





Single-cell transcriptome analysis reveals thyrocyte diversity in the zebrafish thyroid gland

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Abstract

The thyroid gland regulates growth and metabolism via production of thyroid hormone in follicles composed of thyrocytes. So far, thyrocytes have been assumed to be a homogenous population. To uncover heterogeneity in the thyrocyte population and molecularly characterize the non-thyrocyte cells surrounding the follicle, we developed a single-cell transcriptome atlas of the region containing the zebrafish thyroid gland. The 6249-cell atlas includes profiles of thyrocytes, blood vessels, lymphatic vessels, immune cells, and fibroblasts. Further, the thyrocytes show expression heterogeneity, including bimodal expression of the transcription factor *pax2a*. To validate thyrocyte heterogeneity, we generated a CRISPR/Cas9-based *pax2a* knock-in line that monitors *pax2a* expression in the thyrocytes. A population of *pax2a*-low mature thyrocytes interspersed in individual follicles can be distinguished. We corroborate heterogeneity within the thyrocyte population using RNA sequencing of *pax2a*-high and *pax2a*-low thyrocytes, which demonstrates 20% differential expression in transcriptome between the two subpopulations. Our results identify and validate transcriptional differences within the presumed homogenous thyrocyte population.

Keywords CRISPR/Cas9; heterogeneity; single-cell; thyroid gland; zebrafish

Subject Categories Development; RNA Biology

DOI 10.15252/embr.202050612 | Received 9 April 2020 | Revised 30 September 2020 | Accepted 6 October 2020 | Published online 3 November 2020

EMBO Reports (2020) 21: e50612

Introduction

The thyroid gland produces hormones thyroxine (T4) and triiodothyronine (T3) that regulate body metabolism, growth, and

development. Thyroid dysfunction afflicts almost 100 million people worldwide (Taylor *et al*, 2018). Hypothyroidism can be efficiently managed by lifelong hormone replacement therapy, while hyperthyroidism is treated with antithyroid medication, surgery, or ablation, depending on the underlying disorder. Congenital hypothyroidism if left untreated may result in profound adverse effects on development, including mental retardation, goiter, or dwarfism.

The thyroid gland is an endocrine organ with an intricate structure enabling production, storage, and release of the thyroid hormones. It contains numerous variable-sized spherical follicles composed of thyroid follicular epithelial cells or thyrocytes. The thyrocytes generate the thyroid hormones in a multi-step process. They secrete and store thyroglobulin (TG) in the lumen of the follicles. Additionally, they intake iodide from the blood via sodium-iodide symporter (NIS/Slc5a5). At the interface between thyrocytes and the lumen, thyroid peroxidase (TPO) expressed by the cells catalyzes the coupling of iodide to tyrosyl residues of TG. Iodinated TG is absorbed back into the thyrocyte and cleaved by cysteine proteases in lysosomes to form T4 and T3 (Brix *et al*, 1996). Though the machinery responsible for the production of thyroid hormones by thyrocytes is well established, it remains unknown whether all the thyrocytes resident in a follicle are equally capable of generating thyroid hormones. In other words, the extent of molecular homogeneity between individual thyrocytes has not yet been investigated.

Additionally, the thyroid gland contains many cell types with potential roles in modulating thyrocyte functionality. The gland contains an extensive distribution of blood vessels, which carry iodide to the thyrocytes and carry thyroid hormones away from them. The thyroid follicles are separated by a mesenchymal cell population, called connective tissue septa, which also divides the gland into lobules. The mammalian thyroid gland also contains parafollicular epithelial cells, or C cells, that synthesize and secrete the hormone calcitonin. These parafollicular epithelial cells are, however, located outside the thyroid gland in fish and amphibians

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(Alt *et al*, 2006). Further, the presence of immune cells and innervation has been demonstrated within the thyroid gland (Nonidez, 1931; Linehan *et al*, 2001). Though we have a considerable understanding of these cell types on a histological level, we still lack the molecular characterization of the thyroid gland cell ensemble. This extends to an incomplete appreciation of the impact of the diverse cell populations on thyroid follicular cell physiology.

To uncover the diversity within the thyrocyte population and further characterize the surrounding tissue at cellular resolution, we develop the first atlas of the thyroid gland at single-cell resolution. For this, we build on the progress in single-cell transcriptomics (Svensson *et al*, 2018) to transcriptionally profile thousands of individual cells isolated from the thyroid gland of juvenile and adult zebrafish. We demonstrate that these profiles comprehensively represent the cells present in the zebrafish thyroid gland. Utilizing the expression profiles of discrete cell populations, we build an intercellular signaling network to uncover communication between thyrocytes and the surrounding tissue. Further, we demonstrate the segregation of thyrocytes into two transcriptionally distinct subpopulations based on the expression level of a transcription factor. Finally, to enable easy access to the data, we have made the zebrafish thyroid gland atlas available for online browsing.

Results

Single-cell transcriptomics of the zebrafish thyroid gland

The zebrafish thyroid gland is composed of follicles scattered in the soft tissue surrounding the ventral aorta (Fig 1A and B). Ventral aorta extends from the outflow tract of the zebrafish heart and carries blood from the ventricle to the gills. Dissection of the ventral aorta associated region (detailed in Methods section) provided us with tissue that included the thyroid follicles and parts of zebrafish gills (Fig 1C). Using *Tg(tg:nls-EGFP)* transgenic line, which labels thyrocytes with nuclear green fluorescence (Fig 1D), we estimated presence of $5.9 \pm 1.9\%$ thyrocytes within the dissociated region (Fig 1E).

To generate the molecular catalogue of the thyroid gland at cellular resolution, we sampled the organ from two ages of zebrafish: 2 months post-fertilization (mpf) and 8 mpf (Fig EV1A). The time points span juvenile to adult transition in zebrafish, with animals containing fully differentiated functional organs at both stages. By 2 mpf, the juvenile animals have completed morphogenesis, but are yet to reach sexual maturity. The animals sampled at 2 mpf were on

average 2.6 cm in length and 123.8 mg in weight. In contrast, fish at 8 mpf are sexually mature adults, with an average length of 3.5 cm and an average weight of 294.4 mg (Fig EV1B and C). To characterize the organ cell types in an unbiased manner, we dissected out the entire thyroid gland (Fig 1B and C) from six animals at each stage and prepared the single-cell suspension for cDNA library preparation. To guide thyroid gland dissection, we utilized the *Tg(tg:nls-mVenus-T2A-NTR)* zebrafish reporter line (McMenamin *et al*, 2014) that labels thyrocytes with bright yellow fluorescent protein (Fig 1B'). The micro-dissected tissue was dissociated using enzymatic digestion. The single-cell suspension was stained with calcein, which specifically labels live cells with blue fluorescence. The live cells were then enriched using FACS (Fig 1F–G) to limit false-positive signals from dead and/or ruptured cells (AlJanahi *et al*, 2018). Thyrocytes consisted of around 4% of the alive cells at both stages, comparable to the percentage obtained by immunofluorescence analysis (Fig 1E). Twelve thousand live cells, pooled from six animals, were collected in separate tubes according to age and profiled using droplet-based high-throughput single-cell RNA sequencing provided by 10× Genomics (Macosko *et al*, 2015; Zheng *et al*, 2017). Droplet-based methods encapsulate cells with single-Poisson distribution (Zheng *et al*, 2017). This leads to approximately 50% cell capture rate, which is the ratio of the number of cells detected by sequencing and the number of cells loaded. The 10× Genomics pipeline uses molecule and cell-specific barcoding allowing transcript quantification without amplification bias (Kivioja *et al*, 2011; Islam *et al*, 2014). Using the Cell Ranger bioinformatics pipelines, the resulting Next-Generation Sequencing libraries were mapped to the zebrafish genome, de-multiplexed according to their cellular barcodes and quantified to generate gene/cell UMI (unique molecular identifier) count tables. The Cell Ranger pipeline provided us with 13,106 sequenced cells from 24,000 input cells (54.6 % cell capture rate). Quality-based exclusion of single-cell transcriptomes was implemented based on mean library size, percentage of mitochondrial reads, and number of genes detected per cell. On average, we detected 6,012 UMIs and 1,303 genes per cell (Fig EV2). The process recovered in total 6,249 cells out of 13,106 sequenced cells (47.7% retention rate), providing single-cell transcriptomic profiles for 2,986 and 3,263 individual cells for 2 mpf and 8 mpf, respectively.

Identification of cell types present in the zebrafish thyroid gland

To aid with visualization of the zebrafish thyroid gland single-cell RNA-Seq (scRNA-Seq) data, we projected the cellular profiles onto

Figure 1. Isolation of zebrafish thyroid gland.

- A, B A brightfield image showing the zebrafish thyroid gland along with surrounding tissue. The thyroid follicles reside in the soft tissue surrounding the ventral aorta, which extends from the outflow tract of the heart into the gills toward the basibranchial cartilage in the lower jaw. The thyroid follicular cells, or thyrocytes, are labeled in green in the *Tg(tg:nls-mVenus-NTR)* transgenic line (B'). Scale bars: 500 μ m.
- C Maximum intensity projection of 3D confocal stack obtained from the dissected thyroid gland of *Tg(tg:nls-EGFP)* animal and labeled with DAPI.
- D Confocal scan of a transverse section across the dissected thyroid gland from *Tg(tg:nls-EGFP)* animal at 3 mpf. Sections were stained with DAPI to visualize cells surrounding thyroid follicles.
- E Boxplot depicting the proportion of thyrocytes present in transverse sections obtained from three *Tg(tg:nls-EGFP)* animals at 3 mpf. Each dot represents a transverse section. The Tukey boxplot marks the 25th percentile, median, and 75th percentile with whiskers extending from smallest to largest values.
- F, G Representative FACS plot of single cells from *Tg(tg:nls-mVenus-T2A-NTR)* animals at 2 mpf (F) and 8 mpf (G). Calcein (Pacific Blue) labels live cells, while green fluorescence (FITC) labels thyrocytes. Percentage values represent proportion of calcein⁺ thyrocytes within total calcein⁺ cells.

Source data are available online for this figure.

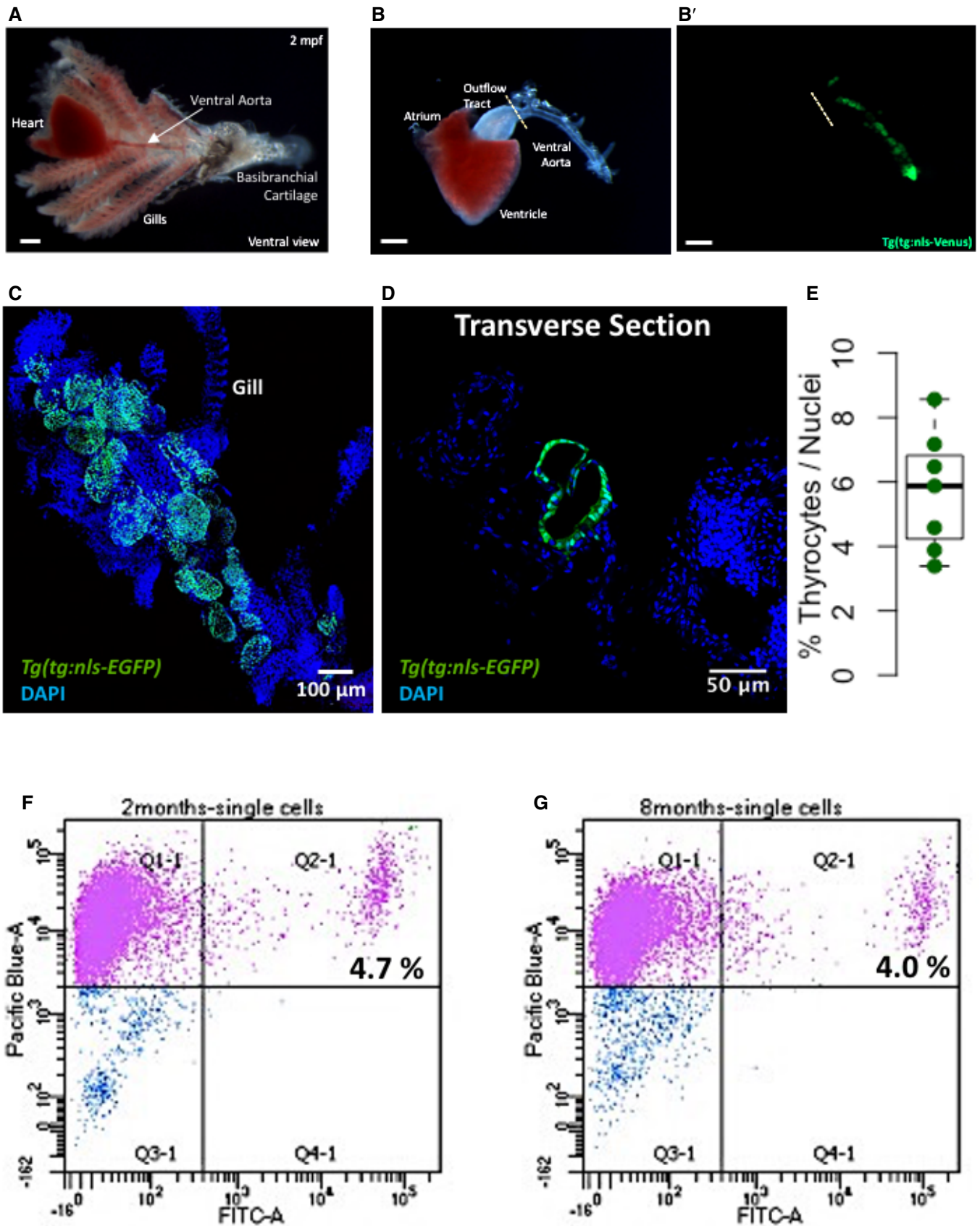


Figure 1.

