A dorsal-ventral gradient of Wnt3a/β-catenin signals controls mouse hindgut extension and colon formation

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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 BenchPressand 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 significant criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Garriock et al provides new insight into the role of canonical Wnt signaling in intestinal patterning and what distinguished small intestine from colon. While it has long been known that a A-P gradient

of Wnt activity establishes hindgut, this study illustrates a novel differential Wnt activity along the D-V axis of the extreme hindgut with Wnt3a, Bcat and Sp5/8 in the hindgut dorsal mesendoderm being required for colon development. A series of conditional LOF and GOF experiments addresses how, and in which tissues this Wnt activity is required. The data is of high quality and overall the results will be very interesting result to those in the field of gut development.

However there are some concerns that related to the analysis and interpretation that need to be addressed in order to fully support the author's conclusions:

Comments for the author

1. The authors conclude that Wnt/Bcat is required in the dorsal endoderm, but not ventral endoderm for colon formation. An alternative explanation also supported by the data is that Bcat is not required in the endoderm at all to make colon (after its earlier posteriorizing role in defining hindgut). The Wnt3a-/- and T:Cre lines both delete Wnt/Bcat early (at the hindgut A-P patterning step) and in the medoderm as well as dorsal endoderm. It is possible that Wnt3a/Bcat are required in mesoderm to make a second signal (BMP, is a likely candidate). Whereas the effect of ectopic Bcat in the Shh+ endoderm may be unrelated to the requirement of Wnt3a and Bcat in the T+ lineage. Bcat GOF in Shh+ epithelium could result in increased proliferation (like it does in many contexts) even though Bcat is not normally active in these cells. It is important to distinguish these possibilities. What about a Sox2CreER;BcatLOF?

2. The authors need to looked at early colon-specific markers such as Satb2, Trh1, Cpn1 and/or HoxD13.

3. The whole mounts of Cdx1-3 do really show whether early A-P patterning in the epithelium is disrupted, since most of the expression is mesodermal. Sections should be shown.

4. The authors make the general comment in the intro that "the mechanisms underlying hindgut tubulogenesis and extension are poorly understood". Which is sort of true, but there are some important things known such the fact that BMP is crucial for colon (DOI:10.1016/j.stem.2017.05.020), and that non-canonical Wnt and Fgf are important for hindgut morphogenesis (DOI:10.1038/s41586-018-0865-9). These are almost certainly involved in the phenomena being studied here so they should be acknowledged.

5. In figure 1 the authors claim that they have identified a "unique progenitor population that co-expressed Wnt3a, Cdx2, Sox2, T and SP8", but these truly distinct from neuromesodermal progenitors (NMPs) because they do NOT express TBX6, as in the previous work from the Yamaguchi group (Garriock et al 2015) and others (Edri et al 2019 multiple papers from the Briscoe Lab) have shown? If that is correct, then showing the lack of TBX6 or lack of other NMP factors/primitive streak markers) seems appropriate.

Reviewer 2

Advance summary and potential significance to field

Wnt signaling is required for early endoderm development, but whether this pathway is also important for downstream hindgut tubulogenesis and colon formation has not been studied. In this manuscript, Garriock et al. use multiple mouse models to show that Wnt signaling is essential for extension of the hindgut endoderm and colon formation. The phenotype is strong and consistent across each model, which strengthens the data and the conclusions.
The authors also find that Wnt3a is expressed specifically in the dorsal hindgut but that ventral hindgut progenitors contribute to the majority of the colon. Overall, this study is very beautifully presented and the results are of high quality. The findings are significant since they really shed light on colon formation including lineage tracing to show the difference between ventral and dorsal contributions to the colon.

