RYK-mediated filopodial pathfinding facilitates midgut elongation

Sha Wang, James P. Roy, Abigail J. Tomlinson, Ellen B. Wang, Yu-Hwai Tsai, Lisa Cameron, Julie Underwood, Jason R. Spence, Katherine D. Walton, Steven A. Stacker, Deborah L. Gumucio and Terry Lechler
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MS TITLE: RYK-mediated filopodial pathfinding facilitates midgut elongation

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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.

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, Wang et al. took approaches including mouse genetics and live imaging to investigate the Wnt5a mediated gut elongation process. They found that loss of the Wnt-5a receptor Ryk partially recapitulated the phenotypes of Wnt5a-/- mutants. Ryk-/- mutants also showed multiple phenotypes including impaired filopodial growth, increased apoptosis, and

shortened midgut length . In addition, they demonstrated that epithelial Ror2, another Wnt5a receptor, is dispensable for midgut elongation, while mesenchymal Ror2 is required during the primordial stage. They conclude that Wnt5a-Ryk-mediated filopodial pathfinding facilitates midgut elongation. Many discoveries in the current study are based on their previous findings (Wang et al., 2018, Development Cell). Although the data is clear, this story will be further strengthened if following questions can be addressed.

Comments for the author

Major concerns:
1. It will be helpful if the authors examine the function of Ryk in the epithelium using Shh-Cre; Rykf/f mice. Results from Ryk-/- mice cannot rule out the possibility whether Ryk also functions in the mesenchyme.
2. One key question in this manuscript is whether filopodial protrusion is necessary for "pathfinding" or just a random cell behavior in the basal connection process. Experiments like inhibition of filopodial growth will further strength their conclusions.
3. Ryk-/- mutants partially phenocopies the Wnt5a-/- mutants. The minor basal connection defects in Wnt5a-/- (13% in Wnt5a-/- vs. 8.9% in Ryk-/- large clones) may not fully explain the severe midgut length defects (24% of control in Wnt5a-/- vs. 63.8% of control in Ryk-/-). Does the other ligand Ror2 in mesenchyme functions prior to E10.5 ?
4. The author claimed that increased apoptosis is secondary to the pathfinding-failure, which was already mentioned in the previous findings (Wang et al., 2018, Development Cell). The authors should provide further evidence to clarify the relationship of pathfinding-failure cell and the apoptosis cell.

Minor concerns:
1. The proliferation ratio at E10.5 should be provided in Figure 3X.
2. 'Ryk depletion indeed only perturbs the "pathfinding" return' in page 10 is overstated.
3. Some figure numbers are missing, for example, Figure 5B 7&8 and Figure 5B'7&8.

Reviewer 2

Advance summary and potential significance to field

This well-written manuscript by Wang et al. seeks to better understand how Wnt5A regulates the lengthening of the embryonic small intestine.

The study is focused on the analysis of conditional mouse mutants lacking Ror2 and/or Ryk in the epithelial or mesenchymal compartment of the developing intestine at different stages of gut development, in order to determine whether/when each of these candidate Wnt5A receptors might be required for gut elongation, and in which tissue layer of the gut tube. The results show convincingly that Ryk, but not Ror2, is required in the gut epithelium for Phase 1 elongation. (Although Ror2 was previously shown to be required for gut elongation, Wang et al.'s results serve to illuminate the timing and tissue-specificity of Ror2 in the process.)

The manuscript also characterizes the shortened intestine phenotype at the cellular level, building on a very nice previously published study showing that Wnt5A controls an interkinetic nuclear migration (IKNM) mode of proliferation in some cells of the developing intestine, and is required for the growth and maintenance of filopodial protrusions on these apically dividing cells. (They previously showed that pre-existing protrusions tether one daughter cell to the basal side of the epithelium, providing a "conduit" for this daughter to return basally, while the other daughter must generate a new protrusion to "pathfind" its way back to the basal surface. This previous study also revealed that loss of Wnt5A signaling slows gut elongation due to filopodial navigation errors, and subsequent apoptosis, in daughter cells that fail to re-tether.)

In the present study, Wang et al. use live imaging of cultured intestines to provide a higher temporal resolution and more dynamic description of the IKNM cellular events involved in Wnt-deficient intestinal lengthening. Both static and dynamic (ex vivo) imaging indicate that Ryk is required for proper filopodial navigation as Ryk loss of function leads to several interesting

categories of aberrant cellular behavior, resulting in daughter cells being unable to tether to the basal surface, remaining apical, and eventually dying by apoptosis.
Comparison of Ryk cellular phenotypes with those found in Wnt5A mutants (and Ror2/Ryk double mutants) suggests Ryk is indeed the salient receptor facilitating the Wnt5A-mediated IKNM process. As in the previous paper, mathematical modeling simulations confirm that the resultant apoptosis of apical/untethered daughter cells alone can account for the observed reduced intestine length.

