RESEARCH ARTICLE

Multi-omic profiling of pituitary thyrotropic cells and progenitors

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Abstract

Background: The pituitary gland is a neuroendocrine organ containing diverse cell types specialized in secreting hormones that regulate physiology. Pituitary thyrotropes produce thyroid-stimulating hormone (TSH), a critical factor for growth and maintenance of metabolism. The transcription factors POU1F1 and GATA2 have been implicated in thyroprote fate, but the transcriptomic and epigenomic landscapes of these neuroendocrine cells have not been characterized. The goal of this work was to discover transcriptional regulatory elements that drive thyroprote fate.

Results: We identified the transcription factors and epigenomic changes in chromatin that are associated with differentiation of POU1F1-expressing progenitors into thyrotropes using cell lines that represent an undifferentiated Pou1f1 lineage progenitor (GHF-T1) and a committed thyroprote line that produces TSH (TαT1). We compared RNA-seq, ATAC-seq, histone modification (H3K27Ac, H3K4Me1, and H3K27Me3), and POU1F1 binding in these cell lines. POU1F1 binding sites are commonly associated with bZIP transcription factor consensus binding sites in GHF-T1 cells and Helix-Turn-Helix (HTH) or basic Helix-Loop-Helix (bHLH) factors in TαT1 cells, suggesting that these classes of transcription factors may recruit or cooperate with POU1F1 binding at unique sites. We validated enhancer function of novel elements we mapped near Cga, Pitx1, Gata2, and Tshb by transfection in TαT1 cells. Finally, we confirmed that an enhancer element near Tshb can drive expression in thyrotropes of transgenic mice, and we demonstrate that GATA2 enhances Tshb expression through this element.

Conclusion: These results extend the ENCODE multi-omic profiling approach to the pituitary gland, which should be valuable for understanding pituitary development and disease pathogenesis.

Keywords: TSH, Chromatin, bZIP, bHLH, bHTH, POU1F1, GATA2

Background

Recent genome-wide association studies (GWAS) have begun to identify loci that are associated with sporadic pituitary adenomas and variation in normal height, but the genes associated with many of these loci are unknown [1–3]. Nearly 90% of GWAS hits are in noncoding regions, making it difficult to transition from genetic mapping to biological mechanism [4]. Recent studies that identify enhancer regions by undertaking large-scale functional genomic annotation of noncoding elements like Encyclopedia of DNA Elements (ENCODE) have begun to yield a better understanding of some complex phenotypes and diseases. Dense molecular profiling maps of the transcriptome and epigenome have been generated for more than 250 cell lines and 150 tissues, but pituitary cell lines or tissues were not included. This represents a major limitation, as the cell types that comprise the pituitary gland secrete hormones responsible for growth (growth hormone secreted by somatotropes), reproduction (gonadotropins secreted by gonadotropes),

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adrenal gland function and the stress response (ACTH secreted by corticotropes), lactation (prolactin secreted by lactotropes), and thyroid gland function (thyroid-stimulating hormone secreted by thyrotropes). Epigenomic and gene expression data are emerging for somatotropes, gonadotropes, and corticotropes, but there is very little available data on thyrotropes [5–8].

Thyrotropes represent ~5% of cells in the pituitary gland, and their function is to express and secrete thyroid-stimulating hormone (TSH or thyrotropin), which regulates thyroid gland development and thyroid hormone production. These hormones are essential for normal growth and metabolism. Up to 12% of the US population has abnormal levels of thyrotropin [9]. The incidence of secondary hypothyroidism is estimated to be 1:20,000 to 1:80,000 individuals [10]. Research into the regulation of thyrotrope differentiation and function is relevant to this public health problem.

