Homozygous *Ret* LOF leads to precocious differentiation and changes in cell fate of enteric neural crest cells

E. Vincent[1], S. Chatterjee[2], G. Cannon[3], D. Auer[2], H. Ross[4], A. Chakravarti[2], L.A. Goff[1,5]
1) McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, MD; 2) Center for Human Genetics and Genomics, NYU Langone Medical Center, New York, NY;
3) Center for Gastrointestinal Biology and Disease, University of North Carolina School of Medicine, Chapel Hill, NC; 4) University of Maryland, Baltimore, MD; 5) Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD

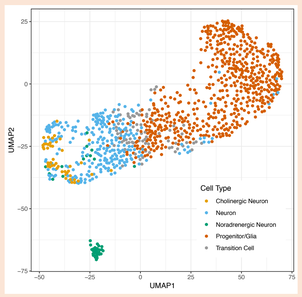
1. Experimental design

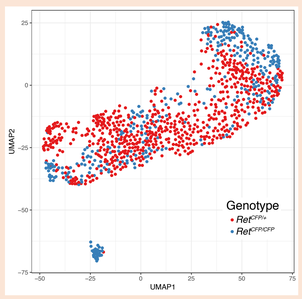
The enteric nervous system (ENS) is formed by neural crest cells (NCC) that migrate from the neural crest through the developing gut tube. The tyrosine kinase receptor *RET* is necessary for the proper formation of the ENS; all enteric NCC express *RET*. Loss-of-function (LOF) mutations in *RET* lead to aganglionic megacolon (Hirschsprung Disease), though the precise mechanisms by which *RET* leads to aganglionosis have not yet been elucidated. *RET* promotes proliferation and maintains cells in an undifferentiated state, therefore we hypothesize that *RET* LOF leads to precocious differentiation of NCC, resulting in depletion of the progenitor pool prior to reaching the distal end of the colon and changes in cell fate.

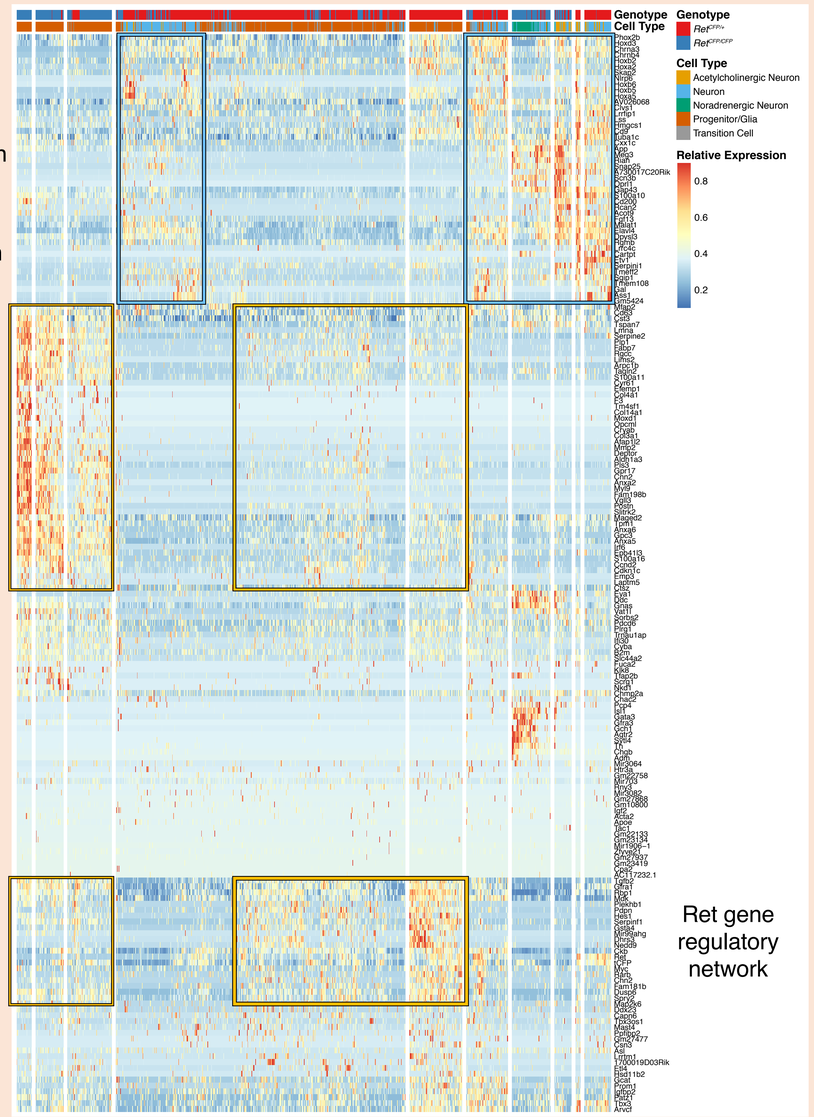
To test our hypothesis we performed single-cell RNA-sequencing (scRNA-seq) on the developing colon of a mouse model of Hirschsprung Disease. The mouse model contains a *Ret*-null allele with a constitutively active fluorescent reporter, which we used to enrich our cell population for enteric NCCs via FACS. Cells were sorted for 2 biological replicates each of 6 conditions: each combination of embryonic day (E)12.5 and E14.5, $Ret^{CFP/+}$ and $Ret^{CFP/CFP}$, and males and females.