The figures and analyses by Wang et al., are all very well done (qualitatively and quantitatively) and nicely illustrate the key conclusions of the manuscript regarding the role of Ryk in early intestine elongation, convincingly identifying Ryk as a likely receptor involved in the process. Overall, this is a solid, well executed study with significant conclusions for the field of gut morphogenesis.

Comments for the author

I have only very minor suggestions:

1) The Discussion would be strengthened by further addressing the implications of the observed distinct spatiotemporal requirements of Ror- vs Ryk-mediated signaling in gut lengthening, e.g., multiple morphogenetic events and tissue layers are likely to contribute to the overall process of gut elongation.

2) The relatively small population of navigation-deficient cells found in Ryk/Wnt5A mutant intestines suggests that other, still unknown, possibly even non-Wnt-mediated, pathways or molecules may also play an essential role in normal gut elongation (via IKNM or non-IKNM mediated mechanisms). This should also be addressed in the Discussion.

First revision

Author response to reviewers' comments

We extend a sincere “thank you” to you and the reviewers for the positive and constructive comments on our manuscript “*RYK-mediated filopodial pathfinding facilitates midgut elongation*”. We have modified the manuscript in accord with these concerns. All new changes to the manuscript are highlighted in red, while underlined areas indicate existing text that also pertain to reviewers’ concerns. A point-by-point response to all comments is provided below:

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, Wang et al. took approaches including mouse genetics and live imaging to investigate the Wnt5a mediated gut elongation process. They found that loss of the Wnt-5a receptor Ryk partially recapitulated the phenotypes of Wnt5a-/- mutants. Ryk-/- mutants also showed multiple phenotypes, including impaired filopodial growth, increased apoptosis, and shortened midgut length. In addition, they demonstrated that epithelial Ror2, another Wnt5a receptor, is dispensable for midgut elongation, while mesenchymal Ror2 is required during the primordial stage. They conclude that Wnt5a-Ryk-mediated filopodial pathfinding facilitates midgut elongation. Many discoveries in the current study are based on their previous findings (Wang et al., 2018, Development Cell). Although the data is clear, this story will be further strengthened if following questions can be addressed.

Reviewer 1 Comments for the Author:

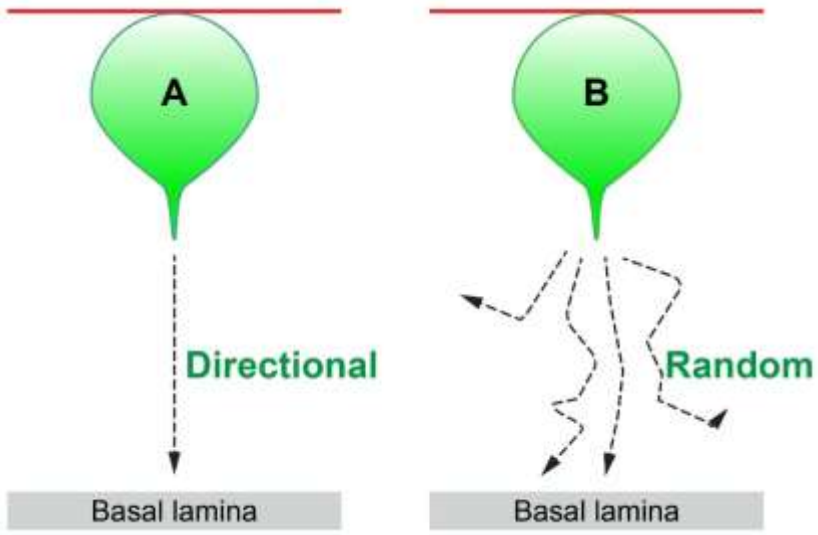
Major concerns:
1. It will be helpful if the authors examine the function of Ryk in the epithelium using Shh-Cre; Rykf/f mice. Results from Ryk-/- mice cannot rule out the possibility whether Ryk also functions in the mesenchyme.