A cascade of transcription factors is responsible for the differentiation of the major pituitary hormone-producing cell types. The transcription factors associated with thyrotrope development and function are Pou1f1, Gata2, Isli1, Pitx1, and Pitx2. The pituitary transcription factor Pou1f1 is essential for the differentiation of growth hormone, prolactin, and TSH-producing cells [11]. It binds to the promoters of Gh, Prl, and Tshb to activate gene expression [12–16]. Defects in the Pou1f1 gene cause severe growth insufficiency and hypothyroidism in humans and mice [11, 17]. Pou1f1 and Gata2 act synergistically to activate Tshb expression through promoter-proximal elements [13, 18]. Defects in Gata2 and Isli1 reduce thyrotrope differentiation in mice, but they do not appear to ablate it [19–21]. Finally, induction of Pitx2 deficiency in thyrotropes using Tshb-cre causes moderate growth deficiency, blunted TSH response to hypothyroidism challenge, and elevated Pitx1 expression [22]. This suggests that Pitx2 has a role in thyrotrope function that overlaps with the related Pitx1 gene [23, 24]. Despite the important role of Pou1f1 in thyrotrope development and function, little is known about the gene regulatory network of Pou1f1 in progenitors or thyrotropes.

Due to the scarcity of the thyrotrope cell type, classical genomic techniques are challenging to apply. Hormone-producing cell lines have been invaluable for understanding changes in chromatin and gene expression that occur during development [5, 25]. To discover thyrotrope-specific regulatory elements and potential drivers of differentiation, we generated and compared the transcriptome (RNA-seq), open chromatin (ATAC-seq), histone modification (CUT&RUN for H3K27Ac, H3K4Me1, and H3K27Me3), and transcription factor binding (CUT&RUN for Pou1f1) in two mouse cell lines, a Pou1f1-expressing pituitary precursor cell line that does not express any hormones, GHF-T1, to a thyrotrope-like cell line, TaT1 [26, 27]. TaT1 cells behave much like endogenous thyrotropes in that they respond to TRH and retinoids, and they secrete TSH in response to diurnal cues [28–30]. Finally, we evaluated putative enhancer elements for function using transfection assays in TaT1 cells and genetically engineered mice. Together, these studies extend ENCODE-like multi-omic analyses to generate reference maps of gene regulation for cell types critical for growth and metabolism.

**Results**

**Comparison of transcriptomes**

To identify candidate factors that drive the differentiation of thyrotropes, we performed RNA sequencing on the GHF-T1 and TaT1 cell lines. There were many differences in their transcriptomes, consistent with their distinctive morphology, growth rate, and hormone secretion properties (Fig. 1a). Eighty-two percent of genes were differentially expressed (FDR < 0.01). Pou1f1 expression levels were nearly twice as high in TaT1 cells (160 FPKM) relative to GHF-T1 cells (85 FPKM). Other SV40 immortalized pituitary cell lines vary ten-fold in Pou1f1 expression levels, but there was no correlation with differentiation state [31]. As expected, Cga and Tshb were expressed in TaT1 (3557 and 11 FPKM, respectively) but negligibly expressed in GHF-T1 cells (1.4 and 0 FPKM, respectively). The GHF-T1 cells had elevated expression of the transcription factors Gli3, PAX3, Foxg1, and others (Table 1). TaT1 cells had elevated expression of Gata2 and Isli1, as expected, and Lhx3, Rxrg, Neurod4, Ins1, and other transcription factors were also expressed at significantly higher levels than in GHFT1 cells. Some of the differences in transcription factor gene expression were dramatic, i.e., in the range of 8–10 fold, but others were more modest, like Isli1 (1.3x). Isli1 is critical for driving thyrotrope fate, but it is expressed in both progenitors and differentiated cells [19].

