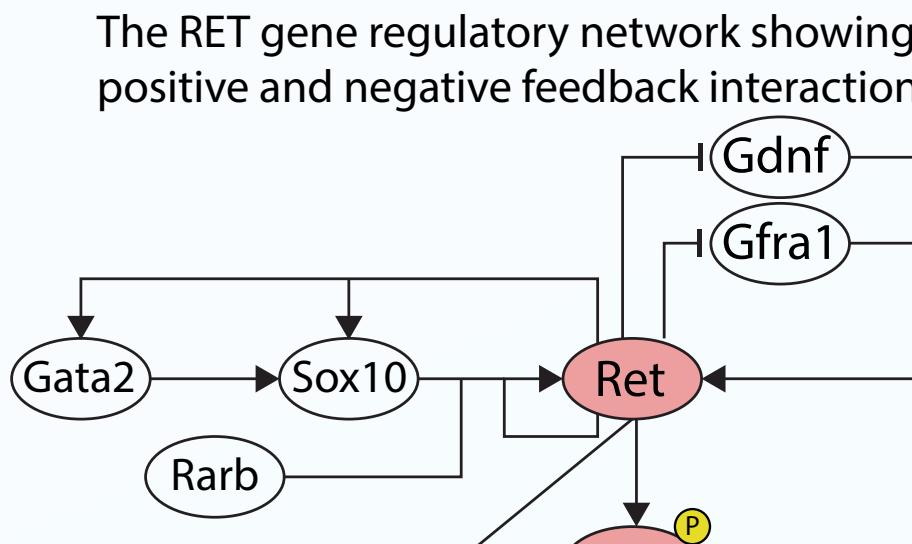
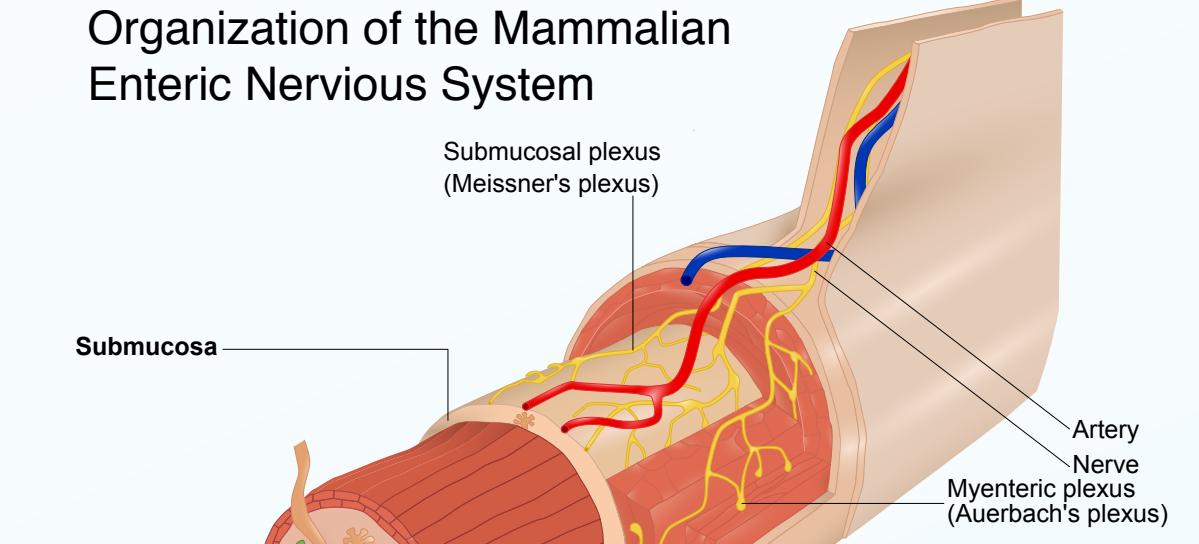