E12.5 / E14.5 X $Ret^{CFP/+}$ / $Ret^{CFP/CFP}$ X Male / Female

2. Effects of *Ret* LOF are cell-type-specific

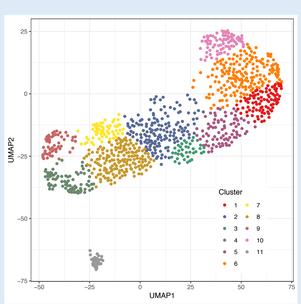


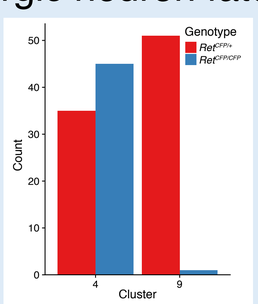


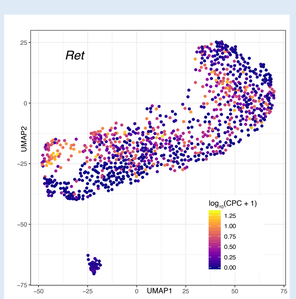


- Dimensionality reduction through uniform manifold approximation and projection (UMAP) reveals contiguous trajectory representing developmental time
- Cell type represents a major axis of variation (left, top)
- Bias wrt genotype is evident in both progenitor/glia population and neuronal populations (left, bottom)
- Differential gene expression analysis (DGEA) wrt genotype (right, q-value < 0.001) reveals *Ret*-null neurons express later *Hox* genes, more mature neuronal markers, and lower levels of motor neuron markers. *Ret*-null glia also express more mature glial markers but show lower expression of genes in the *Ret* gene regulatory network

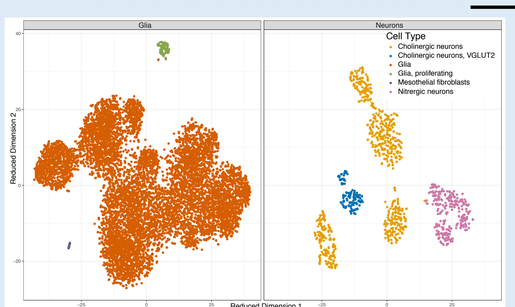
3. Nitrergic neuron fate is inaccessible in the absence of *Ret*

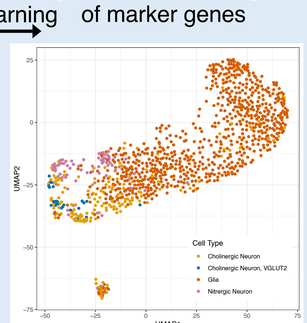






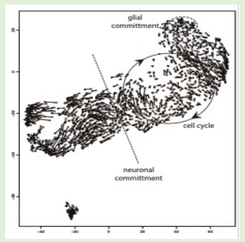
Publicly available, annotated data from adult enteric neural-crest-derived cells → Transfer learning → Cell type classification agnostic of marker genes

