In toto imaging of early enteric nervous system development reveals that gut colonization is tied to proliferation downstream of Ret

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MS TITLE: In Toto imaging of early Enteric Nervous System Development reveals that gut colonization is tied to proliferation downstream of Ret

AUTHORS: Phillip A Baker, Akshaya Venkatesh, Eileen W Singleton, and Rosa A Uribe

To expedite the process I have decided not to wait for the final referee report on the above manuscript. 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 recommend a substantial 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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.

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Reviewer 1

Advance summary and potential significance to field

In this paper baker et al. performed in toto gut imagining in a Transgenic (-8.3phox2bb:Kaede) zebrafish line. Using this transgenic model, they visualized and analyzed the ENCC migration pattern of a Retwmr1/+ line (designed using CRIPR-Cas9) and compared it to Ret+/+ controls. Thee authors used powerful high resolution confocal Time-Lapse microscopy to video and image ENCC migration. The authors used single cell lineage tracking to follow the ENCC migration and proliferation during gut colonization between 48hpf and 96hpf, a developmental timepoint when the entire length of the gut is colonized in control larvae. This experiment was repeated on a minimum number of n=3 for both Retwmr1/+ mutants and Ret+/+ controls. The authors measured and quantified relevant parameters such as, migration speed, distance between ENCCs and proliferation rate. Lastly the authors associate the drop in proliferation and migration in the Retwmr1/+ line with early differentiation of enteric neurons using Pbx3 as a differentiation marker. The results support a model of cell number dependent migration, driven by proliferation as well as the “frontal expansion” model of NCC migration.
This is a well-designed study, using appropriate methods (microscopy, great movie quality, quantification of data and appropriate statistics) looking at the developmental mechanisms of ENCCs gut colonization. The results are in line with some previous published work regarding ENCC numbers and migration (Barlow et al., 2008; Peters-Van Der Sanden et al., 1993; Young et al., 2004), mathematical modeling of ENCC migration (Simpson et al., 2007) and early neuronal differentiation (Jaroy et al., 2019). However, some results regarding early differentiation and proliferation do not line up with results from a different Ret zebrafish line rethu2846/+ (Heanue et al., 2016).

Reference :
Jaroy, E.G., Acosta-Jimenez, L., Hotta, R. et al. “Too much guts and not enough brains”: (epi)genetic mechanisms and future therapies of Hirschsprung disease — a review. Clin Epigenet 11, 135 (2019). https://doi.org/10.1186/s13148-019-0718-x

Comments for the author

Having performed single-cell lineage tracing of ENCCs between 48hpf and 96hpf the authors are in a great position to test the interaction between proliferation and migration. A greater depth of analysis here would be important and a specific statistical analysis to assess this question seems to be a necessary addition to the paper. Spearman’s correlation test can be used to study the relationship between proliferation and migration distance / migration speed. Should this relationship be established in the control, it would be useful to know how it is affected in the Retwmr1/+ fish.
In the control Movie2 there is a sudden burst of migration at 00:07. Could the authors comment on this. Is this linked to a proliferation burst or a sudden elongation of the gut? Could proliferation be added at this stage in figure E.
In the discussion, more emphasis on the possible reasons why two zebrafish Ret models seem to affect different aspects of migration and yet, both results in HSCR phenotype. The limitations of zebrafish as a model for HSCR should be discussed (no Sacral ENCC contribution, difference in gut anatomy, ENS plexuses etc…). Additional discussion of the role of Ret as a protooncogene and the relationship between PBX Homeobox 3 and MEIS transcription factor could be added.

Minor corrections:
“based off” is incorrect English and should be replaced by “based on”. (9 corrections needed, p7, p13, p15, p16, p25, p26).