Introduction

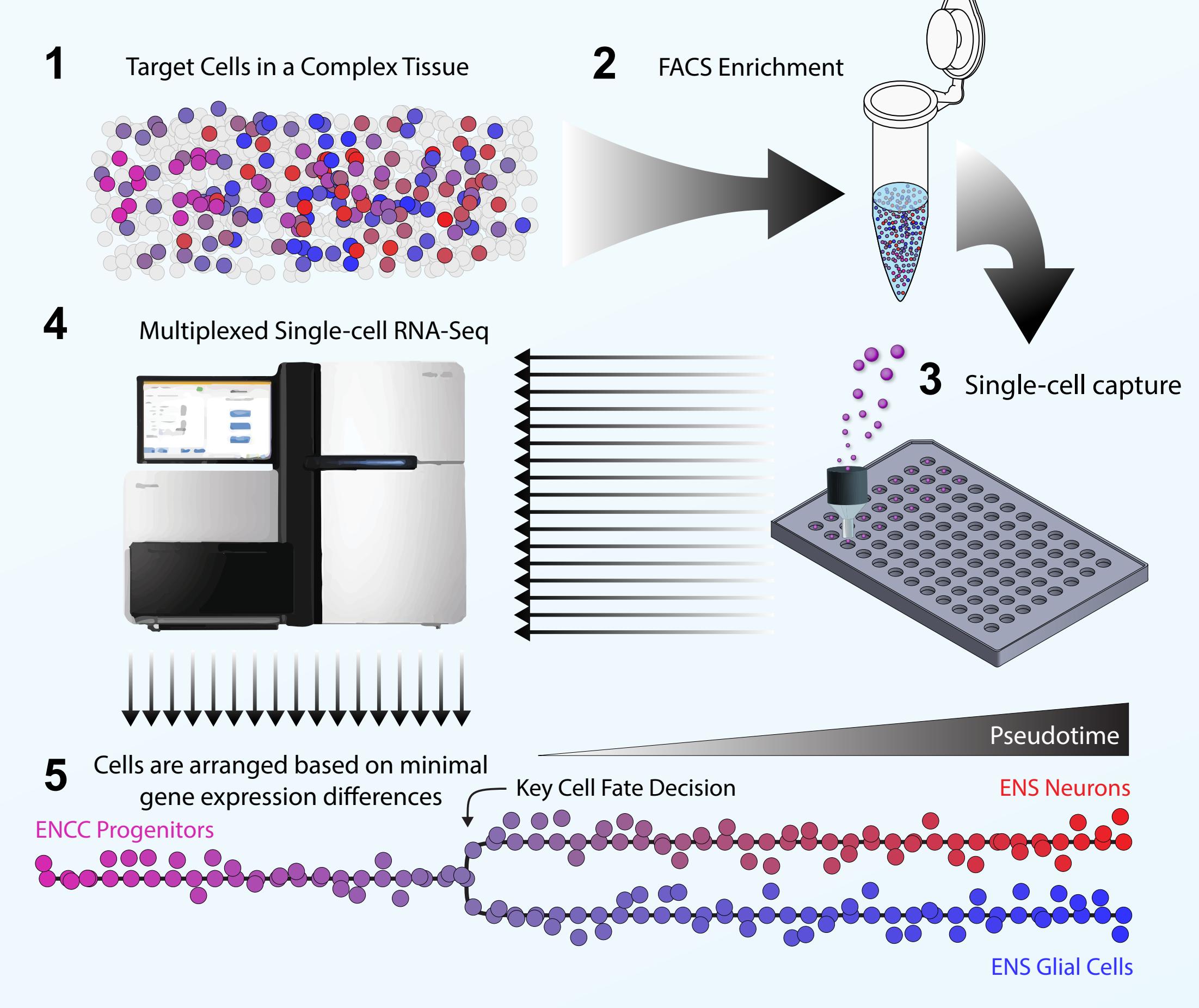
A significant proportion of the genetic risk of Hirschsprung disease (HSCR) in European ancestry subjects arises from common noncoding and rare coding variants at the gene encoding the receptor tyrosine kinase RET. Mouse models have demonstrated that homozygous loss of Ret signaling leads to complete aganglionosis in the gut by immunohistochemical methods. We have previously demonstrated through expression profiling of the developing gut in wildtype and Ret null mice that significant transcriptional changes take place as a result of the loss of Ret signaling which affects multiple pathways and biological process. Due to the nature of bulk RNA-seq, however, it is difficult to delineate cell type-specific transcriptional responses and cell fate decisions attributed to the loss of RET.

Single cell RNA-seq on both unaffected and Ret null cells from the developing mouse gut at embryonic day (E)12.5 has enabled us to systematically characterize the transcriptional signatures of Ret⁺ celltypes within the developing ENS, as well as identify the specific transcriptional responses contributing to the observed changes in cell identity and fate associated with a loss of Ret signaling. The absence of RET leads to significant transcriptional changes in early progenitor cells, which precociously express glial cell markers, as well as in fate committed enteric neuronal cells. RET null enteric nervous system (ENS) cells appear to undergo a fate switch amongst distinct neuronal subtypes as well. Furthermore we observe a population of ENS neurons with sex-specific transcriptional response to the loss of RET, consistent with a known sex-bias in Hirschsprung Disease severity. These results demonstrate that loss of Ret signaling has much broader, and diverse effects on multiple cell types and that these effects are cell-type specific. In addition, the predominant effects do not appear to induce neuronal cell death as previously thought, but may affect proliferation rate and induction of alternate cell fates. These data highlight the diversity of cellular responses to the signaling activity of a specific gene, and provide a roadmap towards uncovering the diversity of ENS cell types, and the cell-autonomous responses of these subtypes in Hirschsprung disease.