Comments for the author

Essential Critiques
- One of the major conclusions of the manuscript is that Wnt3a is required for colon extension and formation. However, images in 2B show small/reduced hindgut region in Wnt3a null embryos at E8.5, then absence of a colon at E13.5. In that regard, statements on lines 168-169 and lines 320-322 appear contradictory. Lines 168-169 say that Wnt3a is not require for hindgut initiation or formation. But the title and lines 320-322 say that Wnt3a is required for formation. This needs to be clarified through the manuscript to alleviate confusion. Do the authors know if the cells are dying or failing to proliferate?
- "Our demonstration that the conditional removal of Ctnnb1 from the dorsal and ventral hindgut using T-Cre leads to colon agenesis, while the deletion of Ctnnb1 in the ventral gut with Shh-Cre does not, strongly suggests that Wnt3a/β-catenin signaling is specifically required in the dorsal hindgut for hindgut extension." The authors also very nicely demonstrate that the majority of the colon progenitors arise from the ventral rather than the dorsal hindgut. The authors propose that the dorsal hindgut domain acts as a signaling center to maintain the ventral hindgut, but this was not directly tested. One question that arises is what happens if Ctnnb1 or Wnt3a is deleted using the Sox2-Cre. Is that consistent with the model?

Minor Critiques
- The introduction of Wnt signaling in endoderm/hindgut development is extremely limited. What about other signaling pathways that may be driving colon development? If that is not known, should be stated. In general, the introduction could build a strong rationale for studying the role of Wnt signaling in colon development.
- Many mouse models are mentioned but not well-defined in the manuscript (i.e. T-CreERT2;Ctnnb1flLOF/Δ on line 194, Shh-CreER on line 244, etc.). For the general reader, better descriptions of the models would be beneficial.
- Line 206: posterior polarity is mentioned but not defined. Perhaps anterior-posterior polarity? Or posterior identity?
- Line 210 is the first mention of hindgut extension stages. Perhaps this could be included in introduction to alleviate potential confusion regarding extension vs formation issue which is argued throughout the manuscript.
- The control mice are not described. Are these Cre-negative? Do they get TAM injections as well?

First revision

Author response to reviewers' comments

We are grateful to the reviewers for their thoughtful analysis and for their positive and supportive comments, which we feel have helped to improve the manuscript. As outlined below in our detailed response to the reviewers, we have addressed every comment and request to the best of our abilities. Note that our responses in this letter are in blue text. Please see the uploaded "Response to reviewers pdf" in Supplementary Information for a properly formatted letter. Substantial changes or additions to the manuscript are presented in red in the revised manuscript.

Reviewer 1 Advance Summary and Potential Significance to Field:

Garriock et al. provides new insight into the role of canonical Wnt signaling in intestinal patterning and what distinguished small intestine from colon. While it has long been known that a A-P gradient of Wnt activity establishes hindgut, this study illustrates a novel differential Wnt activity along the D-V axis of the extreme hindgut with Wnt3a, Bcat and Sp5/8 in the hindgut dorsal mesendoderm being required for colon development. A series of conditional LOF and GOF experiments addresses how, and in which tissues this Wnt activity is required. The data is of high quality and overall the results will be very interesting result to those in the field of gut development.

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Reviewer 1 Comments for the Author:
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We agree that it remains possible that Wnt/bcat activity may be required in the mesoderm to make a second signal such as Bmp instead of, or in addition to, the dorsal endoderm as we suggested. In anticipation of this comment, we set up Sox2CreER;bcatLOF crosses in an attempt to delete bcat activity from the dorsal hindgut but not the mesoderm. The colon formed and was indistinguishable from controls. As the colon does not form in T- Cre;bcatLOF embryos which delete bcat in the mesoderm and dorsal endoderm, the Sox2CreER;bcatLOF negative result is consistent with bcat activity being required in the mesoderm. The Sox2CreER reagent is clearly functional as we have used it to successfully trace the lineage of at least some dorsal endoderm cells and because phenotypes are observed in the brains of Sox2CreER;bcatLOF embryos at E9.5 and E13.5 (see Fig. S5). However, it is important to point out that the endogenous Sox2 protein is expressed in only a subset of dorsal hindgut cells that are Wnt3a+ (see Fig. 1), and Sox2-CreER (which is a knock-in of CreER into the *Sox2* locus) is likewise expressed in a subset of dorsal hindgut cells, judging from the relatively small number of cells that populated the colon in our lineage tracing studies (see Fig. 5A). Thus, Sox2- CreER does not remove b-catenin activity from all dorsal hindgut cells. With the tools at our disposal, we are currently unable to distinguish whether bcatenin is required in the mesoderm or the dorsal endoderm. We have added the new Sox2CreER;bcatLOF data to the manuscript (new Supp. Fig. S5) and have modified the results and discussion accordingly, pointing out these caveats.