Reviewer 2

Advance summary and potential significance to field

The paper by Baker et al is an interesting characterization of the behavior of ENCCS during gut colonization in zebrafish. It describes an imaging approach capturing the entire ENCC population in

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a vertebrate animal and investigates how ENCC colonization is changed in heterozygous carriers for a new ret allele. While the resolution of the imaging is a new step and the ability to capture all ENCCs in a vertebrate, which is a very exciting development, some of the findings are not embedded well in the existing literature and thus feel a bit overstated, as the literature is only sparsely included, e.g. proliferation-driven migration in the ENS, and the first lineage tracing study in zebrafish.

Comments for the author

1) A novel finding of this paper is the complete lineage of ENCCs, but the authors do not elaborate (and show the data) on how the colonization of the gut happens. The lineage tree in Fig. 1F is so small that it is difficult to see how the lineages develop over time – also the y-axis could indicate developmental time so readers could appreciate at what time ENCC lineages expand in zebrafish. What are the lineages created? Which cells create neurons vs glial cells. The authors state this really interesting finding that subsequent generations of ENCCs migrate further posteriorly but the graph in Fig. 4A looks like it's a bit more complicated than that. I would like to see more information on the behavior of ENCCs during gut colonization (especially also the later steps, when ENCCs populate the expanded gut. How are the ENCCs followed when you have a 3D gut? How did the authors differentiate between ENCCs that do not divide anymore vs. differentiated ENS neurons that presumably will still be Kaede positive? How consistent is this between embryos – the authors have only imaged 3 embryos for each condition and that number should be increased to determine if there's variability in this pattern (or the authors should show that there's not a lot).
2) The authors should more clearly state that a model of proliferation driven migration has already been extensively proposed for the ENS. The authors do not include this in their introduction at all, but only cite one paper (Simpson et al., 2007). There's a larger body of literature on this, so the authors should expand on this and more clearly elaborate what the novel findings of their study are in comparison to the known literature.
3) Lineage tracing studies/behavior of ENCCS after cell divisions have been done already in zebrafish ENS – how do the findings from the current paper fit with the known literature?
4) Why do the authors not use the pan-neuronal marker Elavl to show that there's aberrant differentiation in ret HETs. The authors state that vibp is expressed in all early differentiating ENCCs – that's very interesting as it would suggest that most ENCCs at these stages go towards the neuronal lineage? How does this compare with Elavl-labels?
5) The authors state that they see variability in the ret HET phenotypes – does the colonization phenotype correlate with the later neuron phenotype?
6) Fig. 1A: at 48 hpf, only very few Kaede+ cells are visible in the anterior portions of the gut – however, ENCCs already start entering the gut around 36 hpf, so it is surprising that 12hrs later there aren't more cells visible. The authors should compare their findings to other transgenic lines (e.g. phox2bb:EGFP) to show that they are really tracking all ENCCS and not only a subset of them.
7) How was the manual tracking controlled to ensure that single cells were tracked? ENCCs can stretch out quite far and migrate closely together – how did the authors ensure that they are following single cells? I would like to see images with a nuclear marker to demonstrate that single cells can be followed over time.
8) The authors state that they have created a novel ret mutant. This mutant has the same phenotype as already established ret mutants. What's the rationale for establishing a new mutant and not using an already established ret mutants? How was the CRISPR target site selected (e.g. why target exon 8). How do the authors know that this is a functional loss of ret?
9) Fig. 3C, ret HETs already start with less ENCCs, compared to ret+/+ - so couldn't the problem also be that the starting material is less and they just never catch up?
10) Fig. 3 – for clarification, the vanguard cells are changing all the time? Or are there cells that are migrating at the front and stay at the front? Please clarify.
11) How is gut length taken into account? The gut length is not going to be the same in each embryo.

Minor comments
12) Please indicate the number of embryos for each experiment throughout the manuscript.
13) Introduction, line 1-2, the vertebrate ENS only consists of ganglia in some vertebrate species not in all (and definitely not in zebrafish)– please specify this in this sentence or write the information more generally.

