# Early chromatin shaping predetermines multipotent vagal neural crest into neural, neuronal and mesenchymal lineages

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**Keywords**

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**List of Abbreviations**

GRN, Gene Regulatory Network; NC, Neural Crest; VNC, Vagal Neural Crest; ENS, Enteric Nervous System; HH, Hamburger Hamilton; TF, Transcription Factor; DA, Differentially Accessible; DB, Differential Binding; DE, Differentially Expressed

**Abstract**

The enteric nervous system (ENS) predominantly originates from vagal neural crest cells (VNC) that emerge from the caudal hindbrain, invade the foregut and populate the gastrointestinal tract. However, the gene regulatory network (GRN) orchestrating the early specification of VNC remains unknown. Using an *EdnrB* enhancer, we generated a comprehensive temporal map of the chromatin and transcriptional landscape of VNC in the avian model, revealing three VNC cell clusters (neural, neurogenic and mesenchymal), each predetermined epigenetically prior to neural tube delamination. We identify and functionally validate regulatory cores (Sox10/Tfap2B/SoxB/Hbox) mediating each programme and elucidate their combinatorial activities with other spatiotemporally-specific transcription factors (bHLH/NR). Our global deconstruction of the VNC-GRN *in vivo* sheds light on critical early regulatory mechanisms that may influence the divergent neural phenotypes in enteric neuropathies.

## Introduction

Achieving cellular diversity within the enteric nervous system (ENS) requires precisely governed gene regulation to form a complex interconnected network of ganglia[1]. Therefore, gene perturbations during key steps of ENS formation can have detrimental effects, as in the case of Hirschsprung disease (HSCR, OMIM #142623), a congenital intestinal aganglionic malformation where the gut lacks ENS innervation at varying lengths of the large bowel, leading to the loss of motility and a potentially lethal pseudo-obstruction[2]. The increasing catalogue of coding and non-coding mutations[3, 4] implicated in this complex oligogenic enteric neuropathy heightens the need to explore the gene regulatory network (GRN) and the chromatin landscape required for early ENS development.

Decades of experimental embryology using grafting and lineage tracing experiments have demonstrated that neurons and glia of the ENS are largely derived from the vagal neural crest, VNC (arising at somite levels 1-7)[5, 6] with some contribution from sacral NC, caudal to somite 28[7]. Avian and murine models have shown that VNC delaminates from the neural tube around E8.5 in mouse (E1.5-2/HH10 in chicken), enters the developing foregut at E9-9.5 (E3-3.5/HH18 in chicken) and undergoes a long migration rostrocaudally from the foregut to the hindgut to complete its colonisation around E13.5 (or E7.5/HH30 in chicken)[7, 8] (Extended Data Fig. 1). Current gene expression profiles of ENS sublineages were only analysed at later time points[9-13], including recent single-cell datasets of *Sox10*-derived ENS lineages[14] and of post-otic derived *Wnt1*-traced NC[15] (Extended Data Fig. 1). From a regulatory standpoint, NC-GRN analyses to date have covered early cranial[16-18], or only trunk NC[17, 19] restricted to *FoxD3* expressing cells, but non-coding, *cis*-regulatory elements specific to the vagal region remain to be dissected.

The present study addresses this knowledge gap (Extended Data Fig. 1) by characterising both the transcriptional and chromatin landscape in all VNC cells from delamination to gut colonisation. We identified distinct VNC cell populations and functionally validated early regulatory signatures that drove their propensities to differentiate into a particular fate. By correlating motif enrichment on identified enhancer elements with transcriptional profiles and transcription factor (TF) binding maps, we highlighted Tfap2B and Sox10 as key factors that function in combination with other major classes of TFs to drive different lineages and precisely control instructive and repressive programmes. *In vivo* CRISPR/Cas9 knockouts of core factors confirmed their essential inputs into regulatory circuits driving VNC downstream targets. Together, these data validated a comprehensive VNC-GRN governing the very early determination of VNC fate into neural, neurogenic and a previously undescribed mesenchymal lineage within the gut.

## Results

### Chromatin profiling identifies NC-specific *EdnrB* enhancers

As previously described early trunk NC progenitor driver *FoxD3*-NC2[20] does not encompass all VNC[21], we generated chromatin accessibility maps of NC2$^+$ cells FAC-sorted from dissected vagal regions adjacent to somites 1-7 in HH10 chicken embryos (Fig. 1a) to reveal other *cis*-regulatory elements with a broader VNC activity (Figs. 1b, Extended Data Figs. 2a-i). Focusing on *EdnrB*, a gene critical in enteric neurodevelopment[22], six elements identified as differentially accessible (DA) specifically in the VNC (Fig. 1c) drove specific Citrine reporter activity in the embryo (Fig. 1d). Enhancers E1, E2, and E5 controlled expression in NCC, with E3 and E6 active in the neural tube and E4 in the zona pellucida (Fig. 1d).

We selected E2 enhancer for further analysis due to its conservation with mammals, and a strong broad NC-specific activity that persisted at later stages. *In situ* Hybridisation Chain Reaction (HCR)[23] confirmed that Citrine transcripts were distributed within the same cells harbouring E2 fluorescent signal (Fig. 1e). Furthermore, endogenous *EdnrB* transcripts precisely overlapped the pattern of E2 reporter activity, suggesting that this enhancer was a part of the tissue-specific *cis*-regulatory apparatus controlling *EdnrB*, while co-expression with endogenous *Sox10* gene confirmed the NC identity of E2-controlled Citrine-expressing cells (Fig. 1f). Triple reporter assays revealed approximately twice as many E2-*EdnrB*/enh-99-*Sox10* double-positive cells (enh-99 is a global *Sox10* enhancer[18]) compared to triple-positive cells that included NC2, further evidencing that NC2 enhancer alone did not label all VNC (Figs. 1g, h).

### Distinct NC2 and E2 reporter activities during VNC development

Concurrent activities of E2 (Citrine) and NC2 (Cerulean) reporters were assessed at three developmental time points (dissected regions adjacent to somites 1-7 in HH10 and HH18 chicken embryos and dissected embryonic guts at HH25) (Fig. 2a) using optimised co-electroporation assays (Extended Data Fig. 2j). FAC-sorting experiments confirmed that there were approximately twice as many E2-only positive cells (from here on E2) compared to double E2/NC2-positive cells (from here on DP) with no NC2-only positive cells sorted (Fig. 2a', Extended Data Figs. 2k, l). Whole-mount and transverse sections at the axial level adjacent to the somites 3 at HH10 (Fig. 2b) showed migrating DP cells as well as E2-only cells within the dorsal neural tube prior to delamination (Fig. 2b'). At HH18 (Fig. 2c), there was concurrent DP and E2 activity within the dorsal roots, surrounding the foregut, and within the ventral NC stream (Fig. 2c') that was also observed at different axial levels (pharynx and trunk/sacral, Figs. 2c'', c''') with higher observed E2 activity within the

pharyngeal arches (Extended Data Figs. 2m, n). Neither DP nor E2 activity was detected within the neural tube (Figs. 2c', c''). By HH25 (Fig. 2d), distinct E2 versus DP activities were observed within the populations in the stomach and duodenum (Figs. 2d', d'', d'''). Cell counts at HH18 validated FACS experiments (Fig. 2e), indicating a higher proportion of DP cells at the dorsal migratory stream compared to E2 activity at the ventral/pharyngeal arches (Figs. 2c'', c''', e) which closely followed endogenous *EdnrB* expression (Fig. 2f).

**Transcriptional profiling of DP and E2 highlights Sox10-high and Sox10-low populations**

DP, E2, and negative-fluorescence control cells were transcriptionally profiled at the three stages described Extended Data Figs 4b, e). A supervised comparative analysis (Fig. 3a) showed a high *Sox10* and *FoxD3* expression in the DP and a lower *Sox10* expression level with no *FoxD3* in the E2 population. DP-only showed enrichment in neural markers (*Tfap2B*, *Zeb2*) while both DP and E2 displayed neuronal marker genes (*Sox3*, *Elavl4*, *Tubb2B*) at HH18 and HH25. At HH10 and HH18, high level expression of mesenchymal genes (*Prrx1*, *Msx1*) were noted, possibly representing future VNC contribution to neck gland organs or cardiac mesenchyme. However, at HH25 expression of these and other mesenchymal genes (like *Barx1*) persisted in VNC-derived, reporter-positive cells in the gut. These cells did not express gut endodermal markers such as *Sox17* or other vascular markers like *VegfA*, while *Hox* gene expression indicated the cells originated from the correct axial level (Fig. 3a).

RNA-seq expression within the gut was validated using a battery of NC and developmental markers (Figs. 3b-h). Genes specific to *Sox10*$^{+}$ cells included *Msx1, Cdh19, Lmo4,* and *EdnrB*. *Hes4*, *Pdgfa*, *Ets1,* and *Prrx1* showed broad expression in all cells in the gut, while *Sox3* (Fig. 3h) and *Tfap2B* (Fig. 3e) were expressed both in *Sox10*$^{+}$ cells and in discrete surrounding populations of *Elavl4*$^{+}$/*Sox10*$^{-}$ cells (Fig. 3h). *Col9a3*, co-localised with *FoxD3* *(*Fig. 3f), while *Barx1*, a previously described gut mesenchyme marker[24], was found to be expressed within a subpopulation of *Sox10*$^{+}$ cells (Fig. 3d).

**Transcriptional analysis at single-cell resolution reveals three VNC subpopulations**

Further investigation of the VNC transcriptional states using single-cell RNA-sequencing (scRNA-seq) of the entire E2-positive VNC population (Extended Data Figs. 3a, b) revealed four distinct single-cell clusters (Fig. 4a, Extended Data Fig. 3c) and no neural tube cell contamination (Extended Data Fig. 3d). When assessing the top 50 marker genes (Extended Data Fig. 3e), the first single-cell cluster (scC1) expressed mostly mesenchymal differentiation genes (*Prrx1*, *Twist1* etc.). ScC2 cluster was marked by *bona fide* NC genes

(*Sox10*, *FoxD3*, etc.), scC3 mostly by neuronal genes (*Sox3*, *Elavl4*, etc.*)* while scC4 was enriched in expression of housekeeping factors. Neural progenitor markers like *Nes* and *Fabp7* were present in all clusters while *MoxD1*, was expressed only in the neural (scC2) and neuronal (scC3) clusters (Figs. 4b, e). Differentiation pseudotime analysis showed a separate bifurcated split of the neuronal cluster (that could represent the early sympathetic lineage) with the concurrent migration of all three main clusters in the lower branch (that could contribute to the ENS) (Fig. 4c). Ordering single cells according to gene expression highlighted the multipotent nature of the neural and mesenchymal clusters through the additional expression of neuronal genes (*Elavl4* and *Sox3*) while the neuronal cluster signature remained distinct (Fig. 4d, e). HCR analysis at HH25 confirmed co-expression of endogenous *Sox10* with E2 reporter (Fig. 4f), whereas not all E2-positive cells expressed *FoxD3* (Fig. 4g, red arrows) or *Elavl4* (Fig. 4h, red arrows). *MoxD1* was expressed in all E2 cells (Fig. 4i), in keeping with the observation from mouse embryonic *Wnt1*-traced NC[15] (Extended Data Fig. 3f). We next established that both DP and E2 populations formed neurons as both reporter activities co-localised with βIII tubulin (Fig. 4j, j', j''). However, only DP cells expressed glial fibrillary acidic protein (GFAP) when assessed at a later stage (HH28; Fig. 4k, Extended Data Fig. 1), whereas a mesenchymal subset of E2-derived VNC cells within the gut co-expressed *Sox10* and *Barx1* (Fig. 4l).

To analyse the evolution of cellular diversity between DP and E2 across different stages, we constructed a reference matrix from the three major single-cell clusters (neural, neuronal, and mesenchymal) (Fig. 4m) and deconvolved our bulk temporal RNA-seq datasets (Fig. 4n). The decomposition showed that at all stages, over 50% of the DP population carried a signature of "neural" single-cell cluster and the rest was divided between neuronal or mesenchymal signatures. Neuronal and mesenchymal clusters were only recovered in the E2 datasets, in agreement with the enrichment of neural genes in DP and neuronal/mesenchymal ones in E2 population (Fig. 4o, Extended Data Figs. 3g, h, i), some of which highlighted from the *Wnt1*-traced NC single mouse cells at P21[25] (Extended Data Fig. 3j).

Thus, our transcriptomic analyses have shown that VNC activity can be dichotomised by a *Sox10*$^{high}$/*FoxD3*$^{+}$ and *Sox10*$^{low}$/*FoxD3*$^{-}$ signature, with a differential *FoxD3* expression within the neural cluster (and its absence from future neurons), emphasizing *FoxD3* role of as a key neural progenitor state regulator[26]. Additionally, the notion that *FoxD3* highlighted a subset of VNC was corroborated by the *Wnt1*-traced NC single-cell dataset[15], where we found *EdnrB* and *Wnt1* present in all and *FoxD3* only in selected single-cell clusters (Extended Data Fig. 3f).

**DA regions show functionally variable relationships to VNC gene expression**

To determine the regulatory landscape governing the observed transcriptional heterogeneity, we performed ATAC-seq on DP and E2 populations across the three stages described. Profiles were highly reproducible, displaying similar peak densities and complexity between replicates, which clustered by stage as well as cell type (Extended Data Figs. 4a, c, d, f, g, h), thus allowing identification of distinct DP and E2 regulatory signatures. DA analyses of consensus peak sets displayed significant DA peaks annotated to *bona fide* NC genes at HH10 (*Sox10*, *FoxD3, Tfap2*), whereas the E2 population contained significant DA elements regulating neuroepithelial in addition to mesenchymal transcription factors (TFs) (Figs. 5a-c). These distinct profiles were maintained from HH10 and by HH18 to HH25, other genes associated with differentiation of VNC derivatives or previously described as expressed in the gut much later, like *NeuroD4, NeuroD1*[9]*,* and *Mbp,* became differentially accessible (Fig. 5c). As such factors were not as yet readily expressed (Fig. 3a, Extended Data Fig. 3i), the observed epigenomic dynamics reflected preparation of the chromatin landscape prior to transcription (Extended Data Fig. 5a). Cumulatively, DA peaks showed an increase in the number of elements per gene from HH10 to HH25 (Extended Data Fig. 5b). When DA promoter peaks were assigned to differentially expressed genes genome-wide (p-adjusted<0.05, log2FoldChange>1; Fig. 5d, Extended Data Fig. 4k), we observed a positive correlation with the expression of protein-coding genes that increased over time. This suggested that the regulatory programmes were defined and prepared early during NC specification. While the later conversion of DP to E2-only cells is theoretically possible, this scenario is much less plausible given the extent of the chromatin landscape remodelling that would need to take place over a very short period of time.

**Differential TFs usage is associated with regulators of neural and neuronal cell differentiation**

Homer[27] *de novo* motif enrichment analysis of DA peaks identified Tfap2 and Sox10 motifs as the most significantly enriched in DP, and the SoxB and Homeobox (Hbox)/basic Helix-Loop-Helix (bHLH) in the E2 population (Fig. 5e, Supplementary Table 1). We positioned these binding sites within all the DA elements and ranked them according to their $log_2$FoldChange values obtained in the DA analyses. While at HH10 all four motifs distributed equally across the peaks, by HH18 we observed preferential presence of Tfap2 and Sox10 motifs within DP peaks, whereas SoxB and Hbox/bHLH were biased towards E2 population. At HH25, we detected significant use of Hbox/bHLH motifs within the E2 ($p$<1E-413) and Sox10 within DP elements ($p$<1E-300), whereas preferential usage of SoxB or Tfap2 was no longer observed (Fig. 5f). Higher resolution analysis across 84937 merged

consensus peaks using *k*-means clustering[28] identified 10 cohesive groups of elements (*k*-Clusters 1-10, *k*C1-*k*C10) showing DA dynamics across the stages and cell populations (Fig. 5g). By annotating these clustered elements to the nearest promoter (TSS) and selecting the top 200 associated genes for statistical overrepresentation analysis (Fig. 5h, i), we found that the elements broadly mediated processes of nervous system development (*k*C1, 3, 5-9), negative regulation of neurogenesis (*k*C2), mesenchymal development (*k*C4) and extracellular matrix development (*k*C10).

Upstream *cis*-regulatory codes and the known TF motifs enriched within each identified cluster showed that HMG-box containing TFs including Sox factors were distributed across all clusters, but absent from elements active at later stages (Fig. 5j). Tfap2 factors were enriched specifically within the DP population (notably in the *k*C4 and *k*C8 clusters), whereas members of Hbox, bHLH families of TFs driving differentiation into VNC derivative fates were predominantly enriched at later stages. *De novo* motif analyses within each cluster equally singled-out Tfap2, Sox10, Sox2/3, and Hbox/bHLH as top enriched TFs, thus highlighting them as the core factors driving our DA elements (Fig. 5k).

**Combinatorial regulatory codes reveal dynamic uses of enhancer elements to drive VNC derivative programmes**

TFs tend to work in combination, collaboratively or competitively, to accurately regulate hundreds of genes by binding to their regulatory elements[29]. We therefore performed two-way (2TF) and three-way (3TF) heterotypic co-occupancy analysis and included two other top-scored candidates TFs as per our *de novo* motif analysis: Hbox and nuclear receptors NR(1) (Fig. 6a,b; Supplementary Table. 1). Tfap2 motif co-occupied the same elements as Sox10, Sox, NR(1), and Hbox within the DP population at earlier stages, but within the E2 population, Tfap2 only showed significant co-binding with Hbox ($p<1E-63$) and Sox ($p<1E-42$) at HH10. Given that Tfap2B expression levels were high within both the neural and mesenchymal single-cell cluster cells (Fig. 4b), this finding indicated that different Tfap2 interacting partners drove different lineages. Conversely, significant 3TF combinations with Sox10 (Tfap2:Sox10:Sox, $p<1E-43$; Sox10:Sox:NR(1), $p<1E-46$ and Sox10:Sox:bHLH, $p<1E-21$) only became more apparent at HH25. Interestingly, Sox appeared to require a different partner at each analysed stage, Hbox at HH10, NR(1) at HH18 and bHLH at HH25, suggesting a highly dynamic co-regulatory code. To show binding combinations, we annotated TF binding motifs predicted using the MEME suite[30] on *EdnrB* E1 and E2 enhancers, previously shown to drive NC activity. E2 contained binding sites for Sox10, Tfap2 as well as Rarb and Maf while E1 showed binding sites for Sox, Maf, and Zinc Finger proteins (Fig. 6c) suggesting that these factors may drive *EdnrB* activity specific to the E2-

only population and that Zinc finger factors may be key in the neuronal differentiation process.

Biotin ChIP-seq[26] for Sox10 and Tfap2B was employed to validate binding events and direct regulatory targets across the two VNC populations (Figs. 6d, Extended Data Figs. 5c-e). Differential occupancy analysis confirmed Sox10 binding enrichment in DA regulatory elements controlling genes such as *Ret* and *Gata3*, but also highlighted targets like *Kbp* and *Sema3D*, both of which have been implicated in HSCR[31] (Fig. 6e). Tfap2B additionally directly targeted mesenchymal TFs like Hand1 and MafB (Fig. 6e), with peak profiles confirming its enrichment within the DP population (Fig. 6f) and a greater overlap with DA peaks compared to E2 (Fig. 6g).

We next examined differential binding at two loci previously involved in ENS development[4] and found that while both Sox10 and Tfap2B regulated the upstream *Ret* enhancer active in VNC at HH18, positioned ~14kb away from the TSS (Fig. 6h), *Ascl1* enhancer positioned ~18kb downstream from locus that displayed the neural tube and NC derivative activity at HH18, was only bound by Sox10 (Fig. 6i). Sox10 and Tfap2B also directly bound to open elements near the *Dio3* and *MoxD1* genes (Figs. 6j, k), while the entire 5' intronic enhancer cluster of *Col9a3*, specifically accessible in the DP population, was only bound by Sox10 (Fig. 6l). Thus, the differences in combinatorial TF binding and binding intensities between populations and stages revealed precisely coordinated codes for VNC cell differentiation.

**Functional perturbations of core TFs validate gene regulatory interactions in VNC**

To functionally validate identified upstream core signatures and regulatory connections, we performed *in vivo* knockouts (KOs) of *Tfap2B*, *Sox10*, *Sox3*, and *Msx1* genes in VNC, followed by RNA-seq of KO versus control cells (Fig. 7a, Extended Data Figs. 6a). We used the previously described *Msx1* gRNA targeting essential donor splice site[32], whereas for *Sox3*, *Tfap2B* and *Sox10,* we employed a double gRNA strategy to increase KO efficiency (Figs. 7b, Extended Data Fig. 6b-d). Mis-splicing events showed the efficiency of gRNAs/Cas9 to disrupt the targeted genes (Fig. 7b). We observed less than 50% survival in the case of *Sox3* and *Msx1* KO at HH18, whereas double KO of these genes resulted in less than a 10% survival rate (Fig. 7c). In contrary, *Sox10* KO, *Tfap2B* KO, and double *Sox10/Tfap2B* KO (DKO) embryos had high survival rates likely due to our targeting strategy, only affecting later stage NC cells compared to published null allele KOs[33, 34].

In all KO conditions, we observed an overall downregulation of mesenchymal NC genes and ECM and guidance molecules (Fig. 7d). Direct downstream targets of Sox10 and Tfap2B as

identified by our Biotin ChIP-seq were downregulated in the *Sox10* and *Tfap2B* KOs while pro-neural bHLH were downregulated in the *Sox3* and *Msx1* KOs. A significant increase in neuronal genes was observed in the *Msx1* and the *Sox10*/*Tfap2B* double KO (DKO) but not in *Tfap2B* KO alone, suggesting the upregulation of the neuronal differentiation programme and depletion of the neural programme in the absence of *Sox10* (Figs. 7d, Extended Data Fig. 6e). *Sox3* KO appeared to upregulate *Sox10* but downregulate *Tfap2B*, thereby only affecting a few downstream Sox10 targets independent of Tfap2 regulation. This affirmed the importance of combinatorial TF activities to regulate specific factors within the GRN. As *Sox2* was upregulated in the *Sox3* KO, compensation mechanisms[35] could account for a less severe neuronal expression phenotype. By assessing a selected range of expression patterns in KO embryos using HCR *in situ* (Fig. 7e), we observed dysregulation of selected target gene expression in the pharyngeal arches or the NC dorsal root streams validating the KO experiments. In summary, our data not only functionally probed the core TFs within the VNC-GRN but also highlighted potentially genetic compensatory mechanisms that maintain the network robustness.