We should also point out that Wnt3a and T;Cre may not be active early enough to regulate the early A-P patterning step that defines posterior identity, as the reviewer postulates. Wnt3a isn't expressed in the PS until E7.5 (at least a day after gastrulation has begun) and the persistent expression of *Cdx* genes in posterior domains suggests that AP patterning is maintained in Wnt3a-/- mutants. Although the T-Cre transgene is active prior to the onset of Wnt3a expression, it is also activated well after gastrulation has begun (Anderson et al., 2013), and has not been clearly shown to target the caudal definitive endoderm at the AP patterning stage.

To address the suggestion that Wnt/bcatenin could be required in the mesoderm (or endoderm) to make a second signal such as Bmp, we monitored Bmp activity by examining the expression of phospho-Smad1/5 (Munera et al., 2017) in cross-sections of the E8.5 caudal embryo by immunofluorescence. pSmad1/5 expression was not observed in the dorsal hindgut, but was readily detected in the ventrolateral hindgut, demonstrating distinct differences in Bmp signaling across the DV axis of the hindgut. The absence of Bmp activity in the dorsal hindgut despite the strong Bmp activity in the dorsally adjacent PSM suggests that the dorsal hindgut is refractory to Bmp signaling. Interestingly, we could detect little to no pSmad1/5 expression in the hindgut in the absence of Wnt3a suggesting that Wnt3a coming from either the paraxial mesoderm (which is absent in Wnt3a-/- mutants) or from the dorsal endoderm is required for Bmp synthesis, or for the ventrolateral hindgut to respond to Bmps. Satb2 is thought to be a downstream target of Bmp signals (Munera et al., 2017) and, consistent with the ventrolateral hindgut expression of pSmad1/5, is restricted to the ventral HG. Surprisingly, Satb2 remained ventrally expressed in Wnt3a mutants suggesting that it is maintained in the ventral HG by signals other than Wnt3a and Bmps. In the course of these studies, we found that Foxa2 expression in the hindgut also differs across the DV axis, and that this dorsal hindgut expression is perturbed in the Wnt3a mutant. Together, this analysis suggests that the Bmp pathway is downstream of Wnt3a, and that dorsal hindgut progenitors depend upon Wnt3a.

This work is added to the manuscript as a new Figure 4. We feel this work strengthens the manuscript and we are grateful to the reviewers for their suggestions.

2. The authors need to looked at early colon-specific markers such as Satb2, Trh1, Cpn1 and/or HoxD13.
As discussed above, we examined the expression of Satb2 and Foxa2 (new Fig. 4, see above). We also looked at Hoxd13 in wt and Wnt3a-/- embryos at E8.5. *Hoxd13* expression is down-regulated but still readily detected in the caudal-most embryo and hindgut. This new data has been presented in Supp. Fig. S4, and is consistent with a role for Wnt3a in expansion rather than posterior determination.

3. The whole mounts of Cdx1-3 do (not) really show whether early A-P patterning in the epithelium is disrupted, since most of the expression is mesodermal. Sections should be shown.

We examined the expression of *Cdx2* in situ in optical sagittal sections of control and Wnt3a-/- mutant embryos by HCR and displayed it as a supplemental Fig. S4. Optical sections clearly show that *Cdx2* is expressed in the caudal embryo in all three germ layers, including the hindgut epithelium and that this expression is maintained in the Wnt3a-/- mutant hindgut. Our work suggests that the A-P patterning of the hindgut epithelium is not perturbed by the absence of Wnt3a.

