]

It is also important to note that our distinct (MO and CRISPR) reagents phenocopy each other, providing additional confidence that the phenotype is specific to loss of Sim2 function.

We now include additional control data (NEW Figure S3D) confirming that, as expected, right sided injection of *sim2* MO has virtually no effect on stomach curvature. (The *sim2* CRISPR is injected at the one-cell stage, so side-specific injections are not relevant for the gene-editing experiment.) We now mention this additional control in the text (lines 171-2).

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Minor points

1. When is Sim2 first expressed in the gut epithelium? A developmental profile would be helpful. Some comments on when and where Sim2 is expressed in mammals would also be helpful.

We now include additional RNA *in situ* hybridization data documenting our earliest observations of asymmetrical expression of *sim2* in the left side of the *Xenopus* stomach (NF32; NEW Figure S1). This early *sim2* domain overlaps with the well- characterized asymmetrical expression of *pitx2* (which we and others have shown begins ~NF24; not shown), as it does later (see Figure 1).

We also searched for relevant *single-minded* gene expression patterns in other vertebrate species on MGI, Geisha, Zfin, etc. but could not find tissue sections at appropriate stages, informative angles, and/or through the stomach, so we are unable to rule in or out whether *Sim2* is expressed asymmetrically in other animal models. The few published images of *Sim2* expression in sections through the human stomach are oriented sagittally, so there is no way to rule in or out whether the expression is LR asymmetric.

2. Insight into its potential transcriptional targets that may be related to cell polarity and cell-cell adhesion would provide mechanistic insight into what Sim2 is directly regulating and how its disruption leads to the stomach epithelial phenotype.

Others have identified potential direct targets of Sim2 in cultured cells (*PLoS ONE* 10(5): e0126475). Excitingly, some of these genes are among those we have found to be LR asymmetrically expressed in the frog stomach, including a well-known EMT marker (e.g., vimentin) and Zic2, a molecule previously associated with earlier phases of LR development in mammals (*Sci Rep* 2018 8(1):10439; *Genesis*. 2013, 52 (6): 626–35). We now mention these potential Sim2 targets in the text (line 232-236), and look forward to following up on the function of these and other molecules in stomach morphogenesis in future studies.

3. Figure 1G: the text reads (lines 98-102) that sim2 is bilaterally expressed in organs like the lungs, pronephros, etc. however the in situ hybridization sections in Figure 1G show expression in the lungs and pronephros on only one side of the embryo.

Because these organs are small relative to the stomach, and it is not always possible to get sections that are oriented exactly transversely, identical regions of both the left and right lungs and pronephric tubules may not be visible in every section.
Indeed, this is why we show multiple sections through the stomach (e.g., Figure 1), i.e., to convincingly demonstrate that any asymmetries observed in one section are not the result of a skewed section angle, and that *sim2* is indeed expressed LR asymmetrically through the entire organ. We now include additional images which fortuitously include both left and right pronephric tubules (NEW Figure S1E) and left and right lungs (NEW Figure S1F) in the same section, to better illustrate that *sim2* is, in fact, expressed bilaterally in these organs.

Reviewer 2 Comments for the Author:
1) Lines 162–164 describe the change in cellular morphology between the sim2 CRISPants and the controls. Is this contrasting phenotype consistent with what is seen in the Pitx2 knockouts? Does only removing sim2 recreate the phenotype at the cellular morphology level as the loss of a member at the beginning the whole cascade? This is to say, does the single gene convey all of the asymmetric information, or are there likely other players yet to be described?

Loss of Sim2 function does seem to disrupt endoderm cell morphology in a very similar manner as loss of Pitx2 function (i.e., as previously published in Davis et al., 2017). We now mention this in the text (line 227-228). This result is consistent with *sim2* being asymmetrically expressed in the left foregut at very early stages of stomach morphogenesis (i.e., by NF32; see NEW Figure S1), overlapping with the well-characterized left *pitx2* domain, well before organ curvature becomes evident at NF39.

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Interestingly, Sim2 has been hypothesized to associate with pioneer factors and bind super-enhancers in other contexts (*PLoS ONE* 10(5): e0126475), thus, this molecule is a good candidate for coordinating multiple downstream events. We now mention this point in the text (line 239-240).

2) Lines 201–202 state that "Interestingly, sim2 is insufficient to drive stomach curvature itself." However, the experiment cited— overexpression of Sim2 on the right side of the stomach—is insufficient to make this claim. An additional experiment in which Sim2 is overexpressed on the right and inhibited on the left (using one of the techniques in the paper that allows for spatial specificity in expression manipulation) could yield informative results.