We fully agree that tissue-specific RYK depletion using *Shh*^{Cre}; *Ryk*$^{fl/fl}$ or *Twist2*^{Cre}; *Ryk*$^{fl/fl}$ mice would directly address this question. However, when we were carrying out this project, *Ryk*$^{fl/fl}$ mice were not available in our lab nor in JAX. In December 2019, Dr. Yuko Nakamichi generated a *Ryk*$^{fl/fl}$ mouse strain (Kim et al., 2019), but only ES cell lines are available at this time (http://www.findmice.org/summary?gaccid=MGI:101766)*. It would require a significant amount of time for us to import these cells, generate the mouse lines and perform the suggested experiments. Therefore, in the discussion, we point out that, as the reviewer notes, "experiments with constitutive *Ryk* knockout mice alone cannot definitively exclude an additional role for mesenchymal RYK signaling related to midgut elongation" (line 352-354, underlined). We also explain why our data strongly suggest that RYK function is critical in the epithelium during Phase I midgut elongation (line 354-360, underlined). Finally, in the schematic summary (Fig. 7F), we marked the role of mesenchymal Ryk in Phase I elongation as not determined (ND).

*Update: on September 7th, 2020 (after the submission of this letter), Dr. Nakamichi informed us that *Ryk*$^{fl/fl}$ mice are available in her laboratory (in Japan).

2. *One key question in this manuscript is whether filopodial protrusion is necessary for "pathfinding" or just a random cell behavior in the basal connection process. Experiments like inhibition of filopodial growth will further strength their conclusions.*

This is a good point that deserves additional explanation. Theoretically, making a basal connection could be accomplished by directionally oriented filopodial growth (pathfinding via some directional cue) or filopodial growth without a direction (random growth) with eventual "connection" of the filopodium with the basal lamina. In both cases, filopodial growth in length is essential since the nucleus of the newly born daughter is apical and cannot move basally until a basal connection is established.



After observing thousands of cells, including both fixed cells (Fig. 5B,B') and live cells (Fig. 6B,C), we find that, in the control midgut epithelium, the singular growing filopodium emanates from the very basal end of the cell body and consistently grows radially toward the basal lamina with high fidelity, indicating that filopodial projection is not a random behavior but is basally-oriented.

In contrast, in the absence of RYK or WNT5A, some cells fail to form a filopodium (Fig. 5D,H2); some cells project more than one filopodial protrusion beyond the very basal end of the cell body (Fig. 5E,H3,J1,J2); some cells project filopodia of various lengths in random directions (Fig. 5E1,2,3,4,5,8; I3;J1,3). These diverse defects (summarized in Fig. 7G) cannot be simply explained by a failure of filopodial growth alone. Many known attractive guidance cues in neurons act by promoting selective extension of the filopodia towards the cue, whilst ensuring the formation of filopodia is decreased in other directions (Stoeckli, 2018; Ye et al., 2019). The defects observed in *Ryk*$^{-/-}$ *or Wnt5a*$^{-/-}$ epithelia are most likely the consequence of missing

attractive guidance cues.

Inhibition of filopodial growth to further explore these behaviors is an excellent idea, which we did consider. Based on the knowledge of filopodial growth in other systems, treatment with an Arp2/3 inhibitor (CK666) or Formin inhibitor (SMIFH2) that blocks actin polymerization could inhibit filopodial formation/growth (Hetrick et al., 2013; Rizvi et al., 2009; Yang and Svitkina, 2011). However, both of these inhibitors can also perturb cell divisions (Isogai et al., 2015; Sun et al., 2011) and affect nuclear trafficking as well (Thiam et al., 2016; Yanakieva et al., 2019; Zhang et al., 2019), which makes them less than ideal tools, especially for the highly proliferative gut epithelium. Since it has been reported that Cdc42 can induce filopodial formation (Krugmann et al., 2001) and control filopodial activity in axonal growth (Kim et al., 2002), we did test the role of Cdc42 in the pseudostratified midgut epithelium using *Shh*$^{Cre/+}$; *Cdc42*$^{fl/fl}$ and *Shh*$^{CreERT2/+}$; *Cdc42*$^{fl/fl}$; *Rosam*$^{TmG/+}$ mice, but no obvious defects in midgut length nor filopodial behaviors were seen (data not reported).

3. Ryk-/- mutants partially phenocopies the Wnt5a-/- mutants. The minor basal connection defects in Wnt5a-/- (13% in Wnt5a-/- vs. 8.9% in Ryk-/- large clones) may not fully explain the severe midgut length defects (24% of control in Wnt5a-/- vs. 63.8% of control in Ryk-/-). Does the other ligand Ror2 in mesenchyme functions prior to E10.5?

The reviewer is correct that the basal connection defects (13% in *Wnt5a*$^{-/-}$ vs. 8.9% in *Ryk*$^{-/-}$ large clones) do not fully explain the severe midgut length defects at E14.5. This is because the large clonal data only reflects the subset of defects that occur between E11.5/12 and E14.5 (tamoxifen is given at E11.5 or 12). In contrast, in the constitutive knockouts, the severe midgut length defects (24% of control in *Wnt5a*$^{-/-}$ vs. 63.8% of control in *Ryk*$^{-/-}$)" were measured at E14.5, and therefore include all defects that occurred in both the primordial stage and entire Phase I.