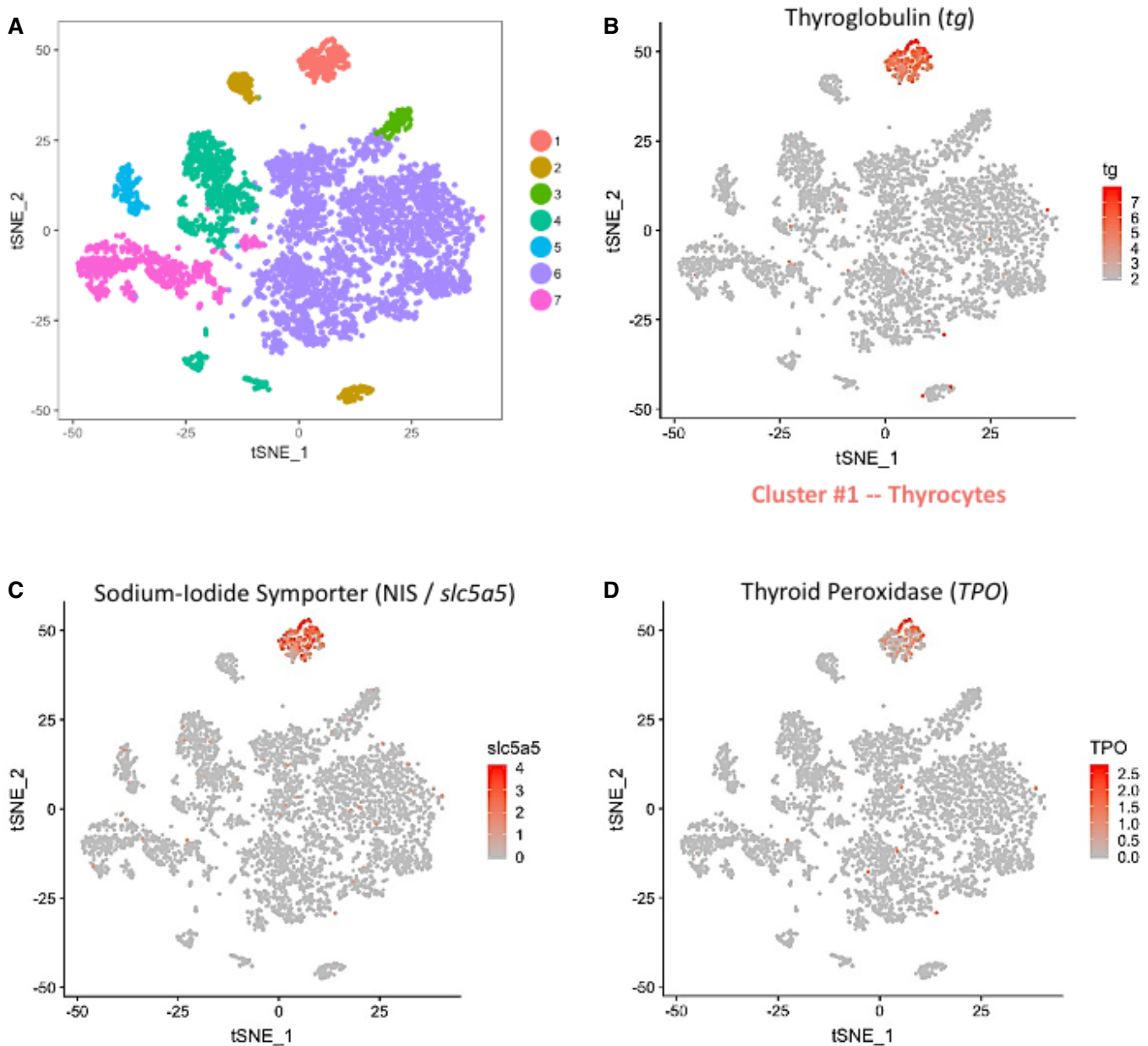


Figure 2. Single-cell RNA-Seq of the zebrafish thyroid gland.

A t-SNE plot displaying the 6,249 single cells profiled in the zebrafish thyroid gland atlas. The colors represent cell clusters denoting a specific cell type.

B–D Cluster #1 represents the thyrocytes that express *tg*, *slc5a5* (NIS) and *tpo*. The color scale represents the normalized expression counts for each gene ranging from lowest (gray) to highest (red).

t-distributed stochastic neighbor embedding (t-SNE) plots, a non-linear dimensionality reduction technique (Maaten & Hinton, 2008) (Fig 2A). Using unsupervised graph-based clustering, we identified seven clusters for the thyroid gland. Using the expression of genes involved in thyroid hormone production, we could identify one of the clusters as thyroid follicular cells (Fig 2B–D). Specifically, the cluster displayed high relative expression of *tg* gene, which was further enriched by background correction (Appendix Figure S1), thereby demonstrating that the cells represented differentiated thyroid follicular cells. The cluster, labeled as thyrocytes, contains

267 cells. This represents 4.2% of the total cells recovered after quality control, similar to the proportion of thyrocytes quantified in the dissociated tissue by imaging and FACS (Fig 1E–G), suggesting lack of thyrocyte loss during the sequencing procedure.

To define the identity of the remaining cell clusters, we generated cluster-specific marker genes by performing differential gene expression analysis (Fig 3A) (Table EV1). For four clusters, the marker genes included one or more known cell type-specific identifiers. This included *gpr182* for endothelial cells; *acta2* for musculature; *fer1gl* for immune cells; and *ponzr3* for cells from zebrafish gills

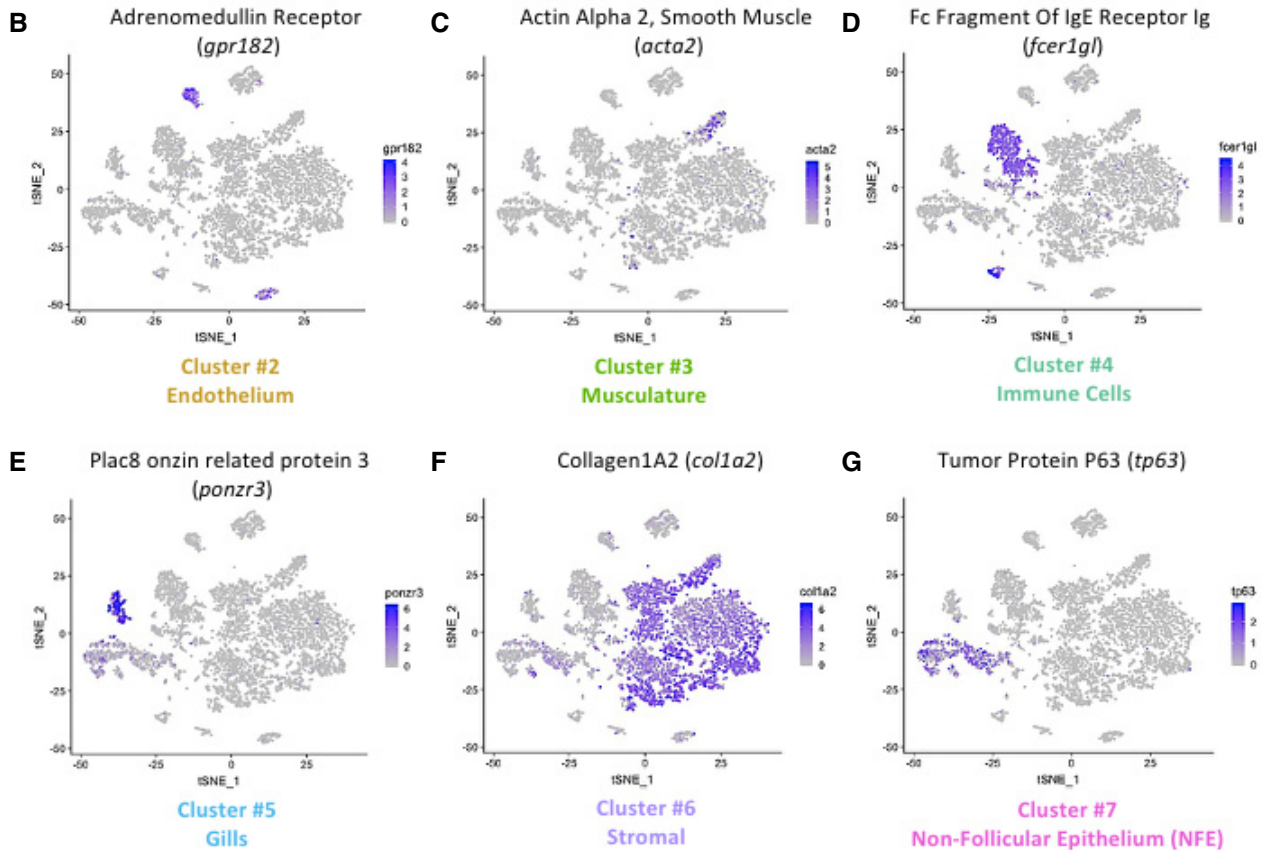
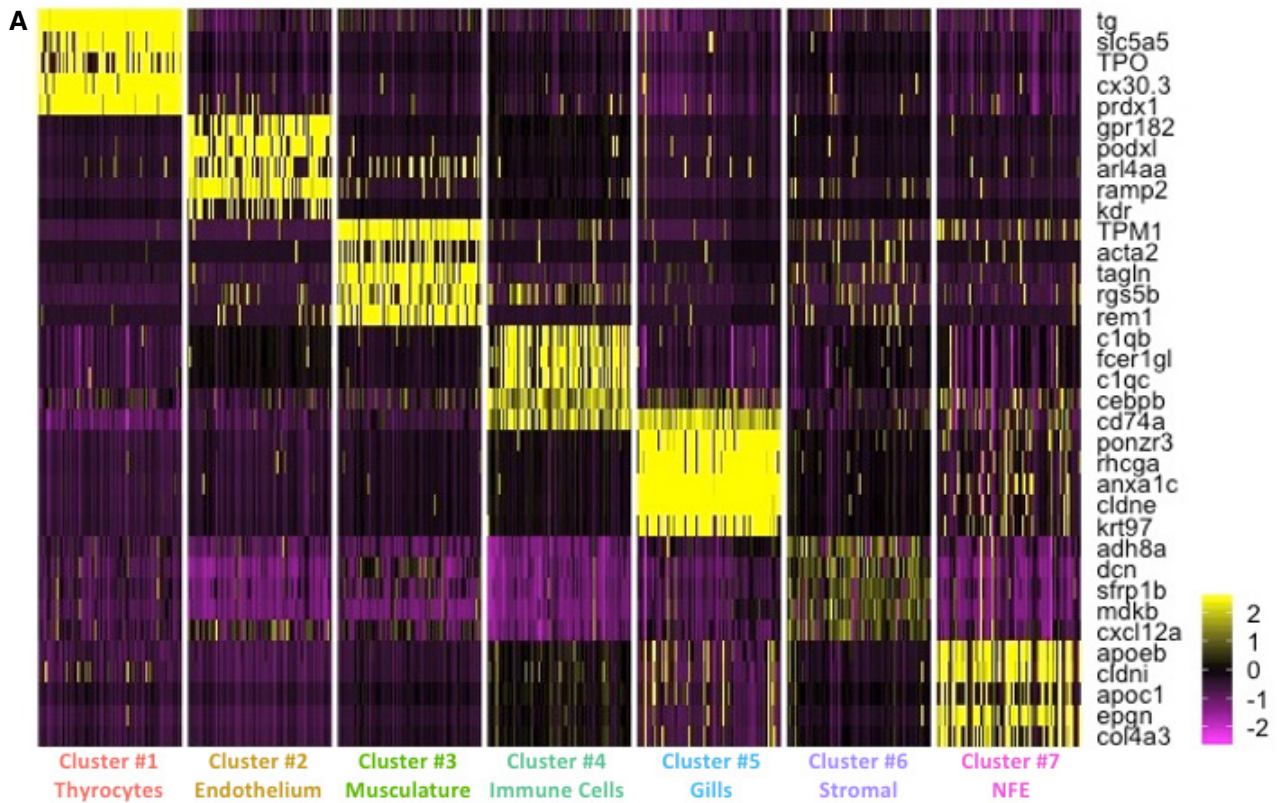


Figure 3.