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14) Introduction line 21, what do you mean with canonical enteric specification markers: all these genes are also expressed in other parts of the peripheral nervous system.
15) Introduction lines 24/25: as outlined, for example in Olden et al., 2008, enteric neurons start to already differentiate at 54 hpf. Please correct the information.

First revision

Author response to reviewers' comments

Response to Reviewers

We are thankful for *Development*'s interest and careful consideration of our work. We especially thank reviewers for their constructive suggestions. We wish to share our point-by-point revisions and responses for addressing the referee's comments made for resubmission.

Per Reviewer 1 Comments:

1) "Having performed single-cell lineage tracing of ENCCs between 48hpf and 96hpf, the authors are in a great position to test the interaction between proliferation and migration. A greater depth of analysis here would be important and a specific statistical analysis to assess this question seems to be a necessary addition to the paper. Spearman's correlation test can be used to study the relationship between proliferation and migration distance / migration speed. Should this relationship be established in the control, it would be useful to know how it is affected in the Retwmr1/+ fish."

   Spearman correlation analysis was performed on our datasets (**Fig. R1**). No apparent correlation was seen between cell divisions per hour vs Vanguard speed (**A,B**) or Vanguard distance traveled (**C,D**). This is likely due to the complex and dynamic nature of ENCC and differentiating enteric neuron proliferation in the zebrafish ENS between 48-96 hpf. We believe this time window constitutes two phases of cell proliferation: 1) Cell proliferation during ENCC migration 48-72 hpf. 2) Enteric neurons continue proliferating as the ENS grows with the developing zebrafish larvae. It is possible that more nuanced patterns exist. For example, one observation in Fig. R1A, is that while cell division rates reach as high as nearly 10 divisions per hour in controls, in mutants we never see mitotic rate greater than ~6. Additionally, qualitatively, we note that earlier timepoints (darker blue on hpf color key, Fig. R1) trend with higher speeds, while later time points (lighter blue) trend with slower cell migration speeds. An in depth future study and analyses will help to parse out specific patterns. We thank the reviewer for bringing this question to our attention.

2) "In the control Movie2 there is a sudden burst of migration at 00:07. Could the authors comment on this Is this linked to a proliferation burst or a sudden elongation of the gut? Could proliferation be added at this stage in figure E."

   We appreciate reviewer 1 bringing this to our attention. What appears to be sudden burst of migration in the final time frames of control movie 2 is neither proliferation burst or elongation of gut, but rather, a drifting of the zebrafish within the agar towards the very end of the 48 hour long imaging cycle. This complication can occur given the long duration of imaging, but we have been careful to select only high-quality imaging data for analysis. To rectify any aberration in quantification caused by this drift, we have normalized this movement within the IMARIS software by moving the Origin Reference Frame to maintain a constant position within the tissue per these final timepoints when the drift occurred. This normalized the individual cell dynamics in relation to the drift within these last time-points. The new data points were reanalyzed and plotted. Updated graphs were replaced in **Fig. 3F,G**, **Fig. 4A** and **Fig. 5A,B**. The changes from this normalization are indistinguishable from previous figures due to the small amount of drift that occurred at the end of control

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movie 1 and 2. Control Movies 1 and 2 were also re-recorded to include the normalized reference origin frame.

3) "In the discussion, more emphasis on the possible reasons why two zebrafish Ret models seem to affect different aspects of migration and yet, both results in HSCR phenotype. The limitations of zebrafish as a model for HSCR should be discussed (no Sacral ENCC contribution, difference in gut anatomy, ENS plexuses etc…). Additional discussion of the role of Ret as a protooncogene and the relationship between PBX Homeobox 3 and MEIS transcription factor could be added."

These topics are discussed in greater detail within the discussion section of the manuscript. Discussion paragraph 4 on page 12 addresses different findings between two zebrafish *ret+/-* models. We note that while similar changes in ENCC speeds were shared between the two models, proliferation was measured using differing assays and at different time points. Discussion paragraph 2, on page 10 address limitations of zebrafish as a model for ENS study. Discussions regarding Ret as a protooncogene and relationships between *Pbx3* and *Meis* have been added to the end of the discussion section, page 8. We thank the reviewer for bring these to our attention.

