A distinct transcriptome characterizes neural crest-derived cells at the migratory wavefront during enteric nervous system development

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DOI: 10.1242/dev.201090

Editor: Francois Guillemot

Review timeline

Original submission:	1 July 2022
Editorial decision:	1 August 2022
First revision received:	15 November 2022
Editorial decision:	11 January 2023
Second revision received:	31 January 2023
Accepted:	3 February 2023

Original submission

First decision letter

MS ID#: DEVELOP/2022/201090

MS TITLE: Enteric neural crest-derived cells at the migratory wavefront are transcriptionally distinct and require DUSP-mediated ERK suppression for enteric nervous system development

AUTHORS: Rhian Stavely, Ryo Hotta, Nicole Picard, Richard Guyer, Ahmed A Rahman, Meredith Omer, Adam Soos, Emoke Szocs, Jessica Mueller, Allan M Goldstein, and Nandor Nagy

I have now received the reports of three referees on your manuscript and I 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, two of the three referees express great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, referee 1 recommends that you perform a single cell RNA-Seq analysis of wavefront and trailing cells , and that you provide a validation of differential expression by RNA in situ hybridisation; referee 2 requests that you further analyse the experiment of inhibition of Dusp6 activity; and referee 3 requests extensive re-writing of the manuscript, to provide more rational for the design of the experiments, more details in the method section, etc. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their 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.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Nagy and colleagues transcriptionally profile enteric neural crest cells as the migrate through the gut, comparing the transcriptional profile of cells at the wavefront with those at the trailing edge. They find that the transcriptional signature of wavefront cells and find that is enriched in genes characeristicsof uncommitted progenitors at both embryonic and postnatal stages. They then focus on the gene Dusp6 which is one of the genes enriched in wavefront cells, and show that loss of function of DUSP in an ex ovo assay abrogates enteric progenitor migration in an ERK dependent fashion.

Comments for the author

This is a nicely done study with interesting findings. However there remain a few issues that would need to be addressed, most prominently concerning rationale for using bulk RNA-sequencing and validation of gene expression. Major issues are:

1. Given that there is likely to be hetereogeneity both in the wavefront and trailing cells, it is is puzzling that the authors used bulk RNA-sequencing rather than single cell RNA-seq. This further complicates their analysis since they are often comparing their datasets to single cell RNA-seq datasets. They should repeat at least one of their embryonic timepoints using scRNA-seq to determine the degree of heterogeneity in the wavefront population.

2. The authors identify several genes enriched in the wavefront and validate expression and test function of Dusp6. While it would be unreasonable to ask for functional testing of more of these genes, the authors should at least include in situs validating their expression patterns in the wavefront compared with trailing cells.

3. Figure 1C-C'": while Nestin is said to be enriched in "wavefront" cells, the signal above the mesentery in the "trailing" region looks strong to me. Please clarify.

4. Which enriched genes are common between enteric wavefront cells and cranial neural crest wavefront cells described by Morrison et al.?

5. Figure 2A-C: the comparison between PNS and the present data is not convincing. Please clarify how you calculated the WF score based on wavefront DEGs as opposed to gene counts.

6. The requirement for Dusp6 ex vivo is a very nice addition. Is there is a Dusp6 flox allele available to perform a knockout via Wnt1-cre for in vivo assay?

Reviewer 2

Advance summary and potential significance to field

This manuscript performs scRNAseq analysis on enteric neural crest, comparing cells in the migratory front with trailing cells. They found several differences in the transcriptomic of these two populations that are consistent with their distinct cellular activities.

Comments for the author

Migration of enteric neural crest cell is an important topic of research with critical clinical implications. The observation that leader or front cells behave different to trailing cells during the colonization of the gut by neural crest cells, is widely documented. I do not see what significant biological questions this scRNAseq analysis is addressing, as it is manly a confirmation of what was already known about the biology of these two cell populations. For example, the authors find that wave front cells express higher levels of mRNA linked to proliferation, but it was already known that migration of enteric neural crest requires high levels of proliferation, and that most of the proliferation takes place in the leader cells; or the finding that front cells express high levels of genes associated with migration is not surprising at all, as these are highly migratory cells, etc

The last part of this manuscript is about the role of Dusp6 on enteric cell migration. This is done very superficially, as they only show that chemical inhibition of Dusp6 shows reduced staining for the front cells. Is this an effect on the number of cells? The migratory abilities of the cells? The invasiveness of the cells? The EMT on these cells? Etc.

Reviewer 3

Advance summary and potential significance to field

Stavely et al have generated the first transcriptional profile of enteric neural crest derived progenitors (ENDCs) that are migrating at the front of the population of cells that colonizes the fetal bowel. The team elected to profile these cells at 11.5dpc in the mouse using a bulk sequencing approach. The goal of the study was to determine whether this population of ENDCs at the migration wavefront during colonization exhibits a unique transcriptional signature. The study compares transcriptional profiles from flow-sorted wavefront progenitors to the population of cells behind the migrating wavefront in a region slightly more proximal to identify differentially expressed genes that may be participating in the colonization process. The authors then perform comparisons with other datasets including a single cell ENS RNA-Seq data set from Morarach et al., 2021 and another bulk RNA-Seq data set from their own group (Stavely et al., 2021) that includes primary postnatal ENS cell types as well as cells dissociated from cultures of postnatal neurospheres that they call “postnatal progenitors”. The study includes identification of Dusp6 as a gene that exhibits differential expression in the wavefront ENDCs and uses a DUSP inhibitor (BCI) in an effort to assess whether Dusp6 is essential for hindgut colonization.

Despite the issues with the manuscript in its current form, this study IS important as it offers an opportunity to understand how ENDCs have such vast migratory potential and how they respond to exogenous cues (GDNF, Edn3) during migration. However, the article could be greatly improvided through added clarity of writing and better placement for context regarding what is already known in the field about ENS development.

Comments for the author

Primary Concerns:

There are several aspects in the manuscript that need to be addressed to make the findings of value to the ENS field and the larger Development community. These include: lack of rationale in the text for the focus on the wavefront progenitors in the cecal region; lack of detail in the methods sections for cell isolations, bioinformatics, and information regarding the specificity of the BCI inhibitor; overstatement of findings particularly the chick and mouse comparison studies; lack of any study limitations that should be added to the Discussion.

1 – The rationale and arguments for the initial profiling of the wavefront cells is lacking and not well referenced. The study is unique and the first of its kind to profile a population of wavefront progenitors. However, the authors do not clearly state why they choose this particular population or this stage of development. They could have chosen to profile wavefront ENDCs earlier in development when colonization is in the foregut. Importantly, because work from Enomoto’s group has conclusively shown that transmesenteric progenitors provide the greatest percentage of

neurons in the colon (Nishayama et al., 2012) it is not clear how valuable profiles from wavefront progenitors in the cecal region that are paused there due to very high levels of GDNF will be. If the approach taken was pursued because it offered a more consistent isolation during development, the article should state that AND acknowledge that other sources of colonizing progenitors that populate the colon do exist.

2 - The authors need to be much more explicit and clear about what they are doing in the methods and results section with regards to several of the datasets they are using. For instance the Cao et al., 2019 data includes both peripheral neurons and glia as well as enteric lineages. It is not clear why the present authors choose to use the entire PNS dataset instead of the ENS lineages. Do they get the same results when they perform the same comparisons with only te ENS lineages? If so or if not, this needs to be stated in the article. At a minimum, the authors must justify why they use the specific data subsets and not others. At first mention in the text each dataset should be referenced. As currently structured the reader must go dig around in the methods to figure out what the authors are doing. In particular the current state of the article makes it very difficult to determine what bioinformatic comparisons are being made and how some of the figures (e.g., Figure 5B, 5D) are being generated. The current article requires a good deal of understanding of prior ENS work to discern that the authors are not really using “postnatal progenitors” (Stavely et al., 2021 data set) for comparison with their wavefront ENDC RNA-Seq data but rather dissociated cells from cultured neurospheres that were derived from postnatal ENS. This lack of clarity could greatly muddle interpretations and the field going forward.

3 – the GSEA analysis is not well described and as written the manuscripts lacks sufficient detail to allow readers to assess how rigorously the study has been done or how to reproduce it in future. See additional specific points about this are listed below.

4 - None of the caveats of the mouse wavefront versus chick cranial neural crest comparisons are stated either in the results or the Discussion.

5 – The specificity of the DUSP inhibitor (BCI) must be documented. If this has previously been shown in prior literature then that literature should be cited. If specificity of BCI for DUSP6 has NOT previously been shown, then the authors need to devise an additional approach either using siRNA or lentiviral knockdown to support their claim that Dusp6 is required for migration of fetal ENDCs. This is important as multiple other Dusp genes are detected at high levels in the RNA-Seq data of this study.

6 - The discussion section lacks any mention of limitations of the study. In particular the group has profiled cells at the “wavefront” in the cecum. These cells are stalled in this area due to extremely high levels of GDNF and then the colon is further colonized by cells the enter the hindgut through transmesenteric migration as previously shown by Enomoto’s group. So, the question emerges of whether this population of wavefront cells is the most relevant to have profiled for future work to alleviate defects of colonic enteric populations? No other limitations for any aspects of the study are mentioned and there are limitations to the analysis and to the interpretation of the data. There are several and these should be stated so the broader audience of Development is aware.

7 - The study lacks source data and supplementary files to support several figures within the article. Minimally the authors need to include the GEO expression number for the raw bulk RNA-Seq data. This is presently lacking from the manuscript. Typically the authors provide a GEO “reviewer key” so the consistency and quality of the data being submitted can be assessed by reviewers.

8 - Supplementary tables that support figure panels 1E, 3E, and 5B are required so readers can appreciate the genes that underlay these comparisons and how the figures were derived. At present the Venn diagrams are not very informative without these supplementary files.

Other points to be addressed:

1 – There are several areas in the text where terms or approaches used are not defined or are used incorrectly:
a. the authors need to define terms at first use, such as “DEG analysis” on the bottom of page 5.

b. The authors use GSEA as a part of their analysis of RNA-seq data. When they list this, they do not describe what the acronym means or what the importance of it is.
c. The authors state “a PPI network analysis was performed using the DEGs”. Neither PPI nor DEGs are defined terms in the manuscript.
d. The authors use “E15-E18” at multiple points their text. The stages they are analyzing are not “embryonic” stages and are fetal stages when organogenesis is occurring so they should be using days post coitus (dpc) throughout the manuscript text and figures.
e. “wave front score” and “trailing score” in figure 2 panels B-C’ should be defined for the broader audience of Development f.

2 – It would benefit the audience to clearly state in the results section that this is “bulk RNA-seq from pooled flow-sorted ENCCs” because single cell RNA-Seq is now so common that a novice reader may presume otherwise. The authors should also state the limitations of comparing their bulk RNA-Seq data to other single cell RNA-Seq data sets in the Discussion section.

3 – The authors need to include unique identifiers for all mouse strains so that others can definitively know which lines they are using. Use of RRID or Mouse Genome Database numbers would accomplish this. For instance, it appears that the PLP1-EGFP strain is Tg(Plp1-EGFP)10Wmac , but this should be confirmed and added for each strain. The strain backgrounds on which the mouse lines are maintained should also be included as splice variants across strains can greatly influence detection of some transcripts and splice variants.

4 – Animal experiments should include the light schedule of housing, source of chow (vendor & catalog number) as well as bedding type and housing hardware source. Time of day of flow sort isolations should also be indicated as circadian rhythms have the potential to influence gene expression profiles.