Figure 3. Gene expression signature of the different cell types in the zebrafish thyroid gland.

- A Heatmap depicting five genes specifically expressed in each one of the seven clusters of the zebrafish thyroid gland atlas. The color scale represents expression as a z-score ranging from -2 (purple) to 2 (yellow). NFE: Non-Follicular Epithelium.
- B–G t-SNE plots overlaid with the expression of a gene specific to each of the cluster. The endothelium cluster (cluster #2) is a mix of blood vessels and lymphatic vessels (see Appendix Fig S4), while the immune cell cluster (cluster #4) is a mix of macrophages, neutrophils, and lymphocytes (see Appendix Fig S5). The color scale represents the normalized expression counts for each gene ranging from lowest (gray) to highest (blue).

(Fig 3B–E). Based on these cell identifiers, the atlas includes 233 endothelial cells, 135 muscle lineage cells, 914 immune cells, and 199 cells from zebrafish gills. Notably, the endothelial cell cluster includes blood vessels (*flt1* and *kdrl*) and lymphatic vessels (*mrc1a*, *prox1a*, *flt4*, and *lyve1b*) (Fig EV3); while the immune cell cluster includes macrophages (*mpeg1.1* and *mfap4*), neutrophils (*lyz*), and lymphocytes (*il4*, *il13*, and *il11b*) (Fig EV4).

For the remaining two clusters (number six and seven), we identified marker genes that hinted toward identity of the cell type. Specifically, *col1a2* and *tp63* enriched in cluster number six and seven, respectively (Fig 3F–G), are known markers of fibroblasts (Denton *et al*, 2001; Sánchez-Iranzo *et al*, 2018) and epithelial tissue (Barbieri & Pietenpol, 2006; Reischauer *et al*, 2009; Lisse *et al*, 2016). We performed gene ontology (GO) enrichment analysis of the marker genes to aid with classification (Appendix Fig S2). Cluster six demonstrated an enrichment of "extracellular matrix structural constituent", "connective tissue development", and "extracellular space", confirming the presence of tissue fibroblasts in this cluster. Thus, we labeled cluster six as "Stromal" cells. Cluster seven displayed an enrichment of "cell motility", "cell migration", and "epithelium development", suggestive of epithelial cells. Notably, cluster seven displayed expression of cytokeratins (*krt5*, *krt14*) and E-cadherin (*cdh1*) (Table EV1). Hence, we labeled cluster seven as "Non-Follicular Epithelium (NFE)", to distinguish them from the thyroid follicular epithelial cells. Our data contains 3670 stromal cells and 831 non-follicular epithelial cells.

We validated the presence of blood vessels, macrophages, and stromal cells in the thyroid gland using tissue-specific transgenic lines (Fig 4A–C). Immunofluorescence (IF) analysis demonstrated physical proximity between thyrocytes and blood vessels (Fig 4A). Notably, we observed a subset of macrophages in direct contact with thyroid follicles (Fig 4B). In addition, we visualized NFE by immunostaining against TP63 antibody (Fig 4D), which revealed NFE scattered throughout the gills and in the region adjacent to the follicles. Thus, the IF analysis successfully confirmed the presence of different cell types identified in the single-cell atlas.

Our marker gene identification further established additional genes enriched in a single cell type in the thyroid gland (Fig 3A) (Table EV1). For instance, we identified *cx30.3*, a connexin gene, and *prdx1*, a gene involved in the antioxidant response, to be specifically expressed in the thyrocytes. To enable further investigation of the clusters and gene expression profiles, we have developed an interactive webtool for online browsing (<https://sumeet.shinyapps.io/zfthyroid/>).

Development of autocrine and paracrine signaling networks in the thyroid gland using known ligand–receptor interactions

Having defined the cell types of the thyroid gland, we quantified potential cell–cell interactions between thyrocytes and all cell types

present in the organ (Fig 5A) based on a reference list of approximately 3,100 literature-supported interactions containing receptors and ligands from receptor tyrosine kinase (RTK), extracellular matrix (ECM)-integrin, chemokine, and cytokine families (Ramilowski *et al*, 2015). Although anatomical barriers between cell types are not modeled in this analysis, we restricted the analysis to secreted ligands for NFE, stroma, and gills—cell types that are physically separated from thyrocytes (Fig 4C–D). For the remaining cell types, secreted and cell membrane-tethered ligands were considered. The expression patterns of ligand–receptor pairs revealed a dense intercellular communication network (Fig 5B). The network consisted of 272 ligands expressed on different cell types with a corresponding receptor expressed on the thyrocytes (Table EV2). For instance, the stromal cells express the ligand *lpl* (Lipoprotein Lipase) that signals through the *lrp2a* (zebrafish homologue of Megalin) receptor (Fig 5C). Stromal and smooth muscle cells express *dcn* (Decorin) whose receptor *met* is expressed by thyrocytes. Further, the ligand *cyr61* is broadly expressed in the thyroid gland and adjoining gills, with one of its receptors, *itgb5*, an integrin isoform, expressed specifically by the thyrocytes. The identified interactions also include autocrine signaling. For example, the ligand *sema3b* and its receptor *nrp2a* are both present on thyrocytes. GO analysis for identified ligand–receptor pairs revealed genes involved in "PI3K-Akt signaling pathway", "MET signaling", and "integrin binding" (Appendix Fig S3).

Thyrocytes display transcriptional heterogeneity

Next, we characterized the transcriptional differences within the thyrocyte population. For this, we bioinformatically isolated the thyrocytes and performed analysis of genetic entropy, a measure of the degree of transcriptional uncertainty. This revealed transcriptional heterogeneity in 231 genes in the thyrocyte population (Fig 6A) (Table EV3). Genes displaying statistically significant entropy (P -value < 0.05) included *pax2a* and *ctsba*. Notably, for the thyrocyte population, the expression of *tg* displays a unimodal distribution, while the expression of *pax2a* and *ctsba* displays bimodal distribution (Fig 6B, Appendix Fig S4).

Cathepsin B (mammalian homologue of *ctsba*) is a cysteine protease that is involved in the processing of iodinated thyroglobulin to T4 and T3 in the thyrocyte lysosomes (Brix *et al*, 1996, 2001). Moreover, fusion of Cathepsin B and EGFP has been previously used to track thyroid hormone processing lysosomes in rat thyroid epithelial cell lines (Linke, 2002).

The gene *pax2a* belongs to the PAX (paired box DNA binding) domain containing family of transcription factors. The heterogeneity in *pax2a* expression is notable, as *pax2a* is an important regulator of thyrocyte development (Wendl *et al*, 2002). Zebrafish thyroid primordium expresses *pax2a* at 24 hpf (Wendl *et al*, 2002), which is required for specification of the thyroid follicles (Pfeffer *et al*, 1998;

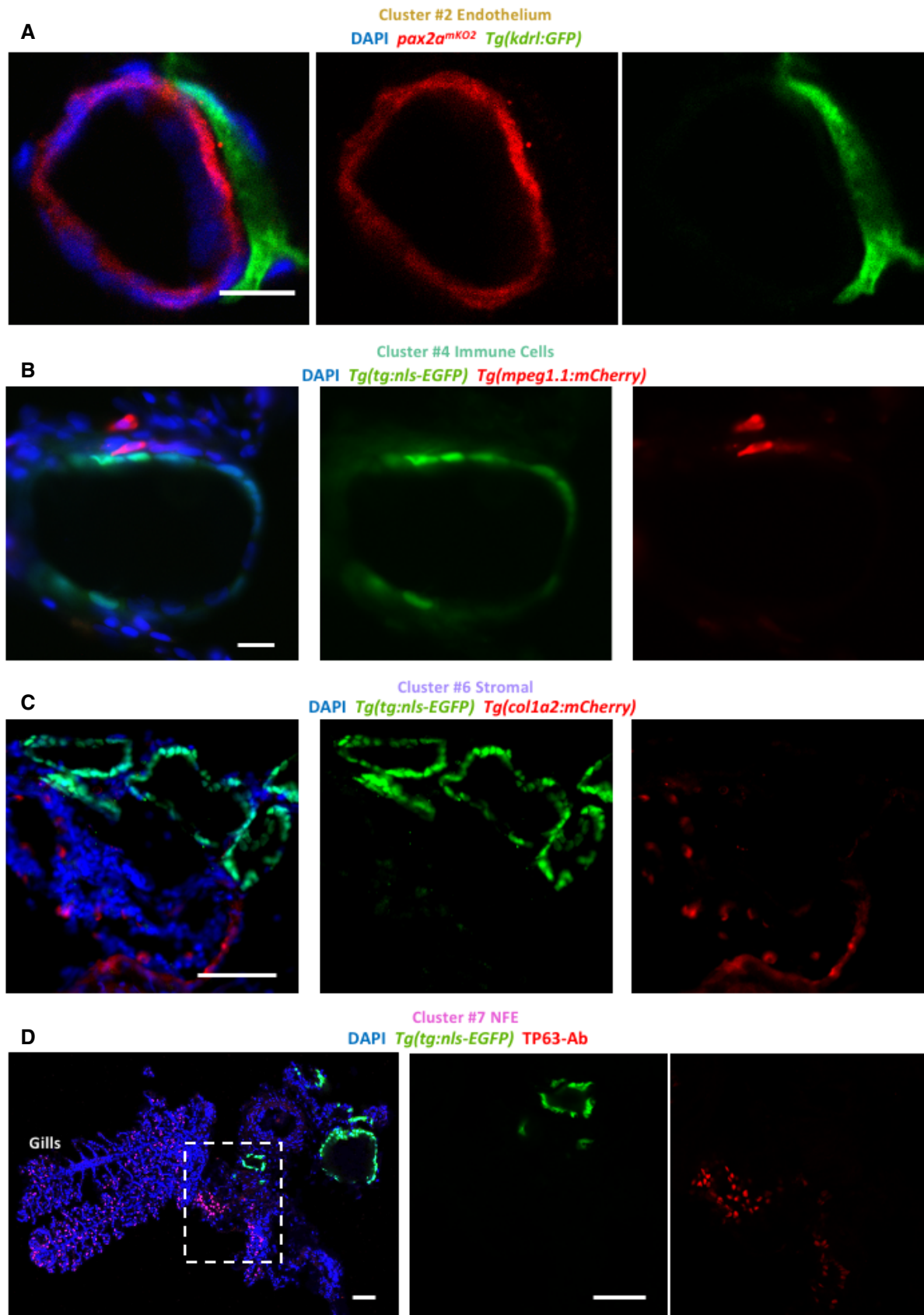
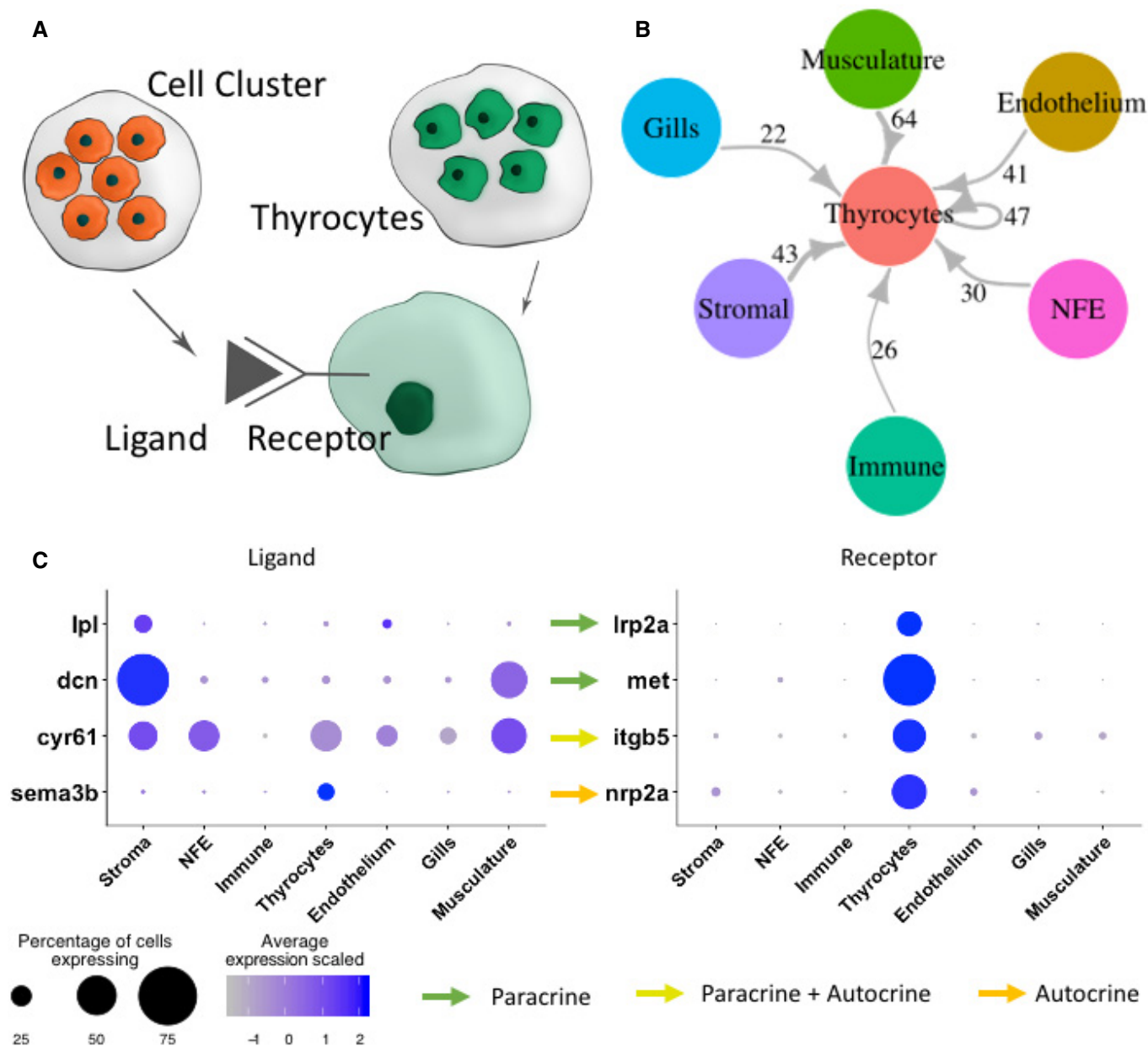