However, as we discussed, minor basal connection defects in Phase I can indeed lead to amplified effects in SI length because, during Phase I, nearly all epithelial cells undergo continuous cell cycling to expand the epithelial population (and length) (line 431-433, underlined). An average 16 hr-long cell cycle (Wang et al., 2018) ensures that one cell starting at E10.5 will undergo 5–6 cell divisions by E14.5, creating 32-64 progeny. Because of this exponential expansion, losing a relatively minor proportion of cells due to failed basal re-connection in Phase I can have a robust effect on overall SI length, as illustrated by the mathematical modeling shown in Fig. 7E.

Yes, the reviewer is correct that mesenchymal ROR2 plays a role in gut elongation before E10.5; these data are illustrated in Fig. 2 and summarized in Fig. 7F.

4. The author claimed that increased apoptosis is secondary to the pathfinding-failure, which was already mentioned in the previous findings (Wang et al., 2018, Development Cell). The authors should provide further evidence to clarify the relationship of pathfinding-failure cell and the apoptosis cell.

We would argue that the live imaging data provide a very clear relationship between pathfinding failure and apoptosis. With a longer recording times, we observed repeatedly that those post-mitotic cells that fail to return in time eventually fragment and disappear (indicated by yellow arrowheads in Fig. 6F,N), a sign of apoptosis. In contrast, labeled cells that manage to return in time very rarely enter apoptosis. We point out in the text that "we observed that those pathfinding daughters that fail to return in time eventually fragmented, a sign of apoptosis, in both Mode I and II" (line 311-312, underlined) and "live imaging indicates that failure of the pathfinding process cell leads to apoptosis (Fig. 6F,N)" (line 321-322, underlined).

In fixed daughter pairs of *Ryk*$^{-/-}$ epithelium, we also observed cases in which one daughter exhibits pathfinding defects and contains small vesicles, a clear sign of a dying cell, while the other (connected) daughter is intact (Fig. 5E8), as well as cases in which both basally-unconnected daughters are fragmented (Fig. 5I5). Finally, we used the apoptosis marker Cleaved Caspase 3 on cross-sections of *Ryk*$^{-/-}$ midguts (Fig. 7B',C'). These studies also reproducibly showed that apoptotic fragments were increased preferentially at the apical side or in the lumen, a finding fully consistent with the idea that cells which fail to establish a basal connection cannot return their nucleus to the basal side and eventually undergo apoptosis, as

illustrated in Fig. 7G.

Minor concerns:
1. The proliferation ratio at E10.5 should be provided in Fig. 3X.
Thank you for this suggestion. The quantification of mitosis ratio at E10.5 is now included in Fig. 3X.

2. *'Ryk depletion indeed only perturbs the "pathfinding" return' in page 10 is overstated.*
This may be a wording issue. After observing the dynamics of live Mode I pairs and analyzing the returning time of conduit cells and pathfinding cells (Fig. 6R), no obvious abnormalities in conduit cells' behaviors were noted. Therefore, we concluded that "Ryk depletion indeed only perturbs the 'pathfinding' return". In light of the reviewer's concern, we have now adjusted this statement to read: "*Ryk* depletion perturbs the "pathfinding" return, but not the "conduit" return" (line 314-315).

3. *Some Fig. numbers are missing, for example, Fig. 5B 7&8 and Fig. 5B'7&8.*
We apologize for this omission. These missed figure numbers have now been added.

Reviewer 2 Advance Summary and Potential Significance to Field:
This well-written manuscript by Wang et al. seeks to better understand how Wnt5A regulates the lengthening of the embryonic small intestine.

The study is focused on the analysis of conditional mouse mutants lacking Ror2 and/or Ryk in the epithelial or mesenchymal compartment of the developing intestine at different stages of gut development, in order to determine whether/when each of these candidate Wnt5A receptors might be required for gut elongation, and in which tissue layer of the gut tube. The results show convincingly that Ryk, but not Ror2, is required in the gut epithelium for Phase 1 elongation. (Although Ror2 was previously shown to be required for gut elongation, Wang et al.'s results serve to illuminate the timing and tissue-specificity of Ror2 in the process.)