5 – There are multiple aspects of the methods in which detail is lacking and should be corrected for publication to improve rigor and offer better opportunity for reproducibility. These include:
a. Were ENCCs sorted directly into trisol? Details of isolation methods are lacking and should be expanded. What metrics did the authors use to ascertain whether the RNA they sequenced was of sufficient quality?
b. How many embryos were pooled to produce each RNA-Seq replicate?
c. The authors need to include a PCA plot that compares the trailing and wavefront cells as a quality control metric to show show the trailing and wavefront replicates are distinct from one another and yet are similar between replicates.
d. This statement lacks sufficient detail for others to understand what was done or be able to repeat the work and needs to be elaborated for future reproducibility “A subset of cells representing the developmental trajectory of glia and neurons from neural crest cells of the peripheral nervous system (PNS) were utilized for downstream analysis.” What was the basis of selection of these cells (marker gene expression, features, etc).
e. For future reproducibility the authors need to add additional detail describing what imaging parameters were used for in vivo quantitation. “. . . All-in-one microscope using consistent imaging parameters.” What microscope (vendor and catalog) and what imaging parameters (filters used, magnification, range of exposure times etc).
f. It’s not clear how they are performing the comparison of the ENDC mouse data to the cranial NCC data from chick. Greater detail of how this was accomplished is required.
g. In figure 2 panels B – C’ the authors describe “module scores for wavefront ENCC genes”. How are these calculated? This information should be stated in the figure legend, or results section briefly then detailed in the methods.
h. Details of reagents used for library construction are lacking and should be included in the methods.

6. Some of the genes identified as potentially important wavefront genes (Ppp2r2b, CYTB, Cadm1, Rbp1, Dcx, Scg3, Onecut2,Lgals1, Elavl4, Sox10,Plp1) exhibit notable differences in expression BETWEEN individual wavefront runs. For instance Ppp2r2b is at 112 cpm in wavefront 1 run and only at 53 cpm in wavefront 2 run. Such notable differences in expression levels implies differences in either embryo staging, isolation process, or numbers of cells sorted. This should be mentioned and discussed. Such variation could be contributing to failure to detect other relevant genes. The

possibility that other genes not detected in this particular wavefront subset of cells could be essential in colonic colonization should be stated in the Discussion.

6. In the abstract the statement “The wavefront signature was enriched in uncommitted progenitors at E15-18 and in postnatal ENCC progenitors, indicating that some genes remain conserved during and after development.” is oddly worded. The use of the word “conserved” is incorrect and this should be rephrased. “maintained” or “consistently expressed” would be a more appropriate word choice.

7. There are multiple issues with the GSEA analysis in the results section.
a. When first mentioning GSEA as a part of their RNA-seq analysis, the authors should introduce what the tool does so that this analysis is better appreciated by the broad readership of Development.
b. When performing their GSEA analysis, the authors rely on ranked gene expression ranked by LogFC. This is not always the best option, especially if there is a large difference between the p-value and LogFC (for example, a gene with high p-value meaning less significant but a large LogFC might inflate the importance of the gene). It would be much more rigorous to rank by both the LogFC and the p-value. If this approach changes the outcomes, the authors should adjust relevant tables.
c. How this data is presented should be more clearly described. It’s very confusing that the authors shift to talking about comparison of mouse versus chick data without clear description of what they are doing and the caveats of this approach. It is very possible that such cross species comparison could be missing genes due to annotation differences and this limitation should be mentioned in the Discussion.
d. Most readers of Development are unlikely to be familiar with GSEA plots and data so the burden is on the authors to better explain in the results section the visual results they have included in their figures and their interpretations particularly for figures 4A and 5A.

8. Use of the term “ENCC” is an inaccurate acronym. Cells are no longer “neural crest” once they leave the region of the neural tube although they are derivatives of the neural crest throughout their subsequent migration and differentiation. It is strongly recommended that the authors use the more accurate terminology of Enteric Neural Crest-Derived Cells (ENDC) since they are studying derivatives of the neural crest in the ENS.

9. Upon comparing the gene signatures from enteric wavefront and trailing populations with those from cranial neural crest, the authors conclude there are “unique migratory mechanisms between NCC populations”. However, they do not show this is the case and should correct their phrasing to state “there are distinct patterns of gene expression between cranial and enteric wave-front populations” because that is what their analysis shows and they do not have any experimental support for unique migratory mechanisms.

10. The wording of the section on page 7 that begins “To explore the properties of wavefront ENCCs, a PPI network analysis was performed . . .” is very confusing and required reiterative reading to grasp what the group is doing. The authors should break down their analysis into a stepwise description and ask for non-expert readers to read/review for better accessibility of this aspect prior to resubmission.

11. There are several places in the text where the wording is loose and could be misinterpreted or lacked sufficient citations.
a. For instance “In previous studies, no clear distinctions between neural progenitors and glia have been observed” is ambiguous and could be misinterpreted or taken out of context. The beginning of this section should be revised to be more detailed, emphasize transcriptional differences among enteric progenitors, and include relevant references!
b. also in the sentence “Comparison of gene expression profiles between postnatal enteric progenitors and the primary ENS”. What is meant by “primary ENS”? This is unclear and could be misconstrued. It took this reviewer a while to determine that
c. The statement “These genes are also highly expressed in postnatal glia and appear to reflect the gradual maturation of early embryonic progenitors to glia, which notably retain neurogenic potential after birth, albeit more restricted” needs to include appropriate citations to support what is being stated.

d. The text lines “the neuroblast specific gene Miat was observed in the maturing trailing signature and is considered to be an important functional long noncoding RNA in neuronal stem cell specification in other regions” needs appropriate references to support the statement that Miat is “is considered to be an important functional long noncoding RNA in neuronal stem cell specification in other regions”.
e. The authors state “We hypothesized that DUSP6 signaling regulates hindgut colonization” but the rationale for why the focus on hindgut in this statement is not apparent.

12. The authors are relaying on transgenes like Tau-EGFP to label differentiating neurons and state “Expression patterns were validated in mice postnatally, confirming appropriately labeled neurons (GFP+)”. Figure 4D simply shows co-localization of Wnt1-cre lineages with Tau-EGFP+ cells and the resolution is such in the images shown that one cannot conclude if these cells are neuronal. The data shown is all relative to Wnt1-cre:Rosa labeling and does not illustrate cell type specificity. If they are going to use this line, they must either site the literature that unequivocally shows this transgene is expressed in differentiating enteric neurons or perform a HuC/D or other pan-neuronal stain with this transgenic reporter. Because EGFP signal takes a while to accumulate, it is not clear why they are relying on this reporter instead of using an early neuron IHC label like HuC/D or peripherin?

13. Similarly in relying on the Plp1-EGFP transgene as a “glial marker”, the authors are making a leap of faith that Plp1-EGFP is accurately reporting glial cell fate. In the images shown for postnatal Plp1-EGFP, the distribution pattern does appear to be that of glial cells, although it would be preferable for the study to show this using co-localization with a glial restricted marker using immunolabeling at higher resolution. Moreover, if Plp1-EGFP is a “glial” reporter then one would expect to see it expressed at a delay as glial markers emerge after neuronal markers according to data published years back by Heather Young’s group (J. Comp Neurol 456:1-11, 2003). Instead the authors show emergence of the Plp1-EGFP reporter signal within the wavefront population. So it is possible that this transgene is not truly reflecting glial identity or that it is transiently activated in progenitors then becomes restricted to glia postnatally. Oddly the authors data in Figure 4 panels J and K does show that some of the Plp1-EGFP+ cells ARE TuJ1+ indicating that this transgene cannot be relied upon as a “glial marker”. These issues must be addressed to avoid muddying terminology and understanding in the field.

14. The authors describe other “glial markers” in the heatmap panel B of Figure 4 with no provided explanation or basis for why these specific genes are considered “glial markers”. Additional information is required to justify how the genes listed are specific to enteric glia. Minimally citation of prior literature that explicitly shows these genes are expressed in enteric glia should be added.

15. As written the manuscript lacks appropriate citation of datasets within the results section that the authors have incorporated into their analysis. At first mention in the results where the authors state they are assessing differentially expressed genes from wavefront populations using GSEA in the E15-18 dataset that appears to derive from Morarach et al., 2021 they should site that adjacent in the results so readers are not having to go find this in later article sections.

16. The authors are using datasets that originate from neurospheres that were cultured from postnatal ENS and are calling this “postnatal progenitors”. This loose use of terminology could mislead readers and needs to be more clearly described in the results section of titled The Wavefront-Trailing ENCC gene signature is predictive of progenitor and restricted cell populations post colonization To their credit the authors do include relevant details sufficient to find this in prior publication that is cited in the methods. However, readers should not have to go back to prior papers to assess what is being done.

17. The expression profile of DUSP6 that is presently relegated to a supplementary figure (S2) should be moved to the main text. Additional expression data for Dusp6 should also be added, including a feature plot (UMAP) showing the distribution of its expression similar to that shown for Miat in figure 5 panel F. The authors should include Dusp6 as a line in panel Figure 5D. It’s not clear why this was not done initially. The logic and stepwise comparisons that the authors followed to identify Dusp6 as a priority candidate gene for subsequent study are lacking.

18. The authors are examining Dusp6 function in mouse cells and identified it in mouse cells. They should pay attention to use mouse appropriate nomenclature with designation of “Dusp6” as the gene throughout the text. There are several places where it’s clear they are referring to the gene and yet they are using protein nomenclature (DUSP6).

19. In the first paragraph of the results section focused on DUSP6 requirement, the authors evaluate DUSP6’s role in ENCC colonization through the “DUSP inhibitor BCI”. Towards the end of this same section, they refer to BCI as a “DUSP6 inhibitor”. How specific is BCI as an inhibitor of DUSP6? Does BCI inhibit all DUSP proteins or is it limited/targeted to DUSP6? Citations from the literature should be included that clarify this aspect.

20. When comparing their bulk RNA-seq data to the 15 and 18 dpc ENDCs scRNA-seq data, the authors use a combined dataset with both ages instead of the two ages separately. When the 15.5dpc signature alone is used does it have a stronger signature for the wavefront DEGs?

21. It is not clear what data set is being used to generate the image shown in Figure 5 panel D.

22. The authors appear to have duplicated a sentence in the introduction “Interestingly, key ligands such as GDNF can have dual roles in migrating ENCCs by being chemoattractive, providing trophic support and promoting neural differentiation, with excess of the latter arresting colonization”. The introduction should be

23. For Figure 5B the figure legend states “Venn-diagram of leading-edge genes in the E11.5 wavefront signature (ENCC immaturity) upregulated in the E15.5-18.5 ENCC progenitor population and postnatal ENCC progenitors”. But there are only two circles to the Venn Diagram that is shown. So the authors needs to clarify how they got to this outcome.

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
In this manuscript, Nagy and colleagues transcriptionally profile enteric neural crest cells as the migrate through the gut, comparing the transcriptional profile of cells at the wavefront with those at the trailing edge. They find that the transcriptional signature of wavefront cells and find that is enriched in genes characteristics of uncommitted progenitors at both embryonic and postnatal stages. They then focus on the gene Dusp6 which is one of the genes enriched in wavefront cells, and show that loss of function of DUSP in an ex ovo assay abrogates enteric progenitor migration in an ERK dependent fashion.