**Reconstructing the VNC-GRN**

We have shown that a heterogeneous population of VNC delaminating from the neural tube (Fig. 8a, left) can be dichotomised based on their *Sox10* and *FoxD3* expression patterns, with *Sox10*$^{high}$/*FoxD3*$^{+}$ population maintaining its multipotent neural ability to form all three lineages while the *Sox10*$^{low}$/*FoxD3*$^{-}$ population is restricted to the neuronal and mesenchymal fates (Fig. 8a, right). To explore distinct chromatin signatures and uncover larger co-binding dynamics of TFs mediating the VNC-GRN, we performed a comprehensive high-resolution survey of 77 vertebrate TF motifs ($p<0.0001$, Binomial test) across our DA elements. We assimilated the data and reverse engineered the gene regulatory circuits governing expression of selected factors like *Ednrb* (Figs. 8b, Extended Data Figs. 7a-b) to define the hierarchies within the VNC-GRN (Fig. 8c).

To further highlight detail of VNC regulation, we chose the Hes family members, key regulators of neurogenesis[36] shown here to be expressed in *Sox10*$^{+}$ cells within the gut (Fig. 3c). By integrating enhancer binding motif enrichment (Fig. 8d) with TF binding information (Fig. 8e), we ascertained the that while *Hes* is directly activated by both Tfap2 and SoxB, it is also indirectly controlled by Sox10, which directly activates its transcriptional repressors *Etv* and *Olig* (Fig. 8f). This circuit was functionally validated by our KO experiments (Fig. 8g), highlighting the complex dysregulation of factors involved. Therefore, our datasets have allowed us to dissect the global VNC-GRN and individual gene regulatory circuits that drive VNC cell fate decisions at a greater resolution.

## Discussion

### Genome-wide analyses parse VNC diversity driven by core TF regulators

The VNC not only majorly contributes to the ENS but also gives rises to derivatives in the thymus[37], thyroid gland[38], heart[39], lungs[40], sympathetic ganglia[41], and pancreas[42]. By dissecting the regulatory circuitry that generates VNC-derived glia, neurons or mesenchyme, we uncovered a vast matrix of gene regulatory interactions that precisely activate specific programmes and repress undesired fates using a combination of multiple TFs to guide the cell fate decisions. Our study offered mechanistic insight into the elegant fate-mapping experiments performed by Nicole Le Douarin and colleagues, who found that quail VNC (somites 1-7) grafted to replace the host chick NC at the adrenomedullary level (somites 18-24), still ended up invading the gut of the host and differentiating into enteric ganglia[5, 43] . The intrinsic bias to colonise the gut regardless of the anatomical level at which the VNC are grafted suggested a degree of predetermination before delamination[5, 43].

We focussed our efforts on incorporating chromatin accessibility and gene interactions to define VNC-GRN and identify Tfap2, Sox, Hbox and bHLH families of TFs as core regulators orchestrating the delineation of VNC. We highlighted the role of Tfap2 in combination with Sox10 in driving the neural fate, the role of Tfap2B with other TFs in driving cardiac crest/arches/mesenchymal development and that of SoxB playing a critical role for the neuronal derivatives.

### VNC contributes to mesenchymal cells within the ENS

Soldatov et al.[15] highlighted a mesenchymal fate split in post-otic mouse NC at E9.5 (HH18 in chicken) and described key regulators like *Prrx1* and *Twist1* within this NC cell fate cluster, similar to our findings. Here, we have shown that VNC expressed mesenchymal genes at HH18 but importantly, that these genes were also specifically maintained within the VNC-derived cells in the gut at HH25, highlighting NC-derived mesenchymal cells during ENS and gut development. Indeed, VNC cells have previously been shown to be a mediator of mesenchymal-epithelial interactions to control stomach size, patterning, and differentiation[44]. Additionally, VNC contribution to the mesenchyme of the glands in the neck and pericytes surrounding capillaries and connective tissue in the region[45] hints at the possibility that these VNC-derived mesenchymal cells are critical for maintaining ENS structural integrity.

### VNC-GRN informs on tissue-specific regulation of *Ednrb* and *Ret*

From a gene regulatory perspective, our analyses highlight how key ENS and HSCR disease risk genes, such as *EdnrB* and *Ret* are regulated. By integrating our datasets, we deconstructed the *EdnrB* GRN circuit showing direct and indirect control from core TFs, thus explaining its differential regulation in other NC lineages and persistence of *EdnrB* expression in these cell types in *Sox10*-null mouse embryos[46]. Similarly, our *Ret* enhancers harboured binding sites for Sox10, Gata and Rarb that were previously described to be an important triad in the *RET* GRN[3] in addition to other motifs such as Maf, in keeping with a recent study that found MafB regulated *Sox10*, which in turn regulates *Ret*, during cardiac NC development[21].

By assimilating all our validated genome-wide *omics* datasets, we have uncovered the early establishment of the VNC developmental *cis*-regulatory landscape underlying a paradigm of core regulatory circuitries with distinct and dynamic conglomeration of TF signatures to drive cell identity, thus enabling future studies focusing on deciphering specific lineage subtypes.

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**Author Contributions**

Conceptualisation, I.L., T.S.S.; Investigation, Validation and Formal Analysis, I.L.; Writing I.L., T.S.S.; Visualisation I.L., T.S.S.; Supervision, T.S.S.; Funding Acquisition, T.S.S., I.L.

**Competing interests**

We have no financial or non-financial competing interest.

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405 **Figure legends**

406

407 **Figure 1. Defining regions of differential chromatin accessibility within VNC identifies**
408 **NC specific *EdnrB* enhancers.**

409 (a) *Ex ovo* electroporation of NC2:Citrine construct at HH4. Embryos were incubated until
410 HH10 to reveal Citrine expression in the VNC (A'). Vagal region from somites 1-7 from
411 approximately 90 embryos (Red box) was dissected and dissociated prior to FAC-sorting
412 Citrine$^{+}$ cells. ATAC-seq was performed on 2500 live sorted cells. Scale bar = 100 μm
413 (b) Volcano plot showing merged peaks from triplicates of ATAC-seq experiment
414 differentially accessible in NC2 (green) versus negative cells (brown). Analysis using
415 DiffBind identified peaks with statistically significant enrichment; *p*-values obtained using
416 Wald test with Benjamin-Hochberg correction for multiple testing (False Discovery Rate,
417 FDR) were plotted against the FoldChange on a log scale.
418 (c) Genome browser screenshot of the *EdnrB* locus spanning approximately 150kb showing
419 RNA-seq and ATAC-seq tracks, differentially accessible peaks, as well as human and
420 mouse conservation chains. Six *EdnrB* enhancers, E1-E6, are highlighted in blue.
421 (d) Live embryo confocal image of HH10 embryos electroporated *ex ovo* with
422 enhancer:Citrine constructs. Orientation A, anterior, P, posterior, D, dorsal, V, ventral. 6
423 embryos/experiment
424 (e) *In situ* HCR of an electroporated embryo with E2:Citrine showing co-localisation with
425 Citrine and endogenous *EdnrB* gene expression. 6 embryos/experiment
426 (f) *In situ* HCR of an electroporated embryo with E2:Citrine showing co-localisation with
427 endogenous *EdnrB* and *Sox10* gene expression. 6 embryos/experiment
428 (g) Live embryo confocal image of E2:mCherry, NC2:Cerulean and enh-99:mCherry
429 (marking *Sox10*-expressing cells). White arrowheads mark E2/enh-99 positive cells but NC2
430 negative. 3 embryos/experiment
431 (h) Activity and overlap of three NC enhancers, E2:mCherry, NC2:Cerulean and enh-
432 99:mCherry indicated as a percentage of all fluorescent NC cells in the vagal region (somite
433 levels 1-7). n=3 independent embryos imaged and quantified separately using confocal
434 images. Error bars indicate Standard Deviation. Scale bars = 100 μm.

435

436 **Figure 2. Two distinct NC populations; DP and E2 are revealed by differential**
437 **enhancer activity**

438 (a) E2:Citrine and NC2:Cerulean constructs were co-electroporated *in ovo* into the neural
439 tubes of HH8/9 chick embryos. Vagal regions at HH10 and HH18 (somite 1-7 level, in
440 yellow) and whole guts at HH25 (in yellow) from approximately 90

embryos/stage/experiment were dissected and dissociated, and single cells suspensions subjected to FAC-sorting. (a') Two distinct population of cells; one E2/NC2 double positive and one E2-positive only with no NC2 were isolated by FACS. No NC2-only cells were detected. n=10 independent FACS experiments from pooled embryos. Error bars indicate Standard deviation.
(b) Live embryo images of double enhancer electroporations at HH10 followed by sections at the approximate level of somite 3 (B', dashed line) 5 biologically independent embryos.
(c) Live embryo images of double enhancer electroporations at HH18 and section (c') were taken at the approximate level of somite 3 highlighting crest specific expression with higher magnification images of the dorsal path (c'') and ventral region (c'''). drp, dorsal root pathway, nt, neural tube, vp, ventral pathway, nc, notochord, ao, dorsal aorta, np, nodosa placode, oes, oesophagus. 5 biologically independent embryos.
(d) Whole mount dissected gut at HH25 and sections (d') showing positive enhancer cells within the oesophageal wall with higher magnification images of an area within the stomach (d'') and duodenum (d'''). Yellow arrowheads indicating E2-only cells. 5 biologically independent embryos.
(e) Cell counting (HH10 WM: n=5, HH18 dorsal WM; n=3, HH18 ventral WM; n=2, HH18 dorsal section; n=4, HH18 ventral section; n=5, HH25 WM; n=4). For HH18, cell counts were carried out on whole mount (WM) and sections, split into the dorsal aspect of the migrating stream and ventral aspect. Error bars indicate standard deviation and values expressed as a percentage of fluorescent cells.
(f) HCR at HH18 of E2:Citrine enhancer colocalising with endogenous *EdnrB* gene expression. 3 independent embryos. Scale bars = 100 μm.

**Figure 3. Distinct transcriptomic profiles of VNC derivatives**
(a) Supervised clustering of RNA-seq average gene transcripts per million (TPM) shown for genes classified by different annotated functions. Each column represents the average TPM values calculated from duplicate experiments. (See Extended Data Figs. 4b, e for hierarchical clustering and principal component analysis of RNA-seq replicates showing reproducibility and profile alignment by stage and cell population and Source Data Fig. 3 for raw count data including separate DESeq2 comparison analyses).
(b-h) High magnification 63X confocal images of HCR *in situ* expression patterns on dissected guts performed in at least 3 independent HH25 embryos showing co-expression of genes previously undescribed in the context of ENS development that include *Msx1, Pdgfra, Hes4, cdh19, Barx1, Prrx1, Tfap2B, Lmo4, Col9a3, FoxD3, PdgfA, Ets1, Elavl4* and *Sox3* with known ENS markers *Sox10* and *EdnrB*. Scale bar = 10 μm

**Figure 4. Single-cell analysis parse three distinct VNC lineages, revealing relative proportions in RNA-seq datasets by deconvolution analysis**

(a) tSNE plot depicting clustering of 570 single cell transcriptomes obtained from FAC-sorted dissected vagal neural crest regions of 30-40 HH18 embryos expressing E2:Citrine.

(b) Bubble plot depicting relative expression of selected top genes identified in each cluster. Bubble size reflects percentage of cells per cluster expressing the gene.

(c) Pseudotime trajectory of single-cell clusters.

(d) Gene expression heterogeneity of vagal NC cells at single-cell level.

(e) Violin plots of marker and common gene expression in transcripts per million (TPM) across clusters plotted on a log scale. Dots (n) represent individual cells with the median representing the highest probability expression within the cluster.

(f-i) Expression of *Sox10*, *FoxD3*, Elavl4 and *MoxD1* by HCR with E2:Citrine activity within the gut of 3 HH25 embryos. Red arrows point to cells not expressing the particular gene. Scale bar = 10 μm.

(j) Whole-mount antibody staining with anti-Tuj1 on dissected guts of HH25 embryos co-electroporated with E2:Citrine/NC2:mCherry. Higher magnification views of the lower oesophagus (g') and stomach (g'') highlighting E2-only cells (yellow arrowheads) or NC2-only cells (red arrowheads) colocalising with Tuj1. Greater proportion of E2-only compared to NC2 cells within the gut is detected. 3 embryos; Scale bar = 100 μm

(k) Whole-mount antibody staining with anti-GFAP on dissected guts of HH28 embryos co-electroporated with E2:Citrine/NC2:mCherry. Higher magnification views show GFAP colocalising with DP (E2 and NC2) cell while E2-only cells do not express GFAP. 2 embryos. Scale bar = 100 μm

(l) HCR of HH25 dissected stomach showing co-localisation of *Barx1* and *Sox10* with E2 activity in at least three E2-positive cells. 3 embryos; Scale bar = 50 μm

(m) Reference matrix used to deconvolve RNA-seq datasets depicting expression of top single-cell-cluster markers, re-scaled using average counts across the cluster.

(n) Relative fraction of E2 and DP populations assigned to each single-cell-cluster identity.

(o) Volcano plot showing differentially expressed genes at HH25 highlights neural, neuronal and mesenchymal signatures. Adjusted *p*-values (padj) obtained using Wald test with Benjamin-Hochberg correction plotted against FoldChange on a log scale.

**Figure 5. Analysis of differential TF motifs within differentially accessible elements uncovers Tfap2, Sox10, Sox2/3, and Hbox/bHLH as core TFs**

(a-c) Volcano plots highlighting top DA peaks from DiffBind analysis across triplicates at HH10, HH18 and HH25. Negative FoldChange indicates peaks enriched in the DP and positive FoldChange peaks enriched in E2-only population. *P*-values obtained using Wald test with Benjamin-Hochberg correction (False Discovery Rate, FDR) plotted against the FoldChange on a log scale.

(d) $R^2$ score calculated from the coefficient of determination between effect sizes of significantly DA promoters and significantly differentially expressed (DE) genes closest to those promoters. See Extended Data Fig. 4k for individual values.

(e) Motif enrichment search strategy.

(f) *De novo* motif occurrence within ranked merged DA peaks across triplicates associated with merged DE genes across duplicates. Each coloured bar represents a peak containing the newly nominated top motif: Tfap2, Sox10, Sox3 and Hbox/bHLH. If the peaks contain both motifs represented, lines of the respective colour are plotted next to each other. See Extended Data Fig. 4i, j for total number of DA peaks per condition. *P*-value obtained in DESeq2 DA analysis using Wald test with Benjamin-Hochberg correction.

(g) *K*-means clustering of DA regions across stages and samples highlighting number of elements and peak density profiles. Each cluster is accompanied by the average gene TPM across analysed stages.

(h, i) Genes assigned to the top 100 elements per *k*-cluster and significantly enriched Gene Ontology terms obtained using statistical overrepresentation ($p<0.01$); *p*-values calculated with binomial distributions and Bonferroni correction for multiple hypothesis testing.

(j) Top known upstream motif enrichment as predicted by Homer analysis of merged DA peaks (no. elements stated in Fig. 5g) across triplicates with the colour gradient indicating the $-\log_{10}$pvalue of enrichment analysis over total background peaks using binominal *p*-testing and the bubble size indicating the percentage of target peaks with given motif and positions, clustered according to TF families.

(k) Top *de novo* motifs enriched in merged DA peaks (no. elements stated in Fig. 5g) across triplicates within each cluster obtained by Homer; *p*-values determined by binominal test, using all peaks as background.

**Figure 6. Combinatorial analysis of top *de novo* motif reveals dynamic use of enhancers to drive cell lineage specification validated by Sox10 and Tfap2B differential binding**

(a, b) 2-way and 3-way co-occupancy analysis of top *de novo* motifs. *P*-values were calculated using two-tailed Chi-squared test with Bonferroni correction.

(c) Motif binding analysis within +/-200 bp from the centre of the peak for *EdnrB* E1 and E2 enhancers.

(d) BiotinChIP-seq experimental strategy. E2 enhancer driven Avi-Tagged *Sox10* and *Tfap2B* constructs are co-electroporated with ubiquitously-driven NLS-BirA-mCherry into the neural tube of chicken embryos at the premigratory NC stage (HH8/9) and cells are harvested at HH18.
(e) Differential binding (DB) analysis of Sox10 (magenta) and Tfap2B (dark green) BiotinChIP-seq showing peaks with Fold change >1 and FDR <0.05. Adjusted *p*-values (FDR) obtained using Wald test with Benjamin-Hochberg correction are plotted on a log scale in function of fold enrichment. Positions of bound elements associated to key regulators are indicated.
(f) Heatmaps depicting profiles of differentially bound BiotinChIP-seq peaks within DP and E2 populations. Gradient scales indicate baseline-normalised read counts.
(g) Percentage overlap of DB ChIP-seq peaks and DA ATAC-seq elements within DP and E2 populations.
(h) *Ret* putative enhancer, ~14 kb upstream of the *Ret* TSS, drives Citrine expression within trigeminal ganglia (tg), rhombomere 4 (r4), pharyngeal arches (pa), and dorsal root streams along somites (som) 1-7 (6 embryos). Multiple motifs including Sox10 and Tfap2 are predicted within +/-200bp from the peak centre. Bar plot shows the average gene TPM from RNA-seq analysis. Error bars indicates SEM. Scale bar = 0.5 mm
(i) *Ascl* putative enhancer, ~18 kb downstream from *Ascl* TSS shows activity within the neural tube (nt), pharyngeal arch (pa), mandibular arch (ma), trigeminal ganglia (tg), and dorsal root streams from somites 1-7 (6 embryos). Multiple motifs including Sox are revealed within +/-200bp from the peak centre. Bar plot shows the average gene TPM from RNA-seq analysis. Error bars indicates SEM. Scale bar = 0.5 mm
(j,k,l) ATAC-seq and biotinChIP-seq genome browser tracks for *Dio3* (j), *MoxD1* (k) and *Col9a3* (l) highlighting elements differentially bound by Sox10 and Tfap2B.

**Figure 7. Functional validation of upstream TFs in VNC specification analysed at HH18.**

(a) CRISPR/Cas9 mediated strategy for upstream core TF knockout (KO). Cas9-2A-Citrine and sgRNA expression constructs are co-electroporated into neural tube of HH8/9 chicken embryos *in ovo* and incubated until HH18. The region indicated in green in the schematic is dissected, neural tube removed and dissociated cells FAC-sorted to isolate Citrine-positive cells for use in gDNA validation assays and RNA-seq.
(b) Sashimi Plots of control and experimental targeted gene loci with CRISPR guide RNA target highlighted in red. *Sox10* KO showed efficient loss of exon while *Tfap2b*, *Sox3* and *Msx1* showed an increase in number of new splicing events particularly at the gRNA sites.

(c) Bar plots showing embryo survival across multiple KO experiments. N=4 independent experiments including 30 embryos each, except for Sox10, Tfap2B and Sox10/Tfap2B double KOs (DKO) for which n=3 experiments was used. Error bars indicate standard deviation. The percentage of survival from the total number of embryos electroporated were calculated and displayed.
(d) Unbiased clustering of RNA-seq gene expression in transcripts per million (TPM) across all KO and control conditions, shown across replicate samples. Expression of selected TFs, mesenchymal, signalling or extracellular matrix genes is depicted.
(e) HCR imaging on selected TFs performed on the same embryo after KO, where the Left represents the non-electroporated endogenous gene pattern and the Right represents the experimental, KO side (3 biological replicates per KO). Scale bar = 50 μm.

**Figure 8. Deconstruction of the VNC GRN**
(a) Summary graphic of migrating VNC showing mix heterogeneity of cells leaving the neural tube at HH10 and regionalising at HH18 (Left). VNC cell lineage decisions showing dichotomy of cells through *FoxD3* expression, offering a *Sox10*$^{high}$ and *Sox10*$^{low}$ population (Right). nt=neural tube, nc=notochord, dp=dorsal path, vp=ventral path, da=dorsal aorta
(b) *EdnrB* GRN deconstructed from motif binding analysis shown in Figure 5c along with other enhancers, biotinChIP-seq dataset and RNA-seq KO experiments in Extended Data Fig 7. *Ednrb* is indirectly controlled by Tfap2, while Sox10 and SoxB directly input into its enhancers along with MafB, Twist, Fox and Ets.
(c) Proposed VNC GRN highlighting the core TFs and other factors in grey feeding into three lineages; neural, mesenchymal and neuronal.
(d) Motif binding sites on DA elements focusing on a selected number of TFs showing motifs for Tfap2, Etv, Hes and Olig.
(e) UCSC genome browser screenshot depicting tracks of biotinChIP-seq and ATAC-seq experiments highlighting differentially binding and differentially accessible elements within *Hes6*, *Hes5*, *Etv1* and *Olig2* loci.
(f) Proposed Hes GRN circuit with indirect regulation by Sox10 mediated via Msx1, Etv and Olig, which feed directly into *Hes* enhancers. *Hes* receives direct regulatory inputs by SoxB and Tfap2B.
(g) Heatmap of differential gene expression following knockout of core TFs validating proposed Hes GRN. FoldChange values are calculated by DESeq2. In *Sox10* KO, *Hes* is upregulated, while its direct transcriptional repressors (Etv and Olig) are downregulated. Upon its upregulation, Hes feeds back to repress Etv and Olig, thereby this feedback loop is dysregulated when *Tfap2B* and *SoxB* are knocked out.