To uncover pathways with altered gene expression in these cell lines, we performed GO term (gene ontology) enrichment analysis on the top 5% of the most differentially expressed genes (by log-2 fold-change) in both lines [32, 33]. The GO terms enriched in GHFT1 cells were broadly related to development and morphogenesis (Table 2). Genes contributing to these GO terms include genes from the GLI family that are targets of hedgehog signaling (Gli2, Gli3, Glipr1, Glipr2, Glis2, Glis3), BMPs (Bmp1, Bmp4, Bmp5, Bmp1a, Bmp2r, Bmp6, Bmp2k), and FGFs (Fgf5, Fgf7, Fgf8, Fgf10, and Fgf21). Elevated expression of these factors in GHFT1 cells is consistent with the underlying the importance of sonic hedgehog, BMP, and FGF signaling in early pituitary development [34–36]. In contrast, the genes with elevated expression in TaT1 cells were enriched for GO terms related to nervous system development and synapse formation. This may be attributable to the fact that the hormone secretory cells in the pituitary gland are excitable and
fire action potentials, which are altered in response to hypothalamic input and exhibit characteristic patterns of hormone release [37]. In addition, stimulus-secretion coupling also involves interconnection of homotypic networks [38]. Some genes enriched in TαT1 cells that contribute to these neuronal-like GO terms are neurexin 1 (Nrxn1), genes of the glutamate receptor family (Grin1, Grina, Grin2d, Grin3a), and the synaptic regulator Unc13a. KEGG pathway enrichment analysis revealed an increase in neuroactive ligand-receptor interaction in TαT1 cells, consistent with the enrichment in GO terms found.

The function of several members of the bHLH family of transcription factors, including Ascl1, Neurod4, and Neurod1, has been investigated in pituitary development [39]. Seventy-one of the ninety-three known bHLH factors are differentially expressed between GHF-T1 and TaT1 cells (FDR < 0.05); Neurod4 and Ascl1 are expressed at higher levels in TαT1 cells, consistent with the enrichment in GO terms found.

We compared gene expression profiles that we obtained from GHF-T1 and TaT1 cells with those of other SV40-transformed pituitary cell lines, Pit1-zero and Pit1-triple cells (Fig. 1b) [31]. Pit1-zero cells express Pou1f1, but none of POU1F1’s downstream hormone genes, whereas Pit1-triple cells express Pou1f1 and all three POU1F1-dependent hormones, GH, PRL, and TSH. In the TαT1 cell line, we found a statistically significant increase in the expression of sodium channel genes (p value = 0.002) and potassium channel genes (p value = 2.9e−05), but not in calcium channel genes as a group (p value = 0.26) (Fig. 1c–e). The most highly expressed sodium channel genes in TaT1 cells are Scn1a, Scn8a, and Scn3a. Three sodium channel genes (Scn1a, Scn8a, and Scn9a) are also expressed in the Pit1-Triple cell line, the only other...
hormone-expressing cell lineage we studied. The most highly expressed potassium channel genes in TaT1 cells are Kcnc3, Kcnq2, Kcnk1, and Kcnk2. G protein-gated ion channels are involved in regulated hormone secretion. This marked increase in ion channel genes in the TaT1 cells is consistent with their GO terms associated with synapses and neuron formation and function. The only calcium channel gene with differential expression was Cacna1g, which is highly expressed in TaT1 cells relative to the other three cell lines.

Chromatin landscape around thyrotrope-signature genes
To assess genome-wide changes in the chromatin landscape associated with thyrotrone differentiation, we performed Cleavage Under Target and Release using Nuclease (CUT&RUN) for three major histone marks: H3K27Ac, H3K4Me1, and H3K27Me3 [41]. The presence of both H3K27Ac and H3K4Me1 marks active enhancers, while H3K27Me3 marks repressed regions [42–44]. We also performed an Assay for Transposase-Accessible Chromatin with High-Throughput Sequencing (ATAC-seq), a method for profiling regions of accessible chromatin, which are often regulatory [45]. The results for three selected genes, Isl1, Gli3, and Rxrg, are shown in Fig. 2. We chose Isl1 because of its important role in thyrotrone and gonadotrope fate [19], Gli3 for its role in hypothalamic and pituitary development in humans, [17], and Rxrg because of its role in thyrotrone function and retinoic acid regulation of gene expression [46, 47]. Also, Gli3 and Rxrg are the most and second-most differentially expressed transcription factors in the GHFT1 and TaT1 cells, respectively. These data (called tracks) reveal that Isl1 is expressed in both cell lines and has extensive H3K27Ac, H3K4Me1, and ATAC-seq signal across the locus, revealing putative active enhancers and areas of open chromatin. For example, the stretch of H3K4Me1 and H3K27Ac signal covering the last intron and penultimate exon of Isl1 could be an enhancer (Fig. 2a).