Figure 4.

Figure 4. Immunofluorescence-based visualization of cell types surrounding zebrafish thyroid follicles.

A–D Images show immunofluorescence labeling of thyroid gland from adult zebrafish. Transverse sections were utilized for imaging. The organ was isolated from tissue-specific transgenic lines to allow marking of a particular cell type adjacent to the thyroid follicle. Blood vessels were marked using *Tg(kdr):EGFP* (A), macrophages using *Tg(mpeg1.1:mCherry)* (B), and stroma using *Tg(col1a2:mCherry)* (C). Thyrocytes were labeled with *pax2a^{mko2}* expression in (A) (described in Fig 7) and *Tg(tg:nls-EGFP)* expression in (B, C). (D) NFE was labeled using antibody against TP63 in sections of the thyroid gland isolated from *Tg(tg:nls-EGFP)* animals. Gills are marked based on their morphological appearance. DAPI labels nuclei. Scale bars: 10 μ m (A, B), 50 μ m (C, D).

**Figure 5. Connectome of the zebrafish thyroid gland identifies a dense intercellular signaling network.**

A To build a connectome for the atlas, the ligands expressed specifically in each cell type were matched with their corresponding receptors in the thyrocytes.
 B A highly connected intercellular interaction network is identified by the connectome. The number of ligand–receptor pairs identified between two cell types is denoted alongside the arrows. For NFE, Gills, and Stromal cells, the connectome was restricted to secreted ligands.
 C A dotplot depicting examples of paracrine and autocrine signaling in the thyroid gland. The size of the dots represent percentage of cells expressing the gene in a particular cluster, while the color scale represents average expression of the gene in the cluster after scaling.

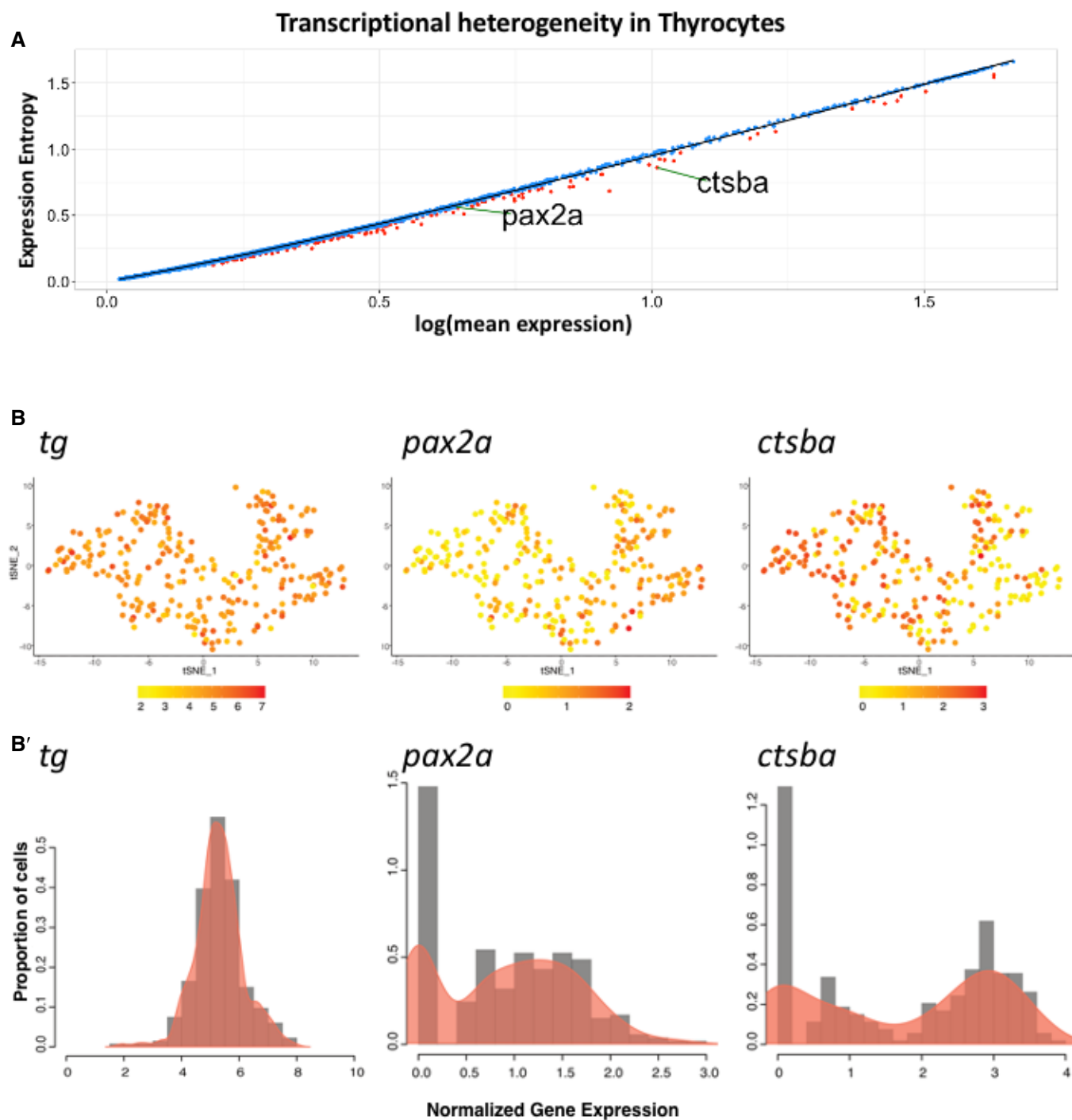


Figure 6. Thyrocytes display transcriptional heterogeneity.

A Dot plot depicting expression entropy on y-axis against average gene expression on x-axis for the thyrocyte population. Each dot depicts a gene, with red dots depicting genes that show statistically significant ($P < 0.05$) difference in entropy from expected value. Expected value is represented by black regression line. *pax2a* and *ctsba* are marked on the graph.

B t-SNE plot of the thyrocyte cluster with expression of *tg*, *pax2a*, and *ctsba*. The color scale represents the normalized expression counts for each gene ranging from lowest (yellow) to highest (red). (B') Histogram overlaid with density plots depicting the distribution of normalized gene expression for *tg*, *pax2a*, and *ctsba*.

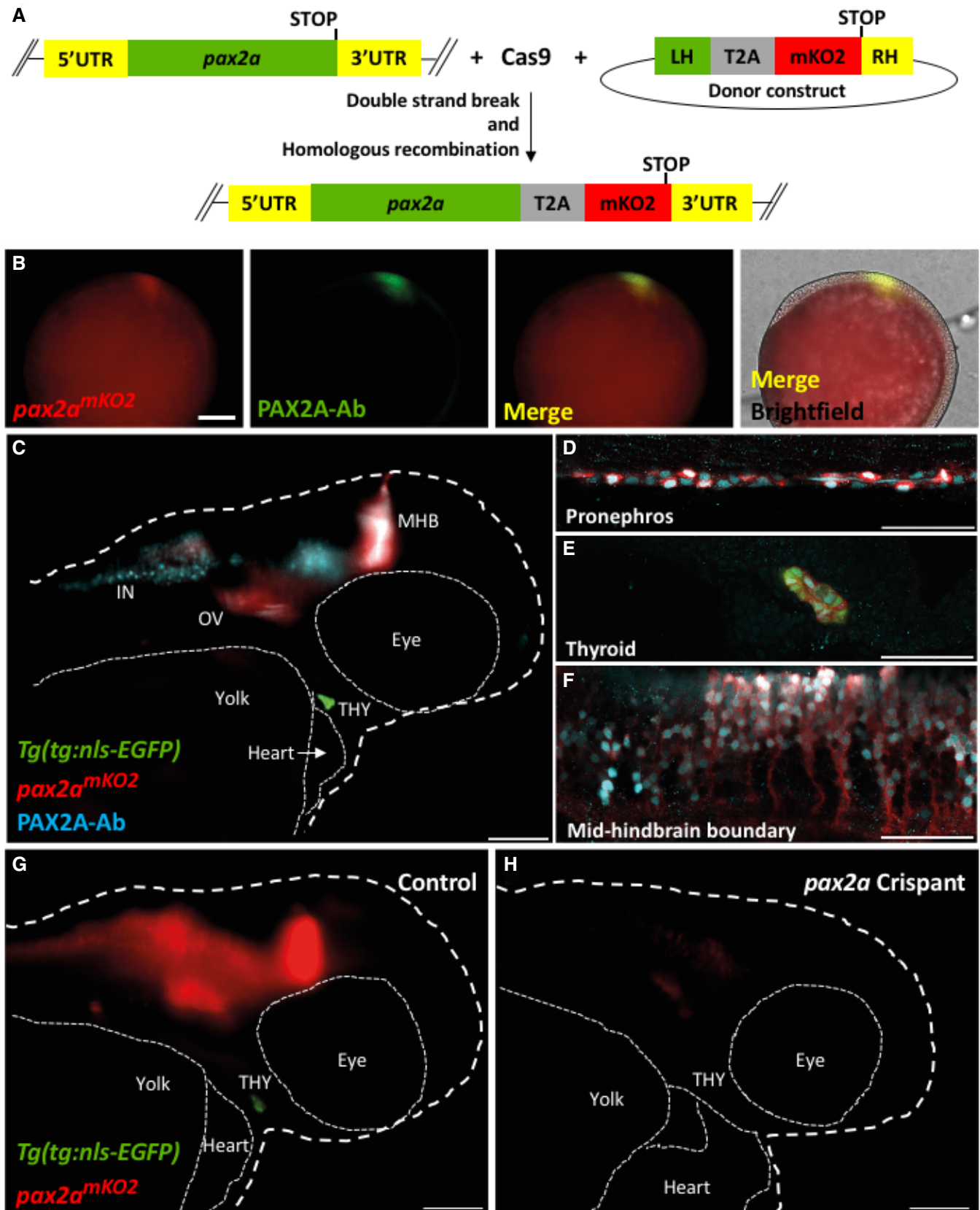


Figure 7.