Comments for the author

This is a nicely done study with interesting findings. However there remain a few issues that would need to be addressed, most prominently concerning rationale for using bulk RNA-sequencing and validation of gene expression. Major issues are:

1.Given that there is likely to be heterogeneity both in the wavefront and trailing cells, it is is puzzling that the authors used bulk RNA-sequencing rather than single cell RNA-seq. This further complicates their analysis since they are often comparing their datasets to single cell RNA-seq datasets. They should repeat at least one of their embryonic timepoints using scRNA-seq to determine the degree of heterogeneity in the wavefront population.
We agree that scRNA-seq would be a great addition to the study and was an idea we initially considered in great detail. There were a few reasons we were unable to take this approach. In the study of Morarach 2021 Nat Neurosci the authors are only able to isolate ~ 3000 Wnt1-tdT cells from the entire small bowel of 15.5 dpc and 18.5 dpc embryos. Even in these older embryos the cell resolution was too low to define heterogeneity in progenitor populations. In our study of ENCCCs in trailing and wavefront regions of the 11.5 dpc gut we utilized just 0.5 mm of intestinal

tissue from each section and therefore the cell yield is very low. We actually attempted the experiment with 10 breeding pairs but to get enough embryos at the same time to do 10X experiments after accounting for cell viability, droplet capture efficiency and inevitable cells with poor reads, achieving an appropriate number of wavefront cells for a meaningful analysis was extremely difficult and unlikely to provide much information on heterogeneity of the wavefront ENCDCs given the prior Morarach study.
Morarach, K., Mikhailova, A., Knoflach, V., Memic, F., Kumar, R., Li, W., Ernfors, P. and Marklund, U. (2021). Diversification of molecularly defined myenteric neuron classes revealed by single-cell RNA sequencing. Nature neuroscience 24, 34-46.

2.The authors identify several genes enriched in the wavefront and validate expression and test function of Dusp6. While it would be unreasonable to ask for functional testing of more of these genes, the authors should at least include in situs validating their expression patterns in the wavefront compared with trailing cells.
We performed immunohistochemistry to validate the expression patterns of the protein products of several of these genes, including Cdh11, Fbn2 and Synd-4 (new Figure 2). We also tried the following antibodies but were unsuccessful in detecting a signal that we could confidently say was expressed in ENCDCs; anti-Heparan Sulfate Proteoglycan 2/Perlecan antibody (cat.no.: ab2501; Abcam; 1:100), anti-Crym Polyclonal Antibody (cat. no.: PA5-53920; ThermoFisher Scientific; 1:20) and anti-vimentin (cat.no.: sc-7557; SantaCruz Biotech; 1:100).

3.Figure 1C-C'": while Nestin is said to be enriched in "wavefront" cells, the signal above the mesentery in the "trailing" region looks strong to me. Please clarify.
We apologize for this confusion and agree that Nestin expression is uniform throughout the intestine, and this matches the RNA-seq data suggesting there is no statistical difference in Nestin expression between wavefront and trailing ENCDCs. The text has been altered to make our interpretation clearer. This also helps emphasize our points later in the manuscript that cells behind the wavefront still possess stem cell qualities and furthermore provides rationale for our quantification of GFP as a surrogate marker of Mapt and Plp1 expression, which do show differences between the wavefront and trailing ENCDCs, as shown in Figure 4.

4.Which enriched genes are common between enteric wavefront cells and cranial neural crest wavefront cells described by Morrison et al.?

Thank you for this question. We have added this analysis to supplementary material as being cross-species the analysis has its limitations. Despite this, the results show statistically significant similarities between the wavefront and trailing cells from both datasets which is noteworthy. Individual genes are reported in Table S2. Compared to the trailing signatures only a handful of genes are upregulated in both wavefront populations.

5.Figure 2A-C: the comparison between PNS and the present data is not convincing. Please clarify how you calculated the WF score based on wavefront DEGs as opposed to gene counts.

The addmodulescore is a function within the Seurat package that generates scores based off the combined expression of gene sets compared to computed sets of equivalent control genes to each cell individually. Scores are unitless but positive scores indicate the degree of overexpression compared to random chance. The methods were originally derived from Tirosh et al. (2016) before being implemented into the Seurat package. This provides an overall estimation of wavefront and trailing signatures that account for differences in the complexity and quality of individual expression data between cells which is a problem for scRNA-seq datasets. For example, one limitation of scRNA-seq that isn't an issue with bulk RNA-seq is that most genes within a cell have 0 counts which does not necessarily mean the cells don't express the gene but the sequencing depth per cell is limited due to the nature of the technology. By evaluating whole gene sets the problem with the heterogeneity of reads can be overcome and also provides a nice summary. In the updated manuscript we have now provided heatmaps that reflect the largest and most consistent genes driving changes to these scores in Figures 3, 5, S2 and data in Tables S3, S4, S5 S7.
Tirosh, I., Izar, B., Prakadan, S. M., Wadsworth, M. H., Treacy, D., Trombetta, J. J., Rotem, A., Rodman, C., Lian, C. and Murphy, G. (2016). Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science (New York, N.Y.) 352, 189-196.

6.The requirement for Dusp6 ex vivo is a very nice addition. Is there is a Dusp6 flox allele available to perform a knockout via Wnt1-cre for in vivo assay?
This would be a fantastic addition to the manuscript. Unfortunately, we were unable to find any Dusp6 floxed mice, but BCI has been used as a specific DUSP6 inhibitor in many high-profile studies in other fields of research (Molina et al. 2009; Li et al. 2012). In the updated manuscript we performed two additional models to assay the role of DUSP6 on migration, and these are shown in Figure 7H-I, Figure 8 A-D, Figure S4 and Supplementary Movie 1-2.
Molina G, Vogt A, Bakan A, Dai W, De Oliveira PQ, Znosko W, Smithgall TE, Bahar I, Lazo JS, Day BW. 2009. Zebrafish chemical screening reveals an inhibitor of Dusp6 that expands cardiac cell lineages. Nature chemical biology 5: 680-687.
Li G, Yu M, Lee W-W, Tsang M, Krishnan E, Weyand CM, Goronzy JJ. 2012. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. Nature Medicine 18: 1518-1524.

Reviewer 2 Advance summary and potential significance to field
This manuscript performs scRNAseq analysis on enteric neural crest, comparing cells in the migratory front with trailing cells. They found several differences in the transcriptomic of these two populations that are consistent with their distinct cellular activities.

Reviewer 2 Comments for the author
Migration of enteric neural crest cell is an important topic of research with critical clinical implications. The observation that leader or front cells behave different to trailing cells during the colonization of the gut by neural crestcells, is widely documented. I do not see what significant biological questions this scRNAseq analysis is addressing, as it is manly a confirmation of what was already known about the biology of these two cell populations. For example,
The authors find that wave front cells express higher levels of mRNA linked to proliferation, but it was already known that migration of enteric neural crest requires high levels of proliferation, and that most of the proliferation takes place in the leader cells

We agree with the reviewer that noting the highly proliferative nature of ENCDCs is not new – our rationale for including this is to show that our results are consistent with the literature. There are few studies providing quantitative evidence of differences in the proliferation rates between wavefront and trailing ENCDCs, however Simpson et al. 2007 show enhanced proliferation in wavefront ENCDCs in avian models. We have made references to the previous literature clearer in the revised manuscript.
Simpson, M. J., Zhang, D. C., Mariani, M., Landman, K. A. and Newgreen, D. F. (2007). Cell proliferation drives neural crest cell invasion of the intestine. Dev Biol 302, 553-568.

The finding that front cells express high levels of genes associated with migration is not surprising at all, as these are highly migratory cells
We also agree that it isn't surprising that wavefront cells are showing genes associated with migration because they are migratory. Nevertheless, there is conflicting evidence that the wavefront cells are unique as it has been reported that transplantation of trailing cells to the wavefront result in hindgut colonization ex vivo (Simpson et al. 2007). Our transcriptional approach confirms that wavefront ENCDCs are indeed different in vivo. Regardless, it isn't known what the genes are that make wavefront cells more migratory and how we can study this model system to learn about colonization, issues with migration leading to disease and potentially how this knowledge can assist in the development of therapies in the future. To our knowledge, this is the first study to transcriptionally profile the migratory wavefront during ENS development.
Simpson, M. J., Zhang, D. C., Mariani, M., Landman, K. A. and Newgreen, D. F. (2007). Cell proliferation drives neural crest cell invasion of the intestine. Dev Biol 302, 553-568.

The last part of this manuscript is about the role of Dusp6 on enteric cell migration. This is done very superficially, as they only show that chemical inhibition of Dusp6 shows reduced staining for the front cells. Is this an effect on the number of cells? The migratory abilities of the cells? The invasiveness of the cells? The EMT on these cells? Etc.

We thank the Reviewer for this comment. Although we use SOX10 staining in the avian experiments, the mouse experiments use tdTomato expression driven by Wnt1-cre which is not

immunolabeled, so a lack of staining in the wavefront is not a contributing factor to those observations. We have provided additional assays to further explore the role of Dusp6 in ENCDC migration. This includes staining of the proliferation marker Phosphohistone H3 during avian hindgut colonization and quantification of ENCDC migration from avian midgut explants on fibronectin in response to GDNF, including live cell imaging. BCI (DUSP6 inhibitor) significantly reduced ENCDC proliferation in the avian hindgut; however, ERK pathway inhibition restored cell migration without ameliorating the reduction in cell proliferation, suggesting a direct role in cell motility. Live cell imaging recordings of midgut explants show that cells migrating out of explants in response to GDNF return to the explants after DUSP6 is inhibited with BCI. Cells proceed to migrate out of the explants again after BCI is removed. These findings suggest that although DUSP6 has a role in proliferation, the effect on invasion that is mediated by the ERK pathway is directly associated with cell migration. These new data can be found in Figure 7H-I, Figure 8A-D, Figure S4 and Supplementary Movie 1-2.

Reviewer 3 Advance summary and potential significance to field
Stavely et al have generated the first transcriptional profile of enteric neural crest derived progenitors (ENDCs) that are migrating at the front of the population of cells that colonizes the fetal bowel. The team elected to profile these cells at 11.5dpc in the mouse using a bulk sequencing approach. The goal of the study was to determine whether this population of ENDCs at the migration wavefront during colonization exhibits a unique transcriptional signature. The study compares transcriptional profiles from flow-sorted wavefront progenitors to the population of cells behind the migrating wavefront in a region slightly more proximal to identify differentially expressed genes that may be participating in the colonization process. The authors then perform comparisons with other datasets including a single cell ENS RNA-Seq data set from Morarach et al., 2021 and another bulk RNA-Seq data set from their own group (Stavely et al., 2021) that includes primary postnatal ENS cell types as well as cells dissociated from cultures of postnatal neurospheres that they call “postnatal progenitors”. The study includes identification of Dusp6 as a gene that exhibits differential expression in the wavefront ENDCs and uses a DUSP inhibitor (BCI) in an effort to assess whether Dusp6 is essential for hindgut colonization.

Despite the issues with the manuscript in its current form, this study IS important as it offers an opportunity to understand how ENDCs have such vast migratory potential and how they respond to exogenous cues (GDNF, Edn3) during migration. However, the article could be greatly improved through added clarity of writing and better placement for context regarding what is already known in the field about ENS development.

Reviewer 3 Comments for the author
Primary Concerns:
There are several aspects in the manuscript that need to be addressed to make the findings of value to the ENS field and the larger Development community. These include: lack of rationale in the text for the focus on the wavefront progenitors in the cecal region; lack of detail in the methods sections for cell isolations, bioinformatics, and information regarding the specificity of the BCI inhibitor; overstatement of findings particularly the chick and mouse comparison studies; lack of any study limitations that should be added to the Discussion.

1 – The rationale and arguments for the initial profiling of the wavefront cells is lacking and not well referenced. The study is unique and the first of its kind to profile a population of wavefront progenitors. However, the authors do not clearly state why they choose this particular population or this stage of development. They could have chosen to profile wavefront ENDCs earlier in development when colonization is in the foregut. Importantly, because work from Enomoto’s group has conclusively shown that transmesenteric progenitors provide the greatest percentage of neurons in the colon (Nishayama et al., 2012) it is not clear how valuable profiles from wavefront progenitors in the cecal region that are paused there due to very high levels of GDNF will be. If the approach taken was pursued because it offered a more consistent isolation during development, the article should state that AND acknowledge that other sources of colonizing progenitors that populate the colon do exist.