We visualized the expression and chromatin architecture around genes that are differentially expressed in the precursor and differentiated cell lines. Gli3 is strongly expressed in GHFT1 cells and has many H3K27Ac, H3K4Me1, and ATAC-seq peaks, revealing active enhancers in areas of open chromatin (Fig. 2b). By contrast, Gli3 is not expressed in TaT1 cells. The chromatin surrounding Gli3 in the TaT1 cells is devoid of H3K27Ac, H3K4Me1, and ATAC-seq peaks and is covered with H3K27Me3, a mark of active repression. This shows that Gli3 is actively repressed in the TaT1 cell

Table 1 Differentially expressed transcription factors (FDR < 5 × 10−14)

| GHFT1 cells | Log2 fold-change | Rank | TaT1 cells | Log2 fold-change | Rank |
|-------------|-----------------|------|------------|-----------------|------|
| Gli3        | 11.90           | 1    | Lhx3       | 11.04           | 8    |
| Pax3        | 10.95           | 12   | Rexg       | 10.74           | 25   |
| Zfhx4       | 10.74           | 21   | Insr1      | 10.67           | 30   |
| Foxg1       | 10.59           | 26   | Neurod4    | 10.56           | 37   |
| Zfp57       | 10.51           | 29   | Sox3       | 10.23           | 55   |
| Hoxa13      | 10.29           | 40   | Fov        | 10.18           | 57   |
| Kdm5d       | 10.19           | 48   | Myt11      | 9.93            | 72   |
| Hmg12       | 10.15           | 53   | Sct2       | 9.90            | 77   |
| Mx2         | 10.11           | 54   | Zfp641     | 9.39            | 126  |
| Rux1        | 10.07           | 55   | Sct1       | 9.15            | 145  |
| Rhox10      | 9.94            | 60   | Zic3       | 8.96            | 168  |
| Mecom       | 9.84            | 68   | En2        | 8.95            | 169  |
| Tead2       | 9.65            | 80   | Lin28b     | 8.82            | 184  |
| Vax1        | 9.58            | 85   | Pax5       | 8.61            | 205  |
| Tcf7l1      | 9.57            | 87   | Zfp709     | 8.52            | 218  |
| Hoxa1       | 9.52            | 91   | Prdm16     | 8.30            | 249  |
| Tcf24       | 9.46            | 95   | Pou2f2     | 8.15            | 268  |
| Hoxc9       | 9.35            | 104  | Zim1       | 7.92            | 301  |
| Maf         | 9.32            | 106  | Nihh1      | 7.85            | 312  |
| Pou3f3      | 9.16            | 112  | Foxl2      | 7.82            | 317  |
| Gli2        | 9.13            | 117  | Isl1       | 1.3             | 3896 |

Daly et al. BMC Biology (2021) 19:76
line. Conversely, Rrxg, a gene whose deletion in mice is associated with thyroid hormone resistance [46], is highly expressed in TaT1 cells but not in GHFT1 cells (Fig. 2c). Consistent with this, in TaT1 cells the Rrxg locus is decorated with H3K27Ac, H3K4Me1, and ATAC-seq peaks, whereas the GHFT1 line has no such peaks, and shows active repression of Rrxg, with a broad H3K27Me3 signal. Additional tracks for selected genes that are expressed at similar levels, higher in GHFT1, or higher in TaT1 are presented in Supplemental Figures 2, 3 and 4, respectively.

We used ChromHMM to annotate different chromatin states based on H3K27Ac, H3K4Me1, H3K27Me3, and ATAC-seq signal [48]. Iterating over increasing numbers of possible states, we found that 11 states best captured the chromatin architecture within these two cell lines (Supplemental Figure 5). Of these states, two had both H3K4Me1 and H3K27Ac, predicting active enhancers. The difference between the two states was the presence or absence of an ATAC-seq signal, meaning one state represented open, active enhancers, while the other represented active enhancers in a more closed state.