Figure 7. *pax2a*^{mKO2} knock-in line faithfully reports *pax2a* expression and knock-down.

- A Schematic of the knock-in strategy used to generate the *pax2a*^{mKO2} line. Double strand break was induced between the penultimate codon and the STOP codon of *pax2a* gene using CRISPR/Cas9. DNA repair integrates the donor construct at the site of double strand break, resulting in a *pax2a* reporter line. The donor construct contains T2A-mKO2 reporter cassette flanked by left homology (LH) and right homology (RH) arms.
- B Whole mount immunofluorescence of 9.5 hpf *pax2a*^{mKO2} embryos stained with anti-mKO2 antibody (red) and anti-PAX2A antibody (green). Anterior is to the left, and dorsal side is to the top. Scale bar: 0.1 mm.
- C Whole mount immunofluorescence of 55 hpf *pax2a*^{mKO2}; *Tg(tg:nls-EGFP)* stained with PAX2A antibody (PAX2A-Ab) displays an overlap of mKO2 and PAX2A-Ab signal. The otic vesicle (OV), mid-hindbrain barrier (MHB), interneurons (IN), and thyroid gland (THY) is labeled. White dashed line represents the outline of the zebrafish larvae. Scale bar: 100 μ m. Anterior to the right.
- D–F Confocal microscopy imaging of a sagittal section of a 55 hpf *pax2a*^{mKO2} (red); *Tg(tg:nls-EGFP)* (green) embryos immunostained with PAX2A antibody (cyan) showing co-localization of mKO2 and *pax2a* in the pronephros (D), thyroid gland (E), and mid-hindbrain barrier (F). In the thyroid gland, mKO2 (red), PAX2A-Ab (cyan), and thyrocyte-specific GFP (green) show co-localization. Scale bar: 50 μ m.
- G, H Snapshots from live imaging of 55 hpf *pax2a*^{mKO2}; *Tg(tg:nls-EGFP)* embryos injected with sgRNA targeting *pax2a* coding sequence. The anterior part of a representative control embryo (G) is shown alongside a representative crispant (H). Crispants display a strong reduction of mKO2 fluorescence, as well as an absence of GFP signal suggesting absence of thyroid (THY) tissue. White dashed line represents the outline of the zebrafish larvae. Scale bar: 100 μ m. Anterior to the right.

Porazzi et al, 2009). Consequently, zebrafish lacking *pax2a* fail to develop thyroid follicles (Wendl et al, 2002), which is similar to the Pax8 knockout phenotype in mouse (Mansouri et al, 1998). The low expression of *pax2a* in a subset of thyrocytes suggests the presence of a thyrocyte subpopulation with a distinct gene expression signature.

Generation of *pax2a* knock-in reporter line

To validate the heterogeneity among the zebrafish thyrocytes, we focused on the expression of *pax2a* transcription factor. We generated a knock-in line by inserting monomeric Kusabira-Orange 2 (mKO2) fluorescent protein to the 3' end of the endogenous *pax2a* genomic location (Fig 7A). The mKO2 coding sequence was inserted in-frame with the C terminus of *pax2a*, separated by a T2A-cleavable linker. This leads to generation of two proteins, PAX2A and mKO2, from the *pax2a* regulatory sequence. The two proteins could display differential protein turnover characteristics. The *pax2a*^{Pax2a-T2A-mKO2} (abbreviated as *pax2a*^{mKO2}) reporter expression overlapped with PAX2A antibody staining in a majority of regions at 9.5 h post-fertilization (Fig 7B). Moreover, the knock-in line displayed mKO2 fluorescence in the otic vesicle, mid-hindbrain boundary, optic stalk, pronephros, and the thyroid gland (Fig 7C–F, Movie EV1, Appendix Fig S5), mimicking known expression of *pax2a* during zebrafish development (Kesavan et al, 2017). The difference in levels of PAX2A antibody staining and mKO2 fluorescence could be attributed to different half-lives of the two proteins. In order to assess whether the dynamics of mKO2 expression would follow modifications in the expression of endogenous *pax2a*, we used CRISPR/Cas9 technology to generate F0 knockouts (also known as Crispant (Trubiroha et al, 2018)) of *pax2a* gene in our *pax2a*^{mKO2} line. The crispants displayed defects in thyroid morphogenesis (Fig 7G and H), mimicking the phenotype of *pax2a* loss-of-function mutation (Wendl et al, 2002). Live imaging of crispants at 55 hpf revealed strong decrease of mKO2 expression (Fig 7G and H), thereby corroborating the faithful recapitulation of *pax2a* expression by the newly generated reporter line.

Segregation of thyrocyte subpopulations based on *pax2a* reporter expression

Upon investigating the fluorescence expression of the *pax2a* reporter in the thyroid gland of adult zebrafish, we found strong

and specific expression of *pax2a* reporter in the thyrocytes lining the thyroid follicles (Fig 8A–D). Although a majority of thyrocytes displayed uniform expression of *pax2a* reporter, we could identify a small population of *pax2a*^{mKO2}-Low thyrocytes (Fig 8B–D). The *pax2a*^{mKO2}-Low thyrocytes were not segregated, but scattered throughout the gland, thereby suggesting a mixing of the two thyrocyte subpopulations.

To validate *pax2a* expression heterogeneity at a protein level, we performed immunostaining against PAX2A in thyroid glands obtained from *Tg(tg:nls-EGFP)* animals (Fig 8E). For antibody staining, we utilized 8 μ m thin sections of the thyroid gland to ensure uniform antibody penetration to all cells. Confocal imaging of the stained sections demonstrated the presence of PAX2A-Low and PAX2A-High thyrocytes (Fig 8F). Notably, both PAX2A-Low and PAX2A-High cells display *tg* promoter-driven EGFP expression, thereby confirming their differentiated status.

Further, to quantify the proportions of *pax2a*^{mKO2}-Low and *pax2a*^{mKO2}-High thyrocytes, we performed FACS analysis on *pax2a*^{mKO2}; *Tg(tg:nls-EGFP)* double transgenic line (Fig 9A–C). The *Tg(tg:nls-EGFP)* zebrafish line labels the thyrocyte population in green fluorescence (Trubiroha et al, 2018). We restricted our analysis to the thyrocyte population by gating for GFP⁺ cells in the thyroid gland (Fig 9A). Within the thyrocyte population, the cells displayed a normal distribution of GFP fluorescence; however, thyrocytes could be split into two subpopulations based on the levels of *pax2a* reporter expression (Fig 9B and C). Specifically, 75% of thyrocytes (202 out of 268 cells) displayed *pax2a*^{mKO2}-High fluorescence, while 25% of thyrocytes (66 out of 268 cells) displayed *pax2a*^{mKO2}-Low fluorescence levels.

Next-generation sequencing of *pax2a*^{mKO2}-High and *pax2a*^{mKO2}-Low thyrocytes

To investigate molecular factors that differ between *pax2a*^{mKO2}-High and *pax2a*^{mKO2}-Low thyrocytes, we performed bulk RNA sequencing on FACS-enriched subpopulations (Fig 9C). Three biological replicates were sequenced at a depth of 10 million reads for each subpopulation. During analysis, quality control was performed by filtering genes that displayed expression lower than 0.5 counts per million (CPM) in all six samples. This provided us with expression levels of 10,062 genes for differential gene expression analysis, which revealed differential expression of 2062 genes (Fig 9D) (Table EV4). This corresponds to

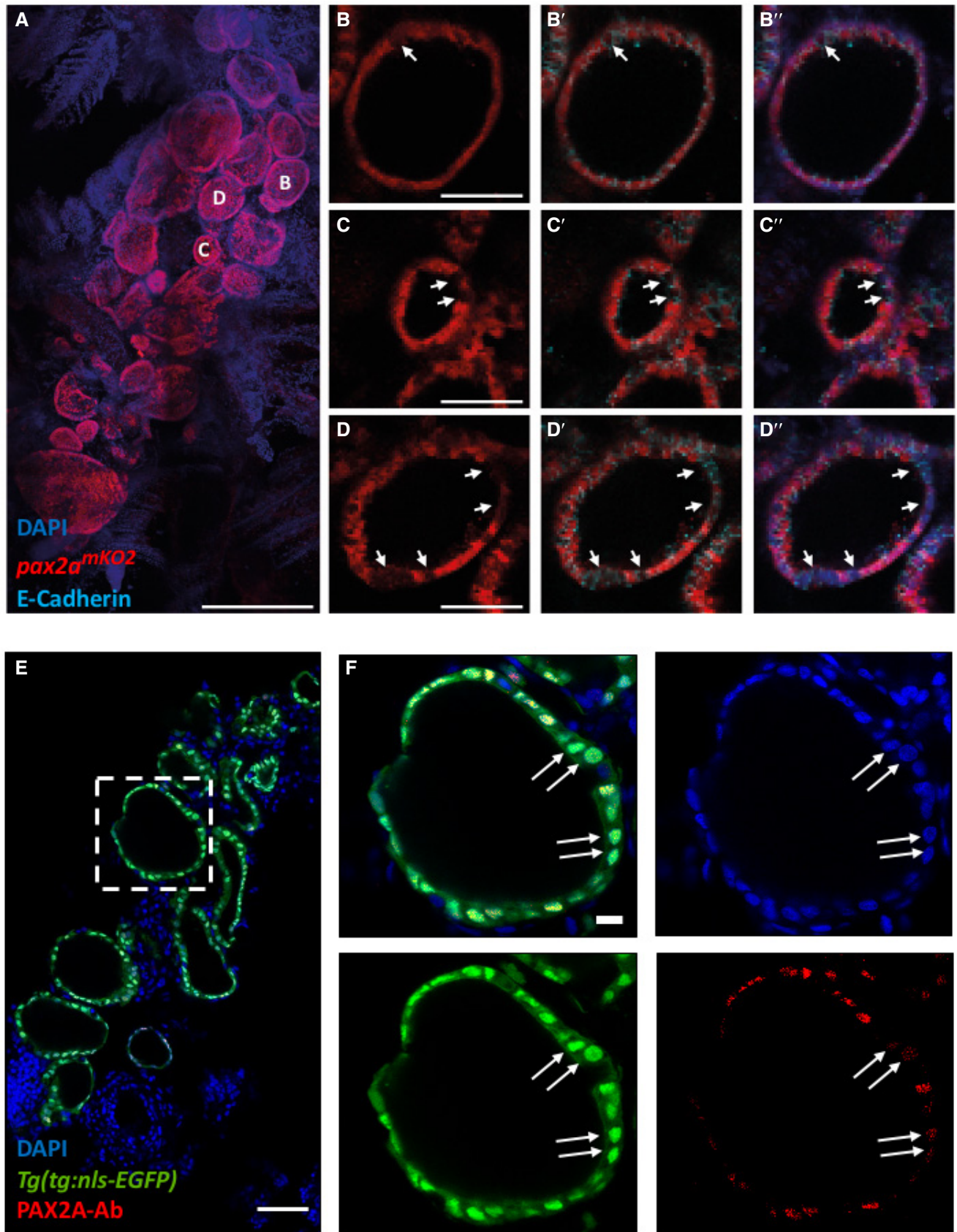


Figure 8.

Figure 8. Immunofluorescence-based validation of thyrocyte heterogeneity.