We thank the Reviewer for this important comment. As noted by the Reviewer, complex developmental mechanisms are responsible for the migration of ENCDCs through the gut, including the presence of chemoattractant molecules, such as GDNF, which is specifically expressed in the ceca mesenchyme before hindgut mesenchyme. We chose mice at 11.5 dpc for several reasons: According to Young et al (2014) the speed and directional migration of ENDCs between 750 and 1,500 μm from the wavefront migrate significantly slower than those within 450 μm of the most caudal cell. Therefore, we needed a stage that provided us sufficient distance between wavefront and trailing regions, and this was possible at 11.5 dpc, but not 10.5 dpc. The length of colonized gut at 10.5 dpc is shorter than 1000 μm, preventing isolation of the trailing region from this younger embryonic stage.

According to Druckenbrod and Epstien 2005 Dev Biol: At E10.5, the wavefront in the ileum advances caudally at an average speed of 45 μm/h (±10.8 SE, n = 4), but decreases to 32 μm/h at E11.25 as it approaches the nascent cecum. Despite the ENCDCs slowing down their caudal advance, the cells still move (show active migration to colonize the complete cecum) before they start to colonize the hindgut.
We agree with the Reviewer that trans-mesenteric ENCDCs are also essential for hindgut ENS formation, and similar to Schwann cell precursors (Uesaka et al. 2015, J Neurosci) contribute to the distal two-third of the mouse hindgut ENS. Comparative RNAseq analysis of these ENS precursors with ENCDCs migrating through the cecum would be an informative and exciting topic for the next study.
As suggested, the text has been amended to highlight to the readership the rationale for these experiments and considerations/limitations of the study.
Young, H. M., Bergner, A. J., Simpson, M. J., McKeown, S. J., Hao, M. M., Anderson, C. R. and Enomoto, H. (2014). Colonizing while migrating: how do individual enteric neural crest cells behave? BMC Biology 12, 23.
Druckenbrod, N. R. and Epstein, M. L. (2005). The pattern of neural crest advance in the cecum and colon. Dev Biol 287, 125-133.
Nishiyama, C., Uesaka, T., Manabe, T., Yonekura, Y., Nagasawa, T., Newgreen, D. F., Young, H. M. and Enomoto, H. (2012). Trans-mesenteric neural crest cells are the principal source of the colonic enteric nervous system. Nature neuroscience 15, 1211-1218.
Uesaka, T., Nagashimada, M. and Enomoto, H. (2015). Neuronal Differentiation in Schwann Cell Lineage Underlies Postnatal Neurogenesis in the Enteric Nervous System. The Journal of Neuroscience 35, 9879.

2a The authors need to be much more explicit and clear about what they are doing in the methods and results section with regards to several of the datasets they are using. For instance the Cao et al., 2019 data includes both peripheral neurons and glia as well as enteric lineages. It is not clear why the present authors choose to use the entire PNS dataset instead of the ENS lineages. Do they get the same results when they perform the same comparisons with only the ENS lineages? If so or if not, this needs to be stated in the article. At a minimum, the authors must justify why they use the specific data subsets and not others.
Thank you for this comment. In the Cao et al., 2019 dataset neural crest developmental trajectories are derived from single cell sequencing of the entire embryo with no spatial data, so there is likely to be an element of guess work in the annotation by the authors. To provide confidence in the data, we have performed data integration of the predicted enteric developmental trajectories of Cao et al. with the Morarach et al 2021 dataset, which is specific to ENCDCs, to try and resolve which Cao trajectories were definitely enteric. This seemed to work well to parse out the correct data as we were able to determine that out of Cao et al's two 'enteric_glia_and_Schwann_cell' trajectories only one was consistent with ENCDCs. After validating this, we repeated the analysis using clusters specific to ENCDCs and early NCCs from 9.5 dpc to show how genes change after differentiation into ENCDCs and their subsequent maturation. Results are similar to the initial analysis, but much improved thanks to this valuable suggestion.
Cao, J., Spielmann, M., Qiu, X., Huang, X., Ibrahim, D. M., Hill, A. J., Zhang, F., Mundlos, S., Christiansen, L., Steemers, F. J., et al. (2019). The single-cell transcriptional landscape of mammalian organogenesis. Nature 566, 496-502.
Morarach, K., Mikhailova, A., Knoflach, V., Memic, F., Kumar, R., Li, W., Ernfors, P. and Marklund, U. (2021). Diversification of molecularly defined myenteric neuron classes revealed by single-cell RNA sequencing. Nature neuroscience 24, 34-46.

2b At first mention in the text each dataset should be referenced. As currently structured the reader must go dig around in the methods to figure out what the authors are doing. In particular the current state of the article makes it very difficult to determine what bioinformatic comparisons are being made and how some of the figures (e.g., Figure 5B, 5D) are being generated.
We apologize for this confusion and have amended the text in the results section to make the bioinformatics processes and datasets used clearer for the reader.

2c The current article requires a good deal of understanding of prior ENS work to discern that the authors are not really using “postnatal progenitors” (Stavely et al., 2021 data set) for comparison with their wavefront ENDC RNA-Seq data but rather dissociated cells from cultured neurospheres that were derived from postnatal ENS. This lack of clarity could greatly muddle interpretations and the field going forward.

This is an excellent point. Neurospheres generated from postnatal ENCDCs have obvious progenitor/stem cell qualities and have a high capacity for proliferation and neurogenesis. Most likely these cells represent enteric glia that revert back to a progenitor-like phenotype when cultured in the right conditions. The distinction between progenitors that can generate neurons and traditional enteric glia is currently not clear in the embryo or postnatal environment. They are transcriptionally very similar, so it seems difficult to provide a strict cut-off. The Stavely et al., 2021 dataset is a bulk RNA-seq dataset of postnatal neurospheres that include all types of ENCDCs, although one would expect progenitors to be enriched in the neurospheres which would subsequently be reflected in the gene expression data. Given the Reviewer’s comment, we repeated the analysis using a single cell RNA-seq dataset of enteric neurospheres generated by our lab to specifically parse out the progenitor population that gives rise to enteric neurons. Hopefully this helps address the limitations of the bulk RNA-seq dataset. We have also consistently included a better explanation of how these cells were obtained, and now refer to them more precisely, so the reader has a clear understanding of what cells we are analyzing.

3 – the GSEA analysis is not well described and as written the manuscript lacks sufficient detail to allow readers to assess how rigorously the study has been done or how to reproduce it in future. See additional specific points about this are listed below.
a. When first mentioning GSEA as a part of their RNA-seq analysis, the authors should introduce what the tool does so that this analysis is better appreciated by the broad readership of Development.
b. When performing their GSEA analysis, the authors rely on ranked gene expression ranked by LogFC. This is not always the best option, especially if there is a large difference between the p-value and LogFC (for example, a gene with high p-value meaning less significant but a large LogFC might inflate the importance of the gene). It would be much more rigorous to rank by both the LogFC and the p-value. If this approach changes the outcomes, the authors should adjust relevant tables.
c. How this data is presented should be more clearly described. It’s very confusing that the authors shift to talking about comparison of mouse versus chick data without clear description of what they are doing and the caveats of this approach. It is very possible that such cross species comparison could be missing genes due to annotation differences and this limitation should be mentioned in the Discussion.
d. Most readers of Development are unlikely to be familiar with GSEA plots and data so the burden is on the authors to better explain in the results section the visual results they have included in their figures and their interpretations particularly for figures 4A and 5A.
a. We have added an explanation of GSEA at first use. b. All GSEA in the study has been repeated with gene lists ranked by the sign(log2FC)×log10(p-value) method. c. We have provided clearer descriptions of comparisons in the results section and added limitations to the discussion. d. We have provided better descriptions of visual results, including the meaning of direction and interpretation of enrichment scores, to improve readability of the manuscript.
4 - None of the caveats of the mouse wavefront versus chick cranial neural crest comparisons are stated either in the results or the Discussion.

Thank you for this comment. As stated by the reviewer above, it is important not to overstate cross-species analysis given the associated limitations such as a lack of conservation in genes, or their functions, between species which needs to be taken into consideration when interpreting this

data. These limitations have now been addressed in the Discussion. However, since we observed common statistically significant relationships between the two datasets, we felt the data was still worth including as a supplementary table. The data for this analysis is provided in Table S2

5 – The specificity of the DUSP inhibitor (BCI) must be documented. If this has previously been shown in prior literature then that literature should be cited. If specificity of BCI for DUSP6 has NOT previously been shown, then the authors need to devise an additional approach either using siRNA or lentiviral knockdown to support their claim that Dusp6 is required for migration of fetal ENDCs. This is important as multiple other Dusp genes are detected at high levels in the RNA-Seq data of this study.

We appreciate this comment. BCI has been shown to be a specific DUSP6 inhibitor and it has been used for this purpose in many studies (Molina et al. 2009; Li et al. 2012). We have updated the manuscript to include this information.
Molina G, Vogt A, Bakan A, Dai W, De Oliveira PQ, Znosko W, Smithgall TE, Bahar I, Lazo JS, Day BW. 2009. Zebrafish chemical screening reveals an inhibitor of Dusp6 that expands cardiac cell lineages. Nature chemical biology 5: 680-687.
Li G, Yu M, Lee W-W, Tsang M, Krishnan E, Weyand CM, Goronzy JJ. 2012. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. Nature Medicine 18: 1518-1524.

6 - The discussion section lacks any mention of limitations of the study. In particular the group has profiled cells at the “wavefront” in the cecum. These cells are stalled in this area due to extremely high levels of GDNF and then the colon is further colonized by cells the enter the hindgut through transmesenteric migration as previously shown by Enomoto’s group. So, the question emerges of whether this population of wavefront cells is the most relevant to have profiled for future work to alleviate defects of colonic enteric populations? No other limitations for any aspects of the study are mentioned and there are limitations to the analysis and to the interpretation of the data. There are several and these should be stated so the broader audience of Development is aware.
Thank you for this suggestion. We have added a section to the Discussion to address these important limitations.

7 - The study lacks source data and supplementary files to support several figures within the article. Minimally the authors need to include the GEO expression number for the raw bulk RNA-Seq data. This is presently lacking from the manuscript. Typically the authors provide a GEO “reviewer key” so the consistency and quality of the data being submitted can be assessed by reviewers.

RNA-seq data have been deposited in the Gene Expression Omnibus database under accession number GSE217757.
To review GEO accession GSE217757: https://secure-web.cisco.com/102gcPAUlptPPTzKo8O1X8IPk5qQoDD8ZJ05GW8mB60_ELqnDRJuGlBB4LejiCsTMvT854_4zBhzraMxDcUHFuEt0gqK7YwM6mVr8bhl1yqUJQ9zDhYnUsEhOfcx15zSIL2frfQdoolLZToVEi24wbyBmId2PSbkXUksHTLtgjSUyU-4UKYhEkBd2adOolZtXgaTLTaKakvdH0zfn2no82mXGIaQX4KwbjOMPtfgIaCVln8fU0WJsh9lvt7B9pw8hSkgEgcixLfF1QD6_1_Dk8uLP_fjwowiU5xi3yZnT1ZtlXsY7pdee8SYjztIUnQS8/https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fgeo%2Fquery%2Facc.cgi%3Facc%3DGSE217757 Enter token yvqluwgunbgjhgp into the box

8 - Supplementary tables that support figure panels 1E, 3E, and 5B are required so readers can appreciate the genes that underlay these comparisons and how the figures were derived. At present the Venn diagrams are not very informative without these supplementary files.