**POU1F1 binding**

We performed CUT&RUN for POU1F1 in both the GHFT-T1 and TaT1 cell lines to identify similarities and differences in POU1F1 binding at these two stages of differentiation. We present three specific examples of POU1F1 binding.

**Pou1f1** has two enhancers, a proximal (5.6 kb), early-stage enhancer bound by PROP1, and a distal (10 kb), late-stage enhancer. POU1F1 binds the distal enhancer and drives its own expression in an auto-regulatory fashion [49, 50]. In both cell lines, CUT&RUN shows extensive POU1F1 binding across the Pou1f1 promoter-proximal region and both the early and late enhancers (Fig. 3a). The Tcf7l1 gene is an example of elevated expression and preferential POU1F1 binding in GHFT1 cells relative to TaT1 cells (Fig. 3b). TCF7L1 is important for hypothalamic-pituitary development and function in humans and mice [51].

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**Table 2 Gene ontology term enrichment**

| Gene | Structure and development | Expression | Gene | Synapse development and function | Expression |
|------|-------------------------|------------|------|---------------------------------|------------|
|      | A | B | C | D | E | GHFT1 | TaT1 | F | G | H | I | J | GHFT1 | TaT1 |
| Cyr61 | X | X | X | X | X | 117.1 | 0.5 |      |      |      |      |      |      |      |
| Edn1  | X | X | X | X | X | 7.3  | 0.0 |      |      |      |      |      |      |      |
| Bmp4  | X | X | X | X | X | 6.8  | 0.0 |      |      |      |      |      |      |      |
| Tgfb2 | X | X | X | X | X | 65.1 | 0.0 |      |      |      |      |      |      |      |
| Fgf10 | X | X | X | X | X | 1.1  | 0.0 |      |      |      |      |      |      |      |
| Gil3  | X | X | X | X | X | 13.4 | 0.0 |      |      |      |      |      |      |      |
| Metf2c| X | X | X | X | X | 7.6  | 0.0 |      |      |      |      |      |      |      |
| Cav1  | X | X | X | X | X | 10.1 | 0.0 |      |      |      |      |      |      |      |
| Bmp5  | X | X | X | X | X | 3.8  | 0.0 |      |      |      |      |      |      |      |
| Ctgf  | X | X | X | X | X | 40.3 | 0.0 |      |      |      |      |      |      |      |
| Gli2  | X | X | X | X | X | 4.2  | 0.0 |      |      |      |      |      |      |      |
| Gata6 | X | X | X | X | X | 1.2  | 0.0 |      |      |      |      |      |      |      |
| Tbx20 | X | X | X | X | X | 2.2  | 0.0 |      |      |      |      |      |      |      |
| Mtx2  | X | X | X | X | X | 20.1 | 0.0 |      |      |      |      |      |      |      |
| Yap1  | X | X | X | X | X | 40.3 | 0.2 |      |      |      |      |      |      |      |
| Shp1  | X | X | X | X | X | 7.1  | 0.0 |      |      |      |      |      |      |      |
| Hlx   | X | X | X | X | X | 1.5  | 0.0 |      |      |      |      |      |      |      |
| Cxcl12| X | X | X | X | X | 4.2  | 0.0 |      |      |      |      |      |      |      |
| Pax3  | X | X | X | X | X | 9.1  | 0.0 |      |      |      |      |      |      |      |
| Fn1   | X | X | X | X | X | 611.8| 1.0 |      |      |      |      |      |      |      |

1 The top five GO terms associated with the top 5% of the most differentially expressed genes (log 2-fold-change) in GHFT1 cells are A = animal organ development, B = anatomical structure development, C = anatomical structure morphogenesis, D = multicellular organism development, E = system development. X's illustrate the association of each gene with a GO term. Expression is given in FPKM.