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## Methods

### Embryo culture and electroporations

Fertilised wild-type chicken eggs were obtained from Henry Stewart & Co (Norfolk) and incubated until the desired stage. Embryos were staged according to Hamburger and Hamilton (HH) (1951) references[47]. All experiments were performed on chicken embryos younger than 12 days of development, and as such were not regulated by the Animals (Scientific Procedures) Act 1986. *Ex ovo* electroporations were performed as previously described[48]. For *in ovo* experiments, HH8/9 embryos were windowed and DNA (2.5 μg/mL) was injected into the lumen of the neural tube prior to passing two sets of electrical current bilaterally. First set of three square 50 ms pulses (12.5 V) with 100 ms rest periods in between were applied, the polarity was inverted before the 2$^{nd}$ set of pulses was applied with the same settings. This approach ensured electroporation of DNA constructs into both sides of the neural tube. However, unilateral electroporations were carried out for the knockout experiments using pcU6_3 sgRNA (Addgene #92359) and pCAG Cas9-2A-Citrine (Addgene #92358) constructs[32] at 1.0 μg/mL. For all experiments, eggs were incubated at 37ºC until desired stages. The study is compliant with all relevant ethical regulations regarding animal research. The age of the embryos prevented any selection by sex.

### Flow cytometry

Dissected regions from electroporated embryos were dissociated with Dispase (1.5 ml/mL in DMEM/10 mM Hepes pH 7.5) at 37ºC for 15 mins with intermittent pipetting and a final 0.05% Trypsin incubation at 37ºC for 3 minutes. The suspension was then added to an excess of Hanks solution (1X HBSS, 0.25% BSA, 10 mM Hepes pH 7.5) buffer. Cells were spun down for 10 mins at 500g, resuspended in Hanks and passed through a 40 μM cell strainer. Pelleted cells were resuspended in 200-300 μL of Hanks prior to being subjected to FAC-sorting using BD FACS-Aria Fusion. We isolated approximately 300 Citrine$^{+}$ NC cells per embryo at HH10, 800-1000 NC at HH18 and 800-1000 NC per dissected gut at HH25.

**ATAC, library preparation and sequencing**

Assay for transposase accessible chromatin followed by sequencing (ATAC-seq) processing following the original protocol with some modifications[18, 49]. In short, FAC-sorted cells were first lysed using IGEPAL solution and transposed using Illumina Nextera Tn5 transposase (FC-121-1030) for 20-30 minutes at 37ºC. Tagmented DNA was amplified using NEB Next High-Fidelity 2X PCR Master Mix for 11 cycles and tagmentation efficiency was assessed using Agilent Tapestation. ATAC-seq libraries were sequenced using paired-end 40 bp reads on the Illumina NextSeq 500 platform to acquire approximately 20-30M fragments per sample. Three biological replicates were used for each stage (HH10, HH18, HH25) and sample (double positive, single E2-only, Citrine-negative control per stage) and sequences within the same batch to prevent batch effects correlated with the biological condition of interest.

**Bulk RNA extraction, RNA-seq library preparation and sequencing**

Total RNAs from FAC-sorted cells were isolated using the Ambion RNAqueous Micro Total RNA isolation kit (Cat #AM1931, ThermoFisher Scientific) and integrity checked using Agilent Tapestation (only samples with a RNA Integrity Number >6 were used for library preparations). Samples were stored at -80ºC until all replicates were collected and sample libraries were prepared on the same day to prevent batch effects. Libraries were prepared using Takara SMART-Seq™ v4 Ultra™ Low Input RNA kit (Cat #634889, Takara Bio Clontech) and sequenced using 40 bp paired-end reads on the Illumina NextSeq 500 platform using TG NextSeq® 500/550 High Output Kit v2 (75 cycles). Two biological replicates were used for each stage (HH10, HH18. HH25) and sample (double positive, single E2-only and Citrine-negative control per stage).

**Single cell 10X preparation and sequencing**

Single-cell RNAseq was performed on the 10X Chromium platform and libraries prepared at the MRC WIMM Institute Single Cell Core Facility using the Chromium Single Cell 3' Library and Gel Bead Kit v2, 4 rxns PN-120267. E2:Citrine was electroporated *in ovo* into the lumen of the neural tube and incubated until HH18 stage. The vagal region from somites 1-7 axial level was dissected and dissociated as described above, prior to FAC-sorting. Approximately 2000 FAC-sorted cells were pooled with a collaborator's experiment using zebrafish cells (carrying mCherry transgene). Final libraries were sequenced on the standalone mode on the Illumina NextSeq 500 platform using TG NextSeq® 500/550 High Output Kit v2 (150 cycles) and settings set to paired-end single index parameters as specified in the manufacturer's protocol.

**Enhancer cloning and preparations**

Putative enhancer elements were amplified from purified chick genomic DNA using primers containing specific sequence tails to the modified pTK:Citrine nanotag reporter vector adapted to type IIs restriction enzyme-based cloning as previously described[18]. Endotoxin-free plasmid preparations (E.Z.N.A. Endo Free Plasmid Mini Kit II, Cat #D6950-02, Omega Bio-Tek or Qiagen endo-free maxi prep kit, Cat#12362, Qiagen) were used for electroporations as described above[32].

**Cryosectioning and immunostaining**

Embryos selected for cryosectioning were fixed in 4% paraformaldehyde (PFA) for 1 hour at RT or overnight at 4ºC. Embryos at HH18 and HH25 were fixed at 4ºC overnight. Fixed embryos were washed in 3 changes of 1X PBS and cryoprotected in 15% sucrose/PBS overnight at 4ºC, brief incubation in 15% sucrose/100% OCT prior to mounting in OCT, stored at -80ºC and later sectioned at 10 μM thickness.

For immunofluorescence assays, slides with collected sections were washed in 1X PBS to remove the OCT. Sections were then blocked using 1% Normal Goat Serum/1% DMSO and 0.1% Triton-X in 1X PBS for at least 20 mins at RT. Slides were rinsed and incubated overnight at 4ºC with primary antibody (1:250 dilution of rabbit anti-GFP Cat #TP401, Torrey Pines Biolabs and 1:200 mouse anti-mCherry Cat#632543, Living Colors®, Takada). Sections were washed in 1X PBS the following day followed by incubation with secondary antibody (Alexa Fluor-488 donkey anti-rabbit IgG diluted at 1:500 in blocking solution and AlexFluor-594 donkey anti-mouse IgG diluted at 1:500 in blocking solution) for at least 2 hours at RT. Sections were then washed extensively in 1X PBS + 0.1%Tween (PBST) and mounted using VectaShield with DAPI (1:1000 Cat#H-1200, Vector Laboratories) and kept at 4ºC prior to confocal imaging.

For whole mount staining, dissected guts were fixed overnight at 4ºC in 4% PFA. Tissues were washed with 1X PBS/1% Triton-X/1%DMSO (PBSDT) and blocked using 1% Normal Goat Serum in PBSDT for at least 20 mins at RT. Embryos were then incubated overnight at 4ºC with primary antibody in PBSDT/Block (anti-GFP) washed extensively in PBSDT the following day prior to incubation with secondary antibody (1:500 Alexa Fluor-647 goat anti-mouse or 1:500 Alexa Fluor® 647 anti-Tubulin B3 (Tubb3/Tuj1) Mouse IgG2a Cat#801209, a kind gift from Tudor Fulga, Oxford, or 1:500 Alexa Fluor® 647 anti-GFAP Mouse IgG2b Clone 2E1.E9 Cat#644706). Embryos were then washed with PBSDT and kept at 4ºC prior to confocal imaging.

### *In situ* Hybridisation Chain Reaction (HCR) assays

HCR kit (v3) containing DNA probe sets, amplifier and hybridisation buffers were purchased from Molecular Instruments for each target mRNA[23]. The protocol provided by the manufacturer was followed with optimisations. Embryos were fixed in 4% PFA for 1 hr at RT or overnight at 4ºC, then bleached in 3% $H_2O_2$/8% KOH until the embryo was cleared of any pigments. Bleached embryos were further fixed in 4% PFA for 20 mins prior to dehydrating in 100% MeOH and stored at -20ºC. Following a rehydration step with graduated series of MeOH/PBST, embryos are digested with 10 μg/mL of Proteinase K for 10 mins (HH10) and 30 mins (HH18) and post-fixed in 4% PFA for 20 mins. Embryos were equilibrated with 5X SSCT and incubated with the probes in hybridisation buffer at 37ºC overnight. Probes were removed and after a series of washes incubated in hairpin solution with amplifying buffer overnight at RT prior to whole mount imaging. For *in situ* hybridisation with the eGFP HCR targeted against the reporter fluorophore, an additional DNAse digestion step was carried out after the rehydration step using Ambion DNAseI 50 U/mL in 10X DNAseI buffer for 1 hour at 37ºC to remove the remaining episomal plasmid from the embryo. For immunofluorescence after HCR, dissected gut tissues were fixed in 4% PFA for 20 mins followed by several washes of PBST before blocking with PBSDT for 1 hour prior to following the protocol above.

### Generation of E2 driven Avi-tagged Sox10 and Tfap2B constructs

Two different modified pTK plasmids were used for the final construct for N-term or C-term cloning. The C-term plasmid has additional cloning sites for NcoI and SnaBI after the chimeric intron followed by linkers, a FLAG tag, a TeV recognition peptide and the AviTag before the polyA region. The N-term plasmid has the AviTag after the chimeric intron followed by a TeV recognition peptide, FLAG tag, an EcoRV restriction enzyme site, followed by a 2A:Citrine. The E2 enhancer was initially cloned into both N-term and C-term plasmids using the aforementioned BsmBI cloning protocol. Subsequently, full length Sox10 cDNA was cloned using InFusion® method into the NcoI-SnaBI linearised C-term plasmid while full length Tfap2B cDNA cloned into the linearised EcoRV N-term plasmid (both to avoid the Avi-tag near the predicted DNA binding sites).

### Biotin ChIP-seq

Biotin ChIP-seq protocol was slightly modified from previously published version[26]. Avi-tagged constructs (1.0 μg/uL) were co-electroporated with a pCI NLS-BirA-2A-mCherry plasmid (0.5 μg/uL) into the neural tube of HH8 embryos bilaterally. 15 HH18 embryos were harvested and their vagal regions (somites 1-7) dissected, offering an approximately

100,000 cells of interest. Tissues were dissociated in nuclei extraction buffer (NEB: 0.5% NP40, 0.25% Triton-X, 10 mM Tris-HCl (pH 7.5), 3mM $CaCl_2$, 0.25M sucrose, 1mM DTT, 0.2 mM PMSF, 1X Proteinase inhibitor) by gentle pipetting. Cells were cross-linked using 1% formaldehyde at RT for 15 mins and quenched with 125 mM of 1M glycine for 5 mins at RT. Cross-linker was washed out 3 times with 1X PBS/PI (1X PBS, 1X PI, 1mM DTT and 0.2 mM PMSF) centrifuging at 2000xg for 4 mins at 4ºC. Pellets were snap-frozen and stored at -80ºC until further replicates were collected. Pellets were thawed and re-suspended in NEB and washed 1X with PBS/PI prior to nuclei lysis in SDS lysis buffer (0.7% SDS, 10mM EDTA, 50 mM Tris-HCl (pH 7.5), 1X PI). Cross-linked chromatin was sonicated at 12A, 10X (10s ON, 30s OFF) followed by 8A, 4x (30s ON, 30s OFF) and ran on a 1.5% agarose gel to ensure appropriate sheared DNA fragments. Sheared chromatin samples were pre-cleared in pre-blocked Streptavidin beads (Dynabeads M-280 streptavidin beads, Invitrogen) o/n at 4ºC on a rotator. 1/20 of biotinChIP was collected as an input fraction and stored at -80ºC. Beads were washed with SDS Wash Buffer (2% SDS, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) at RT, followed by 4x RIPA washes (50 mM Hepes-KOH (pH 8.0), 500 mM LiCl, 1mM EDTA, 1% NP40, 0.7% Na-Deoxycholate, 1x PI) and 1x Na-Cl TE wash (1x TE, 50mM NaCl) at 4°C. Samples eluted from beads with SDS ChIP Elution buffer (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% SDS) and cross-linked reversed o/n at 65ºC in the thermomixer at 1000 rpm. Chromatin samples were then separated from streptavidin beads. Cellular RNA was digested with RNaseA (0.2 μg/mL) at 37ºC for 1 hour and cellular proteins removed with Proteinase K (0.4 mg/mL) at 55ºC for 2 hours. Samples and input DNA were then extracted by phenol-chloroform. Libraries were prepared using MicroPlex Library Preparation v2 kit (Diagnode) with the number of cycles determined from the amount of starting material. For Sox10 ChIP, 10 cycles were used while for Tfap2B, 14 cycles were used and final libraries quantified and sequenced using NextSeq® 500/550 High Output Kit v2 (75 cycles) on the NextSeq 500 sequencing platform.

**CRISPR-Cas9 editing *in ovo***

The cloning of target guideRNAs into the pcU6_3 sgRNA mini vector (Addgene #92359) and the use of pCAG Cas9-2A-Citrine construct (Addgene #92358) were previously described[32]. To rule out potential off-target effects from non-specific guide targeting, we designed three sgRNAs for the *Sox3* locus using CHOPCHOPv2 platform[50], maximising efficiency and specificity scores with zero predicted off-target effects. The guide RNAs were tested by electroporating the cloned pcU6_3 sgRNA plasmid containing individual guides with Cas9 plasmid *ex ovo* into the epiblast of the HH4 stage embryos and incubating them until HH10 as previously described[32, 48]. Cas9-only assays were used as a control. Following the incubation, individual embryos were dissected and genomic DNA extracted using PureLink™

Genome DNA Mini Kit (Cat#K182001) according to manufacturer's protocol. High Resolution Melt Analysis (HRMA) was used as a selection criterion for efficient/functional sgRNAs (Extended Data Fig. 6c). Primers were designed to generate a 164 bp amplicon spanning the sgRNA cut site. HRMA PCR was performed using Hotshot Diamond PCR Mastermix (Client Lifescience, HS002-TS) together with LC Green Plus dye (BioFire Diagnostics, BCHM-ASY-0005) and reactions performed on a C1000 Touch Bio-Rad thermal cycler. The Bio-Rad Precision Melt Analysis™ software was used to visualise and analyse the data. A shift in temperature-normalise melt curve was used as evidence of heteroduplexes in the edited amplicon compared with the controls. The previously published *Msx1* guide generated in our lab was used[32]. For *Sox10* and *Tfap2B*, two sgRNA were designed per gene locus flanking exon 1 and 2 and exon 3 and 4, respectively.

For functional validation experiments, the constructs were electroporated unilaterally *in ovo* into neural tube of HH8/9 chick embryos at a concentration of 1 μg/μL for both the sgRNA and Cas9-2A-Citrine plasmids. The embryo side that did not receive gene editing components acted as an internal control. After the electroporation, the embryos were incubated for ~36 hours, allowing them to develop until HH18, when embryonic regions at the axial level adjacent to the somites 1-7 were dissected, the neural tube carefully removed and embryonic material dissociated into a single-cell suspension prior to FAC-sorting of Citrine positive (mutant of control cells) for RNA extraction. RNA-seq libraries were prepared as described above. IGV visualisation of mapped transcripts around the excision site provides further validation of the targeted genome engineering events.

**Confocal microscopy**

For live imaging, embryos were dissected from the vitelline membrane, mounted in PBS and imaged using Zeiss 780 Upright confocal microscope with a 10X, 25X (oil) or 63X (oil) objective. Z-stacks and tiling were used to capture the area of interest. For HCR imaging, since the left side of the embryo served as an uninjected control, we imaged both sides of the embryo using the exact same settings to allow for an accurate comparison.

**Statistical analysis and Bioinformatics data processing**

**ATAC-seq pre-processing**

Sequencing files from each sequencing lane were de-multiplexed and resulting files merged. Nextera adaptors sequences were trimmed using TrimGalore (v0.4.1) (settings: --nextera --paired --three_prime_clip_R1 1 --three_prime_clip_R2) prior to being mapped to the chicken genome galGal5 assembly using bowtie (v1.0.0) (settings: -S –X 2000)[51]. Only aligned pairs

were retained based on BAM flags and mitochondrial reads were removed from the BAM file using Samtools (v1.3)[52] and unix awk commands. PCR duplicates were then removed using PicardTools (v1.83) and only uniquely mapped reads were retained. Insert sizes were obtained from respective BAM files using samtools view BAM function and unix commands. All samples displayed the expected periodicity of DNA winding around nucleosomes in genome DNA regions. To assess whether each peak read was unique across samples, the complexity curves were generated using Preseq package (v1.0.2)[53], and the corresponding plots were generated using GraphPrism. All samples displayed high complexity and large proportion of unique reads. Peaks were then extended by 75 bp in each direction using Bedtools prior to being called using MACS (v2.0.10)[54] with the parameter of "–extsize 73 –nomodel" parameters for paired-end reads. For reproducibility analyses, BAM files were first sorted by name instead of the location before being down-sampled to the lowest sequencing read depth using Samtools to remove random read pairs without replacement. A custom Perl script was used to generate smoothened genome browser tracks in BigWig format for data visualisation on the UCSC Genome Browser.

**ATAC-seq peak annotation and statistical analysis of differential accessibility**

R version 3.5.1 (2018-07-02) was used for all subsequent analyses. The DiffBind package (v1.10.2)[55] was used to locate high confidence peaks present in all three replicates. This stringent threshold increases confidence that these peaks are reproducible as each peak must be called in all biological replicates. The consensus peak sets obtained using DiffBind package was subsequently used as reference for downstream analyses. Differential binding accessibility was carried out with the DiffBind package using a negative binomial distribution model implemented in DESeq2 with default parameters and contrast performed includes pairwise comparison with their stage respective negative controls as well as between sample types (i.e. double positive versus E2-only). A highly stringent threshold (FDR <0.01, Fold enrichment >1) was used to define a set of high-confidence DA peaks. Peaks were then annotated using the ChIPseeker R package[56] complemented by the BSgenome.Ggallus.UCSC.galGal5 to identify the number of peaks versus the length of each chromosome, TxDb.Ggallus.UCSC.galGal5.refGene to identify transcription start site peaks and finally the annotation database (org.Gg.eg.db) to allocate Ensembl ID to gene symbols. We first identified a peak as belonging to the promoter of a given gene if it fell within 2kb upstream and 1kb downstream of the TSS. Then a 1Mb window upstream and downstream was used to assign peaks to the nearest TSS.

***K-Means* clustering of ATAC peak datasets**

To compare open chromatin signal across all samples, we performed *k*-means clustering using the seqMINER package (v1.3.4)[57] on downsampled ATAC-seq datasets as input. DA peaks called using DiffBind package, and found enriched in signal when compared to their respective negative samples were used as reference genome coordinates. Signal levels were computed genome-wide over DA peaks (±1.5 kb from the centre), using 15-nucleotide binning step and the *k-means* enrichment linear clustering normalization algorithm, with a target number of clusters set to *k*=10. Peak mean densities were exported and plotted using GraphPrism. Heatmaps were plotted using Deeptools (v2.4.1) package[58] package. Clustered peaks were annotated using ChIPseeker as described above.

**RNA-seq analysis**

RNA-sequencing outputs were mapped to the chick genome (galGal5) using RNA-STAR (v2.4.2a)[59]. Duplicates were marked and removed using PicardTools (v1.83) prior to retrieving corresponding count using Subread featureCounts[60] (settings: -p –B –t exon –g gene_id). Normalised TPM values were used as a measure of gene expression. A heatmap of RNA expression was produced using the correlation of normalised gene-level TPM values across samples with the ComplexHeatmap R package[61]. Differential expression analysis was carried out using DESeq2 (v1.14.1)[62].

**Accessibility and gene expression correlation**

Using a custom python script (http://github.com/tsslab/ENS/custom_correlation.py), each differentially accessible annotated peak was matched to the associated differentially expressed gene to obtain the $\log_2$fold change correlation between chromatin accessibility and gene expression. This final matrix was used for plotting volcano plots and creating a subset of peaks that was not only differentially accessible (FDR <0.05) but also differentially expressed (padj < 0.05). These peaks were also retained in the analysis to determine the total number of elements per peak to generate Fig. 4a.

**Motif and combinatorial analysis**

*De novo* motif search was performed using Homer package[27] with findMotifsGenome.pl script using the parameter of "-size given" and motif length between 6 to 18 bp. A peak set containing all merged peaks across all samples including negative controls were used as the background. *De novo* motifs were annotated using HOMER and TOMTOM[63]. Top motifs were then used for further combinatorial analysis using annotatePeak.pl script with the "-motif" function to search each peak for a given motif within a window of +/- 200 bp from peak centre as previously described[18] to output a file containing motif presence within the peak. Combinations enriched at $\alpha$=5% (two-tailed Chi-squared test) with Bonferroni correction for

multiple hypothesis (m) testing were retained for P-values < $\alpha$/m. $Log_{10}$p-adjusted values were then plotted using ComplexHeatmaps[61]. MEME suite[30] was used to predict binding sites within a particular enhancer element +/- 200bp from peak centre. Motifs were then annotated using TOMTOM with the HOCOMOCO v11 (full)[64] Human and Mouse database. The gene regulatory network model was drawn using BioTapestry software[65].

**Motif occurrences and peak differential accessibility correlation**

Using a custom python script (http://github.com/tsslab/ENS/custom_correlation.py), peaks from DiffBind outputs were then matched with the motif-annotated peaks from Homer output to generate Fig. 5f. Peaks were ranked and filtered using FDR <0.05 and log2FoldChange > or < 1) and then converted to a matrix of motif presence (1) or absence (0). Assigned peaks were then plotted in GraphPrism for visualisation.

**Biotin ChIP-seq analysis**

Sequencing reads were processed similarly to ATAC-seq data by trimming raw reads, mapping to galGal5 genome, duplicates removed prior to peak calling with MACS2. Replicate BAM files were merged using Samtools (v1.3)[52] and downsampled to the input prior to using Deeptools (v2.4.1) package[58] for heatmap and plot profile generation. Differential binding analysis was carrired out with DiffBind R package (v1.10.2)[55].

**10X Chromium single cell RNA-seq analysis**

Single-cell RNAseq reads were demultiplexed using Cellranger (v2.2.0)[66] and mapped to the galGal5 transcriptome generated using the Cellranger “mkref” function. Citrine and mCherry transcripts were added to the galGal5 FASTA file and GTF to allow correct assignment of cells to the appropriate organism. We achieved 95% mapping confidence and Q30 Bases in UMI was 96.20%. There were 1309 median genes per cell, 4064 median UMI counts per cell, 12,601 total genes detected and 233,801 Mean reads per cell. Downstream analysis was carried out using Seurat package (v3.0.0)[67] in R. Raw UMI count matrices were filtered to remove barcodes with fewer than 200 genes, more than 3000 genes expressed and high percentage of UMIs from mitochondrial features (greater than 10%) that gave a total of 570 cells used for clustering (Extended Data Fig. 3f). Expression values for total UMI counts per cell were then normalized and Jack-Straw permutation tests carried out determine significant principal components in the data before performing linear dimensional reduction (resolution = 0.5). Cell clusters were visualised using t-SNE and UMAP plots.