We apologize for this oversight. All data have been provided as supplementary material in Tables S2-S8.
Other points to be addressed:
1 – There are several areas in the text where terms or approaches used are not defined or are used incorrectly:
a. The authors need to define terms at first use, such as “DEG analysis” on the bottom of page 5.

b. The authors use GSEA as a part of their analysis of RNA-seq data. When they list this, they do not describe what the acronym means or what the importance of it is.
c. The authors state “a PPI network analysis was performed using the DEGs”. Neither PPI nor DEGs are defined terms in the manuscript.
d. The authors use “E15-E18” at multiple points their text. The stages they are analyzing are not “embryonic” stages and are fetal stages when organogenesis is occurring so they should be using days post coitus (dpc) throughout the manuscript text and figures.
e. “wave front score” and “trailing score” in figure 2 panels B-C’ should be defined for the broader audience of Development

We appreciate these comments which will improve clarity of the manuscript. a-c. DEG, GSEA and PPI have been defined at first usage d. the terminology days post coitus (dpc) is now used throughout the manuscript when referring to mouse; for chick embryos we have maintained the standard reference to embryonic day X (EX). e. we have provided a better explanation of these scores in the results section.

2 – It would benefit the audience to clearly state in the results section that this is “bulk RNA-seq from pooled flow-sorted ENCCs” because single cell RNA-Seq is now so common that a novice reader may presume otherwise. The authors should also state the limitations of comparing their bulk RNA-Seq data to other single cell RNA-Seq data sets in the Discussion section.
The text had been amended to make this clear in the results section. The limitations and advantages of this approach compared to scRNA-seq have been added to the discussion.

3 – The authors need to include unique identifiers for all mouse strains so that others can definitively know which lines they are using. Use of RRID or Mouse Genome Database numbers would accomplish this. For instance, it appears that the PLP1-EGFP strain is Tg(Plp1-EGFP)10Wmac , but this should be confirmed and added for each strain. The strain backgrounds on which the mouse lines are maintained should also be included as splice variants across strains can greatly influence detection of some transcripts and splice variants.
We appreciate this suggestion. All strain details have been added to the mouse resources table.

4 – Animal experiments should include the light schedule of housing, source of chow (vendor & catalog number) as well as bedding type and housing hardware source. Time of day of flow sort isolations should also be indicated as circadian rhythms have the potential to influence gene expression profiles.

Thank you for the comment. All experiments were conducted in the mornings at the same time to keep experimental consistency. Rodents were housed in Allentown Inc rectangular caging (160 cages per individually ventilated cage racks; which uses blower at 60 air changes per hour) under a 12h:12h light:dark cycle from 7 am – 7 pm. Bedding consisted of Hardwood Sanichip, with Carefresh nesting material, and mice had access to Prolab Isopro RMH 3000 chow mix (ScottPharma) ad libitum. This has been added to the methods section.

5 – There are multiple aspects of the methods in which detail is lacking and should be corrected for publication to improve rigor and offer better opportunity for reproducibility. These include:
a. Were ENCCs sorted directly into trisol? Details of isolation methods are lacking and should be expanded. What metrics did the authors use to ascertain whether the RNA they sequenced was of sufficient quality?
b. How many embryos were pooled to produce each RNA-Seq replicate?
c. The authors need to include a PCA plot that compares the trailing and wavefront cells as a quality control metric to show show the trailing and wavefront replicates are distinct from one another and yet are similar between replicates.
d. This statement lacks sufficient detail for others to understand what was done or be able to repeat the work and needs to be elaborated for future reproducibility “A subset of cells representing the developmental trajectory of glia and neurons from neural crest cells of the peripheral nervous system (PNS) were utilized for downstream analysis.” What was the basis of selection of these cells (marker gene expression, features, etc).
e. For future reproducibility the authors need to add additional detail describing what imaging parameters were used for in vivo quantitation. “. . . All-in-one microscope using consistent imaging

parameters." What microscope (vendor and catalog) and what imaging parameters (filters used, magnification, range of exposure times etc).
f. It's not clear how they are performing the comparison of the ENDC mouse data to the cranial NCC data from chick. Greater detail of how this was accomplished is required.
g. In figure 2 panels B – C' the authors describe "module scores for wavefront ENCC genes". How are these calculated? This information should be stated in the figure legend, or results section briefly then detailed in the methods.
h. Details of reagents used for library construction are lacking and should be included in the methods.

Thank you for these questions. a. cells were sorted directly into trizol and RNA integrity was evaluated using an Agilent Bioanalyzer. This been added to the text b. seven embryos were pooled for each experimental run and this has been added to the methods c. PCA plot has been added to Figure 1E showing similarity between replicates. d. the process of defining cells to be analyzed from the Cao dataset has been revisited based on the reviewers suggestion and has been detailed in Figure 3 and Figure S2 e. Imaging parameters were consistent for all experiments (Plp1GFP - filter: BZ-X filter GFP (model OP87763), resolution: 960x720, objective Lens: PlanApo_ λ 4x 0.20/20.00mm, exposure Time: 4s, gain: +6dB, mercury quantity: 20%, capturing mode: Monochrome 8bit, binning: 2x2; Wnt1-tdT - filter: BZ-X filter TexasRed (model OP87765), exposure Time: 4.5s, with the remaining parameters the same as Plp1GFP). This has been added to the methods. f. greater details have been added to the results and methods of how this analysis was performed g. greater description has been provided in the figure legends, results and methods. h. Library construction details have been added to the methods.

6. Some of the genes identified as potentially important wavefront genes (Ppp2r2b, CYTB, Cadm1, Rbp1, Dcx, Scg3, Onecut2,Lgals1, Elavl4, Sox10,Plp1) exhibit notable differences in expression BETWEEN individual wavefront runs. For instance Ppp2r2b is at 112 cpm in wavefront 1 run and only at 53 cpm in wavefront 2 run. Such notable differences in expression levels implies differences in either embryo staging, isolation process, or numbers of cells sorted. This should be mentioned and discussed. Such variation could be contributing to failure to detect other relevant genes. The possibility that other genes not detected in this particular wavefront subset of cells could be essential in colonic colonization should be stated in the Discussion.
Thank you for this important observation. We have added a discussion of this to the limitation section.

6. In the abstract the statement "The wavefront signature was enriched in uncommitted progenitors at E15-18 and in postnatal ENCC progenitors, indicating that some genes remain conserved during and after development." is oddly worded. The use of the word "conserved" is incorrect and this should be rephrased. "maintained" or "consistently expressed" would be a more appropriate word choice.
We agree and have made this change as suggested.

8. Use of the term "ENCC" is an inaccurate acronym. Cells are no longer "neural crest" once they leave the region of the neural tube although they are derivatives of the neural crest throughout their subsequent migration and differentiation. It is strongly recommended that the authors use the more accurate terminology of Enteric Neural Crest-Derived Cells (ENDC) since they are studying derivatives of the neural crest in the ENS.
We have changed the acronym to refer to Enteric Neural Crest-Derived Cells (ENCDCs) throughout the manuscript.

9. Upon comparing the gene signatures from enteric wavefront and trailing populations with those from cranial neural crest, the authors conclude there are "unique migratory mechanisms between NCC populations". However, they do not show this is the case and should correct their phrasing to state "there are distinct patterns of gene expression between cranial and enteric wave-front populations" because that is what their analysis shows and they do not have any experimental support for unique migratory mechanisms.
We thank the Reviewer for this comment and have revised the statement accordingly.

10. The wording of the section on page 7 that begins “To explore the properties of wavefront ENCCs, a PPI network analysis was performed . . .” is very confusing and required reiterative reading to grasp what the group is doing. The authors should break down their analysis into a stepwise description and ask for non-expert readers to read/review for better accessibility of this aspect prior to resubmission.

We apologize for the lack of clarity. We have paid additional consideration to the stepwise approach to analysis and have improved our explanation of this in the results section. We hope this makes the manuscript more readable to the broad audience of Development.

11. There are several places in the text where the wording is loose and could be misinterpreted or lacked sufficient citations.
a. For instance “In previous studies, no clear distinctions between neural progenitors and glia have been observed” is ambiguous and could be misinterpreted or taken out of context. The beginning of this section should be revised to be more detailed, emphasize transcriptional differences among enteric progenitors, and include relevant references!
b. also in the sentence “Comparison of gene expression profiles between postnatal enteric progenitors and the primary ENS”. What is meant by “primary ENS”? This is unclear and could be misconstrued. It took this reviewer a while to determine that
c. The statement “These genes are also highly expressed in postnatal glia and appear to reflect the gradual maturation of early embryonic progenitors to glia, which notably retain neurogenic potential after birth, albeit more restricted” needs to include appropriate citations to support what is being stated.
d. The text lines “the neuroblast specific gene Miat was observed in the maturing trailing signature and is considered to be an important functional long noncoding RNA in neuronal stem cell specification in other regions” needs appropriate references to support the statement that Miat is “is considered to be an important functional long noncoding RNA in neuronal stem cell specification in other regions”.
e. The authors state “We hypothesized that DUSP6 signaling regulates hindgut colonization” but the rationale for why the focus on hindgut in this statement is not apparent.
We apologize for the confusion and appreciate the opportunity to clarify these statements. a. statements have been revised and references have been added b. this has been removed and explained more clearly as described in response above to comment 2c. c. references have been added d. this statement has been removed from the manuscript for better focus. e. we have amended that text to include “Dusp6 was selected for further analysis from among the genes associated with ENCDC progenitors due to the association of DUSP6 with regulating GDNF-RET and ERK signaling in other cell types, and the importance of the same pathways in ENCDC colonization (Lu et al., 2009; Natarajan et al., 2002)”.

Joseph, N. M., He, S., Quintana, E., Kim, Y.-G., Núñez, G. and Morrison, S. J. (2011). Enteric glia are multipotent in culture but primarily form glia in the adult rodent gut. The Journal of clinical investigation 121, 3398-3411.
Laranjeira, C., Sandgren, K., Kessaris, N., Richardson, W., Potocnik, A., Berghe, P. V. and Pachnis, V. (2011). Glial cells in the mouse enteric nervous system can undergo neurogenesis in response to injury. The Journal of clinical investigation 121.
Morarach, K., Mikhailova, A., Knoflach, V., Memic, F., Kumar, R., Li, W., Ernfors, P. and Marklund, U. (2021). Diversification of molecularly defined myenteric neuron classes revealed by single-cell RNA sequencing. Nature neuroscience 24, 34-46.
Lu, B. C., Cebrian, C., Chi, X., Kuure, S., Kuo, R., Bates, C. M., Arber, S., Hassell, J., MacNeil, L., Hoshi, M., et al. (2009). Etv4 and Etv5 are required downstream of GDNF and Ret for kidney branching morphogenesis. Nat Genet 41, 1295-1302.
Natarajan, D., Marcos-Gutierrez, C., Pachnis, V. and de Graaff, E. (2002). Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development 129, 5151-5160.

12. The authors are relaying on transgenes like Tau-EGFP to label differentiating neurons and state “Expression patterns were validated in mice postnatally, confirming appropriately labeled neurons (GFP+)”. Figure 4D simply shows co-localization of Wnt1-cre lineages with Tau-EGFP+ cells and the resolution is such in the images shown that one cannot conclude if these cells are neuronal. The data shown is all relative to Wnt1-cre:Rosa labeling and does not illustrate cell type specificity. If

they are going to use this line, they must either site the literature that unequivocally shows this transgene is expressed in differentiating enteric neurons or perform a HuC/D or other pan-neuronal stain with this transgenic reporter. Because EGFP signal takes a while to accumulate, it is not clear why they are relying on this reporter instead of using an early neuron IHC label like HuC/D or peripherin?
Thank you for this comment. Tau-EGFP was selected for validation because the gene for Tau (Mapt) was upregulated in trailing ENCDCs and is specific to enteric neurons according to the molecular signatures database. We do not claim this is one of the first genes to be expressed by cells committed to the neuronal lineage with the provided data. But it does validate that many ENCDCs in the trailing region are consistent with a transcriptionally more mature enteric neuron population as the sequencing data suggests. We have provided validation that Tau-EGFP is specific to enteric neurons in a nondiscriminatory manner by immunohistochemistry for HuC/D and beta-tubulin (Tuj1) in Figure 4C. As pointed out by reviewer 1, expression of GFP in the nestinGFP model is uniform between wavefront and trailing ENCDCs so there should be little delay in expression and this model seems appropriate.