2 The top five GO terms associated with TaT1 cells are: F = synaptic signaling, G = trans-synaptic signaling, H = synapse organization, I = nervous system development, J = anterograde trans-synaptic signaling.
Fig. 2 (See legend on next page.)
POU1F1 binding was detected at the Nrxn1 promoter in TαT1 cells relative to GHFT1 cells, and Nrxn1 expression increased from nearly zero in GHFT1 cells to 5 FPKM in TαT1 cells (Fig. 3c). Nrxn1 is critical for proper synapse formation, but the role in pituitary is unknown [52].

Genome-wide analysis of POU1F1 binding in both cell lines revealed that the majority of binding is in putative enhancers and associated with higher levels of gene expression. There are a significant number of unique POU1F1 binding sites in each cell line: only one third of all POU1F1 binding sites are shared between the two lines (Fig. 3d). Only 15–16% of POU1F1 binding sites in GHFT1 cells (10,980 out of 69,644) and TαT1 cells (9360 out of 63,036) are within 1 kb of a transcription start site (TSS), suggesting most POU1F1 binding is at enhancers. Consistent with this, nearly 70% of genes whose promoters are bound by POU1F1 in the differentiated line are also bound by POU1F1 in the precursor line (Fig. 3e). We found that POU1F1 binding is associated with higher levels of gene expression in both cell lines (Fig. 3f).
POU1F1 binding is associated with higher ATAC-seq signals in GHF-T1 cells than in TaT1 cells (Fig. 4a). As expected, sites of POU1F1 binding specific to TaT1 cells are more open in TaT1 cells (Fig. 4b), sites of POU1F1 binding specific to GHF-T1 cells are more open in GHF-T1 cells (Fig. 4c), and shared POU1F1 sites have similar signatures of open chromatin in both cell lines (Fig. 4d).

Active enhancers (states containing both H3K27Ac and H3KMe1 in ChromHMM) are heavily enriched for POU1F1 binding in both cell lines (Fig. 4e, f). GHF-T1 enhancers appear to have greater POU1F1 binding than do TaT1 enhancers. POU1F1 binding that is specific to TaT1 cells is associated with active chromatin states in TaT1, but it is less so in GHF-T1 cells (Fig. 4g). Similarly, GHF-T1-specific POU1F1 binding is broadly associated with active chromatin states in GHF-T1 cells, but it less so in TaT1 cells. Sites bound by POU1F1 in both cell types have similarly active chromatin states, as expected.

To identify transcription factors that may be associated with differential POU1F1 binding between the cell lines,
we analyzed the chromatin states associated with shared and unique POU1F1 binding sites and screened these for binding motifs. We classified genomic sites that had POU1F1 binding exclusively in TaT1 cells, were in active states in the TaT1 cells, and were in repressed states in GHF-T1 cells (repressed to active), POU1F1 binding sites that are shared between GHF-T1 and TaT1 and are in similarly active chromatin in both (Active to Active), and GHF-T1-specific POU1F1 binding sites that are in active chromatin in GHF-T1 sites and are repressed in TaT1 cells (labeled active to repressed). This revealed an expected enrichment in POU1F1 motif density at the center of POU1F1 sites in both GHF-T1 and TaT1 cells (Fig. 4h). There was a striking enrichment of bZIP motifs at the center of GHF-T1-associated POU1F1 binding sites (Fig. 4i), suggesting that bZIP factors influence POU1F1 binding in progenitors. Interestingly, there was remarkable helix-turn-helix motif density at the center of TaT1 POU1F1 binding sites, and even more so at repressed to active sites (Fig. 4j). Similarly, there was increased bHLH motif density at repressed to active sites (Fig. 4k). These data suggest that HTH and bHLH factors mediate POU1F1 binding to novel sites in thyrotropes.