Cell-differentiation trajectories were reconstructed using R package Monocle (v2.10.1)[68] from Seurat objects previously normalised and clustered. Dimensionality reduction was

performed with the DDRTree algorithm using highly varied genes as inputs. Cell trajectories were then reconstructed using orderCells function.

**Deconvolution of bulk RNA-seq datasets**

Using our scRNA-seq data, we selected top marker genes for the three main clusters as listed in Extended Data Fig 3e using a p-adjusted cut-off of <0.05 and $\log_2$ FoldChange of >1 as identified using Seurat package. We calculated the reference matrix using BSEQ-sc[69] package that measured the average expression levels of these marker genes in each cell type. This reference matrix was then used to deconvolve the normalized bulk-RNA seq data counts and estimate cell type proportions using CIBERSORT[70]

**Statistics and Reproducibility**

Bioinformatics statistics were described in individual sections above using the package default statistical tests parameters with a 95% confidence levels for all experiments unless stated otherwise. At least three independent replicates were performed for ATAC-seq and RNA-seq knockout experiments and at least two independent replicates for RNA-seq and Biotin ChIP-seq experiments. Approximately 60-90 HH10 live embryos per replicate were used per experiment for earlier stages and 30-50 live HH18 embryos per replicate and 10-15 live HH25 dissected guts to obtain at least 2500 cells for analysis. No statistical method was used to predetermine sample size. For knockout experiments, no randomisation was carried out and the investigators were not blinded to the outcome. Individual embryos were randomly selected for HCR analysis and confocal microscopy.

**Code availability**

The custom python script used for gene expression correlation is available at https://github.com/tsslab/ENS/. All other code used in the study can be obtained from the corresponding author on reasonable request.

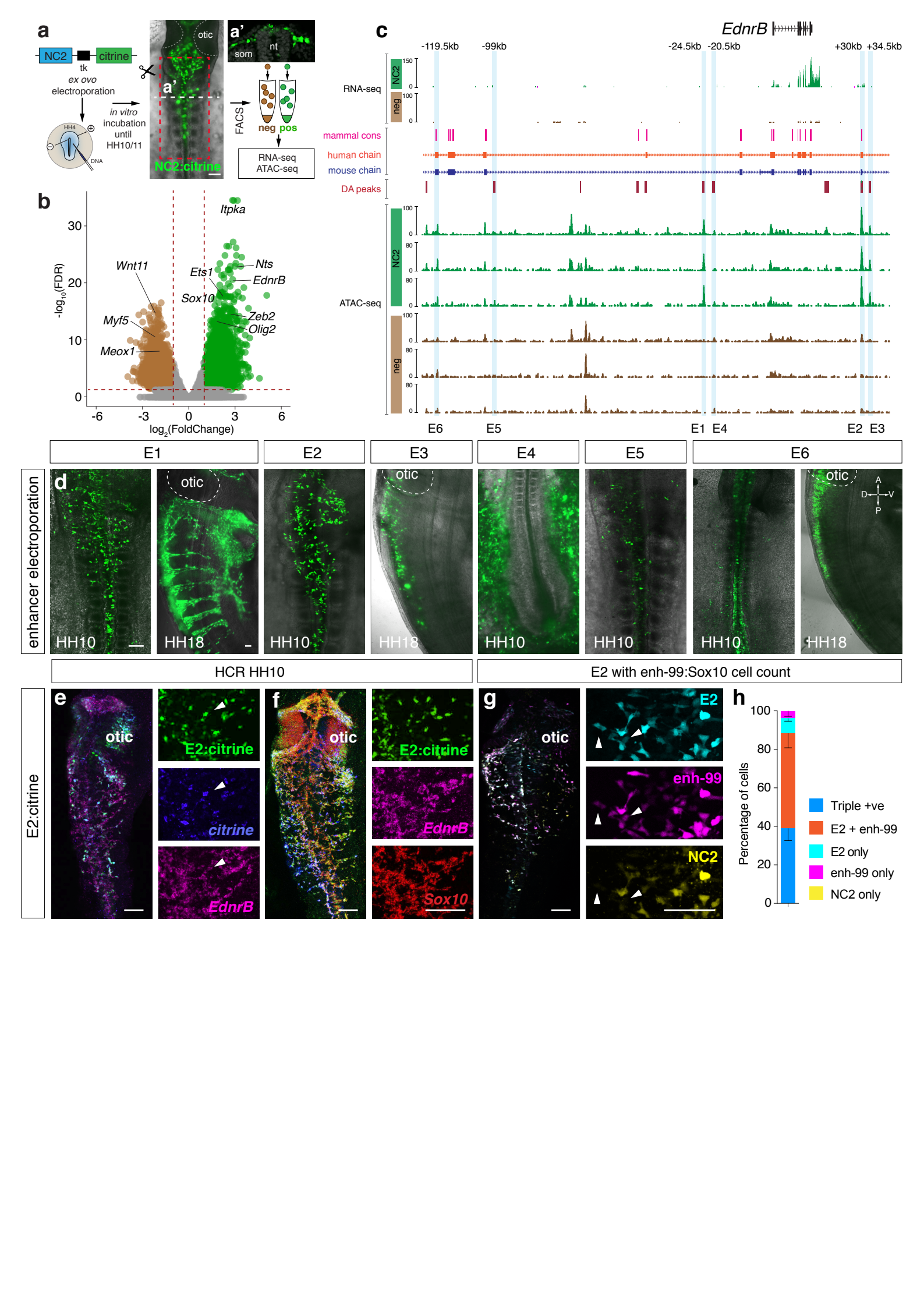
**Data availability**

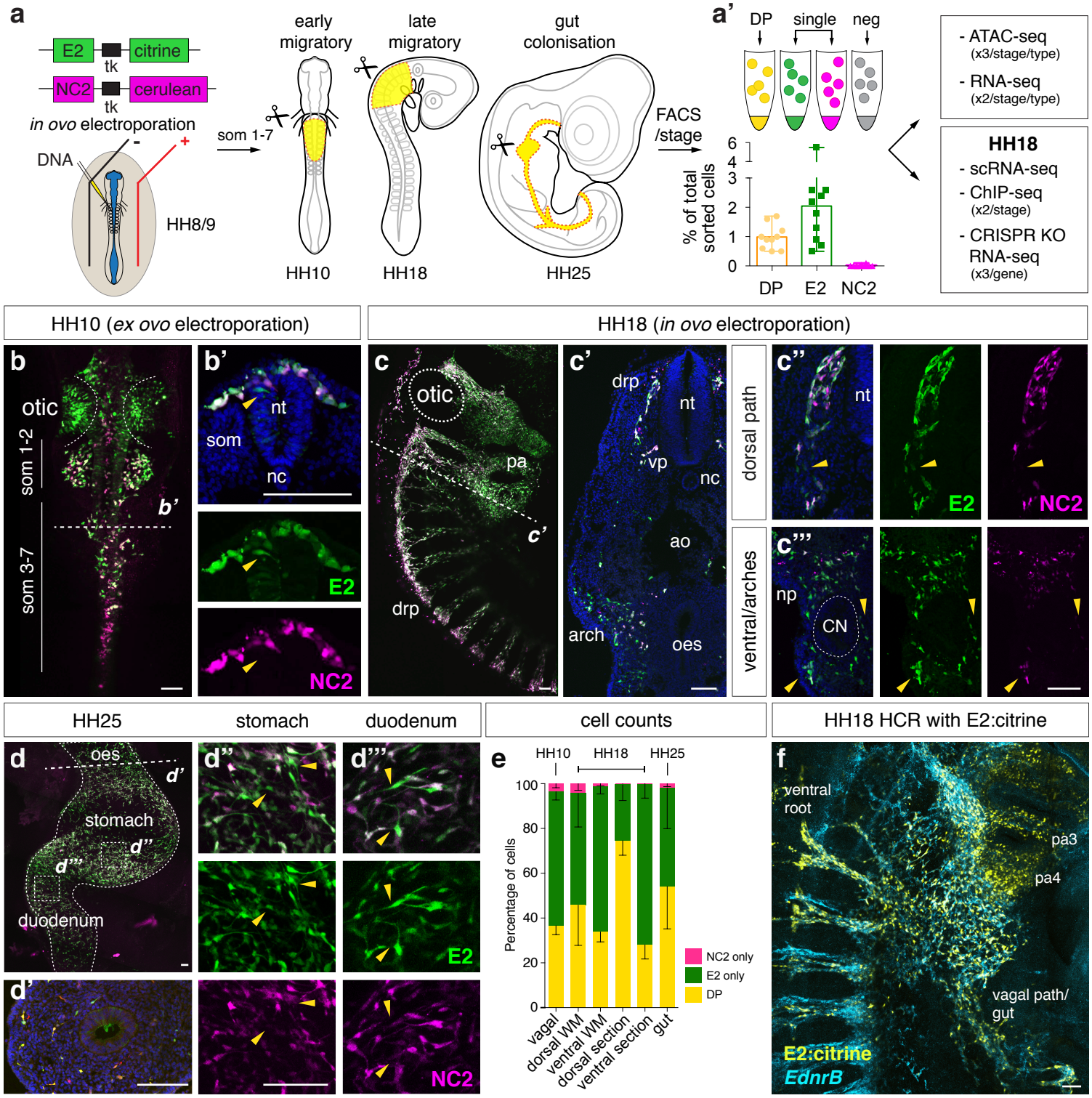
ChIP-seq, ATAC-seq, scRNA-seq and bulk RNA–seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE125711. Previously published sequencing data that were re-analysed here are available under accession codes SRP135960 and GSE129114. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

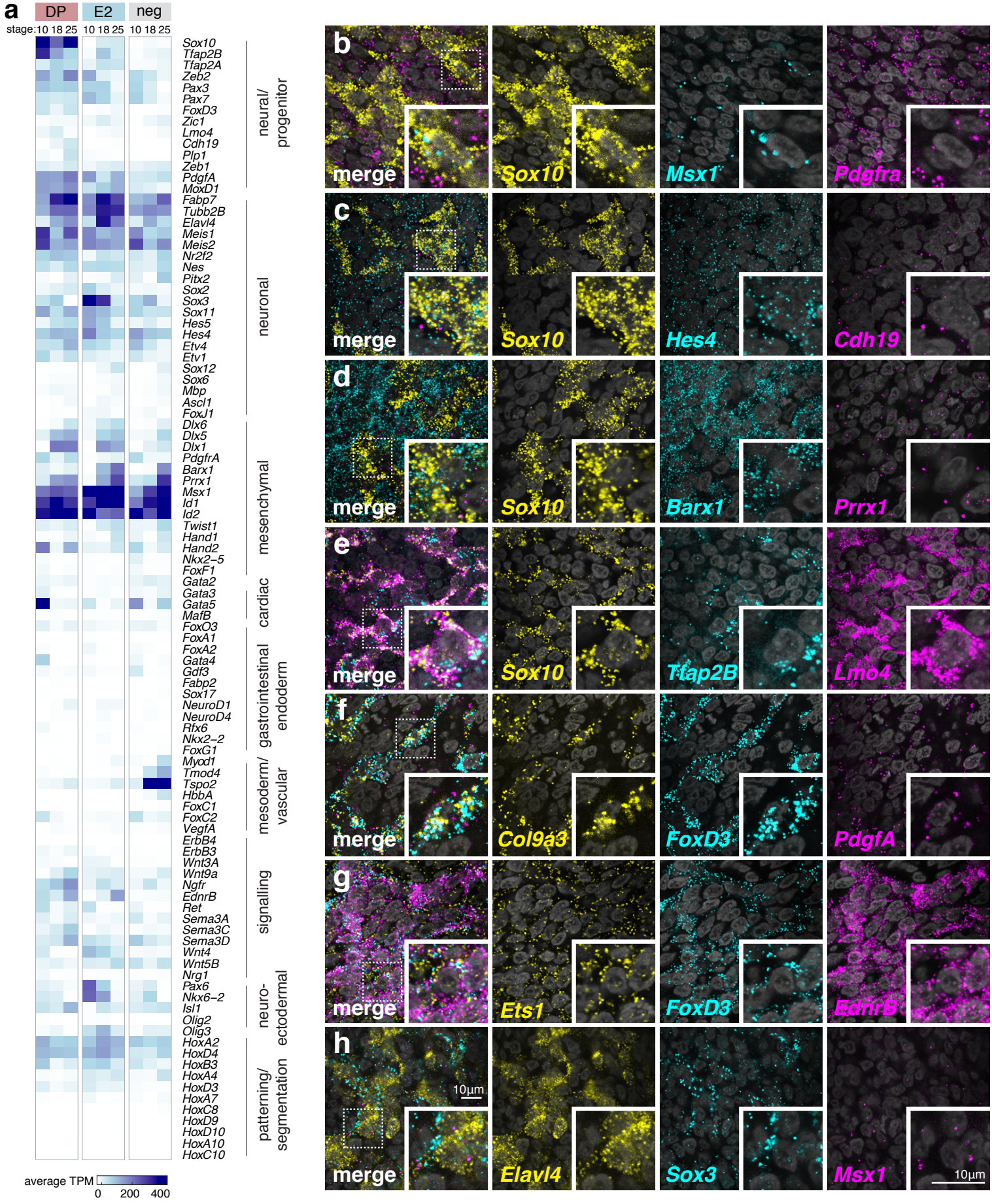
## Method references

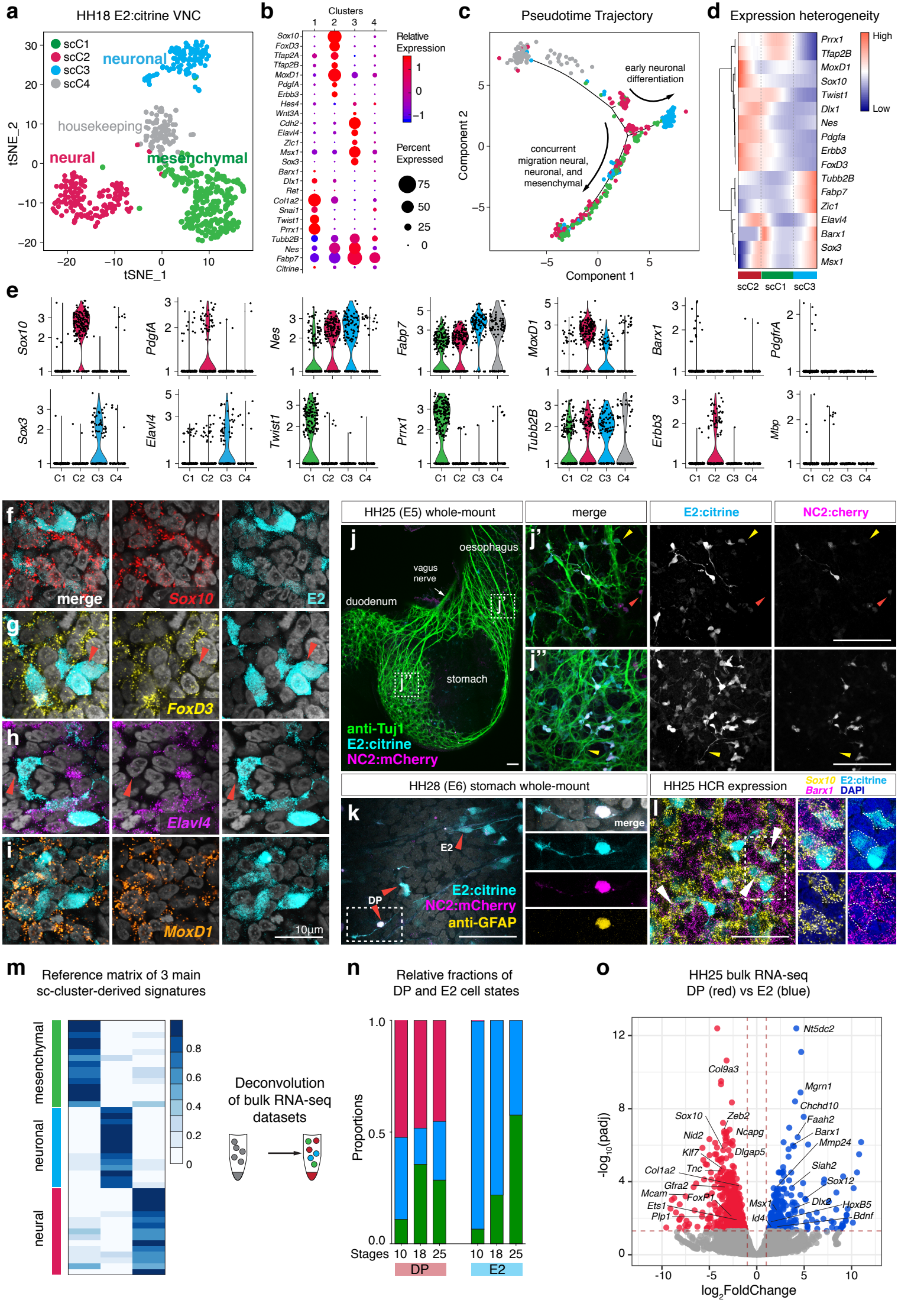

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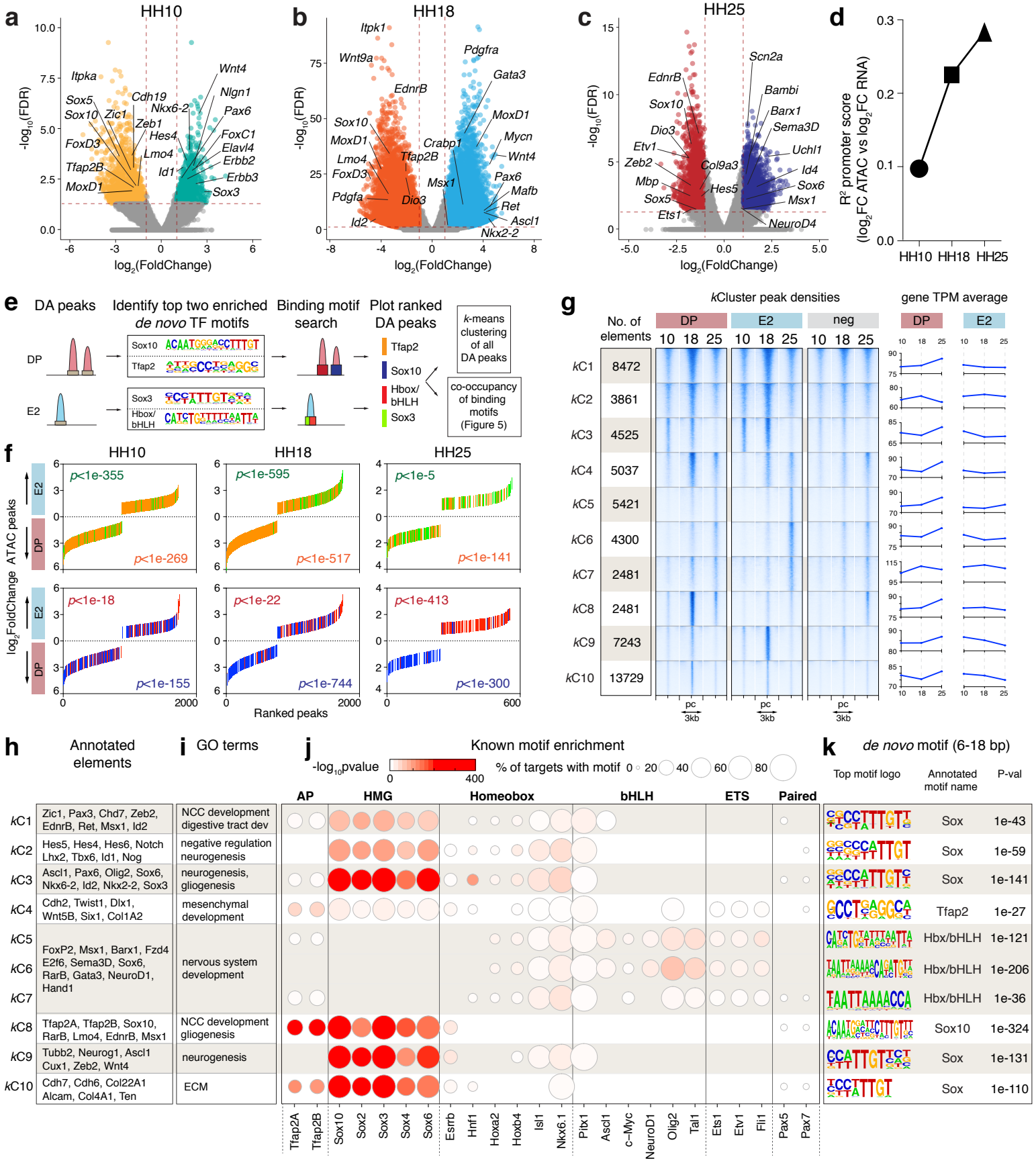
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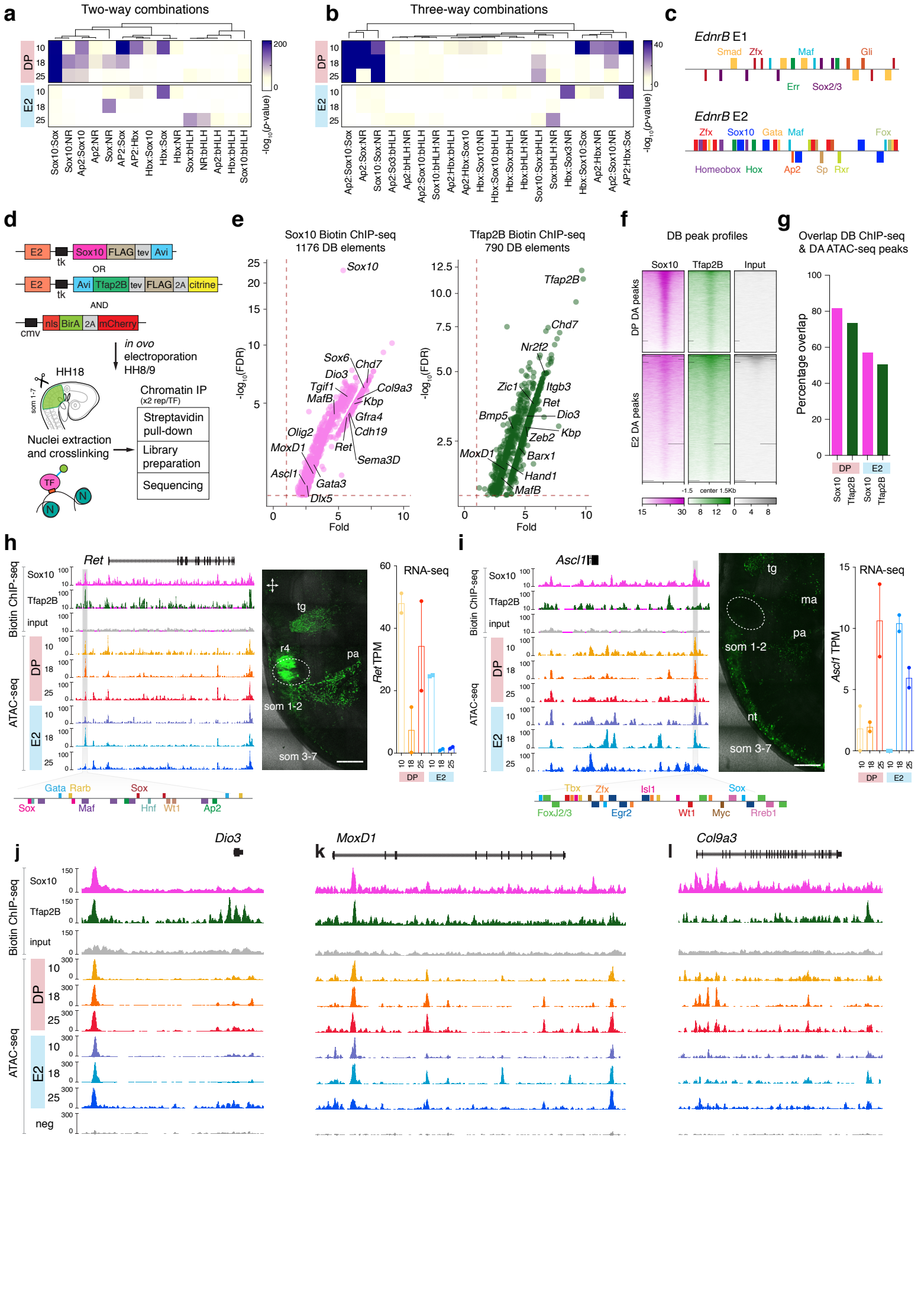