Experimental Questions

- The RET gene regulatory network showing positive and negative feedback interactions

 Organization of the Mammalian Enteric Nervous System

 - Can we reconstruct enteric neural crest cell (ENCC) migration and differentiation in the developing gut that leads to establishment of ENS?
 - Can we identify specific role(s) for RET signaling in this process?
 - Are there cell-type-specific consequences for the loss of RET?
 - Can we identify sex-specific effects that might explain bias in HSCR aganglionosis severity?

Workflow for scRNA-Seq of Ret⁺ ENS



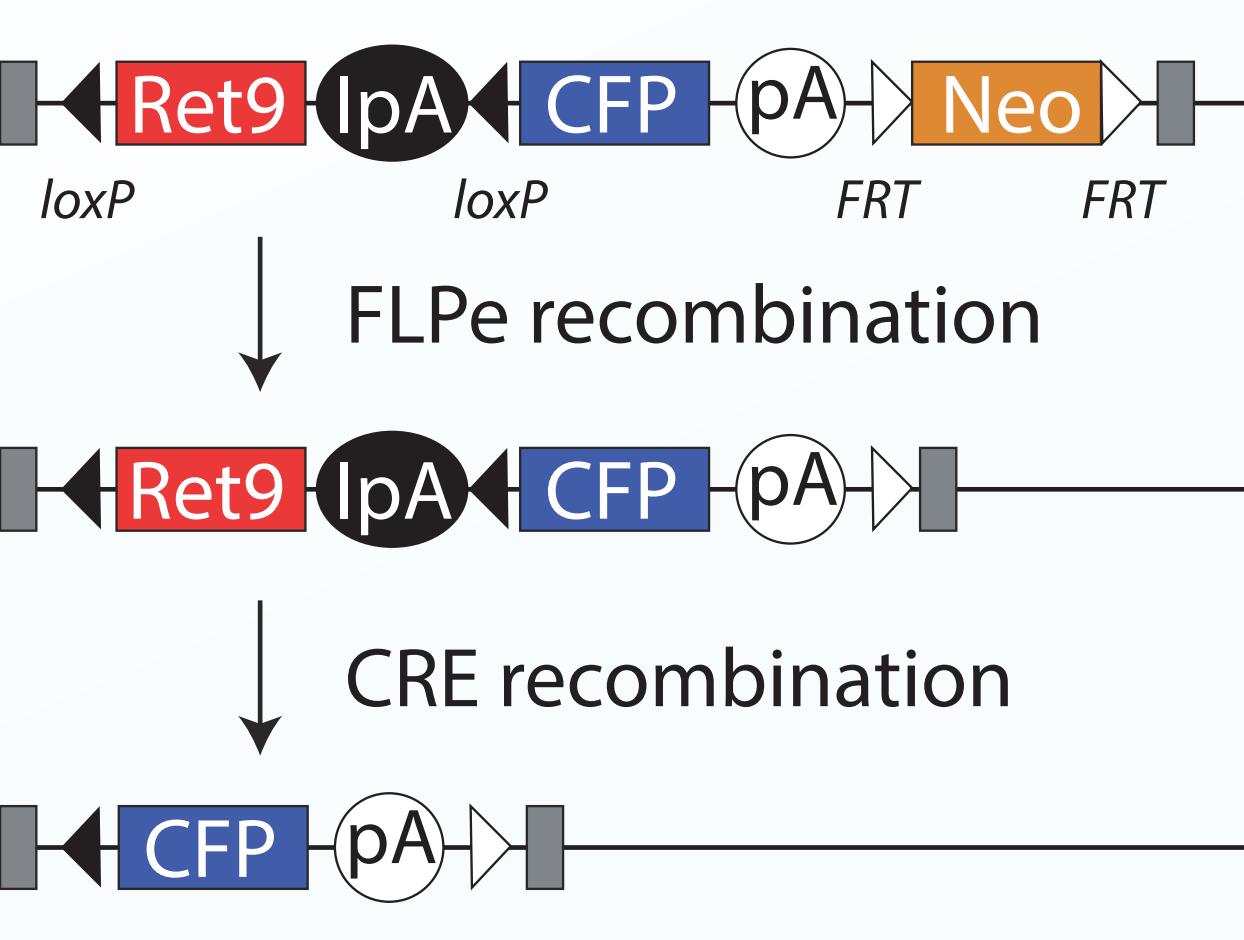
- E12.5 gut was dissected and cells dissociated both physically and enzymatically.
- Single-cells sorted via fluorescence activated cell sorting (FACS).
- Libraries were prepared using a modified Smart-Seq2 template-switching protocol and were sequenced by Illumina.
- Libraries were made for 95 cells from each embryo, resulting in a total of 768 cells from 8 embryos, 2 biological replicates per condition.
- Balanced with respect to sex and genotype
- Data preprocessing:
 - aligned with hisat2 version 2.0.1-beta to mm10
 - quantified with cuffquant v2.2.1 against gencode M8 assembly
 - normalized with cuffnorm v2.2.1 across 742 cells after 26 outliers were removed
 - cells expressing fewer than 700 genes were removed.
- Remaining cells: 185 female hets, 179 female homs, 169 male hets, 128 male homs
- FPKM expression estimates converted to RNA copies per cell via Monocle2 Census approach.
- Batch included as a nuisance variable in all model formulae, as well as the number of genes detected per cell as a proxy for capture efficiency for each cell.