4) Minor corrections (grammar)

All instances of "based off" were corrected to "based on.".

Per Reviewer 2 Comments:

1a) "A novel finding of this paper is the complete lineage of ENCCs, but the authors do not elaborate (and show the data) on how the colonization of the gut happens."

We do respectfully disagree here. We are unsure what further data the reviewer would like to see regarding colonization of gut by the ENCC. Our goal for this study was to define the dynamics of ENCCs. Towards that end, we have examined in detail how colonization of the gut happens and show data throughout the figures to this effect (All control WT data, in addition to the *ret* mutant data). Specifically, we show *in toto* time-lapse movies over the 48 hours of colonization, quantify ENCC numbers during colonization, ENCC speeds during colonization, ENCC displacements over colonization, ENCC proliferation rates during colonization, and ENCC inter-cell distances during the stages of colonization between 48-96 hpf. We have added in new cell track movies, to show qualitative cell tracks of ENCCs over time (Movies 3 and 4). Our data support a model, which we propose as method of colonization (Fig. 5F), in conjunction with prior publications/findings from various other groups, which we cite in our discussion.

1b) "The lineage tree in Fig. 1F is so small that it is difficult to see how the lineages develop over time – also the y-axis could indicate developmental time so readers could appreciate at what time ENCC lineages expand in zebrafish. What are the lineages created? Which cells create neurons vs glial cells."

Thank you for bringing this to our attention. In our revision, we tempered our original intent and use of wording "lineage" by replacing the word with "generation." We want to clarify that mapping neuronal and glial cell type was completely outside the scope of this current work. We have made a larger, higher resolution supplemental figure of the ENCC tree of both control and *ret*$^{wmr1/+}$ time-lapses, with detailed axis labels in the revised paper (**Fig. S1**). The "Generation-Tree" that was previously in Figure 1 was moved (Fig. S1) and the cell tracks in Imaris were expanded for additional vantage points (**Fig. 1D**). Subsequent lettering was revised and all instances of reference to Figure 1 in the manuscript were edited to reflect these changes.

1c) "The authors state this really interesting finding that subsequent generations of ENCCs migrate further posteriorly but the graph in Fig. 4A looks like it's a bit more complicated than that. I would like to see more information on the behavior of ENCCs during gut colonization

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(especially also the later steps, when ENCCs populate the expanded gut. How are the ENCCs followed when you have a 3D gut?"

> We clarify that the movies are 4D, and cell tracking image analysis was performed in 4D (XYZ planes examined, over time (4th dimension)). To clarify this, we have added IMARIS snapshots of cell tracks that are color coded for time viewed from dorsal and lateral orientations to demonstrate how cells were tracked in 4D space (**Fig. 1D**). We have included new animated rotating movies showing cell tracks in 4D that are seen in **Figure 1D** (**Movie 3,4**). Previous Movies 3 and 4 (original submission) were changed to 5 and 6 and all reference to movies in text have been updated. From those new rotation movies, one can appreciate how cell track orientations of ENCCs localize over 4D space. Further in depth analyses of when ENCCs populate the expanded gut is planned for a future study and analyses will help to parse out specific patterns. We thank the reviewer for bringing this question to our attention.

1d) "How did the authors differentiate between ENCCs that do not divide anymore vs. differentiated ENS neurons that presumably will still be Kaede positive?"