4. The authors make the general comment in the intro that "the mechanisms underlying hindgut tubulogenesis and extension are poorly understood". Which is sort of true, but there are some important things known such the fact that BMP is crucial for colon (DOI:10.1016/j.stem.2017.05.020), and that non-canonical Wnt and Fgf are important for hindgut morphogenesis (DOI:10.1038/s41586-018-0865-9). These are almost certainly involved in the phenomena being studied here so they should be acknowledged.

We have significantly expanded the introduction to incorporate the roles of the Fgf and Bmp signaling pathways in hindgut and colon formation. We think that the introduction is substantially improved and thank the reviewers for their suggestions.

5. In figure 1 the authors claim that they have identified a "unique progenitor population that co-expressed Wnt3a, Cdx2, Sox2, T and SP8", but these truly distinct from neuromesodermal progenitors (NMPs) because they do NOT express TBX6, as in the previous work from the Yamaguchi group (Garriock et al 2015) and others (Edri et al 2019, multiple papers from the Briscoe Lab) have shown? If that is correct, then showing the lack of TBX6 or lack of other NMP factors/primitive streak markers) seems appropriate.

We have added a new Supp Fig (Fig S2) that shows that the dorsal hindgut does not express Tbx6 protein nor the NMP marker *Nkx1.2*.

Reviewer 2 Advance Summary and Potential Significance to Field:

Wnt signaling is required for early endoderm development, but whether this pathway is also important for downstream hindgut tubulogenesis and colon formation has not been studied. In this manuscript, Garriock et al. use multiple mouse models to show that Wnt signaling is essential for extension of the hindgut endoderm and colon formation. The phenotype is strong and consistent across each model, which strengthens the data and the conclusions. The authors also find that Wnt3a is expressed specifically in the dorsal hindgut but that ventral hindgut progenitors contribute to the majority of the colon. Overall, this study is very beautifully presented and the results are of high quality. The findings are significant since they really shed light on colon formation including lineage tracing to show the difference between ventral and dorsal contributions to the colon.

Reviewer 2 Comments for the Author:

Essential Critiques
-One of the major conclusions of the manuscript is that Wnt3a is required for colon extension and formation. However, images in 2B show small/reduced hindgut region in Wnt3a null embryos at

E8.5, then absence of a colon at E13.5. In that regard, statements on lines 168-169 and lines 320-322 appear contradictory. Lines 168-169 say that Wnt3a is not require for hindgut initiation or formation. But the title and lines 320-322 say that Wnt3a is required for formation. This needs to be clarified through the manuscript to alleviate confusion.

We thank the reviewer for pointing this out. There appears to be some confusion between hindgut formation, hindgut extension, and colon formation. We have attempted to alleviate confusion regarding these terms in the first paragraph of the introduction. We point out that hindgut formation "starts at E8.25 with the formation of the caudal intestinal portal and the outpocketing of the hindgut endoderm. The posterior extension of the hindgut occurs over several subsequent days (~E8.5-13.5) to generate a tube that will eventually form the colon…". Hindgut extension and colon formation are two related but temporally separate processes, i.e. the extension of the embryonic hindgut is responsible for the formation of the fetal colon. We hope that this helps to clarify the differences between these processes.

Do the authors know if the cells are dying or failing to proliferate?

Hindgut cells are not dying in the absence of Wnt3a, as shown by our examination of cleaved caspase expression in Wnt3a-/- embryos at E8.5 (Garriock et al., 2015). We generated a mitotic index of hindgut cell proliferation from sections of wt and Wnt3a-/- embryos and found that the number of mitotic cells is significantly decreased in the mutants. Thus, the hindgut appears to be failing to proliferate in the absence of Wnt3a. We have added the graph to Figure 2(C).