Cell-type specific responses to loss of Ret signaling in the developing enteric nervous system: Implications for Hirschsprung disease

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A mouse model of Hirschsprung's Disease

Homologous recombinant

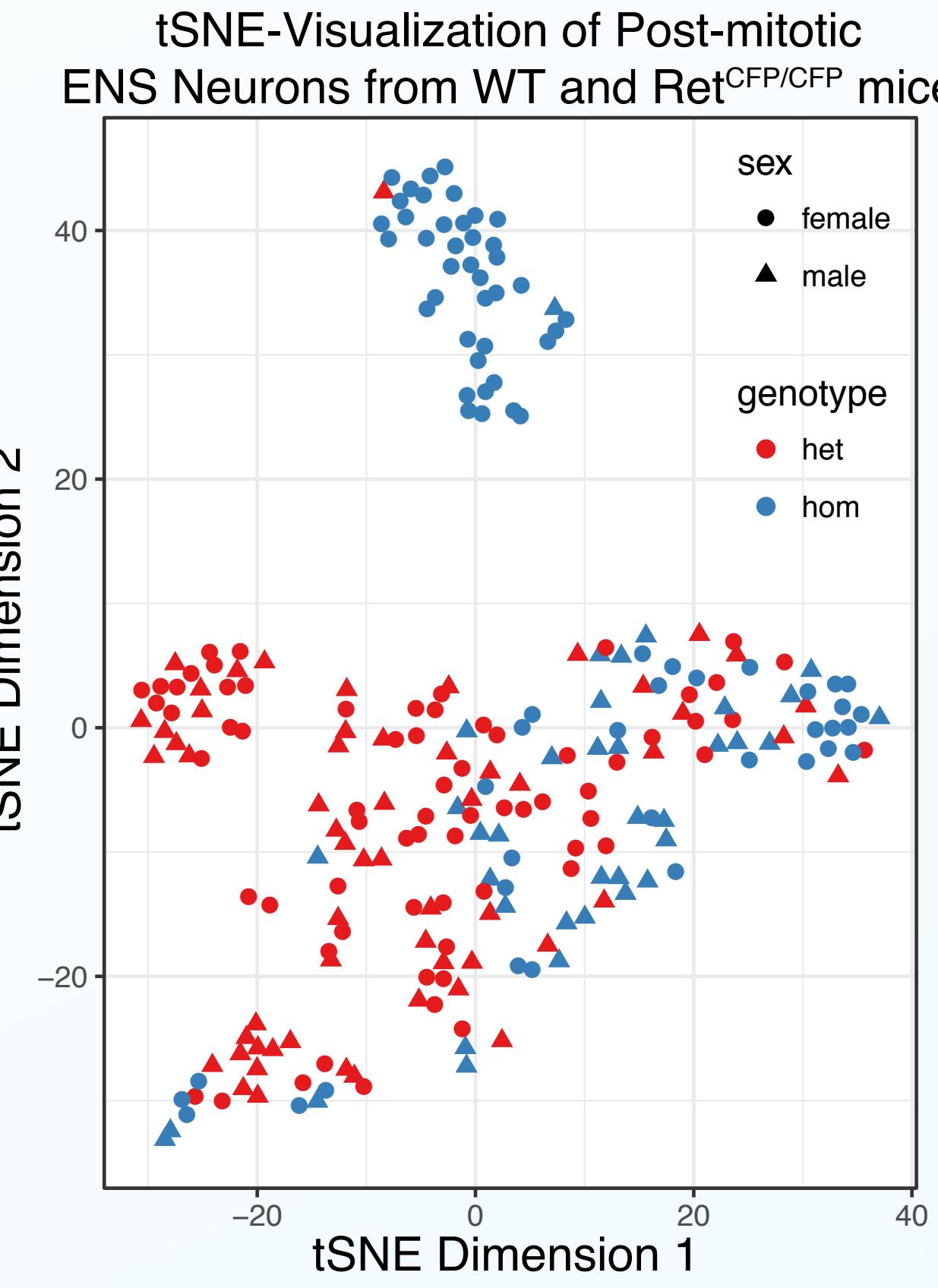


Floxed allele

CFP knock-in (null allele)