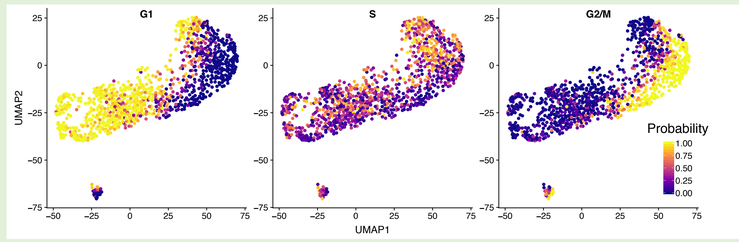


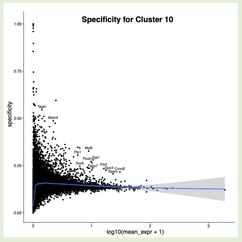


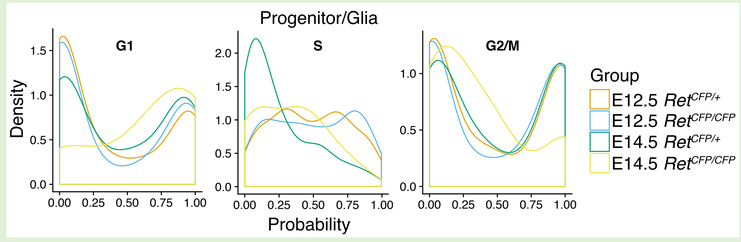
- Branch point in neuronal population forms 2 clusters (top left) showing strong bias wrt genotype:
  - cluster 9 is 98% (51/52) $Ret^{CFP/+}$ cells and
  - cluster 4 is 44% (35/80) $Ret^{CFP/+}$ cells,
  - compared to 66% (664/1003) $Ret^{CFP/+}$ across all cell (top middle)
- Low *Ret* expression in cluster 9, suggesting inactivation of *Ret* promoter, persistent *Ret* expression in cluster 4 (top right)
- We used non-negative matrix factorization to learn patterns of gene expression in publicly available, annotated scRNA-seq data on *Sox10-cre* sorted cells (bottom left)
- We projected our data into the latent spaces (patterns) learned on the public data to calculate pattern usage in our data
- A random forest model was trained on the patterns for the public data and used to classify our data (bottom right) based on projected pattern usage
- Cluster 9 maintains *Ret* expression and is annotated as nitrergic neurons