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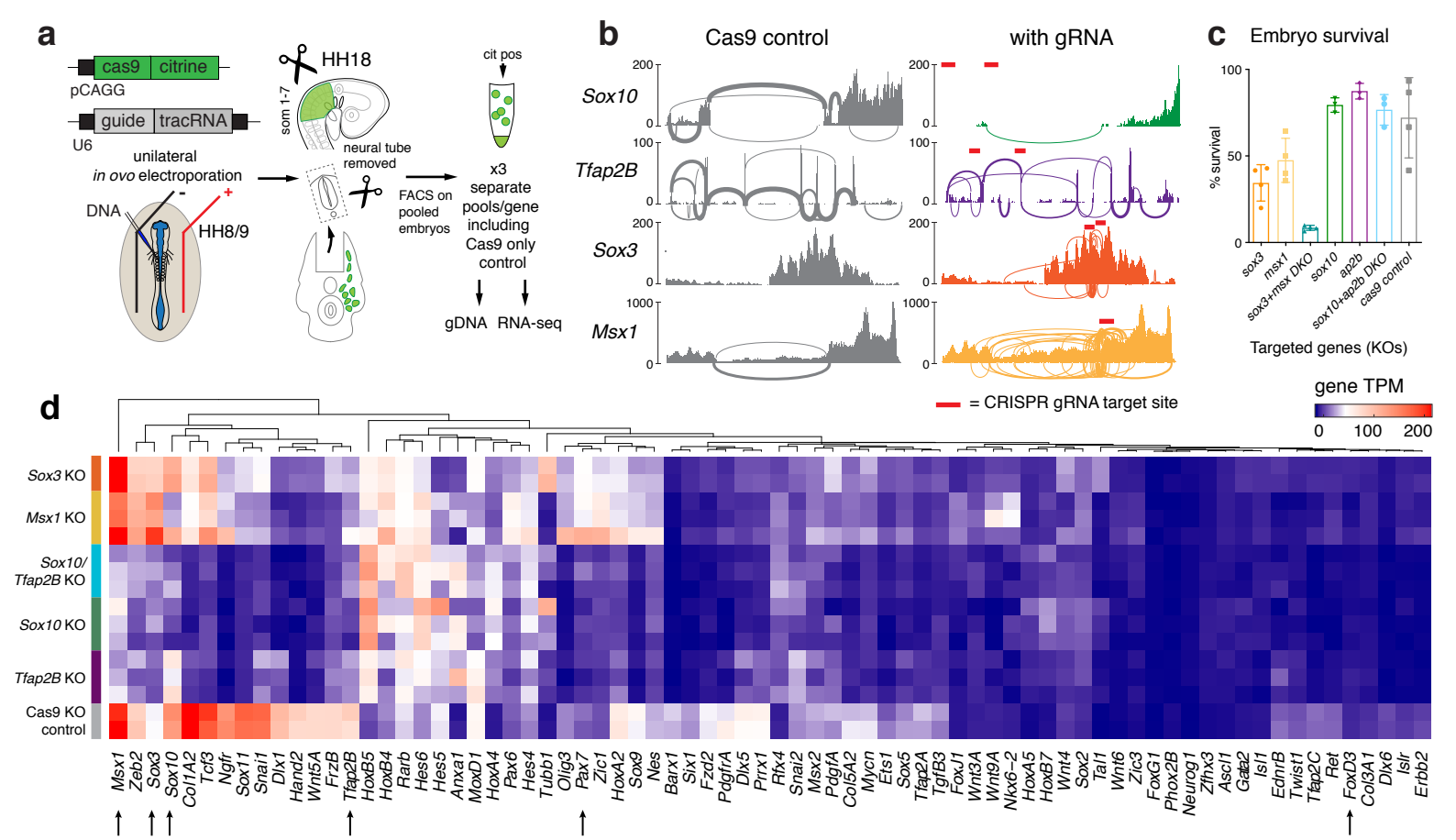

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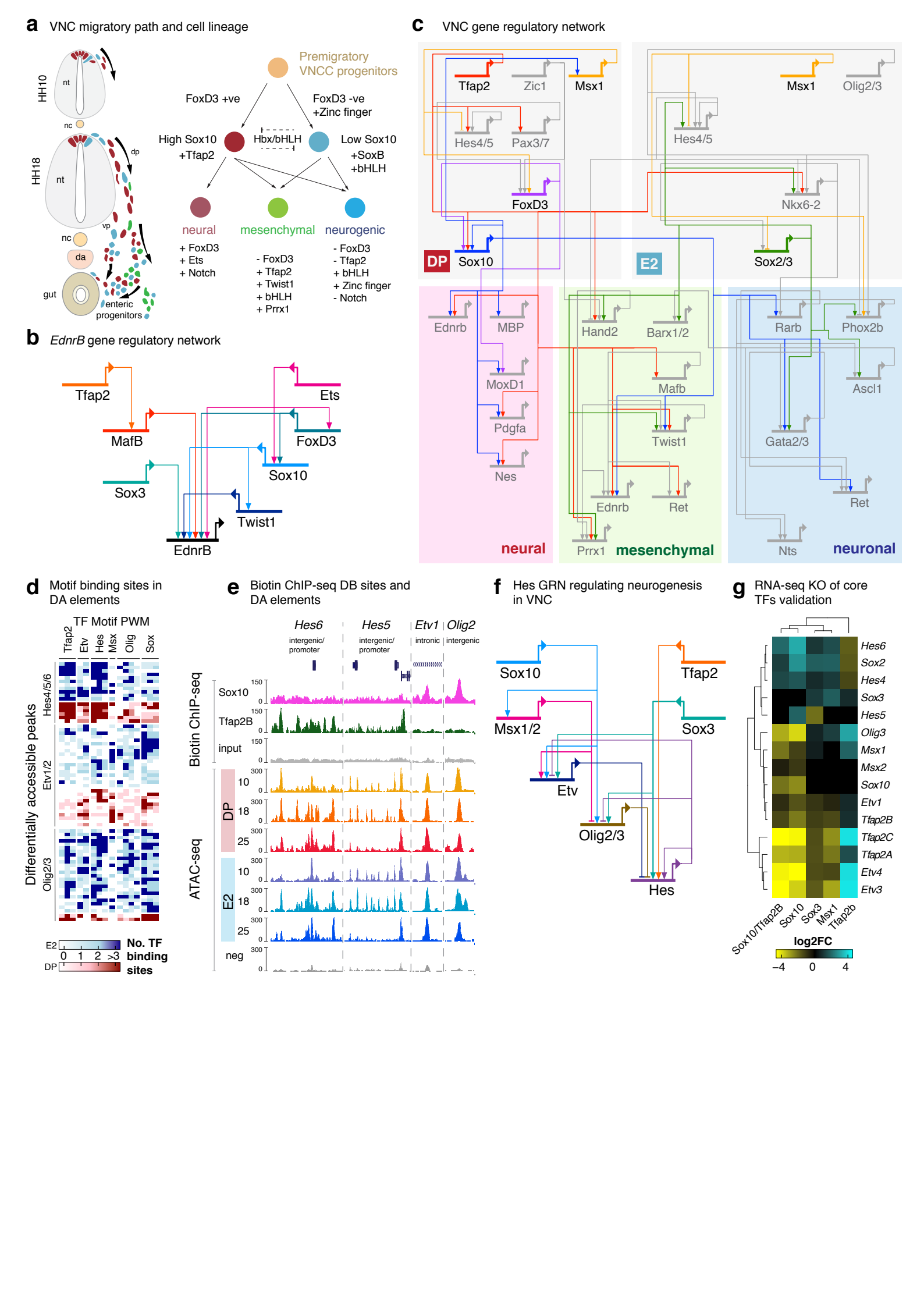

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Meis2
Nr2f2
Nes
Pitx2
Sox2
Sox3
Sox11
Hes5
Hes4
Etv4
Etv1
Sox12
Sox6
Mbp
Ascl1
FoxJ1
Dlx6
Dlx5
Dlx1
PdgfrA
Barx1
Prrx1
Msx1
Id1
Id2
Twist1
Hand1
Hand2
Nkx2–5
FoxF1
Gata2
Gata3
Gata5
MafB
FoxO3
FoxA1
FoxA2
Gata4
Gdf3
Fabp2
Sox17
NeuroD1
NeuroD4
Rfx6
Nkx2–2
FoxG1
Myod1
Tmod4
Tspo2
HbbA
FoxC1
FoxC2
VegfA
ErbB4
ErbB3
Wnt3A
Wnt9a
Ngfr
EdnrB
Ret
Sema3A
Sema3C
Sema3D
Wnt4
Wnt5B
Nrg1
Pax6
Nkx6–2
Isl1
Olig2
Olig3
HoxA2
HoxD4
HoxB3
HoxA4
HoxD3
HoxA7
HoxC8
HoxD9
HoxD10
HoxA10
HoxC10
neural/ progenitor
neuronal
mesenchymal
cardiac
gastrointestinal endoderm
mesoderm/ vascular
signalling
neuro- ectodermal
patterning/ segmentation
average TPM
0 200 400
b
merge
Sox10
Msx1
Pdgfra
c
merge
Sox10
Hes4
Cdh19
d
merge
Sox10
Barx1
Prrx1
e
merge
Sox10
Tfap2B
Lmo4
f
merge
Col9a3
FoxD3
PdgfA
g
merge
Ets1
FoxD3
EdnrB
h
merge
Elavl4
Sox3
Msx1
10µm
10µm


a
HH18 E2:citrine VNC
scC1
scC2
scC3
scC4
neuronal
housekeeping
neural
mesenchymal
tSNE_2
tSNE_1
b
Clusters
1 2 3 4
Sox10
FoxD3
Tfap2A
Tfap2B
MoxD1
PdgfA
Erbb3
Hes4
Wnt3A
Cdh2
Elavl4
Zic1
Msx1
Sox3
Barx1
Dlx1
Ret
Col1a2
Snai1
Twist1
Prrx1
Tubb2B
Nes
Fabp7
Citrine
Relative Expression
Percent Expressed
c
Pseudotime Trajectory
early neuronal differentiation
concurrent migration neural, neuronal, and mesenchymal
Component 1
Component 2
d
Expression heterogeneity
High
Low
Prrx1
Tfap2B
MoxD1
Sox10
Twist1
Dlx1
Nes
Pdgfa
Erbb3
FoxD3
Tubb2B
Fabp7
Zic1
Elavl4
Barx1
Sox3
Msx1
scC2
scC1
scC3
e
Sox10
PdgfA
Nes
Fabp7
MoxD1
Barx1
PdgfrA
Sox3
Elavl4
Twist1
Prrx1
Tubb2B
Erbb3
Mbp
C1 C2 C3 C4
f
merge
Sox10
E2
g
FoxD3
h
Elavl4
i
MoxD1
10µm
HH25 (E5) whole-mount
merge
E2:citrine
NC2:cherry
j
oesophagus
vagus nerve
duodenum
stomach
anti-Tuj1
E2:citrine
NC2:mCherry
j'
j''
HH28 (E6) stomach whole-mount
HH25 HCR expression
Sox10 E2:citrine
Barx1 DAPI
k
E2
DP
E2:citrine
NC2:mCherry
anti-GFAP
l
m
Reference matrix of 3 main sc-cluster-derived signatures
mesenchymal
neuronal
neural
Deconvolution of bulk RNA-seq datasets
n
Relative fractions of DP and E2 cell states
Proportions
Stages 10 18 25 10 18 25
DP
E2
o
HH25 bulk RNA-seq DP (red) vs E2 (blue)
-log10(padj)
log2FoldChange
Nt5dc2
Col9a3
Mgrn1
Chchd10
Sox10
Zeb2
Faah2
Nid2
Ncapg
Barx1
Klf7
Dlgap5
Mmp24
Col1a2
Tnc
Siah2
Gfra2
Sox12
Mcam
FoxP1
Msx1
Dlx2
HoxB5
Ets1
Id4
Plp1
Bdnf


a
HH10
-log10(FDR)
log2(FoldChange)
Itpka
Sox5
Sox10
Zic1
Cdh19
Nkx6-2
Zeb1
Hes4
Lmo4
Id1
FoxD3
Tfap2B
MoxD1
Wnt4
Nlgn1
Pax6
FoxC1
Elavl4
Erbb2
Erbb3
Sox3
b
HH18
Itpk1
Wnt9a
EdnrB
Sox10
MoxD1
Lmo4
FoxD3
Pdgfa
Id2
Tfap2B
Crabp1
Dio3
Msx1
Pdgfra
Gata3
MoxD1
Mycn
Wnt4
Pax6
Mafb
Ret
Ascl1
Nkx2-2
c
HH25
EdnrB
Sox10
Dio3
Etv1
Zeb2
Mbp
Sox5
Ets1
Scn2a
Bambi
Barx1
Sema3D
Uchl1
Id4
Sox6
Msx1
NeuroD4
Col9a3
Hes5
d
R2 promoter score
(log2FC ATAC vs log2FC RNA)
HH10 HH18 HH25
e
DA peaks
Identify top two enriched de novo TF motifs
Binding motif search
Plot ranked DA peaks
DP
E2
Sox10
Tfap2
Sox3
Hbox/ bHLH
Tfap2
Sox10
Hbox/ bHLH
Sox3
k-means clustering of all DA peaks
co-occupancy of binding motifs (Figure 5)
f
HH10
HH18
HH25
ATAC peaks
log2FoldChange
p<1e-355
p<1e-269
p<1e-595
p<1e-517
p<1e-5
p<1e-141
p<1e-18
p<1e-155
p<1e-22
p<1e-744
p<1e-413
p<1e-300
Ranked peaks
g
kCluster peak densities
gene TPM average
No. of elements
DP
E2
neg
kC1 8472
kC2 3861
kC3 4525
kC4 5037
kC5 5421
kC6 4300
kC7 2481
kC8 2481
kC9 7243
kC10 13729
pc
3kb
h
Annotated elements
kC1 Zic1, Pax3, Chd7, Zeb2, EdnrB, Ret, Msx1, Id2
kC2 Hes5, Hes4, Hes6, Notch Lhx2, Tbx6, Id1, Nog
kC3 Ascl1, Pax6, Olig2, Sox6, Nkx6-2, Id2, Nkx2-2, Sox3
kC4 Cdh2, Twist1, Dlx1, Wnt5B, Six1, Col1A2
kC5 FoxP2, Msx1, Barx1, Fzd4 E2f6, Sema3D, Sox6, RarB, Gata3, NeuroD1, Hand1
kC6
kC7
kC8 Tfap2A, Tfap2B, Sox10, RarB, Lmo4, EdnrB, Msx1
kC9 Tubb2, Neurog1, Ascl1 Cux1, Zeb2, Wnt4
kC10 Cdh7, Cdh6, Col22A1 Alcam, Col4A1, Ten
i
GO terms
NCC development digestive tract dev
negative regulation neurogenesis
neurogenesis, gliogenesis
mesenchymal development
nervous system development
NCC development gliogenesis
neurogenesis
ECM
j
Known motif enrichment
-log10pvalue
% of targets with motif
AP
HMG
Homeobox
bHLH
ETS
Paired
Tfap2A
Tfap2B
Sox10
Sox2
Sox3
Sox4
Sox6
Esrrb
Hnf1
Hoxa2
Hoxb4
Isl1
Nkx6.1
Ptx1
Ascl1
c-Myc
NeuroD1
Olig2
Tal1
Ets1
Etv1
Fli1
Pax5
Pax7
k
de novo motif (6-18 bp)
Top motif logo
Annotated motif name
P-val
kC1 Sox 1e-43
kC2 Sox 1e-59
kC3 Sox 1e-141
kC4 Tfap2 1e-27
kC5 Hbx/bHLH 1e-121
kC6 Hbx/bHLH 1e-206
kC7 Hbx/bHLH 1e-36
kC8 Sox10 1e-324
kC9 Sox 1e-131
kC10 Sox 1e-110


a
Two-way combinations
DP
E2
10
18
25
200
0
-log10(p-value)
Sox10:Sox
Sox10:NR
Ap2:Sox10
Ap2:NR
Sox:NR
AP2:Sox
AP2:Hbx
Hbx:Sox10
Hbx:Sox
Hbx:NR
Sox:bHLH
NR:bHLH
Ap2:bHLH
Hbx:bHLH
Sox10:bHLH
b
Three-way combinations
40
0
Ap2:Sox10:Sox
Ap2:Sox:NR
Sox10:Sox:NR
Ap2:Sp3:bHLH
Ap2:bHLH:NR
Ap2:Sox10:bHLH
Sox10:bHLH:NR
Ap2:Hbx:bHLH
Ap2:Hbx:Sox10
Hbx:Sox10:NR
Hbx:Sox10:bHLH
Hbx:bHLH:NR
Sox10:Sox:bHLH
Sox:bHLH:NR
Hbx:Sox3:NR
Hbx:Sox10:Sox
Ap2:Hbx:NR
Ap2:Sox10:NR
AP2:Hbx:Sox
c
EdnrB E1
Smad
Zfx
Maf
Gli
Err
Sox2/3
EdnrB E2
Zfx
Sox10
Gata
Maf
Fox
Homeobox
Hox
Ap2
Sp
Rxr
d
E2
tk
Sox10
FLAG
tev
Avi
OR
Tfap2B
2A
citrine
AND
cmv
nls
BirA
mCherry
in ovo
electroporation
HH8/9
HH18
som 1-7
Nuclei extraction
and crosslinking
TF
N
Chromatin IP
(x2 rep/TF)
Streptavidin
pull-down
Library
preparation
Sequencing
e
Sox10 Biotin ChIP-seq
1176 DB elements
Tfap2B Biotin ChIP-seq
790 DB elements
-log10(FDR)
Fold
Sox10
Sox6
Chd7
Dio3
Tgif1
MafB
Col9a3
Kbp
Gfra4
Cdh19
Olig2
MoxD1
Ret
Sema3D
Ascl1
Gata3
Dlx5
Tfap2B
Chd7
Nr2f2
Zic1
Itgb3
Ret
Bmp5
Dio3
Kbp
Zeb2
MoxD1
Barx1
Hand1
MafB
f
DB peak profiles
Sox10
Tfap2B
Input
DP DA peaks
E2 DA peaks
-1.5
center
1.5Kb
g
Overlap DB ChIP-seq
& DA ATAC-seq peaks
Percentage overlap
DP
E2
Sox10
Tfap2B
h
Ret
Biotin ChIP-seq
ATAC-seq
Sox10
Tfap2B
input
DP
E2
Gata
Rarb
Sox
Sox
Maf
Hnf
Wt1
Ap2
tg
r4
pa
som 1-2
som 3-7
RNA-seq
Ret TPM
i
Ascl1
Biotin ChIP-seq
ATAC-seq
Sox10
Tfap2B
input
DP
E2
Tbx
Zfx
Isl1
Sox
FoxJ2/3
Egr2
Wt1
Myc
Rreb1
tg
ma
pa
som 1-2
nt
som 3-7
RNA-seq
Ascl1 TPM
DP
E2
j
Dio3
Biotin ChIP-seq
ATAC-seq
Sox10
Tfap2B
input
DP
E2
neg
k
MoxD1
l
Col9a3