A–D Analysis of 3 mpf thyroid gland from *pax2a*^{mKO2} zebrafish shows heterogeneity in *pax2a* reporter expression. (A) Whole mount confocal imaging of a 3 mpf *pax2a*^{mKO2} thyroid labeled with mKO2 (red), E-cadherin (cyan, not shown in "A" for clarity reasons) and DAPI (dark blue) for nuclear localization. (B–D) Optical sections of three follicles, with mKO2-Low cells labeled with arrows. E-cadherin (B'–D') and DAPI (B''–D'') staining shows that absence of mKO2 signal does not correspond to an absence of cells. Anterior to the bottom of the pictures.

E, F (E) Confocal image of thyroid gland section from *Tg(tg:nls-EGFP)* at 4 mpf stained with PAX2A antibody and DAPI. The dotted region is displayed at high magnification in (F). Arrows marks thyrocytes displaying low PAX2A staining. Notably, PAX2A-Low thyrocytes display *tg*-driven EGFP expression, demonstrating their differentiated status.

Data information: Scale bars: 250 μ m (A), 50 μ m (B–E), 10 μ m (F).

changes in 20.5 % of detected transcriptome between the two subpopulations.

Specifically, *pax2a*^{mKO2}-High population displayed enrichment in the expression of *pax2a*, mKO2 (driven from *pax2a* locus), *tg* and EGFP (driven from *tg* locus) (Fig 9D). Differential expression of *tg* was observed in spite of the fact that *tg* was detected as one of the top 15 highly expressed gene in both *pax2a*^{mKO2}-High and *pax2a*^{mKO2}-Low populations (Fig EV5). The high expression of *tg* in both populations confirms the identity of the cells as thyrocytes.

In addition, *pax2a*^{mKO2}-High population displayed enrichment in various genes involved in thyrocyte function, including *nkx2.4b*, *slc5a5* (NIS), *TPO*, *tshr*, *ctsba*, and *ctsk* (Cathepsin K) (Fig 9E). Gene ontology (GO) analysis revealed enrichment in genes related to "translation", "macromolecule biosynthetic process", "focal adhesion", and "MAPK signaling" (Appendix Fig S6). The *pax2a*^{mKO2}-Low population, in contrast, displayed enrichment in genes related to "Oxidative phosphorylation", "Inositol phosphate metabolism", and "ATP metabolism". Notably, *pax2a*^{mKO2}-Low population had higher expression of *tgfbr1b* (receptor for TGF- β pathway), *gnai3* (an inhibitor of cAMP activity), *aldob* (enzyme involved in glycolytic process), and *cxcl12a* (a cytokine) (Fig 9E).

In summary, RNA-Seq of thyrocyte population segregated by *pax2a* expression levels, along with the analysis of *pax2a* knock-in line and immunofluorescence imaging of PAX2A protein, validates the identification of thyrocyte heterogeneity within our single-cell RNA-Seq data and clearly demonstrates, for the first time, the presence of transcriptionally diverse subpopulations of thyrocytes present in the thyroid gland.

Discussion

Previous studies on mammalian thyroid gland have identified functional and morphological heterogeneity between the thyroid follicles (Smeds *et al*, 1987; Struder *et al*, 1989; Baptist *et al*, 1995). Using histological analysis, functionally active follicles were identified as being outlined with tall columnar thyrocytes, while inactive follicles were marked by an outline of low cuboidal to almost squamous thyrocytes (Studer *et al*, 1978; Gerber *et al*, 1987). However, two open questions remain unanswered in the field: (a) What are the genes responsible for the functional heterogeneity; and (b) Do all thyrocytes in a follicle display uniform functional capacity. Here, by applying unbiased single-cell gene expression analysis to the thyroid gland for the first time, we have identified transcriptional heterogeneity within the thyrocyte population. Further, we demonstrate that transcriptionally diverse thyrocytes are present in the same

follicle. Thyrocytes differ in the expression levels of a transcription factor *pax2a* and a cysteine protease Cathepsin B (*ctsba*) (Fig 6), among other genes (Table EV3). Cathepsin B is particularly notable as it enables the liberation of thyroid hormone from thyrocytes by proteolytic processing of thyroglobulin (Brix *et al*, 1996, 2001).

We validate the heterogeneity among the thyrocytes using a newly generated knock-in reporter line for *pax2a* gene (Fig 7). The knock-in reporter line was generated using CRISPR/Cas9-based insertion of mKO2 fluorescent protein in the endogenous *pax2a* genomic location. The *pax2a* knock-in line faithfully recapitulates the embryonic expression of *pax2a* gene (Movie EV1, Fig 7B–F). Using the *pax2a* reporter line to characterize the adult thyroid gland, we demonstrate the presence of *pax2a*^{mKO2}-Low thyrocytes in the follicles (Fig 8). Notably, *pax2a*^{mKO2}-Low and *pax2a*^{mKO2}-High thyrocytes are present in the same follicle (Fig 8C, D and F), raising the possibility of contact-mediated interactions between the two subpopulations.

Further, the knock-in line allowed us to segregate the thyrocyte population based on *pax2a* expression levels (Fig 9A–C). RNA-Seq of the two subpopulations revealed enrichment of thyrocyte functional machinery in the *pax2a*^{mKO2}-High population (Fig 9E). Our result suggests that the gland may be divided into functional and resting thyrocytes. It would be of interest to build on this study and investigate the functional and replicative differences among the two subpopulations of thyrocytes. Moreover, it would be important to elucidate the dynamics and plasticity between the two subpopulations as thyrocytes might shuffle between the two states.

Our single-cell transcriptomics atlas provides a comprehensive genomics resource to study the zebrafish thyroid gland in unprecedented detail. We performed unbiased profiling of the thyroid gland, without enrichment for a specific cell type. This allowed us to capture yet poorly characterized cell populations within the thyroid gland. Specifically, we provide the molecular characteristics of the stromal tissue present in the zebrafish thyroid gland. The stromal cells (Fig 4C) display enrichment of extracellular matrix (ECM)-related genes (Table EV1) and are possibly homologous to the mesenchymal connective septa found in the mammalian thyroid gland. The connective septa help cluster the thyroid follicles into lobules. Notably, the expression of *fgf* ligands from the mesenchymal septa cells has been implicated in lobe formation during mouse thyroid gland development (Liang *et al*, 2018). It would be of interest to test whether similar morphological clustering of the thyroid follicles exists in zebrafish, and the role the stromal cells play during development and growth of the thyrocytes.

Our atlas further identifies a non-follicular epithelial (NFE) cell population present near the zebrafish thyroid follicles. A subset of NFE are present in the gills (Fig 4D) and may potentially represent a

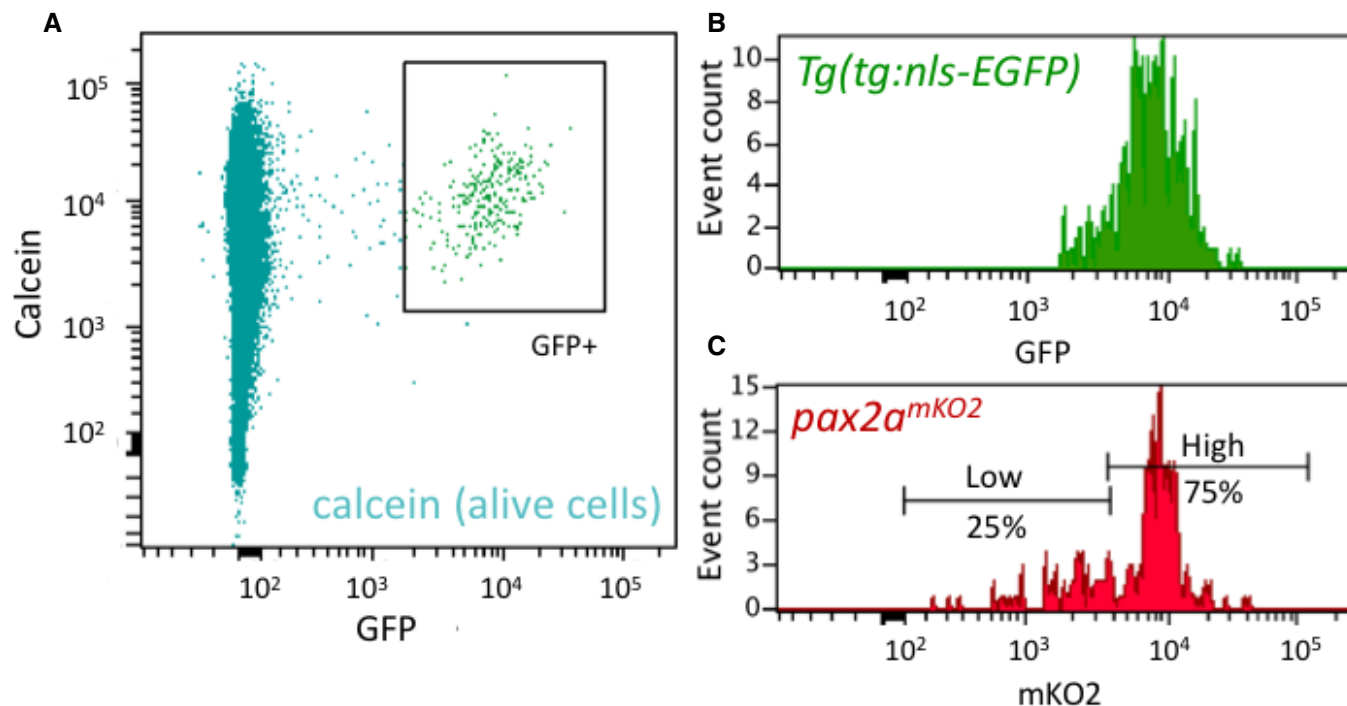
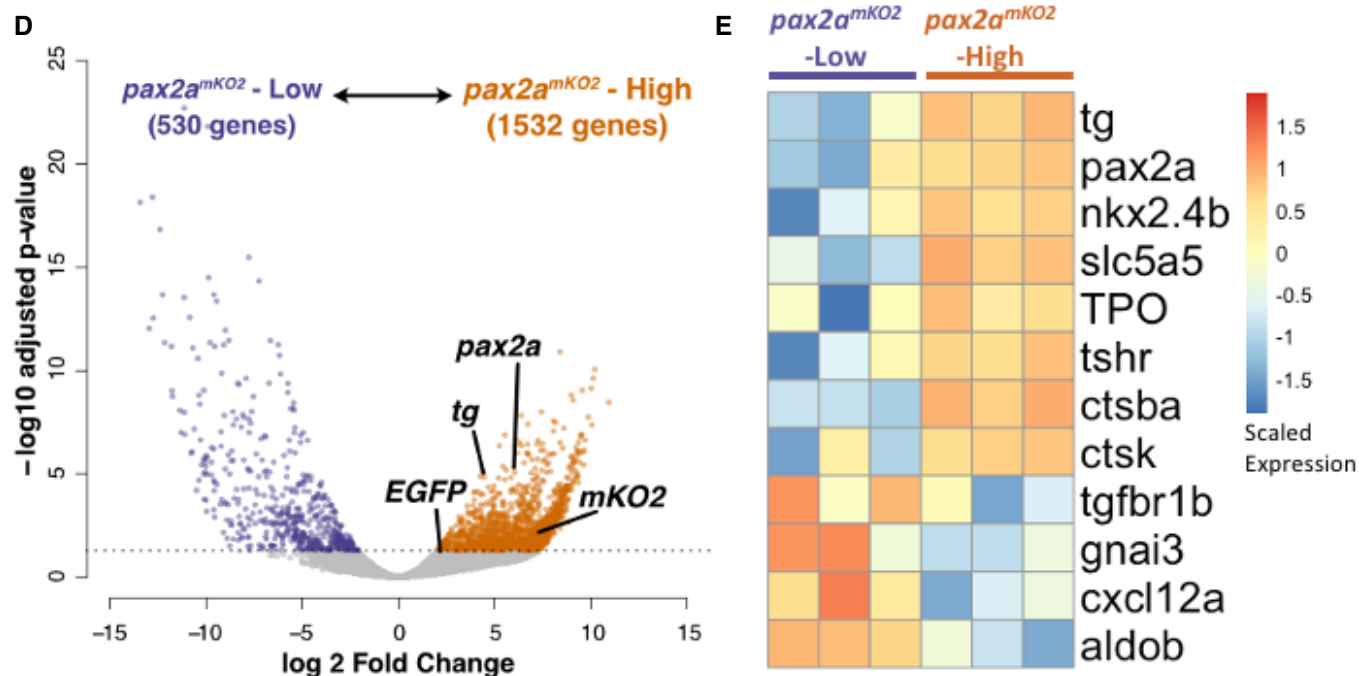
FACS analysis of *Tg(tg:nls-EGFP); pax2a^{mKO2}*RNA-Seq. of *pax2a^{mKO2}*-Low vs. *pax2a^{mKO2}*-High Thyrocytes

Figure 9.