4. *Ret* is required to maintain progenitor state

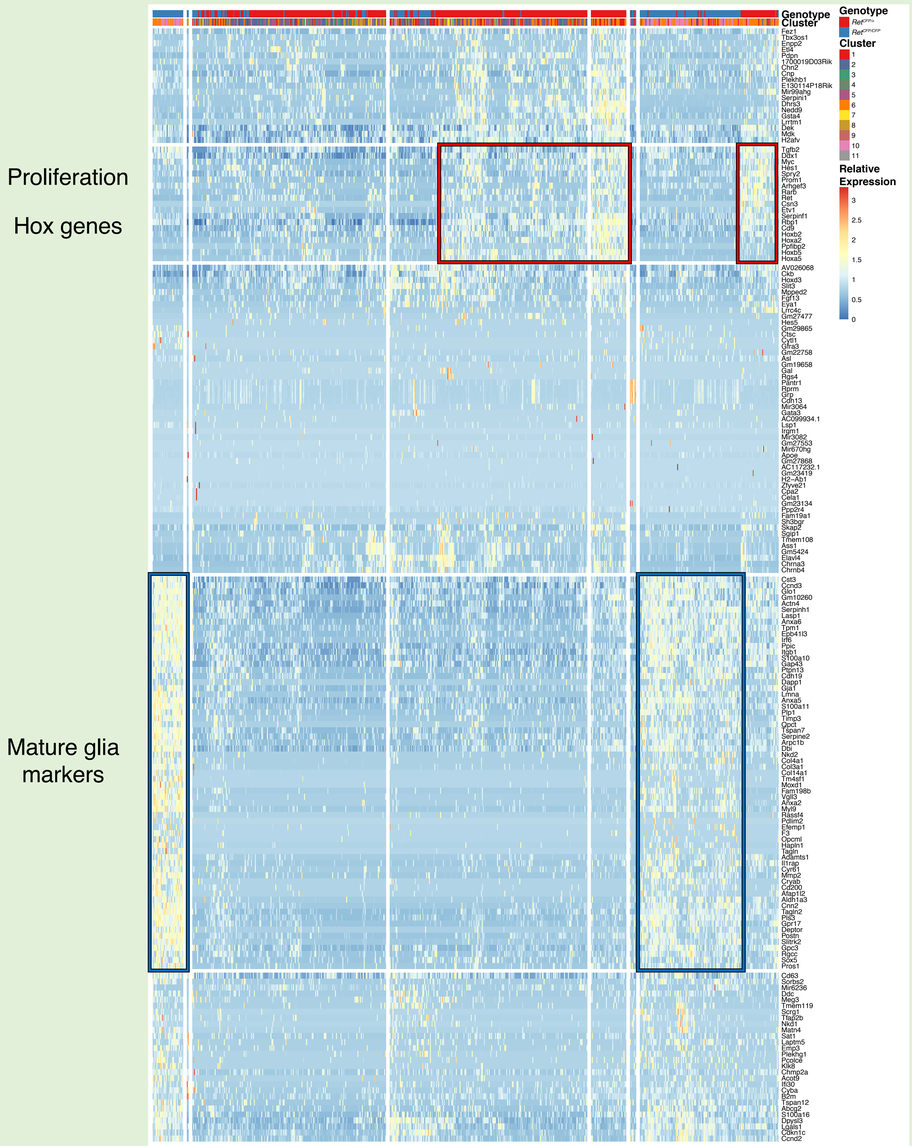








- RNA velocity (above, top left) agrees with cell type annotations based on marker gene expression and transfer learning in that the velocities of neurons point towards the more mature population. Within the glial population it suggests a cycling progenitor population in the center and a smaller population (cluster 10) whose velocity indicates those cells have exited the cell cycle
- Specificity scores for cluster 10, which is 81% (62/77) $Ret^{CFP/CFP}$ cells, reveal expression of mature glia markers is specific to this cluster (above, bottom left)
- Within the progenitor/glia population E14.5 $Ret^{CFP/CFP}$ cells have a higher probability of being in G1 and a lower probability of being in G2/M than other ages and genotypes (above, top and bottom right) suggesting fewer E14.5 $Ret^{CFP/CFP}$ cells are actively proliferating
- DGEA wrt genotype (below, q-value < 0.01) on the progenitor/glia subpopulation shows $Ret^{CFP/+}$ cells express higher levels of earlier *Hox* genes and genes and pro-proliferation genes while $Ret^{CFP/CFP}$ cells express higher levels of mature glial markers. This supports the above data suggesting that $Ret^{CFP/+}$ cells continue to actively proliferate while $Ret^{CFP/CFP}$ cells have begun to commit to a glial fate.



5. Conclusions

1) In the absence of *Ret* both neurons and glia more highly express mature marker genes
2) Nitrergic neurons require *Ret* for fate specification
3) Proportionally fewer *Ret*-null cells are actively cycling, and proportionally more are committed to a glial lineage than $Ret^{CFP/+}$ cells

References:

Fertig *et al.* CoGAPS: an R/C++ package to identify patterns and biological process activity in transcriptomic data. *Bioinformatics* **26**, 2792-3 (2010).
La Manno *et al.* RNA velocity of single cells. *Nature* **560**, 494-8 (2018).
Stein-O'Brien *et al.* Decomposing cell identity for transfer learning across cellular measurements, platforms, tissues, and species. *bioRxiv* preprint doi: https://doi.org/10.1101/395004.
Zeisel *et al.* Molecular Architecture of the Mouse Nervous System. *Cell* **174**, 999-1014 (2018).