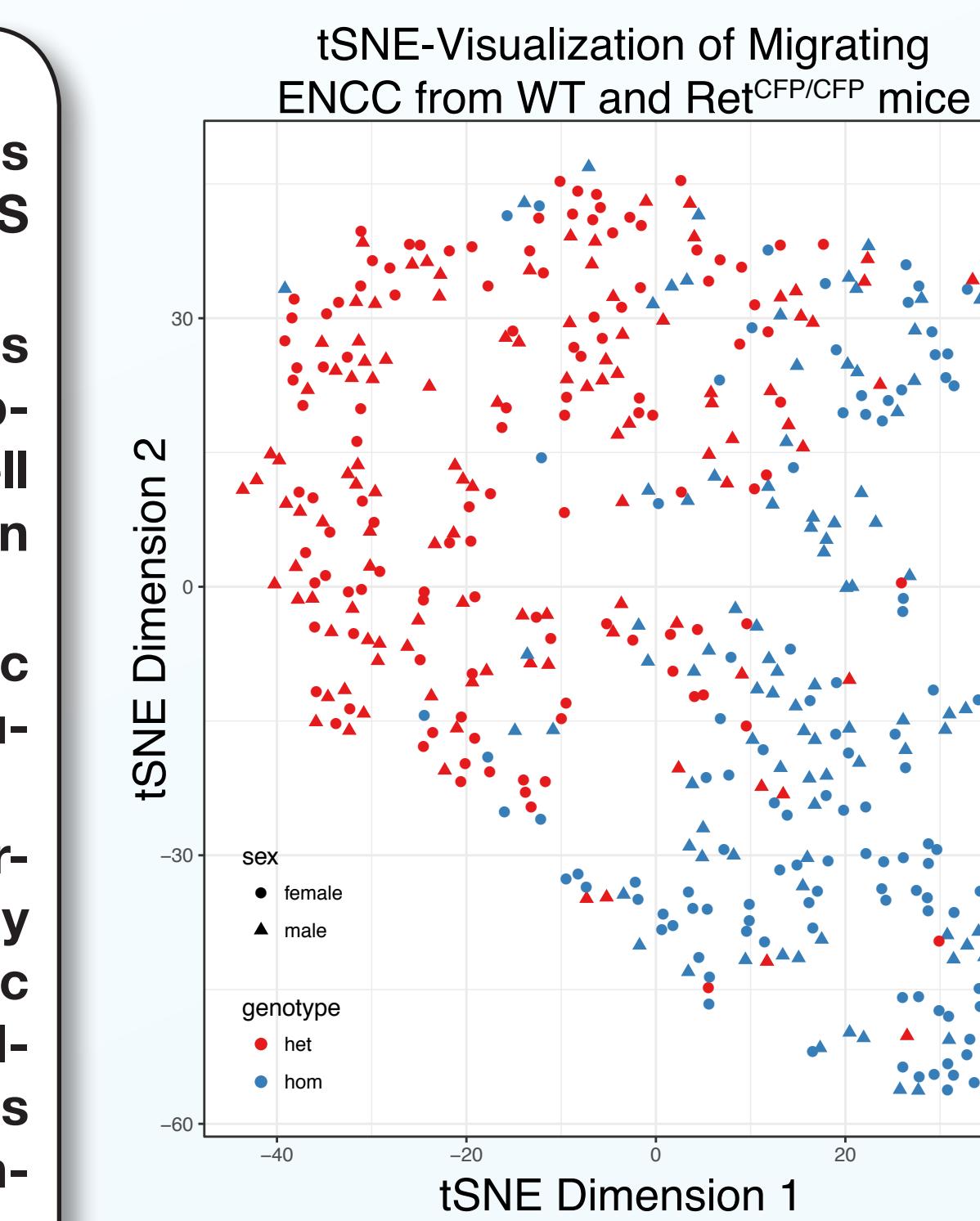
- Cyan Fluorescent Protein (CFP) cDNA inserted into the first intron of Ret using homologous recombination.
- Ret^{CFP/CFP} knockout allele recapitulates the aganglionosis phenotype of HSCR with 100% penetrance.
- We have shown developmental gut expression profiles with defects throughout the Ret GRN in this strain.
- This is an ideal reagent allowing FACS enrich and molecularly analyze Ret expressing cells from the developing mouse gut.

Effect of Ret on postmitotic ENS neurons



- tSNE visualization of high-dispersion genes from the postmitotic ENS Neuron subset.
- Genotypic differences emerge as discrete subtypes of neurons as well as more subtle variation within subtypes.
- Two genotype-specific subpopulations of neurons identified
- Ret-null mice undergo a fate shift away from non-cholinergic (Grp1+, Cck1+) and acetylcholinergic cell types towards catecholaminergic, non-vasodilator-cell types (Th+, Dbh+, CRGP+).