-"Our demonstration that the conditional removal of Ctnnb1 from the dorsal and ventral hindgut using T-Cre leads to colon agenesis, while the deletion of Ctnnb1 in the ventral gut with Shh-Cre does not, strongly suggests that Wnt3a/b-catenin signaling is specifically required in the dorsal hindgut for hindgut extension." The authors also very nicely demonstrate that the majority of the colon progenitors arise from the ventral rather than the dorsal hindgut. The authors propose that the dorsal hindgut domain acts as a signaling center to maintain the ventral hindgut, but this was not directly tested. One question that arises is what happens if Ctnnb1 or Wnt3a is deleted using the Sox2- Cre. Is that consistent with the model?

We have performed this experiment (Sox2-CreER;Ctnnb1^{flLOF}) as outlined above in our response to Reviewer 1. Wnt3a could not be deleted as the available allele is not a floxed conditional allele.

Minor Critiques
-The introduction of Wnt signaling in endoderm/hindgut development is extremely limited. What about other signaling pathways that may be driving colon development? If that is not known, should be stated. In general, the introduction could build a strong rationale for studying the role of Wnt signaling in colon development.

As stated in response to Reviewer 1, we agree with the reviewers about this criticism and have extensively revised the introduction. We hope that they will find the revised introduction greatly improved.

-Many mouse models are mentioned but not well-defined in the manuscript (i.e. T-CreERT2;Ctnnb1flLOF/Δ on line 194, Shh-CreER on line 244, etc.). For the general reader, better descriptions of the models would be beneficial.

We have attempted to better describe the mouse lines that we have used in our studies. New
Lines 190-192 – for T-Cre
Lines 220-222 – we expanded upon the conditional allele of Ctnnb1 and the tamoxifen- inducible T-CreERT2 mouse line
Line 288 – better intro to Sox2-CreER

-Line 206: posterior polarity is mentioned but not defined. Perhaps anterior-posterior polarity? Or posterior identity?

We have taken the reviewers suggestion and changed posterior “polarity” to “identity”.

-Line 210 is the first mention of hindgut extension stages. Perhaps this could be included in introduction to alleviate potential confusion regarding extension vs formation issue which is argued throughout the manuscript.

We first mentioned “extension of the hindgut” in the abstract and go on to distinguish hindgut formation vs hindgut extension in the first paragraph of the introduction as described above.

-The control mice are not described. Are these Cre-negative? Do they get TAM injections as well?

Control embryos are littermates of mutants so all embryos receive TAM treatments. 50% of embryos are Cre-negative. We have provided a more detailed description of the crosses and have described the possible genotypes for control embryos in the Materials and Methods section.

Second decision letter

MS ID#: DEVELOP/2019/185108

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AUTHORS: Robert John Garriock, Ravindra B. Chalamalasetty, JianJian Zhu, Mark W. Kennedy, Amit Kumar, Susan Mackem, and Terry P. Yamaguchi
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 all of my previous concerns with new data and additional explanation. This is a thorough elegant study with high quality data that illuminates a novel role for Wnt3 in colon development. These results will be of high interest to researcher in the field and I support publication.

Comments for the author

well done.

Reviewer 2

Advance summary and potential significance to field

Wnt signaling is required for early endoderm development, but whether this pathway is also important for downstream hindgut tubulogenesis and colon formation has not been studied. In this manuscript, Garriock et al. use multiple mouse models to show that Wnt signaling is essential for extension of the hindgut endoderm and colon formation. The phenotype is strong and consistent across each model, which strengthens the data and the conclusions.
The authors also find that Wnt3a is expressed specifically in the dorsal hindgut but that ventral hindgut progenitors contribute to the majority of the colon. Overall, this study is very beautifully

presented and the results are of high quality. The findings are significant since they really shed light on colon formation including lineage tracing to show the difference between ventral and dorsal contributions to the colon.

Comments for the author

The authors have satisfactorily addressed the reviewers' comments with either additional experiments or additional discussion.