a
cas9
citrine
pCAGG
guide
tracRNA
U6
unilateral
in ovo electroporation
DNA
HH8/9
HH18
som 1-7
neural tube
removed
FACS on
pooled
embryos
cit pos
x3
separate
pools/gene
including
Cas9 only
control
gDNA
RNA-seq
b
Cas9 control
with gRNA
Sox10
Tfap2B
Sox3
Msx1
= CRISPR gRNA target site
c
Embryo survival
% survival
sox3
msx1
sox3+msx DKO
sox10
ap2b
sox10+ap2b DKO
cas9 control
Targeted genes (KOs)
d
gene TPM
Sox3 KO
Msx1 KO
Sox10/
Tfap2B KO
Sox10 KO
Tfap2B KO
Cas9 KO
control
Msx1 Zeb2 Sox3 Sox10 Col1A2 Tcf3 Ngfr Sox11 Snai1 Dlx1 Hand2 Wnt5A FrzB Tfap2B HoxB5 HoxB4 Rarb Hes6 Hes5 Anxa1 MoxD1 HoxA4 Pax6 Hes4 Tubb1 Olig3 Pax7 Zic1 HoxA2 Sox9 Nes Barx1 Six1 Fzd2 PdgfA Dlx5 Prrx1 Rfx4 Snai2 Msx2 PdgfA Col5A2 Mycn Ets1 Sox5 Tfap2A TgfB3 FoxJ1 Wnt3A Wnt9A Nkx6–2 HoxA5 HoxB7 Wnt4 Sox2 Tal1 Wnt6 Zic3 FoxG1 Phox2B Neurog1 Zfhx3 Ascl1 Gata2 Isl1 EdnrB Twist1 Tfap2C Ret FoxD3 Col3A1 Dlx6 Islr Erbb2
e
LEFT
unelectroporated
control
RIGHT
Experiment
KO
HCR imaging
on the
same embryo,
same confocal
settings
ctrl
Tfap2B
KO
Msx1
Sox10
ctrl
Sox3
KO
Msx1
ctrl
Sox10
KO
Msx1
Tfap2B
ctrl
Sox10 + Tfap2B
KO
FoxD3
ctrl
Msx1
KO
Sox3
Pax7


a
VNC migratory path and cell lineage
HH10
HH18
nt
nc
dp
vp
da
gut
enteric progenitors
Premigratory VNCC progenitors
FoxD3 +ve
FoxD3 -ve +Zinc finger
High Sox10 +Tfap2
Hbx/bHLH
Low Sox10 +SoxB +bHLH
neural
mesenchymal
neurogenic
+ FoxD3 + Ets + Notch
- FoxD3 + Tfap2 + Twist1 + bHLH + Prrx1
- FoxD3 - Tfap2 + bHLH + Zinc finger - Notch
b
EdnrB gene regulatory network
Tfap2
MafB
Ets
FoxD3
Sox10
Sox3
Twist1
EdnrB
c
VNC gene regulatory network
Tfap2
Zic1
Msx1
Hes4/5
Pax3/7
FoxD3
DP
Sox10
Ednrb
MBP
MoxD1
Pdgfa
Nes
neural
E2
Msx1
Olig2/3
Hes4/5
Nkx6-2
Sox2/3
Hand2
Barx1/2
Mafb
Twist1
Ednrb
Ret
Prrx1
mesenchymal
Rarb
Phox2b
Ascl1
Gata2/3
Ret
Nts
neuronal
d
Motif binding sites in DA elements
TF Motif PWM
Tfap2
Etv
Hes
Msx
Olig
Sox
Differentially accessible peaks
Hes4/5/6
Etv1/2
Olig2/3
E2
DP
0 1 2 >3
No. TF binding sites
e
Biotin ChIP-seq DB sites and DA elements
Hes6
Hes5
Etv1
Olig2
intergenic/ promoter
intergenic/ promoter
intronic
intergenic
Biotin ChIP-seq
Sox10
Tfap2B
input
ATAC-seq
DP
E2
10
18
25
neg
f
Hes GRN regulating neurogenesis in VNC
Sox10
Tfap2
Msx1/2
Sox3
Etv
Olig2/3
Hes
g
RNA-seq KO of core TFs validation
Hes6
Sox2
Hes4
Sox3
Hes5
Olig3
Msx1
Msx2
Sox10
Etv1
Tfap2B
Tfap2C
Tfap2A
Etv4
Etv3
Sox10/Tfap2B
Sox10
Sox3
Msx1
Tfap2b
log2FC
−4 0 4

# 1. Extended Data

| Figure # | Figure title<br>One sentence only | Filename<br>This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: *Smith_ED Fig1.jpg* | Figure Legend<br>If you are citing a reference for the first time in these legends, please include all new references in the Online Methods References section, and carry on the numbering from the main References section of the paper. |
|---|---|---|---|
| Extended Data Fig. 1 | Summary of ENS development and current genome wide transcriptomic datasets. | Ling_Sauka-Spengler_ExtData_fig_1.eps | Comparative developmental time points during key ENS developmental events summarised from current literature. All the current genome-wide transcriptomic datasets where the majority of studies were carried out in the mouse using either *Wnt1*:Cre or *Sox10*:Cre/Ert2 lines and cells analysed later in ENS development are listed. Red box indicates gap of genome-wide profiles consisting of ATAC-seq, Biotin-ChIP-seq and scRNA-seq unique datasets. Related to Introduction |
| Extended Data Fig. 2 | Quality control for HH10 NC2 ATAC-seq experiments | Ling_Sauka-Spengler_ExtData_fig_2.eps | (a) ATAC-seq fragment lengths.<br>(b) ATAC-seq complexity curves.<br>(c) Hierarchical clustering of ATAC-seq peaks.<br>(d) Principal Component Analysis based on ATAC-seq genome-wide normalise reads. Each point represents one replicate experiment. Three NC2+ and three NC2- experiments are shown. |

| | | | |
|---|---|---|---|
| | | | (e) ATAC-seq TSS peak densities.<br>(f) ATAC-seq peak centre densities.<br>(g) Box and whiskers plot of ATAC-seq total normalised counts per peak (n=7877 peaks) showing min to max counts.<br>(h) Boxplot of Pearson correlation scores between samples with line at median obtained from Deeptools analysis of ATAC samples, n=3 biological triplicates per cell type per stage including negative fluorescent cells.<br>(i) GO-terms of the top 200 genes obtained from DA analysis. Overrepresentation analysis was performed using Panther database and *p*-values were calculated using binominal test with Bonferroni correction for multiple hypothesis.<br>(j) Optimisation of co-electroporation assay. E2:Citrine and E2:Cerulean constructs were co-electroporated at HH4 and embryos were incubated till HH12. Embryos were dissociated and subjected to FAC-sorting. Using appropriate gating strategy, only one cell was recovered in single fluorophore channel. Co-electroporation tests indicated that vast majority of cells receive both plasmids. 60 embryos used fin FAC-sort for each independent experiment.<br>(k-l) Representative FACS plot for negative gating (k) and Citrine and Cerulean positive gating strategy (l). For Citrine only sorts (Figure 1), six independent experiments were carried out.<br>(m) Immunofluorescence for Citrine and mCherry to mark E2- and NC2-driven reporters respectively in a transverse section of a HH18 embryo at the pharynx |

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|---|---|---|---|
| | | | level showing distinct E2-only activity at pharyngeal arch 2 (pa2, pink arrowhead). 3 embryos. Scale bars = 100 μm<br>(n) Whole mount imaging of a HH15 embryo electroporated with E2:Citrine and NC2:mCherry showing lateral view of the cranial and vagal region. Of note, there are NC2:mCherry cells within the cranial region. Pink arrowheads point to distinct E2-only activity in pharyngeal arches. 3 embryos. Scale bars = 100 μm<br>Related to Figures 1 and 2 |
| Extended Data Fig. 3 | Single cell analysis and mining of published single cell datasets | Ling_Sauka-Spengler_ExtData_fig_3.eps | (a) Schematic for the cell preparation of the 10X scRNA-seq experiment<br>(b) QC plots showing RNA features, counts and percentage of mitochondrial reads per cell. Black dotted line indicates cut off for filtering where only cells with >200 and <3000 features (genes) and <10% mitochondrial gene content were selected for analysis. 700 cells obtained in total with the violin plot indicating median and filtered to 570 cells.<br>(c) UMAP representation of 570 single cells assigned to four distinct clusters (scC1-4)<br>(d) Violin plots of the expression levels (log TPM) for *NeuroD1* and *NeuroD4* genes indicate no neural tube contamination of the single cell sample.<br>(e) Top 50 differential gene markers for each cluster plotted on the $-\log_{10}$(padj) values. Adjusted p-value (padj) was calculated using Wald statistics with a negative binominal model and corrected using Benjamin-Hochberg method for multiple hypothesis testing. |

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|---|---|---|---|
| | | | (f) Violin plots depicting selected cluster markers from *Wnt1*-traced NC single-cell datasets from post-otic trunk level at E9.5 (Publicly available data from SRP135960)[15]. Violin Y-axis represents expression levels (log TPM) from Seurat analysis. Individual data points in the plot represent individual cells expressing the gene in that cluster. *EdnrB* is expressed in all clusters similar to *Wnt1* while *FoxD3* is only found in a subset of clusters.<br>(g, h) Volcano plot depicting statistically significant DE genes obtained by RNA-seq comparing DP (left) vs E2 (right) populations at HH10 (g) and HH18 (h). Adjusted p-values (padj), calculated using Wald test with Benjamin-Hochberg correction are plotted against FoldChange enrichment on a log scale. Analysis shows differential enrichment of neural genes in DP and neuronal/mesenchymal ones in E2 population.<br>(i) Heatmap showing the triplicate average gene TPM expression of the selected differentiation markers enriched at HH25 highlighting their expression dynamics across developmental stages and segregation within E2 or DP populations.<br>(j) Heatmap depicting average gene expression levels per single cell clusters from *Wnt1*-traced NC at P21 mouse small bowel myenteric plexus[25] corroborating our data shown in (i).<br>Related to Figures 3 and 4 |

| | | | |
|---|---|---|---|
| Extended Data Fig. 4 | Quality control for ATAC-seq and RNA-seq experiments and correlation of the two datasets | Ling_Sauka-Spengler_ExtData_fig_4.eps | (a) Hierarchical clustering of ATAC-seq datasets.<br>(b) Hierarchical clustering of RNA-seq datasets.<br>(c) Stacked bar plots depicting genomic feature distributions of ATAC-seq peaks as annotated by ChIPseeker package. Annotated features identify a large proportion of peaks as distal intergenic elements.<br>(d) Principal component analysis for ATAC-seq samples. Each point represents one ATAC-seq replicate experiment<br>(e) Principal component analysis for RNA-seq samples. Each point represents one RNA-seq replicate experiment<br>(f) ATAC-seq complexity curves.<br>(g) ATAC-seq fragment length.<br>(h) ATAC-seq peak density profiles.<br>(i) Number of ATAC-seq peak called using MACS and consensus peaksets obtained from all three replicates.<br>(j) Cumulative number of DA peaks per gene.<br>(k) Correlation of DA promoter peaks with DE genes. Simply, promoter peak log2FoldChange scores from DA ATAC-seq Deseq2 analysis and log2FoldChange scores from gene DE RNA-seq Deseq2 analysis were tabulated and gene-matched. The associated Log2FoldChange values (statistically significant with padj<0.05, log2FoldChange>1) were plotted, R-squared coefficient score was generated from the slope and *p*-value from regression analysis (Wald test). Sig, statistically significant.<br>Related to Figures 3 and 4 |

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|---|---|---|---|
| Extended Data Fig. 5 | Common ATAC-seq peaks across stages and single-cell RNA-seq quality control with selected gene expression in individual cells | Ling_Sauka-Spengler_ExtData_fig_5.eps | (a) ATAC-seq UCSC genome browser tracks for *NeuroD4*, *Mbp*, *Sox6* and *Barx1* showing accessible genomic elements. *NeuroD4* elements show an accessibility bias in the E2 population compared to *Mbp* that showed a bias to DP. *Sox6* and *Barx1* displayed opened elements in both populations.<br>(b) The total number of accessible elements across different categories of differentially expressed genes showing dynamic use of elements across stages but also cell types. Size of the bubble indicates total number of differentially accessible elements.<br>(c) Hierarchical clustering of Biotin ChIP-seq peak sets based on Pearson correlation scores from duplicate experiments.<br>(d) Hierarchical clustering of Biotin ChIP-seq peaks within DP peaks based on Pearson correlation scores from duplicate experiments<br>(e) Hierarchical clustering of Biotin ChIP-seq peaks within E2 peaks based on Pearson correlation scores from duplicate experiments.<br>Related to Figure 5 and 6 |
| Extended Data Fig. 6 | CRISPR knockout validation and summary analyses | Ling_Sauka-Spengler_ExtData_fig_6.eps | (a) Principal Component Analysis of all RNA-seq samples. Each dot represents one independent experiment. Duplicate KO experiments were performed per gene. One *Sox3* KO experiment (arrow) was removed as a clear outlier.<br>(b) sgRNA oligo sequence for targeting individual genes. |

| | | | |
|---|---|---|---|
| | | | (c) Temperature-Shifted melt curves representing profiles from Cas9-control individual embryos in red (8 embryos) and *Sox3* KO embryos in green (8 embryos) all showing split profiles, thus suggesting high penetrance of efficient genome editing events Difference in Relative Fluorescence Units (RFU) between experimental and control samples is shown on y-axis.<br>(d) IGV snapshot of BAM-generated mapping around the region of gRNA target sites with the top panel showing one representative replicate of Cas9-only control and the bottom panel showing one representative replicate of the gene knockout. Snapshots obtained at the base of the plots for similar comparison.<br>(e) Summary of differential expression analysis obtained using DESeq2 analysis of KO and control samples for selected genes.<br>Related to Figure 7 |
| Extended Data Fig. 7 | Deconstructing *EdnrB* GRN | Ling_Sauka-Spengler_ExtData_fig_7.eps | (a) TF binding motif enrichment within *EdnrB* DA elements.<br>(b) Results of RNA-seq differential expression analysis comparing KO versus control embryos depicting genes involved in *EdnrB* gene regulatory circuitry.<br>Related to Figure 8 |

## 2. Supplementary Information:

### A. Flat Files

| Item | Present? | Filename<br>This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. The extension must be .pdf | A brief, numerical description of file contents.<br>i.e.: *Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.* |
|---|---|---|---|
| Supplementary Information | No | | |
| Reporting Summary | Yes | nr-reporting-summary_NCB-S39164D.pdf | |

### B. Additional Supplementary Files

| Type | Number<br>If there are multiple files of the same type this should be the numerical indicator. i.e. "1" for Video 1, "2" for Video 2, etc. | Filename<br>This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: *Smith_Supplementary_Video_1.mov* | Legend or Descriptive Caption<br>Describe the contents of the file |
|---|---|---|---|
| Supplementary table | 1 | Ling_Sauka-Spengler_Supplementary_table_1.xlsx | All *de novo* motifs identified by Homer with a p-value of less than 1e-60 were included. Two motifs per gene were added where a larger motif sequence was identified. All position weighted matrices were then |

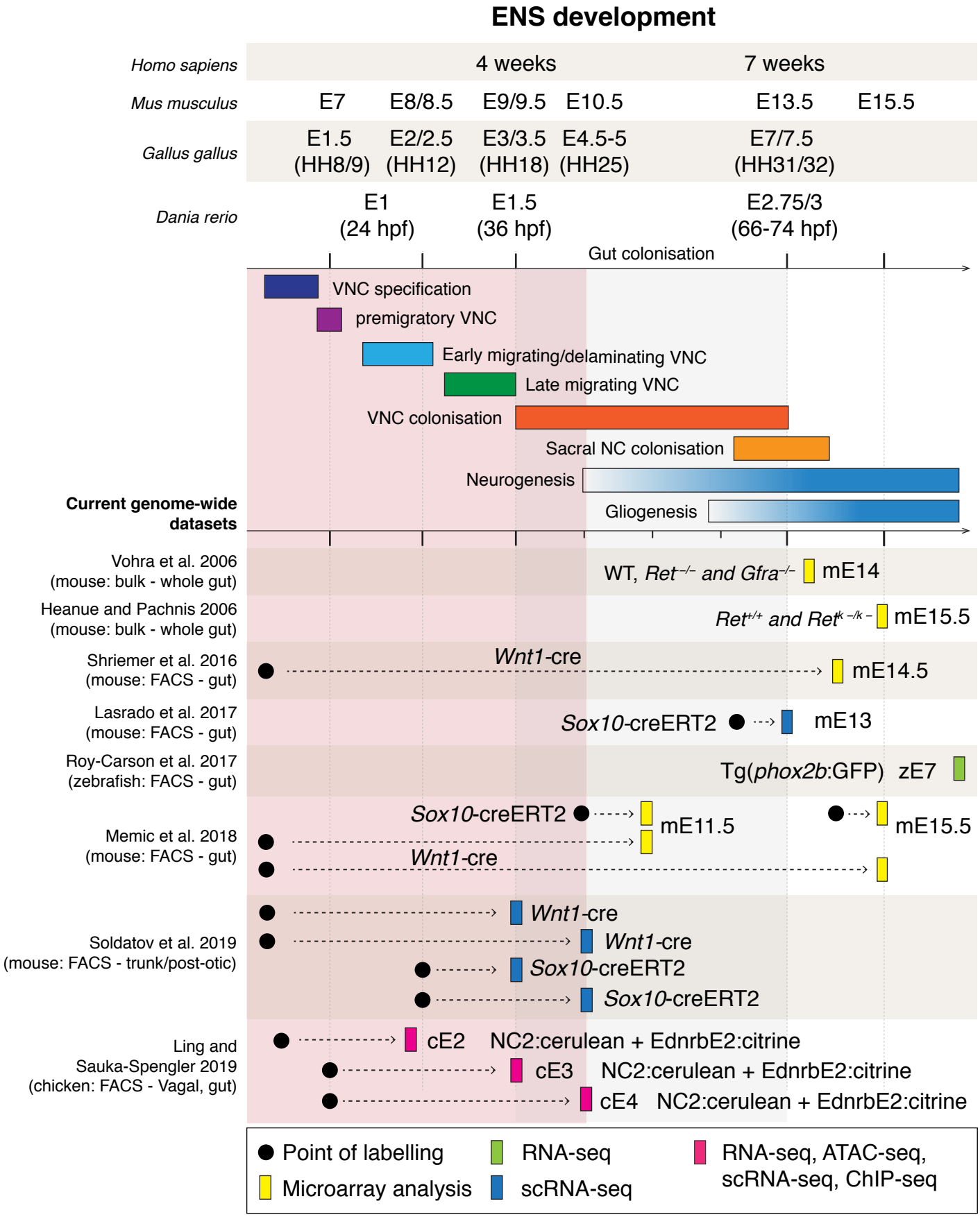
| | | | |
|---|---|---|---|
| | | | annotated using TOMTOM (HOCOMOCO Human Full p <0.0005, q<0.5) and HOMER (score >0.8) to obtain a consensus name. Where there is no consensus annotation reached, a general family of TF name was allocated to the motif. p-adjusted values were obtained to adjust for multiple comparisons.<br>Related to Figure 4 and 5 |

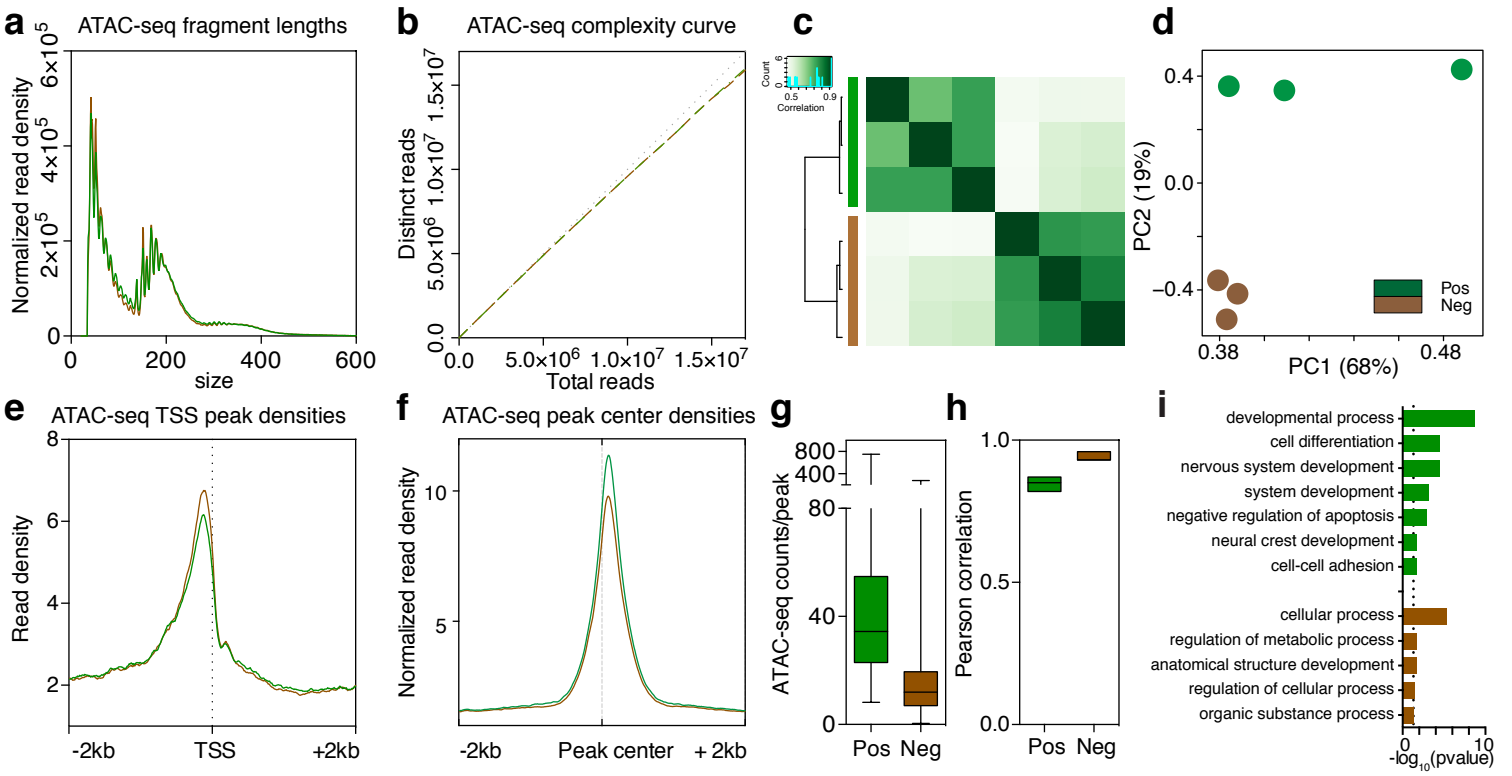
## 3. Source Data

| Figure | Filename<br>This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: *Smith_SourceData_Fig1.xls, or Smith_ Unmodified_Gels_Fig1.pdf* | Data description<br>i.e.: Unprocessed Western Blots and/or gels, Statistical Source Data, etc. |
|---|---|---|
| Statistical Source Data Fig. 1 | Ling_Sauka-Spengler_Raw_data_fig1.xlsx | ATAC-seq counts matrix and cell count |
| Statistical Source Data Fig. 2 | Ling_Sauka-Spengler_Raw_data_fig2.xlsx | Cell counts |
| Statistical Source Data Fig. 3 | Ling_Sauka-Spengler_Raw_data_fig3.xlsx | Featurecounts raw matrix and<br>DESEQ2 analysis from pairwise comparisons |