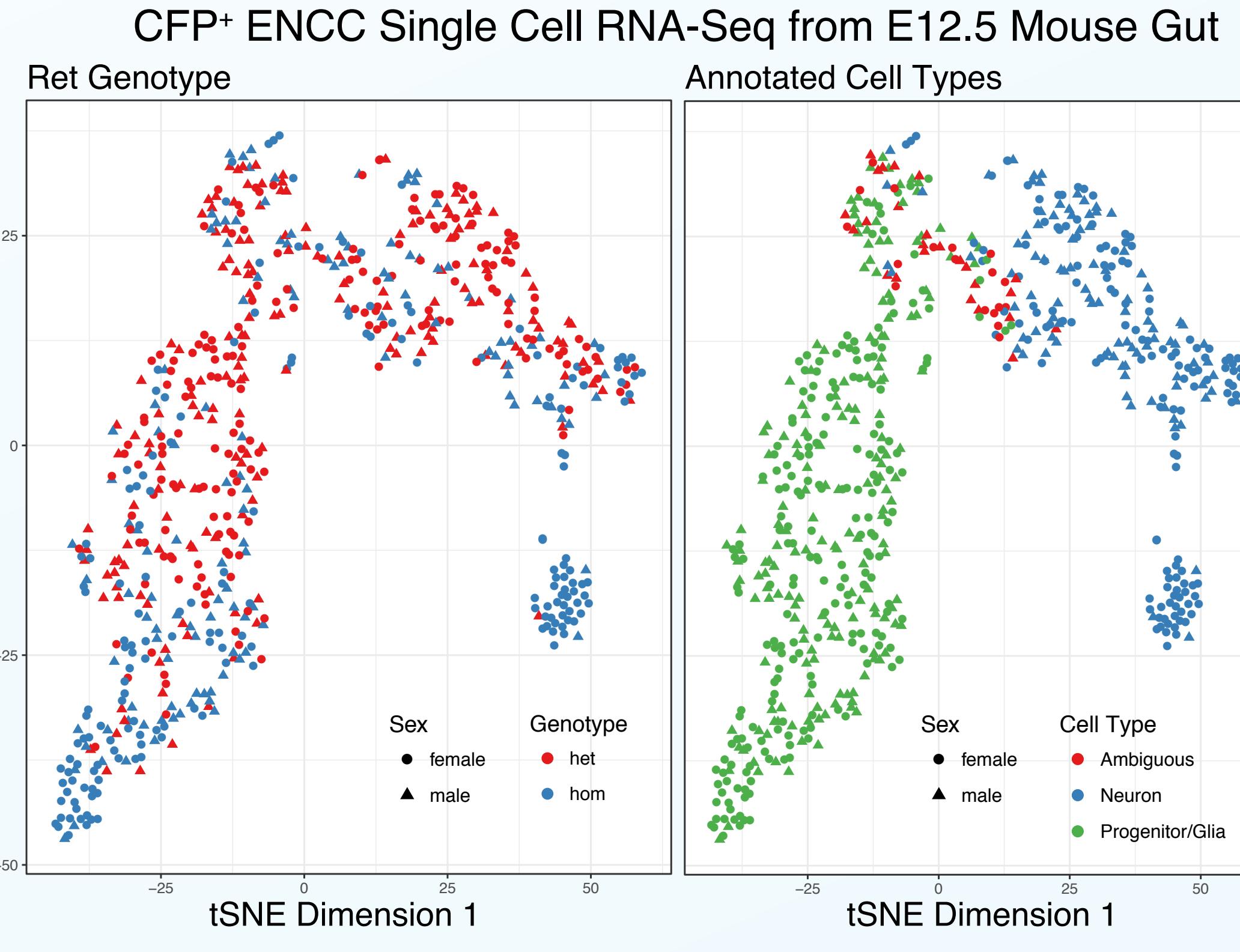
Effect of Ret on ENS Glial cells



- tSNE visualization of high-dispersion genes from ENCC-derived progenitor and maturing glial population.
- Strong systematic bias for gene expression by genotype, but no discrete cellular subtypes emerge.
- Ret^{CFP/CFP} cells shifted towards more mature glial fate suggesting early exit from cell cycle.
- Ret^{CFP/CFP} cells exhibit a more progenitor-like transcriptional signature