The manuscript also characterizes the shortened intestine phenotype at the cellular level, building on a very nice previously published study showing that Wnt5A controls an interkinetic nuclear migration (IKNM) mode of proliferation in some cells of the developing intestine, and is required for the growth and maintenance of filopodial protrusions on these apically dividing cells. (They previously showed that pre- existing protrusions tether one daughter cell to the basal side of the epithelium, providing a "conduit" for this daughter to return basally, while the other daughter must generate a new protrusion to "pathfind" its way back to the basal surface. This previous study also revealed that loss of Wnt5A signaling slows gut elongation due to filopodial navigation errors, and subsequent apoptosis, in daughter cells that fail to re- tether.)

In the present study, Wang et al. use live imaging of cultured intestines to provide a higher temporal resolution and more dynamic description of the IKNM cellular events involved in Wnt-deficient intestinal lengthening. Both static and dynamic (ex vivo) imaging indicate that Ryk is required for proper filopodial navigation as Ryk loss of function leads to several interesting categories of aberrant cellular behavior, resulting in daughter cells being unable to tether to the basal surface, remaining apical, and eventually dying by apoptosis. Comparison of Ryk cellular phenotypes with those found in Wnt5A mutants (and Ror2/Ryk double mutants) suggests Ryk is indeed the salient receptor facilitating the Wnt5A-mediated IKNM process. As in the previous paper, mathematical modeling simulations confirm that the resultant apoptosis of apical/untethered daughter cells alone can account for the observed reduced intestine length.

The Figures and analyses by Wang et al., are all very well done (qualitatively and quantitatively) and nicely illustrate the key conclusions of the manuscript regarding the role of Ryk in early intestine elongation, convincingly identifying Ryk as a likely receptor involved in the process. Overall, this is a solid, well executed study with significant conclusions for the field of gut morphogenesis.

We appreciate the reviewer's thoughtful and careful analysis of the work.

Reviewer 2 Comments for the Author:

I have only very minor suggestions:

1. The Discussion would be strengthened by further addressing the implications of the observed distinct spatiotemporal requirements of Ror- vs Ryk-mediated signaling in gut lengthening, e.g., multiple morphogenetic events and tissue layers are likely to contribute to the overall process of gut elongation.

Thank you for this suggestion. Due to the word limit (7000 words), we unfortunately do not have the room for much further elaboration on these implications. However, we have added the following sentence to address this point: "The dynamic spatiotemporal requirement for distinct WNT5A receptors, as illustrated here, indicates that fetal gut elongation occurs through precise coordination of signals in both epithelial and mesenchymal compartments in a phased manner" (line 371-373).

2. The relatively small population of navigation-deficient cells found in Ryk/Wnt5A mutant intestines suggests that other, still unknown, possibly even non-Wnt-mediated, pathways or molecules may also play an essential role in normal gut elongation (via IKNM or non-IKNM mediated mechanisms). This should also be addressed in the Discussion.

The reviewer is right. During Phase I, only a small subset of "pathfinding" cells encounter navigation errors while the majority still manage to project a filopodial extension properly in the absence of RYK or WNT5A. This suggests that additional uncharacterized guidance cues, WNT or non-WNT, participate in this process. This key point has been added in the discussion (line 403-405).

Ensuring high fidelity in re-establishing the basal connection for post-mitotic cells is a critical step in IKNM mediated mechanisms. However, theoretically, any glitch in cell division, including IKNM-related epithelial proliferation and non-IKNM related mesenchymal proliferation, could also cause shortened midguts. For example, inactivation of Hedgehog pathway largely reduces mesenchymal proliferation and results in severely shortened guts (Mao et al., 2010). Removal of epithelial FGF9 increases TGFß signaling, which drives the differentiation of mesenchymal cells to myofibroblasts, and impairs gut elongation (Geske et al., 2008). These non-IKNM and non-WNT related gut elongation mechanisms have been previously reviewed (Walton et al., 2016; Wang et al., 2019), thus, in the discussion of this manuscript we did not include these but focused on three aspects: 1) how WNT5A signaling spatially and temporally contributes to midgut elongation by engaging RYK and ROR2, 2) what possible pathways act downstream of WNT5A/RYK guidance to mediate filopodial pathfinding, and 3) the potential clinical implication of our findings.

Again, we thank the reviewers for the thoughtful review and helpful comments. We hope we have addressed all reviewers' questions and concerns appropriately and that you will now find the manuscript suitable for publication in *Development*.

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Second decision letter

MS ID#: DEVELOP/2020/195388

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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 our questions. I suggest publication.

Comments for the author

The authors have addressed our questions. I suggest publication.

Reviewer 2

Advance summary and potential significance to field

The scientific advance and significance of the paper are strong and remain unchanged from my previous review.

Comments for the author

I am satisfied with this revised version, in which the coverage of alternative interpretations and caveats has been improved.