13. Similarly in relying on the Plp1-EGFP transgene as a “glial marker”, the authors are making a leap of faith that Plp1-EGFP is accurately reporting glial cell fate. In the images shown for postnatal Plp1-EGFP, the distribution pattern does appear to be that of glial cells, although it would be preferable for the study to show this using co-localization with a glial restricted marker using immunolabeling at higher resolution. Moreover, if Plp1-EGFP is a “glial” reporter then one would expect to see it expressed at a delay as glial markers emerge after neuronal markers according to data published years back by Heather Young’s group (J. Comp Neurol 456:1-11, 2003). Instead the authors show emergence of the Plp1-EGFP reporter signal within the wavefront population. So it is possible that this transgene is not truly reflecting glial identity or that it is transiently activated in progenitors then becomes restricted to glia postnatally. Oddly the authors data in Figure 4 panels J and K does show that some of the Plp1-EGFP+ cells ARE TuJ1+ indicating that this transgene cannot be relied upon as a “glial marker”. These issues must be addressed to avoid muddying terminology and understanding in the field.
Thank you for this important comment. We have provided additional data validating specific co-expression of Plp1 and S100B in the postnatal environment. Plp1 is widely regarded as a pan-enteric glial marker, but little is known about this gene/protein in ENS development (Rao et al 2015). As previously mentioned, glia and the ENCDC progenitors do not show great distinctions on the transcriptomic level. We have provided additional staining at 13.5 dpc and there is no overlap with Plp1 and Tuj1 or HuC/D. Therefore, Plp1 appears to be upregulated as the ENCDCs mature in the non-neuronal population. There is an argument to be made that ‘glia’ is not a developmental cell fate but rather the default setting of ENCDCs if neurogenesis is not induced. As previous studies show glial markers are expressed after neuronal markers, perhaps the advancement of high-throughput technology will allow us to identify other markers expressed earlier. For now, however, we have provided data that validates that trailing ENCDCs increase Plp1 expression, which is associated with maturation into glia. Additional data includes Figure 4G and S3.
Rao, M., Nelms, B. D., Dong, L., Salinas-Rios, V., Rutlin, M., Gershon, M. D. and Corfas, G. (2015). Enteric glia express proteolipid protein 1 and are a transcriptionally unique population of glia in the mammalian nervous system. Glia 63, 2040-2057.

14. The authors describe other “glial markers” in the heatmap panel B of Figure 4 with no provided explanation or basis for why these specific genes are considered “glial markers”. Additional information is required to justify how the genes listed are specific to enteric glia. Minimally citation of prior literature that explicitly shows these genes are expressed in enteric glia should be added.

The enteric glial markers are from the MSigDB annotations (https://www.gsea-msigdb.org/gsea/msigdb/). Specifically, the gene sets from C8: Cell Type Signature, ‘descartes_fetal_intestine_ens_glia’ and ‘descartes_fetal_intestine_ens_neuron’. This has been added to the methods, results, and the figure legends for clarity.

15. As written the manuscript lacks appropriate citation of datasets within the results section that the authors have incorporated into their analysis. At first mention in the results where the authors state they are assessing differentially expressed genes from wavefront populations using GSEA in

the E15-18 dataset that appears to derive from Morarach et al., 2021 they should site that adjacent in the results so readers are not having to go find this in later article sections.
We apologize for this oversight. Results have been edited for clarity and citations and explanations of original datasets have been added.

16. The authors are using datasets that originate from neurospheres that were cultured from postnatal ENS and are calling this “postnatal progenitors”. This loose use of terminology could mislead readers and needs to be more clearly described in the results section titled The Wavefront-Trailing ENCC gene signature is predictive of progenitor and restricted cell populations post colonization. To their credit the authors do include relevant details sufficient to find this in prior publication that is cited in the methods. However, readers should not have to go back to prior papers to assess what is being done.

Thank you for this suggestion. In response to other comments, we have removed analysis using this particular dataset and provided a new analysis of scRNA-seq in postnatal-derived enteric neurospheres with a clearer description of how the cells were derived.

17. The expression profile of DUSP6 that is presently relegated to a supplementary figure (S2) should be moved to the main text. Additional expression data for Dusp6 should also be added, including a feature plot (UMAP) showing the distribution of its expression similar to that shown for Miat in figure 5 panel F. The authors should include Dusp6 as a line in panel Figure 5D. It’s not clear why this was not done initially. The logic and stepwise comparisons that the authors followed to identify Dusp6 as a priority candidate gene for subsequent study are lacking.

We have added an additional figure (Figure 6) and corresponding text to introduce our rationale for further exploration of the role of Dusp6 which includes gene expression in UMAP projections as suggested by the reviewer.

18. The authors are examining Dusp6 function in mouse cells and identified it in mouse cells. They should pay attention to use mouse appropriate nomenclature with designation of “Dusp6” as the gene throughout the text. There are several places where it’s clear they are referring to the gene and yet they are using protein nomenclature (DUSP6).

Thank you for noting this. It has been amended.

19. In the first paragraph of the results section focused on DUSP6 requirement, the authors evaluate DUSP6’s role in ENCC colonization through the “DUSP inhibitor BCI”. Towards the end of this same section, they refer to BCI as a “DUSP6 inhibitor”. How specific is BCI as an inhibitor of DUSP6? Does BCI inhibit all DUSP proteins or is it limited/targeted to DUSP6? Citations from the literature should be included that clarify this aspect.

As noted in response to #5, the specificity and a citation for it have been added.

20. When comparing their bulk RNA-seq data to the 15 and 18 dpc ENDCs scRNA-seq data, the authors use a combined dataset with both ages instead of the two ages separately. When the 15.5dpc signature alone is used does it have a stronger signature for the wavefront DEGs?
We appreciate this valuable suggestion. We evaluated this, but found no differences between 15.5 and 18.5 data. These developmental stages are both stages after completion of ENCDC colonization of the mouse intestine and this may explain why neither age is more reflective of the migratory wavefront gene signature.

21. It is not clear what data set is being used to generate the image shown in Figure 5 panel D.

We apologize for this. Data was acquired from the mouse colon atlas originally produced by Drokhlyansky et al. (2020). The results and figure legend have been amended to make this clearer.
Drokhlyansky, E., Smillie, C. S., Van Wittenberghe, N., Ericsson, M., Griffin, G. K., Eraslan, G., Dionne, D., Cuoco, M. S., Goder-Reiser, M. N., Sharova, T., et al. (2020). The Human and Mouse Enteric Nervous System at Single-Cell Resolution. Cell 182, 1606-1622.e1623.

22. The authors appear to have duplicated a sentence in the introduction “Interestingly, key ligands such as GDNF can have dual roles in migrating ENCCs by being chemoattractive, providing trophic support and promoting neural differentiation, with excess of the latter arresting colonization”. The introduction should be

Some of the comment is missing, but we have revised the introduction accordingly.

23. For Figure 5B the figure legend states “Venn-diagram of leading-edge genes in the E11.5 wavefront signature (ENCC immaturity) upregulated in the E15.5-18.5 ENCC progenitor population and postnatal ENCC progenitors”. But there are only two circles to the Venn Diagram that is shown. So the authors needs to clarify how they got to this outcome.
The Venn-diagram is only of wavefront-associated genes that were differentially expressed in those two datasets. We have revised the text for more clarity and provided the data in supplementary material.

Second decision letter

MS ID#: DEVELOP/2022/201090

MS TITLE: A distinct transcriptome characterizes neural crest-derived cells at the migratory wavefront during enteric nervous system development

AUTHORS: Rhian Stavely, Ryo Hotta, Nicole Picard, Richard Guyer, Ahmed A Rahman, Meredith Omer, Adam Soos, Emoke Szocs, Jessica Mueller, Allan M Goldstein, and Nandor Nagy

I have now received the reports of two of the referees who reviewed the earlier version of your manuscript and I 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 of the reviewers is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of referee 3. 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.

Reviewer 1

Advance summary and potential significance to field

Nagy and colleagues transcriptionally profile enteric neural crest cells as the migrate through the gut, comparing the transcriptional profile of cells at the wavefront with those at the trailing edge. They find that the transcriptional signature of wavefront cells is enriched in genes characeristics of uncommitted progenitors at both embryonic and postnatal stages. They then focus on the gene Dusp6 which is one of the genes enriched in wavefront cells, and show that loss of function of DUSP abrogates enteric progenitor migration in an ERK dependent fashion. The results will be of interest to developmental biologists studying the enteric nervous system development.

Comments for the author

The authors have done a nice job of revising the manuscript and it is now appropriate for publication in Development.

Reviewer 2

Advance summary and potential significance to field

Comments for the author

Reviewer 3

Advance summary and potential significance to field

The study by Stavely et al is novel and informative as it reveals the first transcriptional profiles of enteric neural crest derived cells migrating at the wavefront of the progenitors that colonize the fetal intestine. It is quite interesting that this cell population shares some aspects of gene expression with progenitors migrating at the forefront of cranial neural crest migratory waves. The authors have provided detailed responses to prior review and the revised article is presented at a level where it will be appreciated by the broad readership of Development. Overall, the manuscript is greatly improved and much clearer than the original submission. The efforts the authors have made to clarify prior aspects that were unclear is greatly appreciated. The addition of single cell data from enteric neurospheres generated by their lab to specifically parse out the progenitor population that gives rise to enteric neurons is helpful. However, there are a few issues that remain to be addressed listed below. Please note that it would be very helpful and make review faster if the authors would indicate in their responses where in the text they have incorporated changes or highlight their responses and changes in the manuscript with an alternate font color.

Comments to be addressed are being uploaded as a PDF file so that inset images with annotations are transmitted.

Comments for the author

The study by Stavely et al is novel and informative as it reveals the first transcriptional profiles of enteric neural crest derived cells migrating at the wavefront of the progenitors that colonize the fetal intestine. It is quite interesting that this cell population shares some aspects of gene expression with progenitors migrating at the forefront of cranial neural crest migratory waves. The authors have provided detailed responses to prior review and the revised article is presented at a level where it will be appreciated by the broad readership of *Development*.

Overall, the manuscript is greatly improved and much clearer than the original submission. The efforts the authors have made to clarify prior aspects that were unclear is greatly appreciated. The addition of single cell data from enteric neurospheres generated by their lab to specifically parse out the progenitor population that gives rise to enteric neurons is helpful. However, there are a few issues that remain to be addressed listed below. Please note that it would be very helpful and make review faster if the authors would indicate in their responses where in the text they have incorporated changes or highlight their responses and changes in the manuscript with an alternate font color.

1 – the authors nicely state their rationale for the stages studied in their response to prior review. However, it is not clear where the statements that were made in their response to review are incorporated into the text. The authors should be certain that they fully incorporated the rationale stated in their responses to review that included:

We chose mice at 11.5 dpc for several reasons: According to Young et al (2014) the speed and directional migration of ENDCs between 750 and 1,500 µm from the wavefront migrate significantly slower than those within 450 µm of the most caudal cell. Therefore, we needed a stage that provided us sufficient distance between wavefront and trailing regions, and this was possible at 11.5 dpc, but not 10.5 dpc. The length of colonized gut at 10.5 dpc is shorter than 1000 µm, preventing isolation of the trailing region from this younger embryonic stage. According to Druckenbrod and Epstien 2005 Dev Biol: At E10.5, the wavefront in the ileum advances caudally at an average speed of 45 µm/h (±10.8 SE, n = 4), but decreases to 32 µm/h at E11.25 as it approaches the nascent cecum. Despite the ENCDCs slowing down their caudal advance, the cells

still move (show active migration to colonize the complete cecum) before they start to colonize the hindgut.