> Great question. We have clarified that the goal was to track all Kaede+ cells and their divisions along the length of the gut, to map how the gut is colonized over time (Results; Paragraph 1, line 2-4). An additional reference was added here as well that published data on the *-8.3phox2bb:*Kaede^{+} fishline and its fidelity to label ENCCs by showing co-localization between *-8.3phox2bb:*Kaede, *phox2bb* transcript, and Phox2b protein (Howard et al. 2022).The tracking data made no distinction between differentiated states. Kaede persist throughout ENCC neuronal differentiation.+

1e) "How consistent is this between embryos – the authors have only imaged 3 embryos for each condition and that number should be increased to determine if there's variability in this pattern (or the authors should show that there's not a lot)."

> We have performed Post hoc power analysis and found sufficient statistical power (>0.8) for all tests in which we report statistical significance. We have updated the manuscript to have this information, where we specified this in the statistical analysis section of our methods (page 20).

2) "The authors should more clearly state that a model of proliferation driven migration has already been extensively proposed for the ENS. The authors do not include this in their introduction at all, but only cite one paper (Simpson et al., 2007). There's a larger body of literature on this, so the authors should expand on this and more clearly elaborate what the novel findings of their study are in comparison to the known literature."

> To address this present comment, we have expanded on the literature surrounding this topic in the introduction, paragraph 4, page 3-4 (Kuwata et al. 2019; Harrison et al. 2014). We also have addressed this in the discussion, where we cite numerous papers that link ENCC numbers to migration, which implicate proliferation with migration (Barlow et al., 2008; Peters-Van Der Sanden et al., 1993; Young et al., 2004). We also clearly distinguish the novelties of our study, specifically, the key timepoints where we identified proliferative and migratory deficits, and key spatiotemporal mechanism of ENCC migration, also in the discussion.

3) "Lineage tracing studies/behavior of ENCCS after cell divisions have been done already in zebrafish ENS – how do the findings from the current paper fit with the known literature?"

> We thank the reviewer for bringing this to our attention. We had not seen studies of ENCCs after cell divisions in zebrafish, however after seeing this comment, we noted that Harrison et al., 2014 suggests proliferation is linked to migration, during a specific window of time analyzed. We have included mention of this study in our revised paper, intro paragraph 4, page 3. We have also included additional discussion regarding previous focal "lineage tracing" studies in discussion paragraph 5, page 13

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(Kuwata et al. 2019). We do emphasize that our study is the first to examine the in toto colonization process over the course of 48 hours and utilizes single-cell tracking at high resolution. From our experiments we discovered novel cellular mechanisms underlying enteric phenotypes seen in *ret* mutants, and also discovered new details about how ENCCS migrate to colonize the wild-type gut.

4a) “Why do the authors not use the pan-neuronal marker Elavl to show that there’s aberrant differentiation in ret HETs.”

We have performed wholemount immunohistochemistry using anti-ChAT antibodies (relevant markers to *pbx3b*+ IPAN data shown in figure 6) and anti-Elavl3 at 96 and 120 hpf to compare differentiation and neuronal subtype rates between WT and $ret^{wmr1/+}$. This figure was added to the manuscript and discussed as the final paragraph in the results, page 10 (**Fig. 7**). The methods section was revised to include the details of this experiment.

4b) “The authors state that vibp is expressed in all early differentiating ENCCs – that’s very interesting as it would suggest that most ENCCs at these stages go towards the neuronal lineage? How does this compare with Elavl-labels?”

Indeed, we previously observed strong *vipb* co-expression with *elavl3/4,* and many other neuronal transcripts, from our previously published single-cell RNA-seq data sets of differentiating ENCCs at 68-70 hpf (Howard et al., 2021), showing strong neuronal identities. It is not within the scope of this present study to examine neuronal versus glial lineages, however it is a very interesting question for future study. We thank the reviewer for this question.

5) “The authors state that they see variability in the ret HET phenotypes – does the colonization phenotype correlate with the later neuron phenotype?”

Great question. To address this question, we have plotted 96 hpf HCR data of percent pbx3b+vipb+ cells vs. extent of aganlionosis for $ret^{wmr1/+}$ embryos and performed spearman correlation to test whether the neuron phenotype is correlated with colonization phenotype (**Fig. S3**). We found no correlations between this data and mentioned this in the results paragraph 11, page 10.

