Ret loss-of-function leads to changes in developmental trajectories of single cells in the enteric nervous system of a mouse model of Hirschsprung disease

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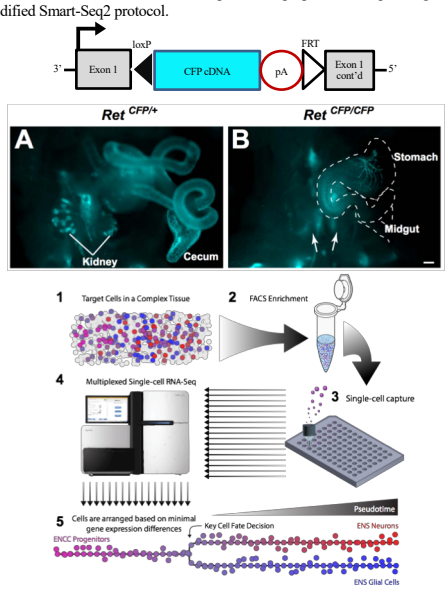
Introduction

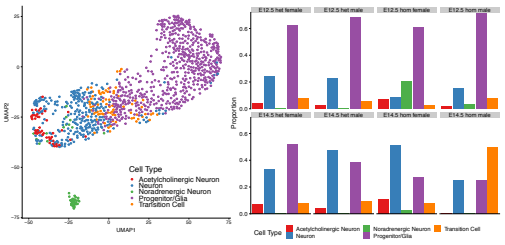
The enteric nervous system (ENS) is formed by neural crest cells (NCC) that migrate from the neural crest through the developing gut tube. From these NCC derive the neurons and glia that form the ganglia of the myenteric and submucosal plexuses. The proto-oncogene *RET* is necessary for the proper formation of the ENS: all NCC migrating through the gut are believed to express *RET*, and loss-of-function (LOF) mutations in *RET* lead to aganglionic megacolon (Hirschsprung Disease) in humans. The precise mechanisms by which *RET* leads to aganglionosis have not yet been elucidated. The normal role of *RET* is to promote proliferation and maintain cells in an undifferentiated state. We therefore hypothesize that *RET* LOF leads to precocious differentiation of NCC, which results in depletion of the progenitor pool prior to reaching the distal end of the colon, and changes in transcriptional trajectories and cell fate.

Experimental Outline

To test our hypothesis we performed single-cell RNA-sequencing (scRNA-seq) on the developing colon of a mouse model of Hirschsprung Disease. The model contains cyan fluorescent protein (CFP) cDNA followed by a poly(A) signal sequence inserted into the first exon of *Ret*. We collected CFP+ cells from male $Ret^{CFP/+}$, female $Ret^{CFP/+}$, male $Ret^{CFP/CFP}$, and female $Ret^{CFP/CFP}$ embryos at embryonic days (E)12.5 and E14.5, which correspond to the middle and end of the migration of NCC through the developing mouse gut. Single cells were sorted into individual wells of 96-well plates and prepared for sequencing with a modified Smart-Seq2 protocol.



Identification of Cell Types



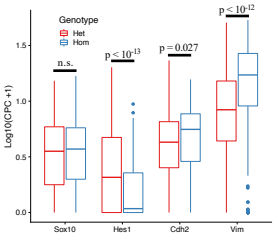
UMAP was used to reduce the dimensionality of the cells to produce the UMAP plots above, which are colored by experimental condition on the left and cell type on the right. Cell types were annotated based on expression levels of select marker genes.

- Neurons: *Snap25*, *Nefl*, *Rtn1*, *Vip*, *Tubb3*, *Nefh*, *Elavl3*, and *Elavl4*
- Noradrenergic neurons: *Isl1* and *Dh*
- Acetylcholinergic neurons: *Ache* and *Slc18a3*
- Progenitors/glia: *Fabp7* and absence of *Tubb3*
- Transition cells: None of the above or multiple of the above

Cell-Type-Specific Effects of *Ret* LOF

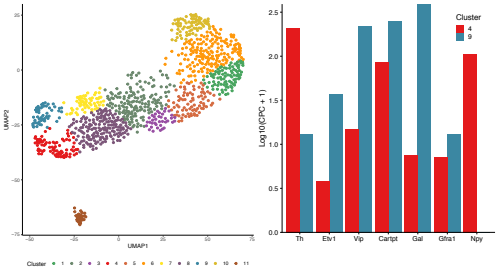
In absence of *Ret*, glial population expresses more mature markers

$Ret^{CFP/CFP}$ glia express higher levels of radial glia markers such as *Cdh2* (N-cadherin) and *Vim* and lower levels of early progenitor markers such as *Sox10* and *Hes1*. This suggests that the **$Ret^{CFP/CFP}$ glia are further along their developmental trajectories than their heterozygous counterparts**, which supports our hypothesis that LOF of *Ret* leads to precocious differentiation of the progenitor pool.