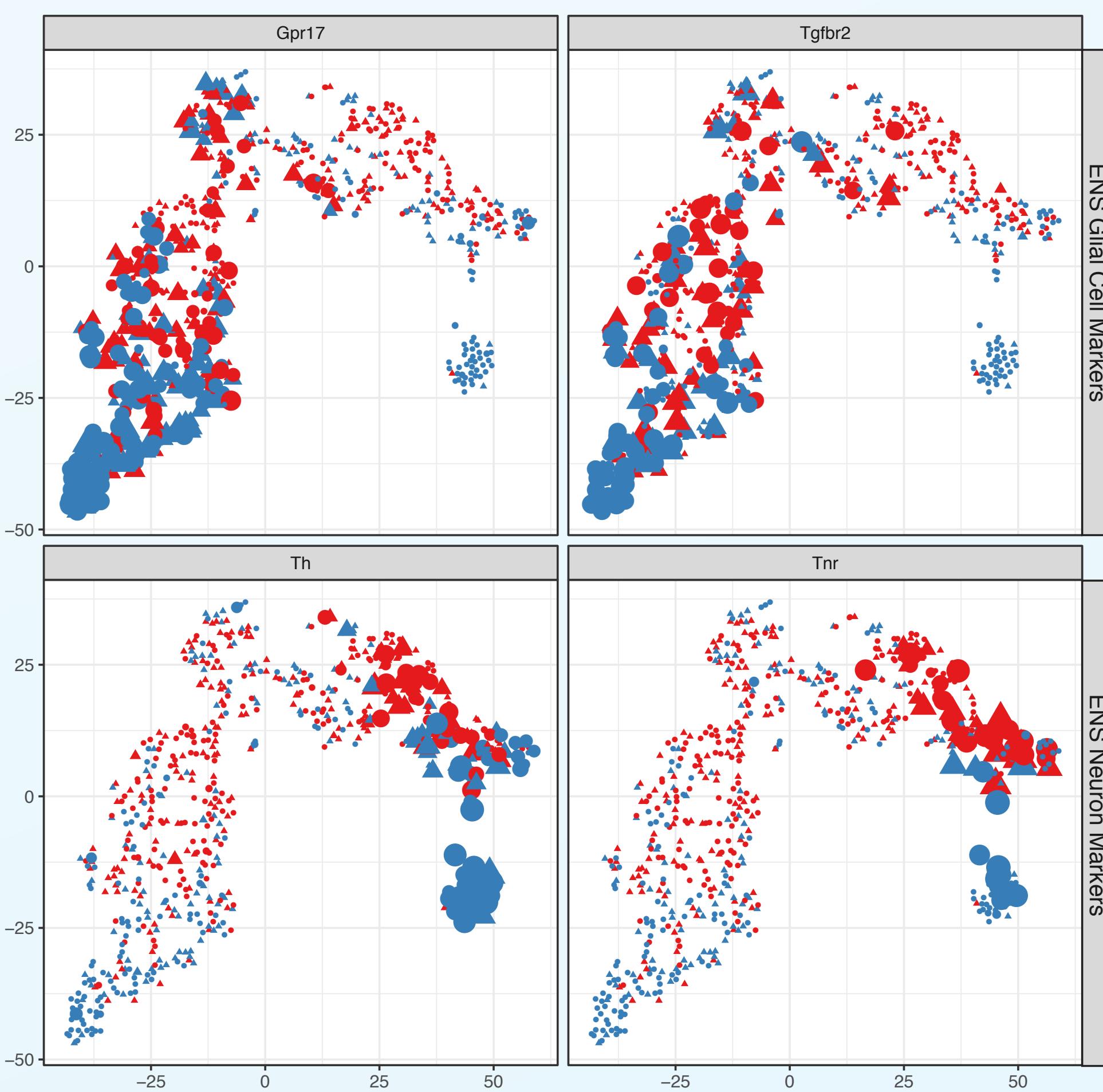
ENCC Progenitors Ret^{+CFP} vs Ret^{CFP/CFP}
Differentially Expressed Genes

Migration and Differentiation (Pseudotime)