6) “Fig. 1A: at 48 hpf, only very few Kaede+ cells are visible in the anterior portions of the gut – however, ENCCs already start entering the gut around 36 hpf, so it is surprising that 12hrs later there aren’t more cells visible. The authors should compare their findings to other transgenic lines (e.g. phox2bb:EGFP) to show that they are really tracking all ENCCS and not only a subset of them.”

We have cited previously published work from our lab demonstrating that the *-8.3phox2bb:*Kaede fish line labels all *phox2bb*+ ENCCs (Howard et al., 2022). We have added in this reference to page 4, beginning of results. The data in Howard et al., shows co-localization between *-8.3phox2bb:*Kaede, Phox2bb immunoreactivity, and *phox2bb m*RNA localization, confirming the fidelity of the line. As well, this line was previously validated by Harrison et al., 2014.

7) “How was the manual tracking controlled to ensure that single cells were tracked? ENCCs can stretch out quite far and migrate closely together – how did the authors ensure that they are following single cells? I would like to see images with a nuclear marker to demonstrate that single cells can be followed over time.”

Our lab created a nuclear reporter fishline (*-8.3phox2bb;*H2A-mCherry;Kaede; unpublished) after the creation of our ret^{wmr1} fishline. We have included a supplemental figure for resubmission that verifies the fidelity of tracking single cells in the Kaede fish line and have quantified H2A-mCherry+ cells at key timepoints, 72 and 96 hpf (**Fig. S5**) showing comparable cell quantifications to original WT data (**Fig. 3C**). Mention of validated cell tracking is referenced in methods page 18.

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8) “The authors state that they have created a novel ret mutant. This mutant has the same phenotype as already established ret mutants. What’s the rationale for establishing a new mutant and not using an already established ret mutants? How was the CRISPR target site selected (e.g. why target exon 8). How do the authors know that this is a functional loss of ret?”

We needed to establish a robust CRISPR mutagenesis pipeline in our lab for the first time and targeting *ret* was an excellent positive control to start with. *ret* CDS was searched for compatible PAM motifs, where we happened to find an optimal motif in exon 8.

To test the functionality of the ret^{wmr1} transcript, we injected WT *ret* mRNA and ret^{wmr1} mRNA into single-cell stage embryos obtained from $ret^{wmr1/+}$ incross and quantified the percent of larvae at 96 hpf that exhibit either total aganglionosis, HSCR-like, or WT phenotypes (**Fig. S2**). We used the specific isoform *ret9*, previously shown to be responsible for *ret* function in zebrafish ENS (Heanue and Pachnis, 2008). We found that WT *ret9* mRNA was able to partially rescue the fraction of larvae displaying both total aganglionois and HSCR-like phenotypes, compared to uninjected animals, while $ret9^{wmr1}$ failed to rescue disease, demonstrating loss of function of the mutant $ret9^{wmr1}$ allele. Results and reference to figure included in results paragraph 3, page 5. We thank the reviewer for this question.

9) “Fig. 3C, ret HETs already start with less ENCCs, compared to ret+/+ - so couldn’t the problem also be that the starting material is less and they just never catch up?”

Yes, indeed Figure 3C shows slightly less cells at the beginning of the time-lapse. We believe this slight discrepancy is likely due to slower migration speeds leading into the gut, resulting in delayed emergence into imaging ROI (**Fig 5 A and B**).
Looking at ENCCs migratory deficiencies prior to 48hpf would be an important study. However, as our study aimed to examine ENCCs during their migration along the gut tube, investigating ENCCs entry into the foregut and early migration is outside the scope of this study. Nonetheless, we have added a statement into our revised discussion paragraph 3, page 12, that acknowledges that early ENCC numbers could also add to the phenotype and could be tested in a future study.