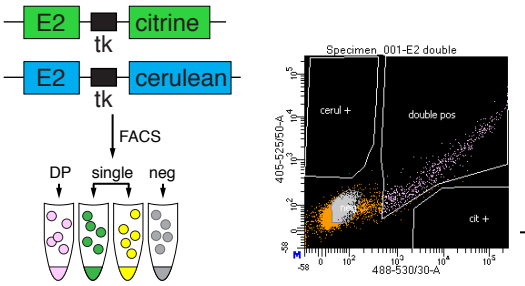
| | | |
|---|---|---|
| Statistical Source Data Fig. 4 | Ling_Sauka-Spengler_Raw_data_fig4.xlsx | Top markers from Seurat analysis including raw matrix file. Source files available on GEO series. |
| Statistical Source Data Fig. 5 | Ling_Sauka-Spengler_Raw_data_fig5.xlsx | ATAC-seq counts matrix |
| Statistical Source Data Fig. 6 | Ling_Sauka-Spengler_Raw_data_fig6.xlsx | Statistical p-values for combinatorial analysis and differential binding DESEQ2 analysis |
| Statistical Source Data Fig. 7 | Ling_Sauka-Spengler_Raw_data_fig7.xlsx | Featurecounts raw matrix |
| Statistical Source Data Fig. 8 | Ling_Sauka-Spengler_Raw_data_fig8.xlsx | Motif occurrences |
| Statistical Source Data Extended Data Fig. 2 | Ling_Sauka-Spengler_Raw_data_ExtData_fig2.xlsx | Source data |
| Statistical source Data Extended Data Fig. 3 | See tabs for source data in Ling_Sauka-Spengler_Raw_data_fig3.xlsx and Ling_Sauka-Spengler_Raw_data_fig4.xlsx | Source data |
| Statistical source Data Extended Data Fig. 4 | Ling_Sauka-Spengler_Raw_data_ExtData_fig4.xlsx | Source data |

| | | |
|---|---|---|
| Statistical source Data Extended Data Fig. 5 | Ling_Sauka-Spengler_Raw_data_ExtData_fig5.xlsx | Source data |
| Statistical source Data Extended Data Fig. 6 | See tabs in Ling_Sauka-Spengler_Raw_data_fig3.xlsx | Source data |
| Statistical source Data Extended Data Fig. 7 | See Ling_Sauka-Spengler_Raw_data_fig3.xlsx and Ling_Sauka-Spengler_Raw_data_fig8.xlsx | Source data |


ENS development
Homo sapiens
4 weeks
7 weeks
Mus musculus
E7
E8/8.5
E9/9.5
E10.5
E13.5
E15.5
Gallus gallus
E1.5 (HH8/9)
E2/2.5 (HH12)
E3/3.5 (HH18)
E4.5-5 (HH25)
E7/7.5 (HH31/32)
Dania rerio
E1 (24 hpf)
E1.5 (36 hpf)
E2.75/3 (66-74 hpf)
Gut colonisation
VNC specification
premigratory VNC
Early migrating/delaminating VNC
Late migrating VNC
VNC colonisation
Sacral NC colonisation
Neurogenesis
Gliogenesis
Current genome-wide datasets
Vohra et al. 2006 (mouse: bulk - whole gut)
WT, Ret−/− and Gfra−/−
mE14
Heanue and Pachnis 2006 (mouse: bulk - whole gut)
Ret+/+ and Retk −/k −
mE15.5
Shriemer et al. 2016 (mouse: FACS - gut)
Wnt1-cre
mE14.5
Lasrado et al. 2017 (mouse: FACS - gut)
Sox10-creERT2
mE13
Roy-Carson et al. 2017 (zebrafish: FACS - gut)
Tg(phox2b:GFP)
zE7
Memic et al. 2018 (mouse: FACS - gut)
Sox10-creERT2
mE11.5
mE15.5
Wnt1-cre
Soldatov et al. 2019 (mouse: FACS - trunk/post-otic)
Wnt1-cre
Wnt1-cre
Sox10-creERT2
Sox10-creERT2
Ling and Sauka-Spengler 2019 (chicken: FACS - Vagal, gut)
cE2
NC2:cerulean + EdnrbE2:citrine
cE3
NC2:cerulean + EdnrbE2:citrine
cE4
NC2:cerulean + EdnrbE2:citrine
Point of labelling
Microarray analysis
RNA-seq
scRNA-seq
RNA-seq, ATAC-seq, scRNA-seq, ChIP-seq




**j** Assessing heterogeneity of double plasmid electroporation



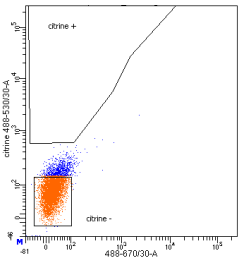




Tube: E2 double

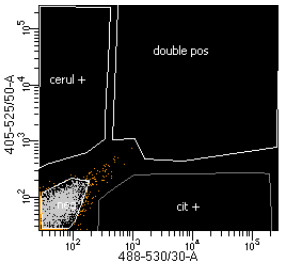
| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 42,315 | #### | 100.0 |
| P1 | 8,469 | 20.0 | 20.0 |
| P2 | 8,179 | 96.6 | 19.3 |
| P3 | 7,851 | 96.0 | 18.6 |
| neg | 4,597 | 58.6 | 10.9 |
| cerul + | 1 | 0.0 | 0.0 |
| double pos | 575 | 7.3 | 1.4 |
| cit + | 0 | 0.0 | 0.0 |

**k** Negative gating strategy



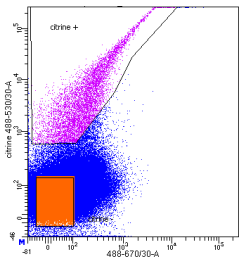

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 29,097 | #### | 100.0 |
| P1 | 10,083 | 34.7 | 34.7 |
| P2 | 7,720 | 76.6 | 26.5 |
| P3 | 6,620 | 85.8 | 22.8 |
| citrine + | 0 | 0.0 | 0.0 |
| citrine - | 5,747 | 86.8 | 19.8 |

Negative gating strategy



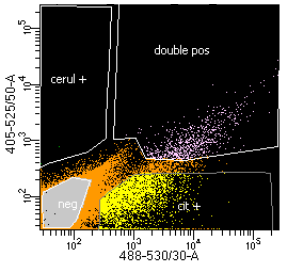

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 10,000 | #### | 100.0 |
| P1 | 5,634 | 56.3 | 56.3 |
| P2 | 4,431 | 78.6 | 44.3 |
| P3 | 4,361 | 98.4 | 43.6 |
| neg | 3,002 | 68.8 | 30.0 |
| cerul + | 0 | 0.0 | 0.0 |
| double pos | 0 | 0.0 | 0.0 |
| cit + | 0 | 0.0 | 0.0 |

**l** FAC-sort citrine positive cells

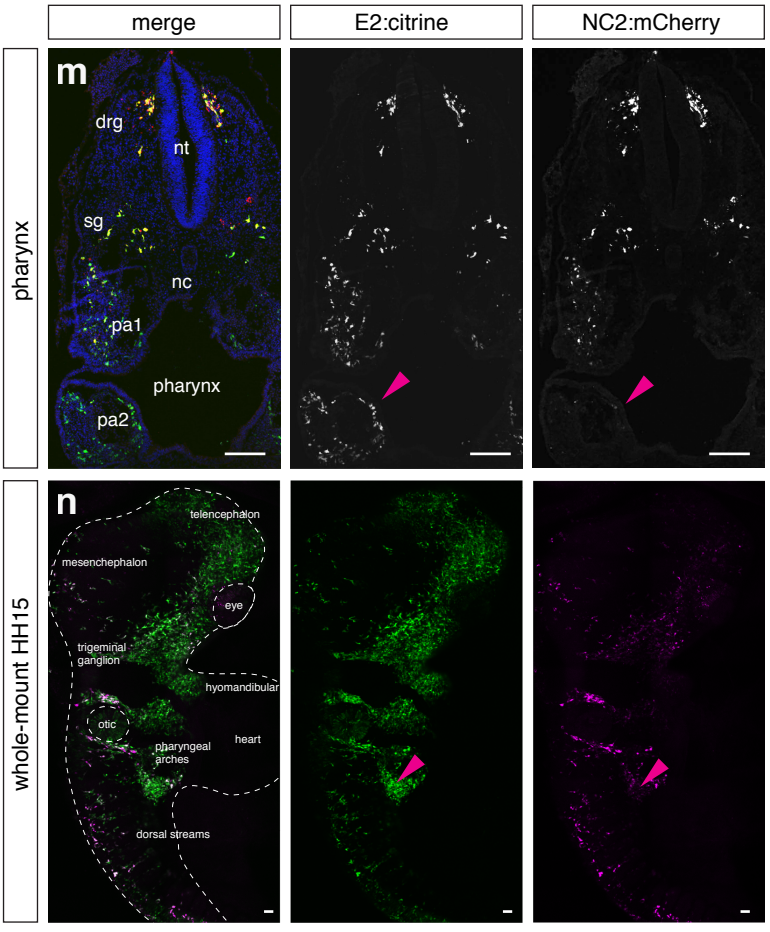


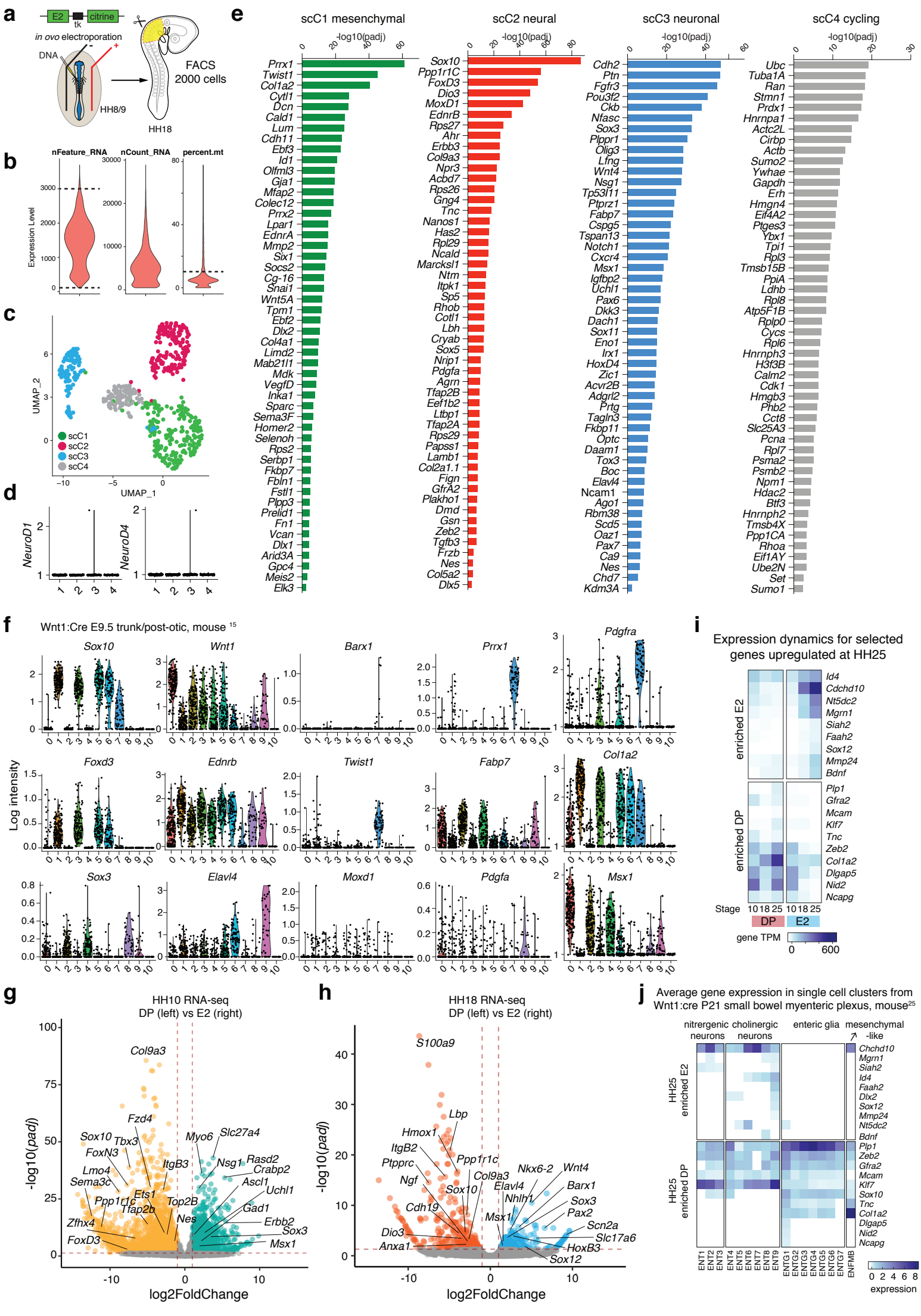

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 1,000,000 | #### | 100.0 |
| P1 | 405,380 | 40.5 | 40.5 |
| P2 | 307,973 | 76.0 | 30.8 |
| P3 | 270,233 | 87.7 | 27.0 |
| citrine + | 6,895 | 2.6 | 0.7 |
| citrine - | 149,057 | 55.2 | 14.9 |

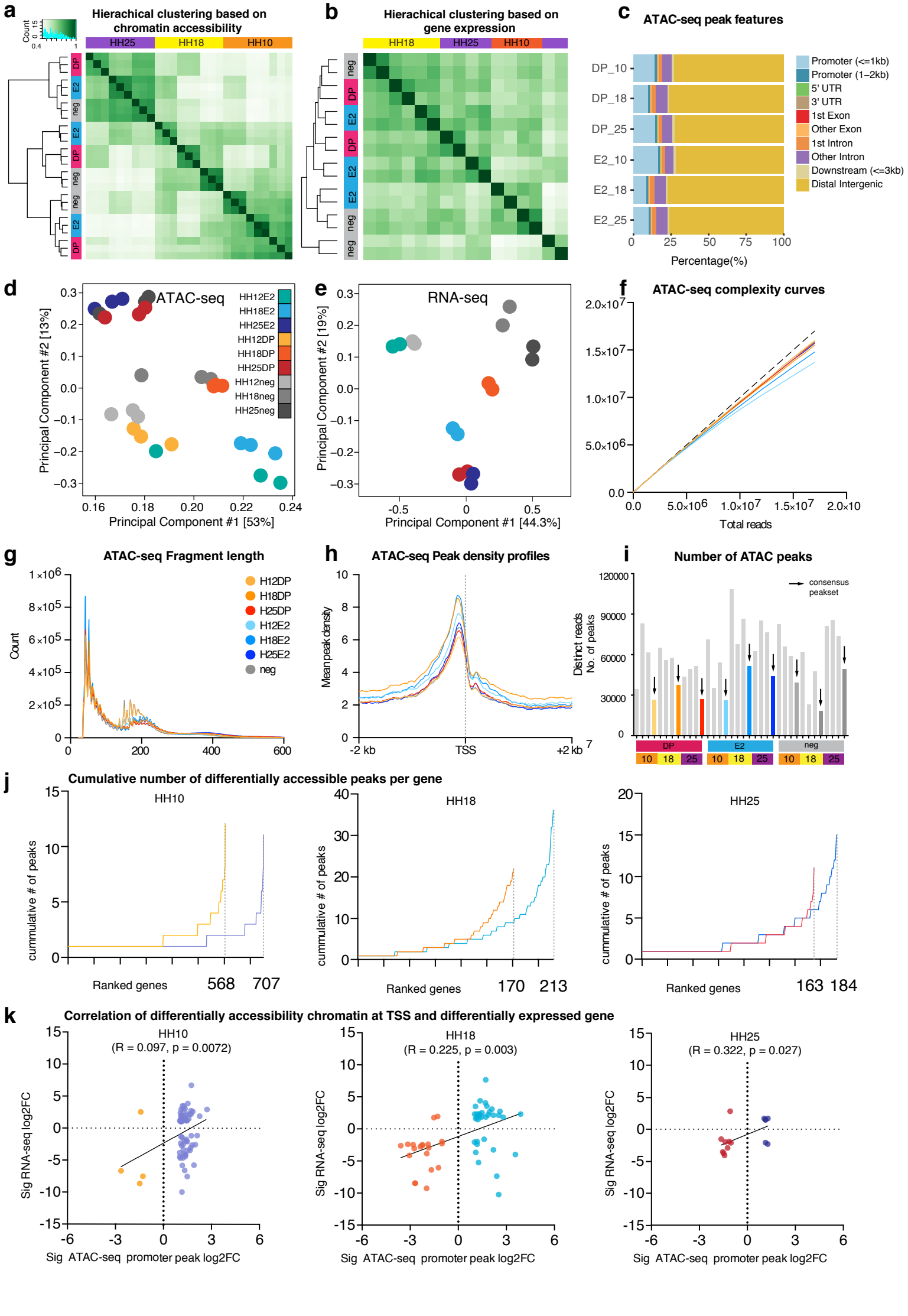
FAC-sort positive cell populations

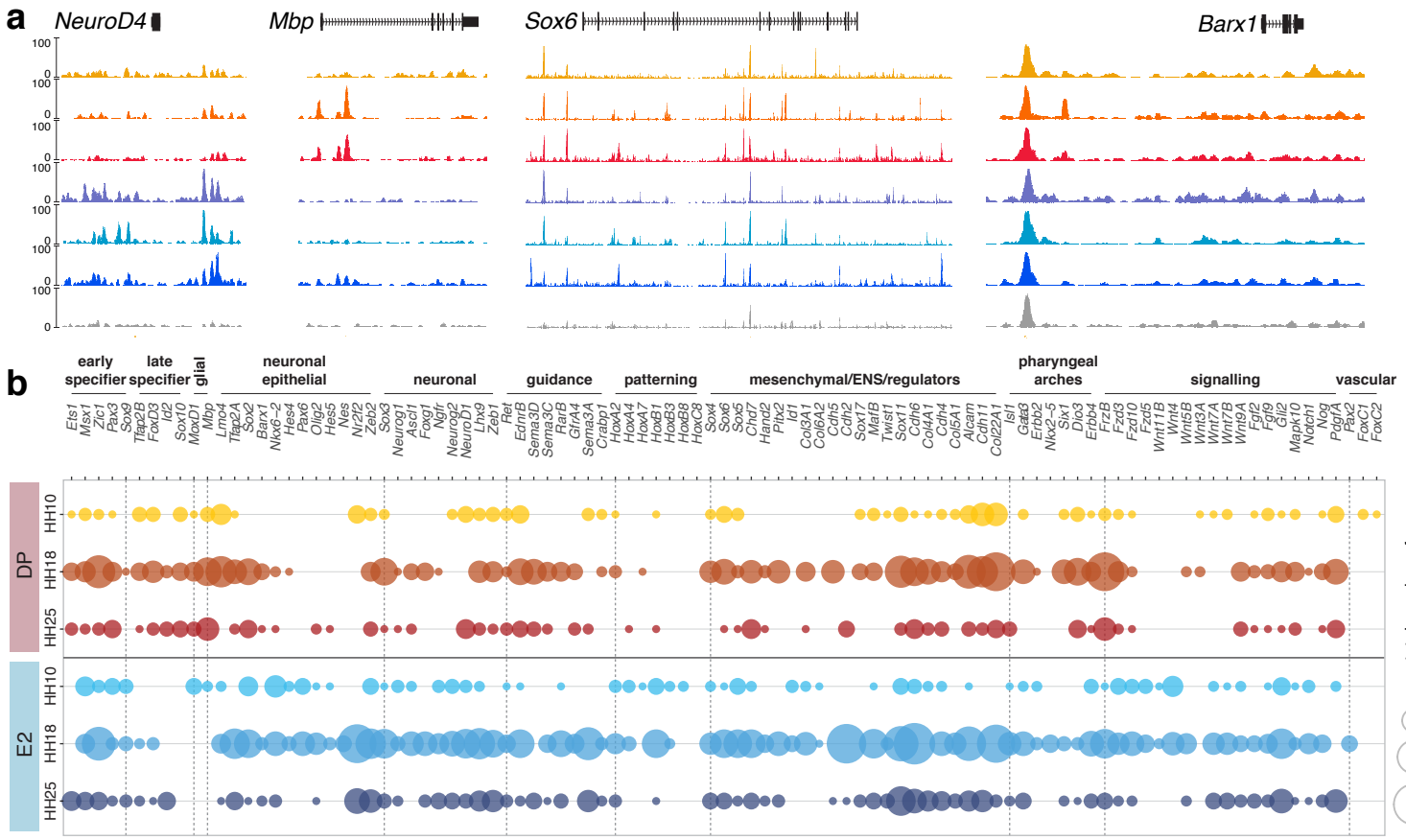


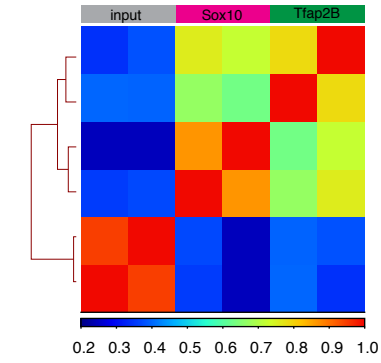

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 372,460 | #### | 100.0 |
| P1 | 171,649 | 46.1 | 46.1 |
| P2 | 137,611 | 80.2 | 36.9 |
| P3 | 132,825 | 96.5 | 35.7 |
| neg | 90,214 | 67.9 | 24.2 |
| cerul + | 2 | 0.0 | 0.0 |
| double pos | 725 | 0.5 | 0.2 |
| cit + | 3,480 | 2.6 | 0.9 |