We previously showed that ectopic Pitx2 activity on the right inhibits stomach curvature, causing the stomach to be straightened (Davis et al., 2017), although not reversed. This suggests that the strongest phenotypic readout we might expect from ectopically expressing factors downstream of Pitx2, such as Sim2, would also be straightening. However, we found that overexpression of Sim2 on the right has no effect on stomach morphology at all, i.e., neither straightening nor even a more shallow curvature. In other words, unlike Pitx2, ectopic expression of Sim2 has no effect within the right stomach wall on its own. Therefore, the experiment cited—overexpressing Sim2 on the right side while inhibiting it on the left—would, in fact, be uninformative regarding whether this factor alone is sufficient to drive stomach curvature (i.e., the phenotype would be predicted to be identical to merely inhibiting Sim2 on the left). As we suggest in the discussion, it is possible that Sim2 requires other conditions or bHLH co-factors present only on the left side (e.g., perhaps also downstream of Pitx2).

3) This paper is eager to draw parallels across animals and claim that the role of sim2 in directing laterally asymmetric development is "deeply conserved." While the paper explores the role of this gene in frogs and cites evidence that its homolog is necessary for asymmetric midgut development in flies, the connections they draw to humans require more evidence. They state on lines 239-240 that "Sim2 is also expressed in the human embryo stomach (although whether it is left-sided is unknown; Rachidi et al., 2005)". The conservation argument would be aided with the presence of expression data (e.g. in situ hybridizations in mouse) showing whether or not the mammalian SIM2 shows LR asymmetric expression in the gut or elsewhere. In addition, as sim2 was found to be symmetrically expressed in frog lungs, it would be interesting to see if this tissue (symmetric in frog but asymmetric in mouse) shows changes in gene expression in the mouse. Conversely, the role of sim2 as a LR asymmetry gene may be conserved in its specificity to the gut.

These are, of course, fascinating questions that we would love to address. However, my lab does not house mammalian models and, with current (COVID-restriction-based) limited access to our own and potential mouse collaborators' labs, we cannot do a thorough enough analysis of mouse embryo expression to rule in or rule out asymmetry in the stomach and/or lungs in a reasonable period of time for resubmission. (We have only within the last 2 weeks been allowed to have the entire staff in our own lab space at one time.) We did search public databases (MGI, Geisha, Zfin, etc.) for relevant *single-minded* gene expression patterns in multiple other vertebrate species but could not find tissue sections at appropriate stages, informative angles, and/or through the stomach, so we are unable to rule in or out whether *Sim2* is expressed asymmetrically in other animal models. The few published images of *Sim2* expression in sections through the human stomach are oriented sagittally, so there is no way to rule in or out whether the expression is LR asymmetric at this time. Nonetheless, we recognize that the expression patterns of *single-minded* genes in other vertebrates, particularly mammals, is highly relevant to our hypotheses regarding evolutionary conservation and the potential human relevance of our discovery, so we have edited the text to reflect this important caveat (line 255-257).

4) Even if additional data were provided regarding human/mammalian function, to call the parallel observations in flies and vertebrates "conserved" is not warranted. "Deep homology" is term applied to homologous genetic circuitry in analogous – but not strictly homologous – settings. When it is not a pathway used to a similar end, but rather the activity of a single gene, it is much more appropriate to frame it as convergent cooption of sim2/singleminded in the two settings. (If

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the fly utilized a pathway including Nodal -> Pitx2 -> Sim, the suggestion of deep conservation would be more justified).

We regret our overeager deep homology arguments and thank the reviewer for the redirection. We have edited the abstract to eliminate unsubstantiated speculation and now offer a more tempered suggestion of convergent cooption (line 209-210).

Second decision letter

MS ID#: DEVELOP/2020/199265

MS TITLE: *Single-minded 2* is required for left-right asymmetric stomach morphogenesis

AUTHORS: Brent H Wyatt, Nirav M Amin, Kristen Bagley, Dustin M Wcisel, Michael Dush, Jeffrey A Yoder, and Nanette M. Nascone-Yoder

ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. The referee reports on this version are appended below.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed my original criticisms with additional data and revisions to the test. This is an excellent brief report describing a novel role for the transcription factor Sim2 in L-R asymmetry.

Comments for the author

I am satisfied with the revisions - well done.

Reviewer 2

Advance summary and potential significance to field

A still unresolved issue in the field of left-right (LR) asymmetric development is what happens at the tissue level after the cascade initiated by Nodal and Pitx2 establishes laterally distinct signaling. In this paper, the researchers are able to identify one gene, Sim2, that acts at the organ level during stomach development in amphibians. They use a suite of manipulations to inhibit and mis-express this candidate, and demonstrate that it has a role in LR asymmetric development with a measurable phenotype. They also convincingly demonstrate that it is a member of the classical LR cascade downstream of Pitx2. Figures are clear and well-annotated, making them easy to understand and the conclusions evident. This work significantly advances the field and will be of interest to vertebrate developmental biologists and those interested in morphogenesis more broadly.

Comments for the author

The authors have adequately addressed the concerns I previously raised. In my view this nice contribution is ready and appropriate for publication.

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