10) “Fig. 3 – for clarification, the vanguard cells are changing all the time? Or are there cells that are migrating at the front and stay at the front? Please clarify.”

Great question. Vanguard cells can change, but not all the time. As vanguard divides, vanguard is always designated as the leader. Therefore, the leader identity will change when the current leader is overtaken. We have added in this information to methods to clarify this.

11) “How is gut length taken into account? The gut length is not going to be the same in each embryo.”

All time-lapses include the same spatiotemporal metrics and we have not observed notable differences in gut length. To explicitly address this, we have measured the gut length of 6 $ret^{+/+}$ and 6 $ret^{wmr1/+}$ larvae at 96 hpf and found little variance and no significant difference between individual gut length (**Fig. R2**; $p=0.68$).

12) Please indicate the number of embryos for each experiment throughout the manuscript.

Figure legends include N for each dataset. Currently, all boxplots of fixed tissue experiments include dotplots that represent individual embryos.

13) Introduction, line 1-2, the vertebrate ENS only consists of ganglia in some vertebrate species not in all (and definitely not in zebrafish)– please specify this in this sentence or write the information more generally.

Ganglia in that sentence has now been replaced with “neurons and glia”.

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14) Introduction line 21, what do you mean with canonical enteric specification markers: all these genes are also expressed in other parts of the peripheral nervous system.

This has been rephrased with "characterized by combinatorial expression of genes".

15) Introduction lines 24/25: as outlined, for example in Olden et al., 2008, enteric neurons start to already differentiate at 54 hpf. Please correct the information.

We have added "ENCCs differentiated into enteric neurons as early as 54 hpf, continuing between 72-120 hpf." Additional reference added (Olsson et al., 2008)

Figure R1

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Figure R2

NOTE: We have removed unpublished data that had been provided for the referees in confidence.

Second decision letter

MS ID#: DEVELOP/2022/200668

MS TITLE: In Toto imaging of early Enteric Nervous System Development reveals that gut colonization is tied to proliferation downstream of Ret

AUTHORS: Phillip A Baker, Rodrigo Ibarra-Garcia-Padilla, Akshaya Venkatesh, Eileen W Singleton, and Rosa A Uribe
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. One of the reviewers had a few suggested textual edits for clarity that you might still address.

Reviewer 1

Advance summary and potential significance to field

This is a well-designed study looking at the developmental mechanisms of ENCCs gut colonization. The results are in line with some previous published work regarding ENCC numbers and migration (Barlow et al., 2008; Peters-Van Der Sanden et al., 1993; Young et al., 2004), mathematical modeling of ENCC migration (Simpson et al., 2007) and early neuronal differentiation (Jaroy et al., 2019).The results support a model of cell number dependent migration, driven by proliferation as well as the "frontal expansion"
model of NCC migration.

Comments for the author

I am happy with the improvement made to this manuscript.

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Reviewer 2

Advance summary and potential significance to field

The paper by Baker et al is an interesting characterization of the behavior of ENCCS during gut colonization in zebrafish. It describes an imaging approach capturing the entire ENCC population in a vertebrate animal and investigates how ENCC colonization is changed in heterozygous carriers for a new ret allele. The resolution of the imaging is a new step and the ability to capture all ENCCs in a vertebrate is a very exciting development.

Comments for the author

The authors have addressed most of my comments, just a few remain:
-abstract: a bit unclear what is meant with "zebrafish produced fewer ENCCS while localized along the gut", I would rephrase this to make clear who is localized along the gut.
- The authors have greatly improved embedding their findings in the current literature, especially regarding the model of proliferation-driven migration of enteric progenitor cells. However, they write on page 7, line 14, that "these data support a model of proliferation driven migration. I think it should be "the model of proliferation driven migration to clarify that this has been proposed before.
- For validating the newly generated ret mutant, why didn't the authors do a complementation cross with the established ret mutant line instead of a general overexpression experiment of ret?

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