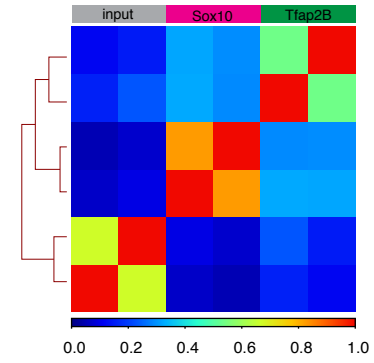


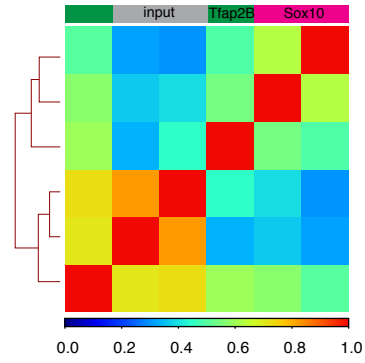

a
E2
tk
citrine
in ovo electroporation
DNA
HH8/9
FACS
2000 cells
HH18
b
nFeature_RNA
nCount_RNA
percent.mt
Expression Level
c
UMAP_2
UMAP_1
scC1
scC2
scC3
scC4
d
NeuroD1
NeuroD4
e
scC1 mesenchymal
scC2 neural
scC3 neuronal
scC4 cycling
-log10(padj)
Prrx1
Twist1
Col1a2
Cytl1
Dcn
Cald1
Lum
Cdh11
Ebf3
Id1
Olfml3
Gja1
Mfap2
Colec12
Prrx2
Lpar1
EdnrA
Mmp2
Six1
Socs2
Cg-16
Snai1
Wnt5A
Tpm1
Ebf2
Dlx2
Col4a1
Limd2
Mab21l1
Mdk
VegfD
Inka1
Sparc
Sema3F
Homer2
Selenoh
Rps2
Serbp1
Fkbp7
Fbln1
Fstl1
Plpp3
Prelid1
Fn1
Vcan
Dlx1
Arid3A
Gpc4
Meis2
Elk3
Sox10
Ppp1r1C
FoxD3
Dio3
MoxD1
EdnrB
Rps27
Ahr
Erbb3
Col9a3
Npr3
Acbd7
Rps26
Gng4
Tnc
Nanos1
Has2
Rpl29
Ncald
Marcksl1
Ntm
Itpk1
Sp5
Rhob
Cotl1
Lbh
Cryab
Sox5
Nrip1
Pdgfa
Agrn
Tfap2B
Eef1b2
Ltbp1
Tfap2A
Rps29
Papss1
Lamb1
Col2a1.1
Fign
GfrA2
Plakho1
Dmd
Gsn
Zeb2
Tgfb3
Frzb
Nes
Col5a2
Dlx5
Cdh2
Ptn
Fgfr3
Pou3f2
Ckb
Nfasc
Sox3
Plppr1
Olig3
Lfng
Wnt4
Nsg1
Tp53l11
Ptprz1
Fabp7
Cspg5
Tspan13
Notch1
Cxcr4
Msx1
Igfbp2
Uchl1
Pax6
Dkk3
Dach1
Sox11
Eno1
Irx1
HoxD4
Zic1
Acvr2B
Adgrl2
Prtg
Tagln3
Fkbp11
Optc
Daam1
Tox3
Boc
Elavl4
Ncam1
Ago1
Rbm38
Scd5
Oaz1
Pax7
Ca9
Nes
Chd7
Kdm3A
Ubc
Tuba1A
Ran
Stmn1
Prdx1
Hnrnpa1
Actc2L
Cirbp
Actb
Sumo2
Ywhae
Gapdh
Erh
Hmgn4
Eif4A2
Ptges3
Ybx1
Tpi1
Rpl3
Tmsb15B
PpiA
Ldhb
Rpl8
Atp5F1B
Rplp0
Cycs
Rpl6
Hnrnph3
H3f3B
Calm2
Cdk1
Hmgb3
Phb2
Cct8
Slc25A3
Pcna
Rpl7
Psma2
Psmb2
Npm1
Hdac2
Btf3
Hnrnph2
Tmsb4X
Ppp1CA
Rhoa
Eif1AY
Ube2N
Set
Sumo1
f
Wnt1:Cre E9.5 trunk/post-otic, mouse [15]
Sox10
Wnt1
Barx1
Prrx1
Pdgfra
Foxd3
Ednrb
Twist1
Fabp7
Col1a2
Sox3
Elavl4
Moxd1
Pdgfa
Msx1
Log intensity
i
Expression dynamics for selected genes upregulated at HH25
enriched E2
Id4
Cdchd10
Nt5dc2
Mgrn1
Siah2
Faah2
Sox12
Mmp24
Bdnf
enriched DP
Plp1
Gfra2
Mcam
Klf7
Tnc
Zeb2
Col1a2
Dlgap5
Nid2
Ncapg
Stage 10 18 25 10 18 25
DP
E2
gene TPM
0
600
g
HH10 RNA-seq
DP (left) vs E2 (right)
-log10(padj)
log2FoldChange
Col9a3
Fzd4
Sox10
Tbx3
FoxN3
Lmo4
Sema3c
Ppp1r1c
Ets1
Tfap2b
Zfhx4
FoxD3
Myo6
Slc27a4
ItgB3
Nsg1
Rasd2
Crabp2
Ascl1
Uchl1
Top2B
Gad1
Nes
Erbb2
Sox3
Msx1
h
HH18 RNA-seq
DP (left) vs E2 (right)
S100a9
Lbp
Hmox1
ItgB2
Ptpprc
Ngf
Ppp1r1c
Col9a3
Sox10
Elavl4
Nhlh1
Cdh19
Msx1
Dio3
Anxa1
Nkx6-2
Wnt4
Barx1
Sox3
Pax2
Scn2a
Slc17a6
HoxB3
Sox12
j
Average gene expression in single cell clusters from Wnt1:cre P21 small bowel myenteric plexus, mouse[25]
nitrergenic neurons
cholinergic neurons
enteric glia
mesenchymal -like
HH25 enriched E2
HH25 enriched DP
Chchd10
Mgrn1
Siah2
Id4
Faah2
Dlx2
Sox12
Mmp24
Nt5dc2
Bdnf
Plp1
Zeb2
Gfra2
Mcam
Klf7
Sox10
Tnc
Col1a2
Dlgap5
Nid2
Ncapg
ENT1 ENT2 ENT3 ENT4 ENT5 ENT6 ENT7 ENT8 ENT9 ENTG1 ENTG2 ENTG3 ENTG4 ENTG5 ENTG6 ENTG7 ENFMB
0 2 4 6 8
expression


a
Hierachical clustering based on chromatin accessibility
Count
HH25
HH18
HH10
DP
E2
neg
b
Hierachical clustering based on gene expression
HH18
HH25
HH10
c
ATAC-seq peak features
DP_10
DP_18
DP_25
E2_10
E2_18
E2_25
Promoter (<=1kb)
Promoter (1–2kb)
5' UTR
3' UTR
1st Exon
Other Exon
1st Intron
Other Intron
Downstream (<=3kb)
Distal Intergenic
Percentage(%)
d
ATAC-seq
HH12E2
HH18E2
HH25E2
HH12DP
HH18DP
HH25DP
HH12neg
HH18neg
HH25neg
Principal Component #2 [13%]
Principal Component #1 [53%]
e
RNA-seq
Principal Component #2 [19%]
Principal Component #1 [44.3%]
f
ATAC-seq complexity curves
Total reads
g
ATAC-seq Fragment length
Count
H12DP
H18DP
H25DP
H12E2
H18E2
H25E2
neg
h
ATAC-seq Peak density profiles
Mean peak density
-2 kb
TSS
+2 kb
i
Number of ATAC peaks
Distinct reads
No. of peaks
consensus peakset
DP
E2
neg
10
18
25
j
Cumulative number of differentially accessible peaks per gene
HH10
HH18
HH25
cummulative # of peaks
Ranked genes
568
707
170
213
163
184
k
Correlation of differentially accessibility chromatin at TSS and differentially expressed gene
HH10
(R = 0.097, p = 0.0072)
HH18
(R = 0.225, p = 0.003)
HH25
(R = 0.322, p = 0.027)
Sig RNA-seq log2FC
Sig ATAC-seq promoter peak log2FC

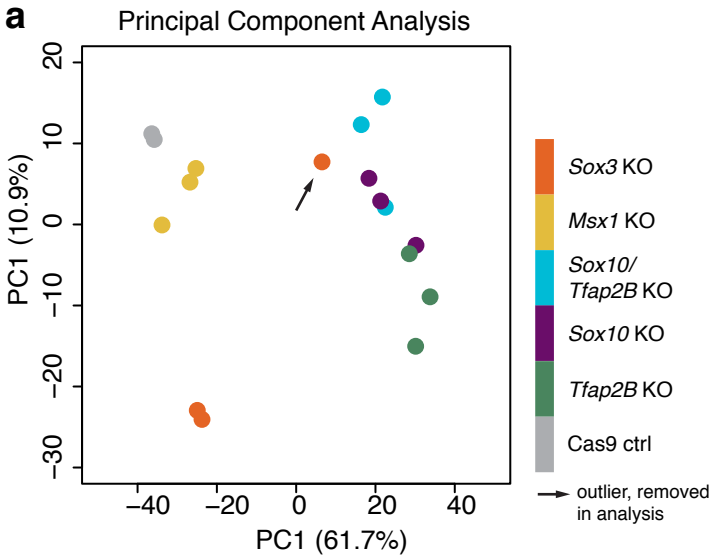

a
NeuroD4
Mbp
Sox6
Barx1
b
early specifier
late specifier
glial
neuronal epithelial
neuronal
guidance
patterning
mesenchymal/ENS/regulators
pharyngeal arches
signalling
vascular
DP
E2
HH10
HH18
HH25
total number of accessible elements
0
10
20
30


c
ChIP-seq Pearson correlation in all DA peaks
input
Sox10
Tfap2B
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0


d
ChIP-seq Pearson correlation in DP DA peaks
input
Sox10
Tfap2B
0.0 0.2 0.4 0.6 0.8 1.0


e
ChIP-seq Pearson correlation in E2 DA peaks
input
Tfap2B
Sox10
0.0 0.2 0.4 0.6 0.8 1.0

**a**



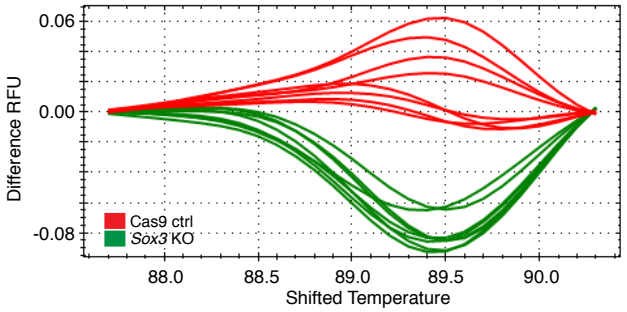

**b**

sgRNA oligo design sequence

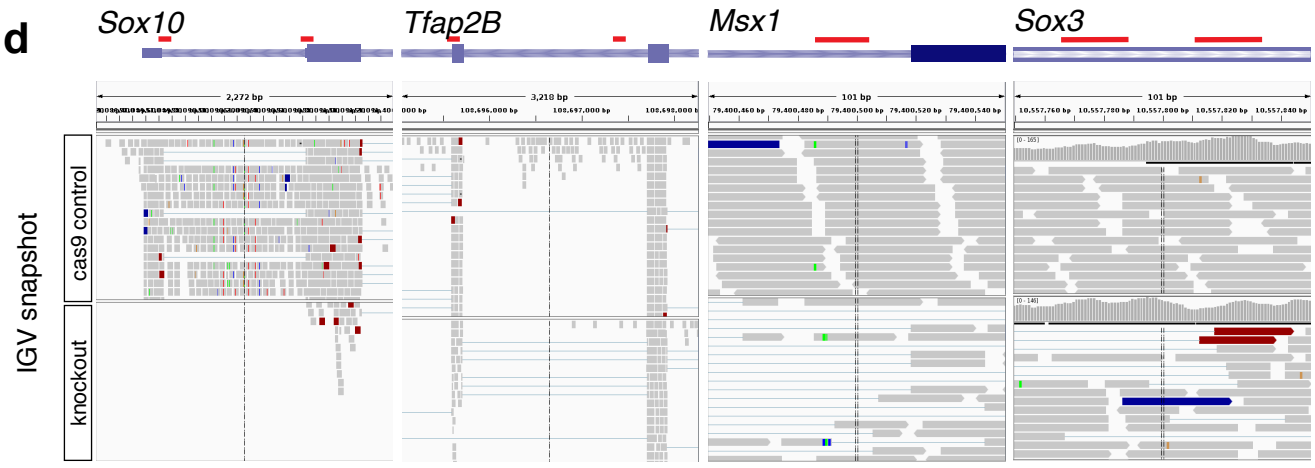
| | | |
|---|---|---|
| *TFAp2b* | sgRNA 1 | GGAGGAGTGCTGAGAAGgta |
| *TFAp2b* | sgRNA 2 | accctcgcttacCTTCCACC |
| *Sox10* | sgRNA 1 | ttccctccccagTGAGAAGA |
| *Sox10* | sgRNA 2 | ggtaggaaaacttacATTGC |
| *Sox3* | sgRNA 1 | GTTCATGTAGGTCTGCGCCG |
| *Sox3* | sgRNA 2 | ATGCACCGCTACGACATGCC |
| *Msx1* | sgRNA | GAGGAGAGGAAGAGACAC |

**c**

Temperature-Shifted Difference Curve




**d**



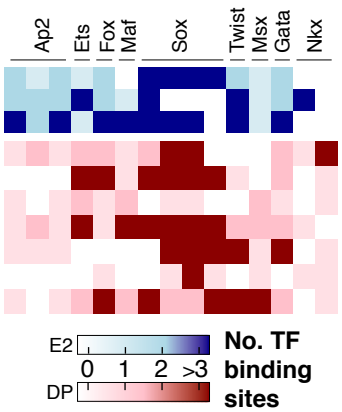

**e**

Summary of DESeq2 log2FoldChange

| KO | *Sox10* | *Tfap2B* | *DKO* | *Sox3* | *Msx1* |
|---|---|---|---|---|---|
| *EdnrB* | ↓ | ↓ | ↓ | ↓ | ↓ |
| *Ret* | ↓ | ↓ | ↓ | ↓ | ↓ |
| *Sox10* | / | ↔ | / | ↔ | ↓ |
| *Sox9* | ↓ | ↓ | ↓ | ↓ | ↓ |
| *Sox3* | ↑ | ↑ | ↔ | / | ↑ |
| *Sox2* | ↑ | ↑ | ↑ | ↑ | ↑ |
| *FoxD3* | ↔ | ↔ | ↓ | ↔ | ↓ |
| *Tfap2B* | ↓ | / | / | ↓ | ↓ |
| *Msx1* | ↓ | ↓ | ↓ | ↑ | / |
| *Pax7* | ↔ | ↔ | ↔ | ↑ | ↑ |

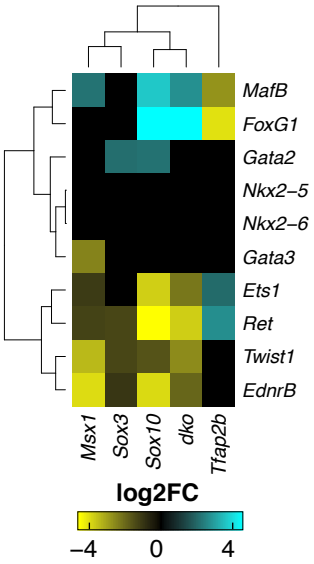
**a**

TF binding motif for
all annotated Ednrb DA elements




**b**

RNA-seq KO validation
of *Ednrb* regulation



| No. | Name | p-value (<1e-60) | Logpvalue | % target | De-novo Motif | TOMTOM (hocomoco Human Full) P <0.0005, q <0.5 | Homer Score >0.8 |
|---|---|---|---|---|---|---|---|
| HH10 DP | AP2 | 1.00E-269 | -6.21E+02 | 31.66 |  | Ap2a, Ap2c, Ap2b | Ap2c, Ap2a, Ap2b |
| | | 1.00E-194 | -4.49E+02 | 22.53 |  | | |
| HH10 DP | Hbx (Hnf1b) | 1.00E-195 | -4.49E+02 | 3.29 |  | Hnf1b, Hnf1a, Zfhx3 | Hnf1, Hnf1b, Hnf1a |
| | | 1.00E-176 | -4.07E+02 | 11.75 |  | Hnf1b, Hnf1a, Foxj3 | |
| HH10 DP | Sox | 1.00E-149 | -3.44E+02 | 42.31 |  | Sox2, Sox17, Sox4, Sox3 Sox13, Sox15, Sox10, Sox9, Sox18 | Sox2, Sox10, Sox3, Sox6 Sox4, Sox9, Sox11 |
| HH10 DP | Sox10 | 1.00E-155 | -3.58E+02 | 15.74 |  | Sox10, Sox9 | |
| HH18 DP | AP2 | 1e-517 | -1.19E+03 | 21.9 |  | AP2a, AP2c, AP2b | AP2a, AP2c, AP2b |
| | | 1.00E-191 | -4.40E+02 | 19.97 |  | AP2b, Ap2c, AP2a | |
| HH18 DP | Sox10 | 1e-394 | -9.08E+02 | 50.42 |  | Sox9, Sox3, Sox10 | Sox10, Sox3, Sox2, Sox Sox4 |
| | | 1e-744 | -1.71E+03 | 17.32 |  | Sox10, Sox9 | |
| HH18 DP | N/A | 1.00E-151 | -3.48E+02 | 25.88 |  | N/A | N/A |
| HH18 DP | NR(1) | 1.00E-117 | -2.70E+02 | 28.05 |  | Pparg, Rxrg, Rxra, Nr4a3 Ppara, Err2, Err3, Rarb | Erra, Nr2f2, Esrrb, Rxra |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HH18 DP | N/A | 1.00E-82 | -1.89E+02 | 31.61 |  | N/A | Sox8, Sox17 |
| HH18 DP | N/A | 1.00E-77 | -1.77E+02 | 31.2 |  | N/A | Zfx |
| HH25 DP | AP2 | 1.00E-141 | -3.27E+02 | 16.37 |  | AP2a, AP2c, AP2b | AP2a, AP2c, AP2b |
| HH25 DP | Sox10 | 1.00E-138<br>1.00E-300 | -3.20E+02<br>-6.91E+02 | 25.93<br>18.6 | <br> | Sox10, sox9 | Sox10, Sox3, Sox6, Sox2 Sox4 |
| HH25 DP | N/A | 1.00E-69 | -1.61E+02 | 39.72 |  | N/A | Sox8, Sox17 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HH10 E2 | Sox3 | 1e-355 | -8.20E+02 | 48.68 |  | Sox3, Sox13, Sox9, Sox2 Sox17, Sox4, Sox10 | Sox3, Sox2, Sox6, Sox1 Sox14, Sox9, |
| HH10 E2 | Hbx (Hnf1b) | 1.00E-245 | -5.64E+02 | 12.08 |  | Hnf1, Hnf1A, hnf1b, | Hnf1b, hnf1a, zfhx3 |
| HH10 E2 | N/A | 1.00E-91 | -2.12E+02 | 2.34 |  | N/A | N/A |
| HH10 E2 | N/A | 1.00E-86 | -2.00E+02 | 62.58 |  | N/A | Sox17 |
| HH18 E2 | Sox3 | 1e-595 | -1.37E+03 | 41.59 |  | Sox3, Sox10, Sox2, Sox4 Sox9, | Sox3, Sox2, Sox10, Sox6 Sox14, |
| HH18 E2 | Hox(1) | 1e-350 | -8.06E+02 | 24.88 |  | Meis1, Hoxa9, Evx1, Hoxc6, Hoxc9, Hoxd9, | Hoxc9, Hoxa9, |
| HH18 E2 | N/A | 1.00E-150 | -3.47E+02 | 45.64 |  | N/A | Sox8 |
| | | 1.00E-146 | -3.36E+02 | 5.71 |  | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HH18 E2 | Rfx | 1.00E-165 | -3.82E+02 | 5.89 |  | Rfx3, Rfx4,Rfx2, Rfx1 | Rfx1, Rfx2, Rfx5 |
| HH18 E2 | Oct4-Sox2-Tcf Nanog | 1.00E-107<br>1.00E-159 | -2.47E+02<br>-3.68E+02 | 23.66<br>3.15 |  | Po2f2<br>Po5f1, Nanog, Sox2 | Oct4, Po2f2<br>Sox2, Oct4, Po5f1 |
| HH18 E2 | NR(2) | 1.00E-81 | -1.87E+02 | 20.81 |  | Ppara, Rxrg, Nr4a2, Rxra | Nr2f2, Rara, Erra, Nr4a2 Esrrb, Rxra, |
| HH25 E2 | Twist | 1.00E-183 | -4.23E+02 | 33.77 |  | Twist1 | Lim3 |
| HH25 E2 | bHLH(1) | 1.00E-110<br>1e-413 | -2.55E+02<br>-9.53E+02 | 31.98<br>25.1 |  | Bhe22, Twist1,<br>Olig2 | Olig2, Tal1, Atoh1, NeuroD<br>Olig2, Olig1, Msx1 |
| HH25 E2 | Hox(2) | 1.00E-106 | -2.46E+02 | 19.68 |  | Meis1, Hoxb7, Hoxc6, Hoxa9, Hoxb8 | Lin39, Pdx1, Hoxb4, Hoxa |