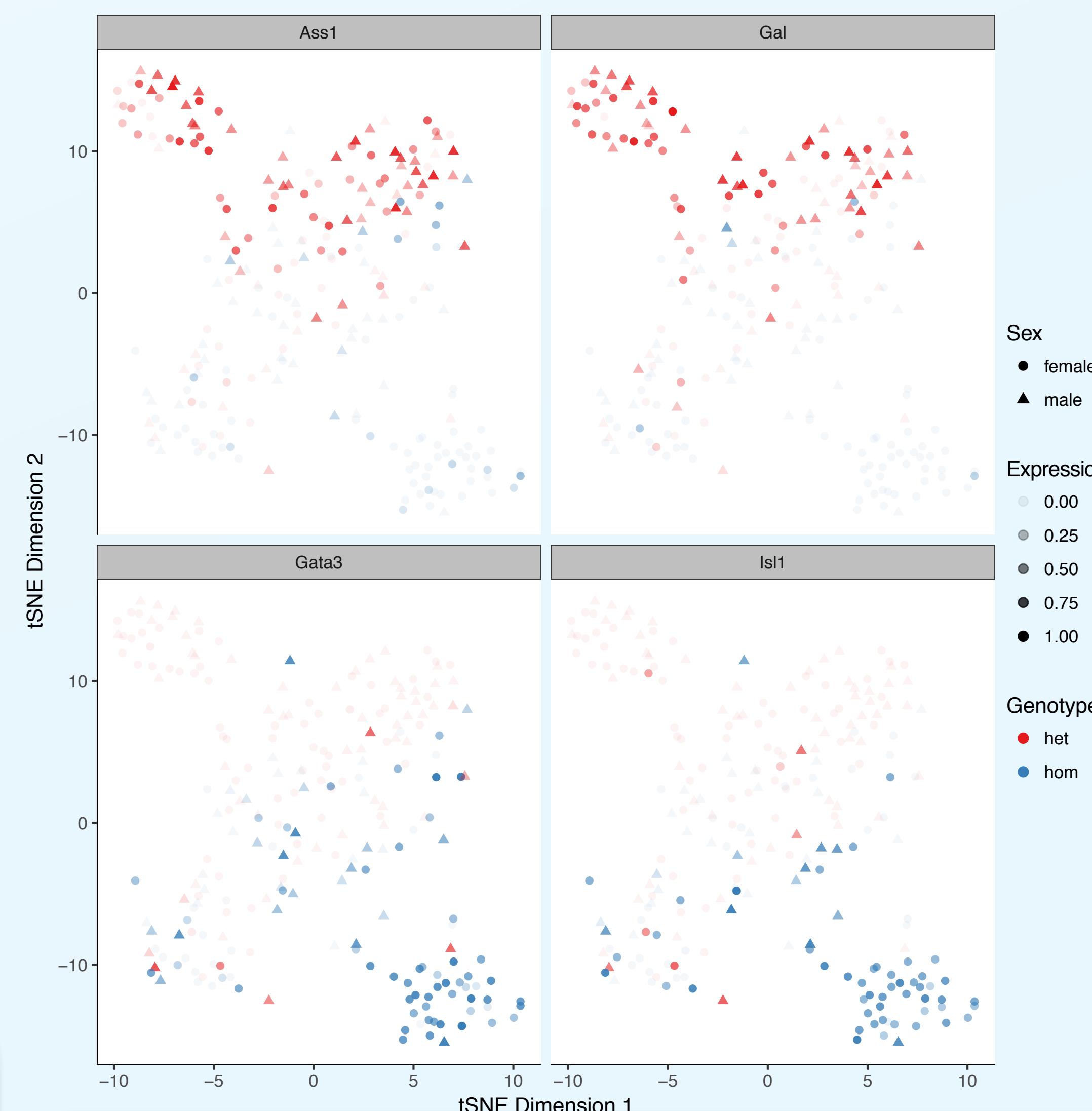
- tSNE visualization of cell-cell variation using variance-stabilized expression estimates from 'high-dispersion' gene set.
- Differentiation trajectories for ENS neurons and glia identifiable.
- Genes varying along this axis are enriched for Cell Cycle, Neurogenesis, and gliogenesis GO terms.
- Trajectories confounded by differentiation state, gut position, and genotypic effects.

Annotation of Major Cell Types and Trajectories



- Known glial and neuronal marker genes are used to annotate each developmental trajectory.
- A cell type hierarchy is created for major cell types, and subclasses of neuron and glial cell types (not shown).
- Currently investigating positional and electrophysiological properties for neuronal cell types.

A sex-specific Ret^{-/-} neuronal subpopulation



- A Female Ret-null specific population of Th+, Isl1+, and Gata3+ neurons may be a compensatory population that mitigates HSCR severity in females.
- Other neuronal subtype populations do not demonstrate sex-specific bias.
- Non-cholinergic secretomotor/vasodilator neurons (Gal+) significantly under represented in Ret-null ENS neurons at E12.5.

Conclusions

- Single-cell analysis of RET⁺ cells in the developing gut can reconstruct both mature cell fate and developmental trajectories in WT and Ret-null mice.
- Loss of RET has independent network effects in both migrating/proliferating ENCCs and mature ENS neurons.
- Identification of a sex-specific ENS dopaminergic cell fate in Ret-null mice.
- Loss of RET shifts ENC neuron identity away from acetylcholinergic and peptidergic, towards catecholaminergic neuronal subtypes.
- Differential expression of cell-cycle genes in Ret-null mice ENCC glia suggests precocious differentiation of progenitor pool may contribute to aganglionosis.
- Downregulation of Hox genes in Ret^{CFP/CFP} and early induction of differentiation may contribute to aganglionosis phenotype.
- Developed interactive visualization/exploration tool for single-cell expression analysis and DE testing. (Inquire for URL)

Acknowledgements

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Postdocs wanted for a variety of projects! Please inquire!