Figure 9. Transcriptome profiling of *pax2a*^{mKO2}-High and *pax2a*^{mKO2}-Low thyrocytes.

- A–C Cells from the thyroid gland of 5 mpf *Tg(tg:nls-GFP); pax2a*^{mKO2} animals were labeled with calcein (live cell marker) and analyzed using FACS. (A) A FACS plot showing calcein on y-axis and GFP on x-axis. The box encompassing the GFP⁺ cells represents the thyrocyte population, which was gated for further analysis. (B) Histogram showing the distribution of GFP intensity in thyrocytes. (C) Histogram showing the distribution of mKO2 intensity in thyrocytes. Thyrocytes were selected by gating for GFP⁺ population. Horizontal lines indicate the mKO2-Low and mKO2-High expression level, with percentage values representing proportion of thyrocytes with mKO2-Low and mKO2-High expression.
- D, E RNA-Seq analysis of transcriptome isolated from *pax2a*^{mKO2}-High and *pax2a*^{mKO2}-Low subpopulations. (D) Volcano plot representing the differential expression of genes between the two thyrocyte subpopulations. Dots for *pax2a*, *tg*, mKO2, and EGFP are marked. Dots for genes that display significant differential expression (DESeq2, adjusted *P*-value < 0.05) are colored. (E) Heatmap displaying the expression of selected genes in the two subpopulations. Biological replicates are represented by individual columns.

progenitor population for the gills, similar to the TP63⁺ basal layer in the zebrafish (Guzman *et al*, 2013) and mammalian (Yang *et al*, 1999) epithelium. We also observe NFE outside the gills (Fig 4D), which may play a different role. It is interesting to note that epithelial cells apart from follicular and parafollicular cells have been observed in the mammalian thyroid gland. In a report from Dr. E. Baber published in 1876 (Baber, 1876), histological examination of the dog thyroid gland displayed the presence of cells “beside the stroma, lymphatics, blood vessels, & cells between the vesicles”. Dr. Baber labeled the cells as “parenchyma” and noted the existence of “numerous cells differing markedly in size and shape from the epithelial cells amongst which they lie” (Baber, 1876). In 1907, Dr. Sophia Getzowa described an epithelial cells containing structure called the Solid Cell Nests (SCN) of the thyroid (Getzowa, 1907). SCN are lumen containing irregular structures located within the thyroid in mammals (Harach, 1988). SCN contain two types of epithelial cells: main cells and C-cells, expressing TP63 and calcitonin respectively (Ríos Moreno *et al*, 2011). Notably, the NFE cells we identified in the zebrafish thyroid gland are marked with TP63 expression (Fig 3G, 4D), raising the possibility of their homology with the main cells of the SCN. C-cells, however, exist in the ultimobranchial bodies, which lies outside the thyroid gland in zebrafish. The ultimobranchial bodies are the zebrafish homologues of parafollicular cells and are located as a pair of follicles on top the sinus venous, adjacent to the atrium and esophagus (Alt *et al*, 2006). Cells adjacent to the atrium were removed during our dissections (Fig 1B). Additionally, NFE cells identified in our atlas do not express the zebrafish homologue of calcitonin (*calca*) (Table EV1). Thus, it is unlikely that the NFE cells we have identified would be related to cells of parafollicular origin. Currently, the developmental origin of NFE cells and their role in thyroid gland is unclear. To study the cell population, transgenic zebrafish reagents driving expression using the *tp63* regulatory region (Rasmussen *et al*, 2015) could be utilized in future.

To survey the communication between thyrocytes, the functional unit of the thyroid gland, and the other cell types present in the thyroid gland, we constructed a cellular interaction network (Fig 5B). The network was built by matching the expression of ligands in the diverse cell types with the expression of receptor in the thyrocytes (Table EV2) (Ramilowski *et al*, 2015). Based on literature survey, we manually identified multiple interacting genes that have been implicated in thyroid diseases. For instance, the ligand Decorin (*dcn*) is expressed by the stromal cells and musculature, with its receptor, MET, present on thyrocytes (Fig 5C). Decorin, a secreted proteoglycan, is considered a “guardian from the matrix” (Neill *et al*, 2012), as it is an antagonist of growth factor signaling. Importantly, Decorin expression has been reported to be downregulated in thyroid cancer

samples (Martínez-Aguilar *et al*, 2016). Thus, stromal cells could modulate Decorin to control thyrocyte growth. Further, interactions for CYR61 (associated with Graves’ Disease (Planck *et al*, 2014)), LRP2/ Megalin (involved in thyroglobulin processing (Marinò & McCluskey, 2000)), and NRP2 (associated with thyroid cancer metastasis (Tu *et al*, 2016)) were identified (Fig 3C). The hypothesis generated by the theoretical ligand–receptor interaction network can be tested *in vivo* in zebrafish or *in vitro* by manipulation of thyrocytes in thyroid organoid models (Antonica *et al*, 2012) to gain valuable insight into thyroid gland homeostasis.

The current atlas is restricted to healthy juvenile and adult thyroid gland. The two stages represent a period of slow growth in zebrafish. Thus, genes driving cellular proliferation might be repressed at these stages. Additionally, the low number of cells per cluster obtained at each stage restricts an in-depth analysis of the transcriptional difference with age. In future, it would be of interest to extend the atlas by increasing cell numbers and by including single-cell transcriptomics from embryonic and old fish, providing a comprehensive resource for development, homeostasis and aging of the thyroid gland. It would be of further interest to profile zebrafish models of thyroid disorder (Anelli *et al*, 2017; Chopra *et al*, 2019) to understand the cellular and molecular changes underlying organ dysfunction. Combined with the power of CRISPR/Cas9 based screen that we have established for the thyroid gland (Trubiroha *et al*, 2018), this resource will provide a roadmap for the functional elucidation of cell type–specific programs during thyroid gland growth and homeostasis.

In summary, our work provides the first molecular map of the zebrafish thyroid gland at cellular resolution. The atlas contains the molecular characterization of the thyroid gland stromal population and identification of non-follicular epithelial cells and demonstrate the transcriptional heterogeneity among zebrafish thyrocytes. Further, by constructing cell–cell communication network, the atlas provides clues into tissue dynamics present within the organ. Finally, the dataset has been made available for browsing via an interactive webtool (<https://sumeet.shinyapps.io/zfthyroid/>). We hope that our efforts will expand the understanding of thyrocytes as a collection of heterogenous endocrine cell population, providing a complex picture of the diversity in thyrocyte identity and function.

Materials and Methods

Cell Zebrafish strains and husbandry

Wild-type or transgenic zebrafish of the outbred AB, WIK, or a hybrid WIK/AB strain were used in all experiments. Zebrafish were raised under standard conditions at 28 °C. Animals were chosen at

random for all experiments. Published transgenic strains used in this study were *Tg(tg:nls-mVenus-T2A-NTR)* (McMenamin *et al.*, 2014), *Tg(tg:nls-EGFP)* (Trubiroha *et al.*, 2018), *Tg(kdrl:GFP)^{la116}* (Choi *et al.*, 2007), *Tg(mpeg1.1:mCherry)^{gl23}* (Ellett *et al.*, 2011), and *Tg(col1a2:LOXP-mCherry-NTR)^{cn11}* (referred as *Tg(Col1a2:mCherry)*) (Sánchez-Iranzo *et al.*, 2018). Experiments with *Tg(tg:nls-mVenus-T2A-NTR)* were conducted in accordance with the Animal Welfare Act and with permission of the Landesdirektion Sachsen, Germany (DD24-5131/346/11, DD24-5131/346/12, DD24.1-5131/476/2, TVV21/2018, and all corresponding amendments). Zebrafish husbandry and experiments with all transgenic lines will be performed under standard conditions as per the Federation of European Laboratory Animal Science Associations (FELASA) guidelines (Aleström *et al.*, 2020), and in accordance with institutional (Université Libre de Bruxelles (ULB)) and national ethical and animal welfare guidelines and regulation, which were approved by the ethical committee for animal welfare (CEBEA) from the Université Libre de Bruxelles (protocols 578N-579N).

Dissection of the zebrafish thyroid gland

The dissection of thyroid gland in zebrafish was performed by using the ventral aorta as a reference (Fig 1A and B). In zebrafish, the thyroid follicles sit loosely in soft tissue around the ventral aorta. Ventral aorta connects to the outflow tract that further joins with the heart ventricle. During dissociation, cells connected to the ventral aorta, including parts of zebrafish gills (Fig 1C) were kept intact to avoid injuring the organ during dissociation.

In detail, zebrafish were euthanized in 0.2% Tricaine (MS-222, Sigma E10521) solution. Using fine forceps, the lower jaw was separated from the upper jaw and disconnected from the gut by pinching near the gills. The dissected tissue was carefully cleaned by removing muscle, skin, pectoral fin, and lateral cartilages of the lower jaw. The cleaned tissue when observed from the ventral side under brightfield clearly shows the ventral aorta as a thick pink blood vessel extending from the heart toward the basibranchial cartilage (Fig 1A). Next, the surrounding gills are pinched off using fine forceps, taking care to keep the ventral aorta intact (Fig 1B). This leaves small parts of gills connected to the ventral aorta (Fig 1C). Lastly, the ventral aorta is disconnected from the outflow tract by pinching with fine forceps (dashed line in Fig 1B).

Single-cell suspension of zebrafish thyroid gland

Single-cell suspension of zebrafish thyroid gland was performed by adapting the cell dissociation protocol outlined in Singh *et al.*, Scientific Reports, 2018 (Singh *et al.*, 2018). In brief, the thyroid gland was collected and dissociated into single cells by incubation in TrypLE (Thermo Fisher, 12563029) with 0.1% Pluronic F-68 (Thermo Fisher, 24040032) at 37°C in a benchtop shaker (Biosan TS-100) set at 450 rpm for 45 min. Following dissociation, TrypLE was inactivated with 10% FBS, and the cells pelleted by centrifugation at 500 g for 10 min at 4°C. The supernatant was carefully discarded and the pellet re-suspended in 500 µl of HBSS (without Ca, Mg) + 0.1% Pluronic F-68. To remove debris, the solution was passed over a 30 µm cell filter (Miltenyi Biotec, 130-041-407). To remove dead cells, calcein violet (Thermo Fisher, C34858) was added at a final concentration of 1 µM and the cell suspension

incubated at room temperature for 20 min. The single-cell preparation was sorted with the appropriate gates, including excitation with UV (405 nm) laser for identification of alive cells (calcein⁺) (Fig 1F and G). FACS was performed through 100 µm nozzle.

Single-cell profiling of the zebrafish thyroid gland

For single-cell RNA-seq of the zebrafish thyroid gland using the 10× Genomics platform, cell suspension was prepared as mentioned above from the thyroid glands of six 2-month post-fertilization and six 8-month post-fertilization *Tg(tg:nls-mVenus-T2A-NTR)* animals. The cell suspension was adjusted with Hanks' Balanced Salt Solution (without calcium and magnesium) to a density of 800 cells/µl and diluted with nuclease-free water according to the manufacturer's instructions to yield 12,000 cells. Subsequently, the cells were carefully mixed with reverse transcription mix before loading the cells on the 10× Genomics Chromium system (Zheng *et al.*, 2017). After the gel emulsion bead suspension underwent the reverse transcription reaction, emulsion was broken and DNA purified using Silane beads. The complementary DNA was amplified with 10 cycles, following the guidelines of the 10× Genomics user manual. The 10× Genomics single-cell RNA-seq library preparation— involving fragmentation, dA tailing, adapter ligation, and indexing PCR— was performed based on the manufacturer's protocol. After quantification, the libraries were sequenced on an Illumina NextSeq 550 machine using a HighOutput flowcell in paired-end mode (R1: 26 cycles; I1: 8 cycles; R2: 57 cycles), thus generating ~45 mio fragments. The raw sequencing data were then processed with the "count" command of the Cell Ranger software (v.2.1.0) provided by 10× Genomics with the option "--expect-cells" set to 10,000 (all other options were used as per default). To build the reference for Cell Ranger, zebrafish genome (GRCz10) and gene annotation (Ensembl 87) were downloaded from Ensembl and the annotation was filtered with the "mkgf" command of Cell Ranger (options: "--attribute = gene_biotype:protein_coding-- attribute = gene_biotype:lincRNA --attribute = gene_biotype:antisense"). Genome sequence and filtered annotation were then used as input to the "mkref" command of Cell Ranger to build the appropriate Cell Ranger Reference.