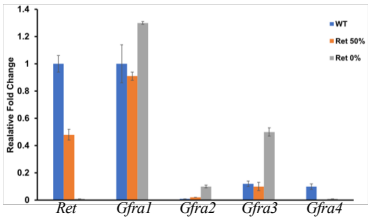
***Ret*-dependent neuronal trajectory**

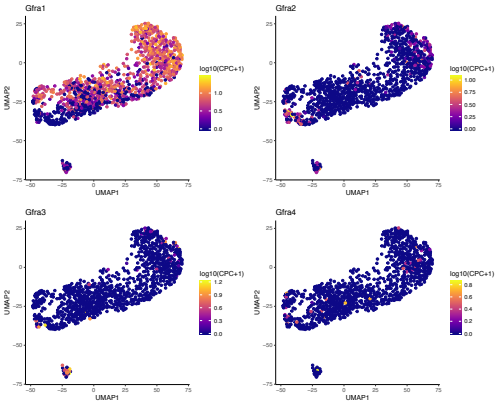
In the bifurcated neuronal population there is clear enrichment of heterozygous cells in the upper branch (one-tailed Fisher's exact test, $p < 10^{-8}$). This suggests that **there are neuronal developmental trajectories that are inaccessible to NCC in the absence of *Ret***, which supports our hypothesis that LOF of *Ret* leads to changes in cell fates.



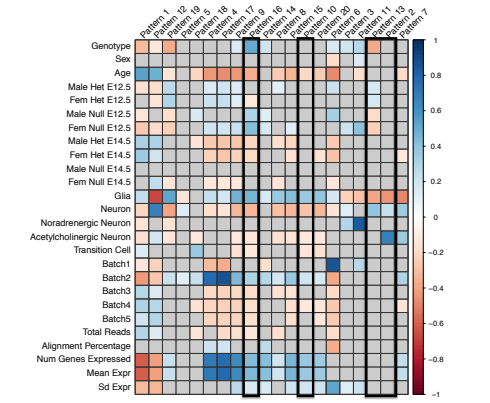
Use of non-canonical *Ret* co-receptors in neurons

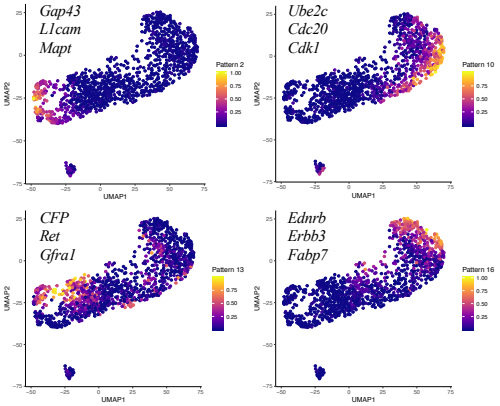
Ret's canonical co-receptor in the gut is Gfra1. Gfra2, 3, and 4 can also act as co-receptors with Ret in other contexts, but are not expressed highly in the gut. Our data from siRNA show that *Gfra1*, *Gfra2*, and *Gfra3* expression all increase in the absence of *Ret*, and our scRNA-seq data show that this change in expression is specific to discrete neuronal populations.





Coordinated Gene Activity in Pattern Sets (CoGAPS)





20 expression patterns were calculated across all cells and all expressed genes. Correlations were calculated between continuous or binarized meta data and cell weights for patterns. Correlations that were not significant at $p < 0.05$ after correcting for multiple testing are coded gray. The pattern weights for four of the patterns are plotted on the UMAP embedding; 3 genes that have high weights and are biologically informative are given for each pattern.

- **Pattern 2 represents neuronal differentiation** and is significantly correlated with neurons, particularly acetylcholinergic neurons.
- **Pattern 10 represents cell cycle progression** and is correlated with age, cell type, and several technical variables.
- **Pattern 13 represents genotype** and is restricted to the *Ret*-dependent lineage of neurons, again supporting our hypothesis that LOF of *Ret* leads to changes in cell fate.
- **Pattern 16 represents maturing glia** and is also correlated with genotype, supporting our hypothesis that *Ret*-null cells go through precocious differentiation.

Major Findings and Future Directions

- **Genotypic bias evident in neurons and progenitors/glia**
  - **There are neuronal trajectories that are inaccessible to NCC in absence of *Ret***
  - ***Ret*-null cells express markers of more mature glia**
- **In the absence of *Ret*, expression of non-canonical *Ret* co-receptors increases in distinct subpopulations**
- **Patterns can be used to determine biological significance of more subtle structure in the data, i.e. cell cycle component of glial population**

References:
Uesaka T. *et al. J Clin Invest.* **118**, 1890-1898 (2008).
Trapnell,C. *et al. Nat. Biotechnol.* **32**, 381-386 (2014).
Stein-O'Brien, G. L. *et al. Bioinformatics* **33**, 1892-1894 (2017).
McInnes, L, Healy, J. *ArXiv e-prints* 1802.03426 (2018).
Picelli, S. *et al. Nature Protocols* **9** 171-181 (2014).