2 - For the integration of Morarach et al., 2021 and the Cao et al., 2019 ENS/ENS+Schwann cell datasets, the authors claim that the "Enteric neuron trajectory_1" integrates with the Morarach et al., 2021 data. From their UMAP, this seems to not be entirely the case, as most of the Cao et al. enteric neuron cluster forms its own cluster, indicated as "Neuron_4" colored blue in Figure 3C. This distinction is even more apparent in Figure 3E. This separate lineage branch is an odd discrepancy that needs to be explained because as presented it does not appear that the authors can state that these data "integrated successfully". The authors might explain this discrepancy between gene expression due to different stage(s) (9.5-13.5 dpc) versus 15.5 and 18.5 dpc for the developing enteric neuronal cells that they appear to be a distinct population when integrated with the Morarach et al., 2021 data. Alternatively, the distinctive branch could be due to differences in data processing standards. Depending on the reprocessing or original processing of the Cao et al. 2019 data, technical/software differences could cause this separation. Whatever the reason, this distinction needs to be clarified and explained so the audience knows the origins of this aspect.

3 - The authors use enteric neuron and glia signatures obtained from MSigDB (on page 10 and Figure 4). This is an appropriate reasonable approach as long the caveats of comparing between species are mentioned. It is not clear that the authors have explicitly stated that comparison of human gene signatures to mouse enteric neurons and glia may not capture genes that are distinct between species or differentially expressed. There is precedence for this in the literature that should be cited as well.

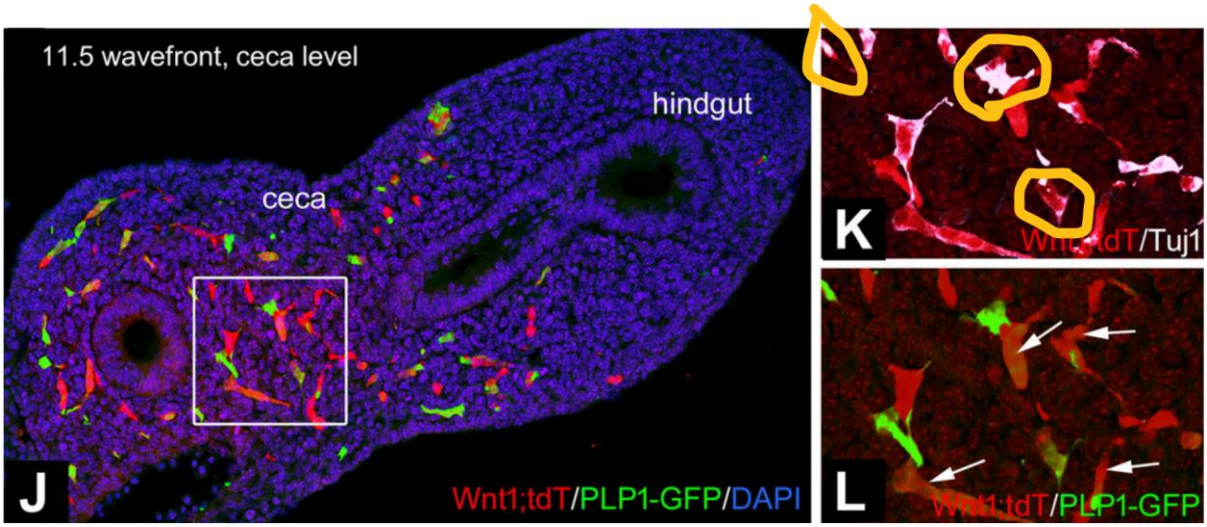
4 - The authors claim that *Miat* is the "only neuroblast specific gene detected in this analysis (Fig. 5E)." However, viewing the heatmap figure of panel 5E with *Miat* expression at the very bottom, the data displayed shows expression of *Miat* in some neuronal clusters as well. So the rationale for this very brief statement with no further mention of *Miat* anywhere else in the paper is not clear. Is the cluster/group of neuronal cells that exhibits *Miat* expression adjacent to the neuroblast clusters in the UMAP dimensionality reduction/pseudotime? Also, Cdkn1c is also fairly highly expressed in neuroblasts and no mention is made of this aspect.

5 - At the bottom of page 14, the authors refer to Figure 6C-D, stating that "ENCDCs in the trailing region at 11.5 dpc and in the proximal hindgut at 13.5 dpc have much lower DUSP6 expression." There is no quantification of DUSP6 protein expression in the data presented. Moreover, the fluorescent images shown do not display clear differences in DUSP6 protein levels that differ between trailing 115dpc and proximal hindgut at 13.5dpc versus wavefront. How differential levels of DUSP6 protein were quantified needs to be stated. This figure also lacks labels for midgut and hindgut regions although it might be reasonable to conclude the midgut is on the left and hindgut on the right based on the adjacent figure. Lastly, the figure description for Figure 6 uses "B" for both B and what is supposed to be C.

6 – The authors have included details of the mouse strains used in Table 1, and some of the background strains are indicated in strain names. However, the strain background on which the authors bred these animals is still lacking and should be included in the manuscript for readership.

7 – The authors responded in part to concerns about the Plp1-EGFP transgene as a "glial marker". They have now included postnatal staining of Plp1-EGFP transgene with HuC/D and S100b (Figure 4, panels G). However, these images are not of sufficient quality to show that the Plp1-EGFP transgene is limited to S100b cells in postnatal ENS. This should be corrected with higher resolution, greater magnification images that show the Plp1-GFP co-localizes with S100b. Oddly, the authors state in their response that "We have provided additional staining at 13.5 dpc and there is no overlap with Plp1 and Tuj1 or HuC/D." but this data is buried in supplemental. The data shown in Figure 4 panels M, N does not include HuC/D or TuJ1 staining of Plp1-EGFP transgene ENS at 13.5dpc so this needs to be corrected and the authors should incorporate the data of S3 panels C, C', D and E into the main figures. This could be accomplished by getting rid of panels H and I in figure 4 that are not as informative for this analysis. The concern still remains that the authors show data at 11.5dpc in figure 4 panels K and L where there IS co-localization of Tuj1 and

Plp1-GFP transgene expression.



Three cells in the field of view that ARE TuJ1 and show Plp1-GFP expression are emphasized with yellow outlines. This aspect needs to be addressed and the authors SHOULD clearly and explicitly state in the text that "it appears that the Plp1-GFP transgene is transiently activated in progenitors then becomes restricted to glia postnatally."

8 - The authors list that in their FindNeighbors and RunUMAP Seurat functions for processing the PNS neuronal and glial trajectories from Cao et al., 2019, that they use the first 15 Principal Components (PCs) as stated on page 26 of the text "Dimension reduction was performed by running the NormalizeData, ScaleData, FindVariableFeatures, RunPCA, FindNeighbors(dims = 1:15) and RunUMAP(dims = 1:15) commands." In typical processing, one first visualizes which PCs will be "useful" using an elbow plot, heatmaps, or jackstraw plots or similar methods. While it may be that the first 15 PCs are fine in this analysis, the authors should provide a sentence or two of rationale, especially if more PCs could potentially contribute to the downstream analysis.

9 - In the methods for single cell processing, the authors state that the "mouse colon atlas" data from Drokhlyansky et al., 2020 was downloaded. However, the authors do not state which runs were downloaded, whether these were human or mouse data, and how these data were processed/reprocessed. These aspects should be included so that readers who are interested in this level of the analysis can reproduce what the authors have done.

10 - The article has a few places where there are odd boxes inserted in the text as if the word processing program has modified the font in error. There should be corrected throughout the text. One example includes on page 40 where the text states: C □-C □ □ □ NestinGFP expression is observed in cells of the mesenteric blood vessel (C □) and colocalized with Wnt1-tdT, characteristic of enteric neural progenitor cells (C □ □-C □ □ □) in

11 - At the bottom of page 9, the authors left an "E11.5" where this should have been corrected to dpc.

12 - Towards the top of page 11, the authors mention the "embryonic gut," which is not accurate. Organogenesis is ongoing, and therefore the term "fetal intestine" is more appropriate here.

13 - On page 14 at the beginning of the "*DUSP6 is required for migration of ENCDCs*" section, the authors refer to the Morarach et al., 2021 dataset as "embryonic." Even if Morarach et al., 2021 refers to this as embryonic, this is not entirely correct, as organogenesis is ongoing. Therefore the more appropriate term to use in the text is "fetal."

Second revision

Author response to reviewers' comments

Responses to Reviewer 3:

1 – the authors nicely state their rationale for the stages studied in their response to prior review. However, it is not clear where the statements that were made in their response to review are incorporated into the text. The authors should be certain that they fully incorporated the rationale stated in their responses to review that included:

We chose mice at 11.5 dpc for several reasons: According to Young et al (2014) the speed and directional migration of ENDCs between 750 and 1,500 μm from the wavefront migrate significantly slower than those within 450 μm of the most caudal cell. Therefore, we needed a stage that provided us sufficient distance between wavefront and trailing regions, and this was possible at 11.5 dpc, but not 10.5 dpc. The length of colonized gut at 10.5 dpc is shorter than 1000 μm, preventing isolation of the trailing region from this younger embryonic stage. According to Druckenbrod and Epstien 2005 Dev Biol: At E10.5, the wavefront in the ileum advances caudally at an average speed of 45 μm/h (±10.8 SE, n = 4), but decreases to 32 μm/h at E11.25 as it approaches the nascent cecum. Despite the ENCDCs slowing down their caudal advance, the cells still move (show active migration to colonize the complete cecum) before they start to colonize the hindgut.

Response: Thank you for this comment. We have added the rationale of the developmental stages to the first paragraph of Results section (page: 5) including the relevant references as suggested.

2 - For the integration of Morarach et al., 2021 and the Cao et al., 2019 ENS/ENS+Schwann cell datasets, the authors claim that the “Enteric neuron trajectory_1” integrates with the Morarach et al., 2021 data. From their UMAP, this seems to not be entirely the case, as most of the Cao et al. enteric neuron cluster forms its own cluster, indicated as “Neuron_4” colored blue in Figure3C. This distinction is even more apparent in Figure 3E. This separate lineage branch is an odd discrepancy that needs to be explained because as presented it does not appear that the authors can state that these data “integrated successfully”. The authors might explain this discrepancy between gene expression due to different stage(s) (9.5-13.5 dpc) versus 15.5 and18.5 dpc for the developing enteric neuronal cells that they appear to be a distinct population when integrated with the Morarach et al., 2021 data. Alternatively, the distinctive branch could be due to differences in data processing standards. Depending on the reprocessing or original processing of the Cao et al. 2019 data, technical/software differences could cause this separation. Whatever the reason, this distinction needs to be clarified and explained so the audience knows the origins of this aspect.

Response: The Cao et al experiments provided no spatial data so there was an element of assumption on their behalf of what populations were enteric. We agree with the reviewer that Enteric neuron trajectory_1 does not completely overlap with the Morarach dataset. However this is the most likely cluster to be enteric neurons in the Cao et al dataset. We have provided an additional supplementary figure where we compared other ’enteric’ clusters of the Cao dataset which are far removed from the enteric neural crest-derived cells of Morarach. As the Enteric neuron trajectory_1 population was the most similar to the Morarach it is safe to assume they are the enteric neurons, which we then use for further analysis (without the Morarach cells).

The aim of this particular experiment was not to analyse the differences between enetric neurons in the Cao and Morarach data, but to identify whether the presumed enteric neuron clusters provided by Cao were accurate. We agree that there are differences between the enteric neurons in both datasets (which shows on the UMAP), which could be attributed to an actual difference in these developmental timeponts, or as suggested by the Reviewer differences in cell isolation, sequencing, or downstream data wrangling. Therefore, we did not compare these neurons between the datasets. Supp. Fig.2 C has been added and we removed the wording ’integrated successfully’.