Analysis of single-cell RNA-Seq of the zebrafish thyroid gland

The raw data generated from 10× Chromium pipeline was clustered using Seurat 2.3.4 (Butler *et al.*, 2018) using the recommended analysis pipeline. Briefly, the raw data as UMI counts were log-normalized, regressed to remove the effect of library size and mitochondrial counts, and scaled. Highly variable genes were identified for PCA analysis and graph-based clustering using shared nearest neighbor (SNN). For clustering, the first five principal components (PCs) were utilized as they displayed significant deviation from uniform distribution as accessed by JackStraw analysis. Further, a resolution of 0.3 for SNN was used for clustering. Marker genes identified for each cluster were used to classify the cell type.

Development of intercellular signaling network

Development of intercellular signaling network for zebrafish was performed as described in Cosacak *et al.* (2019). Briefly, ligands expressed in 20% of a cell population were identified. A connection between cell type and thyrocyte was made if the expression of the

corresponding receptor was identified in 20% of thyrocytes. The connectome contains secreted and membrane-tethered ligands. For cell types that do not physically contact the thyrocytes (gills, NFE and stroma), membrane-tethered ligands were manually removed from the connectome.

Background correction for thyrocyte gene expression

Supervised background correction for the thyrocyte population was performed using DecontX (Yang *et al*, 2020). As input, normalized data and clustering information from Seurat were used. The method using Bayesian approach to model gene expression as a mixture of expression in the expected cell population plus background expression accessed from remaining cell types. Background noise is removed, which likely resembles free mRNA released from injured and ruptured cells. As background correction required clustering information, the corrected data were not utilized for re-clustering to avoid circular use of the data.

Genetic entropy analysis for thyrocyte population

Quantification of genetic entropy was performed using ROGUE (Ratio of Global Unshifted Entropy; preprint: Liu *et al*, 2019). As input, raw counts of thyrocytes that passed quality control were used. Default parameters were used for analysis. The algorithm provided a measure of entropy (degree of uncertainty/ heterogeneity), along with *P*-value, within the population.

Generation of knock-in *pax2a*^{*pax2a-T2A-mKO2*} zebrafish line

For generation of *pax2a* reporter line, we designed a single-guide RNA (sgRNA) targeting the STOP codon of the *pax2a* coding sequence (GCTGCGATGCTAACTAGTGG). We then generated a donor construct in which the sequence encoding for the monomeric Kusabira-Orange (mKO2) protein was fused to a viral 2A peptide linker. This reporter cassette was flanked by left (1,000 bp) and right (2000bp) homology arms of the *pax2a* genomic DNA region around the stop codon therefore preventing the sgRNA from cutting the donor construct. sgRNA design, production, and validation were done as previously described (Varshney *et al*, 2015; Trubiroha *et al*, 2018). Wild-type embryos were injected with 3 nl of the injection mix containing the sgRNA (final concentration 80 ng/μl), the donor construct (final concentration 7.5 ng/μl), the protein Cas9 (recombinant cas protein from *S. pyogenes* PNA Bio CP01, final concentration 100 ng/μl), and KCL (final concentration 200 mM). Upon homologous recombination of this reporter construct in the endogenous locus, *pax2a*-expressing cells were fluorescently labeled by mKO2. This *pax2a*^{*pax2a-T2A-mKO2*} line is referenced as *pax2a*^{mKO2} in the text.

Generation of *pax2a* crispants

Somatic mutagenesis of *pax2a* gene was carried out exactly as mentioned in Trubiroha *et al* (2018). Briefly, sgRNA targeting the exon 2 of *pax2a* was generated as described in the publication. Following the strategy described in the publication, Cas9 protein along with sgRNA was injected in one-cell stage of zebrafish embryos for disruption of *pax2a* gene. Non-injected animals were used as controls.

Tissue collection

To facilitate confocal imaging of the thyroid gland, the organ was manually dissected from fish as previously described and fixed. Fish were killed in Tricaine followed by dissection of the gland, which was fixed by immersion in 4% paraformaldehyde (PFA) + 1% Triton X overnight at 4°C. The gland was washed 2–3 times in PBS to remove PFA before proceeding.

Quantification of proportion of thyrocytes within the dissected tissue

To quantify the proportion of thyrocytes within the dissected tissue, the gland was dissected from *Tg(tg:nls-EGFP)* animals and fixed as described above. The fixed tissue was permeabilized by three washes with 1% PBT (1× PBS + 1% Triton X-100). Nuclei were stained by immersing the tissue in 1 μg/ml Hoechst prepared in 1× PBS for 2 h at room temperature. The tissue was immersed in 30% sucrose solution overnight at 4°C, embedded in Tissue Freezing Medium (Leica 14020108926) and frozen at −80°C. Thin sections (8 μm) were obtained using cryostat (Leica CM3050 S), collected on frosted glass slides (Thermo Scientific 12362098), and covered with glass coverslip of #1 thickness (Carl Roth GmbH NK79.1) using mounting media (Dako S3023). The sections were imaged on Zeiss LSM 780 confocal microscope. Confocal images were analyzed in Fiji using the following step: threshold using "IsoData" to distinguish signal from background, "watershed" transformation to separate joined nuclei and "measure" function to obtain nuclei count. With this, the green channel (number of thyrocyte nuclei) and blue channel (total number of nuclei) was measured for seven transverse sections obtained from three animals. Percentage was calculated by taking the ratio of thyrocyte nuclei to total nuclei.

Immunofluorescence and image acquisition

Whole mount immunofluorescence was performed on thyroid gland collected as described above. The collected samples were permeabilized in 1% PBT (Triton X-100) and blocked in 4% PBTB (BSA). Primary and secondary antibody stainings were performed overnight at 4°C. Primary antibodies used in this study were anti-PAX2A (rabbit, Genetex GTX128127) at 1:250, anti-EGFP (chicken, Abcam ab13970) at 1:1,000, anti-E-Cadherin (mouse, BD bioscience cat 610181) at 1:200, anti-monomeric Kusabira-Orange 2 (mouse, MBL amalgam M-168-3M) at 1:200, anti-monomeric Kusabira-Orange 2 (rabbit, MBL amalgam PM051M) at 1:250, and anti-p63 (mouse, Santa Cruz Biotechnology 4A4) at 1:200. Secondary antibodies at 1:250 dilutions used in this study were Alexa Fluor 488 anti-chicken (Jackson ImmunoResearch laboratories 703-545-155), Alexa Fluor 647 anti-rabbit (Jackson ImmunoResearch laboratories 711-605-152), Alexa Fluor 647 anti-mouse (Jackson ImmunoResearch laboratories 715-605-150), CyTM3-conjugated anti-rabbit (Jackson ImmunoResearch laboratories 711-165-152), and CyTM3-conjugated anti-mouse (Jackson ImmunoResearch laboratories 715-165-150). When needed, nuclei were staining using DAPI at a 1:1,000 dilution. Samples were mounted in NuSieveTM GTGTM Agarose (Lonza cat50080) and imaged on a glass bottom FluoroDishTM (WPI FD3510-100) using a Zeiss LSM 780 confocal microscope or Leica DMI 6000b microscope. ImageJ was

used to add scale bars and PowerPoint was used for adding arrows and labels.

FACS-based reporter analysis

For analyzing the levels of *pax2a^{mKO2}* by FACS, single-cell suspension from the thyroid gland of 5 mpf *Tg(tg:nls-GFP); pax2a^{mKO2}* animals was prepared as described earlier and stained with 1 μ M calcein violet (Thermo Fisher, C34858). Cells were sorted and analyzed using FACS-Aria II (BD Bioscience). Thyrocytes were selected by gating for calcein⁺GFP⁺ population, and mKO2 expression level recorded for analysis.

Gene ontology (GO) analysis

Gene ontology (GO) analysis was performed using DAVID (Huang *et al.*, 2007). The list of genes was uploaded on the web browser of DAVID and statistically significant ($P < 0.05$) GO terms were identified using default parameters.

Transcription profiling and analysis of *pax2a^{mKO2}*-High and *pax2a^{mKO2}*-Low thyrocytes

For transcriptional profiling of *pax2a^{mKO2}*-High and *pax2a^{mKO2}*-Low thyrocytes, mRNA was isolated from cell populations using Relia-PrepTM RNA Miniprep Systems (Promega, Z6011). For this, cells were passed through FACS as outlined above and approximately 100 cells were collected directly in the lysis buffer provided in the kit. mRNA was isolated as outlined in the kit protocol. Three biological replicates for each population.

For sequencing, mRNA was reverse transcribed to cDNA and amplified SMART-seq V4 Ultra Low Input RNA kit for Sequencing (Takara Bio USA, Inc) following the manufacturer's protocol. Amplified cDNA was further processed according to TruSeq Sample Preparation v.2 Guide (Illumina) and paired end-sequenced (2×75 bp) on the HiSeq 2500 (Illumina) at a depth of 10 reads per sample. Raw reads were trimmed using trim-galore (Martin, 2011), mapped using HiSAT2 (Kim *et al.*, 2015) against the GRCz11 zebrafish genome (in which mKO2 and EGFP sequences were added), and counted using FeatureCounts (Liao *et al.*, 2014). For normalization, differential gene expression analysis, and GO analysis, iDEP version 0.92 was utilized (Ge *et al.*, 2018).

Statistical analysis

Statistical analysis was performed using R. Power analysis was not performed to estimate sample sizes. Animals were randomly chosen for experimentation. No animals were excluded from analysis. Blinding was not performed during analysis. Analysis of normal distribution was performed using Shapiro–Wilk test.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE133466 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133466>).

- RNA-Seq data: Gene Expression Omnibus GSE153197 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153197>)
- Interactive browser: Shinyapps (<https://sumeet.shinyapps.io/zfthyroid/>)

Expanded View for this article is available online.

Acknowledgements

We thank members of the Costagliola and Singh laboratory for comments on the manuscript, members of Center for Regenerative Therapies Dresden (CRTD) fish, FACS and sequencing facility, and members of IRIBHM fish facility for technical assistance. We thank J.-M. Vanderwinden from the Light Microscopy Facility and Christine Dubois from the FACS facility for technical assistance at ULB. Bulk RNA sequencing was performed at the Brussels Interuniversity Genomics High Throughput core (BRIGHTcore: www.brightcore.be). We are grateful to Priyanka Oberoi for illustrations. P.G. is Fund for Research in the Industry and the Agriculture (FRIA) Research fellow; M.S. is FNRS Research Fellow (34985615 - THYSCEFA); S.C. is FNRS Senior Research Associate. V.D. acknowledges grants from the Fond Naets (J1813300), the Fondation Contre le Cancer (2016-093) and FNRS (EQP/OL U.N019.19, J006120F). Work by M.B., C.L., and G.K. was supported by grants to M.B. from the Deutsche Forschungsgemeinschaft (BR 1746/11-1) and European Union (European Research Council AdG Zf-BrainReg). N.N. received funding from the DFG–Center for Regenerative Therapies Dresden at TU Dresden and the German Center for Diabetes Research (DZD), as well as research grants from the German Research Foundation (DFG) and the European Foundation for the Study of Diabetes. Work by S.P.S. was supported by MISU funding from the FNRS (34772792 – SCHISM). This work was supported by grants from the Belgian National Fund for Scientific Research (FNRS) (FRSM 3-4598-12; CDR-J.0145.16, GEQ U.G030.19), the Fonds d'Encouragement à la Recherche de l'Université Libre de Bruxelles (FER-ULB).

Author contribution

SPS conceptualized the project. NN, GK, CL, and MB provided reagents and animals for single-cell RNA sequencing. SPS, SR, AK, JB, and AP performed the single-cell RNA sequencing. SPS, SEE, VD, and SC analyzed and interpreted the data. SPS developed the online browser. PG and BH generated the *pax2a* knock-in line. PG and MS analyzed the *pax2a* reporter line and MP-M and IG collected immunofluorescence images. AL and FL performed bulk RNA-Seq experiment, with data analysis being performed by SPS. SPS wrote the first draft and SC, PG, and SEE edited the manuscript. SPS, NN and SC acquired funding for the project. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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