3 - The authors use enteric neuron and glia signatures obtained from MSigDB (on page 10 and Figure 4). This is an appropriate reasonable approach as long the caveats of comparing between species

are mentioned. It is not clear that the authors have explicitly stated that comparison of human gene signatures to mouse enteric neurons and glia may not capture genes that are distinct between species or differentially expressed. There is precedence for this in the literature that should be cited as well.

Response: We have ammended the limitations section of the discussion to address the reviewers comment. Gene sets of human enteric neuron and glial markers were used to assess the overall signature of these cells types in the wavefront and trailing ENCDCs which would not capture the genes distinct between the species. Nevertheless, there appears to be enough overlap of markers of enteric neurons and glia to justify this approach (Drokhlyansky et al., 2020; May-Zhang et al., 2021). This has been added to the text, as have the following references:

Drokhlyansky, E., Smillie, C. S., Van Wittenberghe, N., Ericsson, M., Griffin, G. K., Eraslan, G., Dionne, D., Cuoco, M. S., Goder-Reiser, M. N., Sharova, T., et al. (2020). The Human and Mouse Enteric Nervous System at Single-Cell Resolution. Cell 182, 1606-1622.e1623.

May-Zhang, A. A., Tycksen, E., Southard-Smith, A. N., Deal, K. K., Benthal, J. T., Buehler, D. P., Adam, M., Simmons, A. J., Monaghan, J. R. and Matlock, B. K. (2021). Combinatorial transcriptional profiling of mouse and human enteric neurons identifies shared and disparate subtypes in situ. Gastroenterology 160, 755-770. e726.

4 - The authors claim that Miat is the “only neuroblast specific gene detected in this analysis (Fig. 5E).” However, viewing the heatmap figure of panel 5E with Miat expression at the very bottom, the data displayed shows expression of Miat in some neuronal clusters as well. So the rationale for this very brief statement with no further mention of Miat anywhere else in the paper is not clear. Is the cluster/group of neuronal cells that exhibits Miat expression adjacent to the neuroblast clusters in the UMAP dimensionality reduction/pseudotime? Also, Cdkn1c is also fairly highly expressed in neuroblasts and no mention is made of this aspect.

Response: We agree with the reviewer that although Miat expression is very high in ’neuroblasts’, there is some expression in one of the clusters of developing neurons. It could be speculated that Miat is being ’switched off’ in this cluster however this would require further experimental validation. While we think this is an interesting observation, the implications are unclear and it is not an essential part of the study, therfore we have removed this comment.

5 - At the bottom of page 14, the authors refer to Figure 6C-D, stating that “ENCDCs in the trailing region at 11.5 dpc and in the proximal hindgut at 13.5 dpc have much lower DUSP6 expression.” There is no quantification of DUSP6 protein expression in the data presented. Moreover, the fluorescent images shown do not display clear differences in DUSP6 protein levels that differ between trailing 11.5 dpc and proximal hindgut at 13.5 dpc versus wavefront. How differential levels of DUSP6 protein were quantified needs to be stated. This figure also lacks labels for midgut and hindgut regions although it might be reasonable to conclude the midgut is on the left and hindgut on the right based on the adjacent figure. Lastly, the figure description for Figure 6 uses “B” for both B and what is supposed to be C.

Response: Thank you for these important comments. We apologize for the confusion. Structures were labeled as suggested. We also included new figures (Fig. 6E) to show the heterogeneous DUSP6 expression at 13.5dpc. (Arrow shows DUSP6+/Wnt1+ cell; arrowheads are DUSP6-negative/Wnt1+ cells). We have quantified the fluorescent intensity of DUSP6 expression in 11.5 dpc trailing, wavefront, and 13.5 hindgut (trailing) ENCDCs (see Fig 6F). Fluorescent images were analyzed in Image J, as previously described by Shihan et al (Biochem Biophys Rep. 2021 Mar; 25: 100916.). For the immunofluorescence analysis, mean gray values were analyzed using Image J software. After converting the image to black and white with 8-bit type, the threshold of the image was adjusted (Image-Adjust-Threshold-Apply); then the area of the DUSP6+ ENCDCs was measured and recorded (Analyze-Measure). The mean gray value was automatically calculated using image J. Quantification of the intensity values is shown in Figure 6F. The Results text has been updated accordingly.

6 – The authors have included details of the mouse strains used in Table 1, and some of the background strains are indicated in strain names. However, the strain background on which the authors bred these animals is still lacking and should be included in the manuscript for readership.
Response: As recommended by the Reviewer, Table 1 has been ammended to include the details of the background strains for these mice.

7 – The authors responded in part to concerns about the Plp1-EGFP transgene as a “glial marker”. They have now included postnatal staining of Plp1-EGFP transgene with HuC/D and S100b (Figure 4, panels G). However, these images are not of sufficient quality to show that the Plp1-EGFP transgene is limited to S100b cells in postnatal ENS. This should be corrected with higher resolution, greater magnification images that show the Plp1-GFP co-localizes with S100b.

Oddly, the authors state in their response that “We have provided additional staining at 13.5 dpc and there is no overlap with Plp1 and Tuj1 or HuC/D.” but this data is buried in supplemental. The data shown in Figure 4 panels M, N does not include HuC/D or TuJ1 staining of Plp1-EGFP transgene ENS at 13.5 dpc so this needs to be corrected and the authors should incorporate the data of S3 panels C, C’, D and E into the main figures. This could be accomplished by getting rid of panels H and I in figure 4 that are not as informative for this analysis.
The concern still remains that the authors show data at 11.5 dpc in figure 4 panels K and L where there IS co-localization of Tuj1 and Plp1-GFP transgene expression. Three cells in the field of view that ARE TuJ1 and show Plp1-GFP expression are emphasized with yellow outlines. This aspect needs to be addressed and the authors SHOULD clearly and explicitly state in the text that “it appears that the Plp1-GFP transgene is transiently activated in progenitors then becomes restricted to glia postnatally.”

Response: Thank you. Higher quality images have been provided with arrows indicating overlapping Plp1-GFP and S100b colocalization. We have replaced Fig.4G; removed panel H and I, and incorporated panel C,C',D,E from Suppl. Fig.3 into Fig.4 as suggested. We have a new Fig 4K to show Hu C/D staining of 11 dpc PLP1-GFP gut. We see co-staining with PLP1 and Tuj1 and while we don’t really know the cause, we hypothesize that progenitors can express Plp1-GFP as shown in our previous studies in vivo and in vitro (Belkind-Gerson et al, 2017; Guyer et al, in press). The cells may continue to express Plp1-GFP transiently following neuronal differentiation, as is the case in neurospheres generated from Plp1-GFP mice postnatally. We have ammended the results as follows: ’’Interestingly, Tuj1 could be observed in both Plp1GFP positive and negative cells at the wavefront (Fig. 4I,J); however, there was no overlap between the expression of Plp1GFP and neuronal marker HuC/D at 11.5 dpc (Fig. 4K) or later at 13.5 dpc (Fig. 4M-O), indicating that Plp1GFP is not expressed in maturing enteric neurons.’’ We have also added the following text to the discussion (page 18) ”Plp1GFP however is expressed at low levels in the wavefront, with levels rising along the wavefront-trailing axis of the embryonic gut, consistent with the gene expression data. Given Plp1 expression is restricted to glia in the postnatal environment, these data appear to be indicative of a gradual cell maturation to a more glia-like phenotype in trailing ENCDCs”.

Belkind-Gerson, J., Graham, H. K., Reynolds, J., Hotta, R., Nagy, N., Cheng, L., Kamionek, M., Shi, H. N., Aherne, C. M. and Goldstein, A. M. (2017). Colitis promotes neuronal differentiation of Sox2+ and PLP1+ enteric cells. Scientific reports 7, 1-15.

Guyer, R. A., Stavely, R., Robertson, K., Bhave, S., Mueller, J., Picard, N., Hotta, R., Kaltschmidt, J. A. and Goldstein, A. M. (2023). Single-Cell Multiome Sequencing Clarifies Enteric Glial Cell Diversity and Identifies an Intraganglionic Population Poised for Neurogenesis. Cell Reports (in press).

8 - The authors list that in their FindNeighbors and RunUMAP Seurat functions for processing the PNS neuronal and glial trajectories from Cao et al., 2019, that they use the first 15 Principal Components (PCs) as stated on page 26 of the text “Dimension reduction was performed by running the NormalizeData, ScaleData, FindVariableFeatures, RunPCA, FindNeighbors(dims = 1:15) and RunUMAP(dims = 1:15) commands.” In typical processing, one first visualizes which PCs will be “useful” using an elbow plot, heatmaps, or jackstraw plots or similar methods. While it may be that the first 15 PCs are fine in this analysis, the authors should provide a sentence or two of rationale, especially if more PCs could potentially contribute to the downstream analysis.

Response: Thank you for this comment. These values were selected by the visual aid of elbow plots and determining the first principal component where the percent of total variation was less than 0.1% of the previous principal component, indicating a plateau in meaningful variation. This has been added to the Methods section, page 27.

9 - In the methods for single cell processing, the authors state that the “mouse colon atlas” data from Drokhlyansky et al., 2020 was downloaded. However, the authors do not state which runs were downloaded, whether these were human or mouse data, and how these data were processed/reprocessed. These aspects should be included so that readers who are interested in this level of the analysis can reproduce what the authors have done.

Response: We apologize for this oversight. The processed data files for mouse large intestine (10X) data were downloaded from the Broad Institute single cell portal (https://singlecell.broadinstitute.org/single_cell/study/SCP1038/) including the counts matrix (gene_sorted-mli.matrix.mtx), barcodes (mli.barcodes.tsv) and genes (mli.genes.tsv) files and annotations from the original authors (mli.tsne2.txt). We have amended the Methods to include this.

10 - The article has a few places where there are odd boxes inserted in the text as if the word processing program has modified the font in error. There should be corrected throughout the text. One example includes on page 40 where the text C □-C □ □ □) NestinGFP expression is observed in cells of the mesenteric blood vessel (C □) and colocalized with Wnt1-tdT, characteristic of enteric neural progenitor cells (C □ □- C □ □ □) in

Response: Thank you for pointing this out. Our apologies for this. We have fixed the word processig errors.

11 - At the bottom of page 9, the authors left an “E11.5” where this should have been corrected to dpc.
Response: Thank you. This has been corrected.

12 - Towards the top of page 11, the authors mention the “embryonic gut,” which is not accurate. Organogenesis is ongoing, and therefore the term “fetal intestine” is more appropriate here. 13 - On page 14 at the beginning of the “DUSP6 is required for migration of ENCDCs” section, the authors refer to the Morarach et al., 2021 dataset as “embryonic.” Even if Morarach et al., 2021 refers to this as embryonic, this is not entirely correct, as organogenesis is ongoing. Therefore the more appropriate term to use in the text is “fetal.”

Response: Thank you for this comment. We have revised the manuscript to more accurately reflect the distinction between “embryo” and “fetus.” In this revised manuscript, we use the definitions recommended by Keith Moore’s books on embryology (“Before We Are Born” and “The Developing Human”), namely that once the organs have developed the animal is said to have reached the fetal stage of development. More specifically, as in Roberts Rugh’s classic book on “The Mouse”, we define the fetal stage in mice as starting at 12 days of gestation and continuing until birth. For chicken, we continue to use the term “embryo” throughout development, as is standard.

Third decision letter

MS ID#: DEVELOP/2022/201090

MS TITLE: A distinct transcriptome characterizes neural crest-derived cells at the migratory wavefront during enteric nervous system development

AUTHORS: Rhian Stavely, Ryo Hotta, Nicole Picard, Richard Guyer, Ahmed A Rahman, Meredith Omer, Adam Soos, Emoke Szocs, Jessica Mueller, Allan M Goldstein, and Nandor Nagy
ARTICLE TYPE: Research Article

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