**Stretch enhancers**

Twenty-four percent of the putative enhancers that we identified were in open chromatin in both the precursor and differentiated cell lines, as defined by at least 25% bidirectional overlap (Fig. 5a). There were 15% more areas of open chromatin at putative enhancers in the differentiated, thyrotrope state than the precursor state. The distribution of enhancer sizes was very similar between the two cell lines (Fig. 5b). Enhancers larger than 3 kb in length, called stretch enhancers, represent 5–10% of all enhancers, are typically cell-type specific and often enriched in disease-associated areas [53, 54]. Stretch enhancers represent 4.9% of the enhancer population in the precursor cell lineage and 7.1% of the enhancer population in the differentiated thyrotrope population. This is within the expected fraction, and the increased abundance in TaT1 cells is consistent with their more differentiated state. While GHF-T1 and TaT1 cells share 24% of all enhancers, only 10% of stretch enhancers are shared between the two cell types (Fig. 5a).

We compared the expression of the closest gene to each stretch enhancer and found that expression was highly cell-type specific. Genes closest to precursor stretch enhancers were more highly expressed in the precursor cell line, whereas the genes closest to thyrotrope stretch enhancers were expressed at higher levels in the thyrotrope cell line (Fig. 5c). Genes closest to shared stretch enhancers had similar gene expression in both cell lines.

We sought to determine whether genes associated with thyrotrope function in both mouse and human were closer to stretch enhancers. We generated a list of 25 candidate genes associated with thyrotrope differentiation and/or function (Supplemental Table 3), and we found that the TSSs of all of these genes were within 100 kb of 28 TaT1 stretch enhancers and only 6 GHF-T1 stretch enhancers (Fig. 5d). To determine whether this result was significant, we randomly selected 25 genes 10,000 times, ensuring the genes had similar expression levels, and we counted the number of stretch enhancers within 100 kb of the transcription start site of those randomly selected genes. The randomly selected genes were within 100 kb of 28 TaT1 stretch enhancers only two times out of 10,000, yielding an empirical p value of 0.0002, which confirms the enrichment of TaT1 stretch enhancers at thyrotrope-signature genes.

To probe the potential value of these data for application to human disease studies, we mapped the mouse enhancers in TaT1 and GHF-T1 cells from the mm9 genome onto the human genome, hg19. Because ~90% of GWAS SNPs are intronic or intergenic, and stretch enhancers are heavily enriched for disease SNPs, we expected to implicate thyrotropes in disease phenotypes by uncovering enrichment of disease SNPs in TaT1 stretch enhancers [4, 53]. We used GARFIELD to measure the enrichment of these SNPs in GHF-T1 and TaT1 and stretch enhancers, while accounting for linkage disequilibrium, minor allele frequency, and distance to TSS [55]. We compared their enrichment to stretch enhancers found in heterologous cell lines including but not limited to Islet cells, GM12878 (human B-lymphocyte cells), and K562 (human myelogenous leukemia cells) cells [53]. Supplemental Figure 7 shows the enrichment odds ratios for all GWAS studies and cell line stretch enhancers, as well as their p values. The study that exhibited the greatest enrichment of SNPs in TaT1 stretch enhancers (odds ratio of 4.7) was from GWAS done on the neuroticism sub-phenotype of feeling miserable [56]. While the significance of this is uncertain, untreated hypothyroidism can be associated with fatigue and depression.

**In vitro validation of enhancers**

We sought to test putative enhancers of a sampling of thyrotrope-signature genes, namely Gata2, Cga, and Pitx1, by transient transfection of TaT1 cells. We identified putative regulatory elements as regions with significantly enriched ATAC-seq signals near these genes. The promoter-proximal sequences of each gene were amplified from genomic DNA and fused to a luciferase reporter gene. Putative regulatory elements were amplified from genomic DNA and cloned in both the forward and reverse orientation upstream of the promoter-proximal
region. The transfection efficiency of TaT1 cells is low (~20%), and the results vary, likely due to poor adherence of cells to the plate. Thus, all experiments were performed with six replicates. We detected strong enhancer elements for Gata2 and Pitx1 (Fig. 6). The position (genome coordinates) of cloned promoters and elements is presented in Supplemental Table 4. Gata2 is implicated in Tshb transcription and proper thyrotrope function [13, 18, 21]. Gata2 has two promoters located upstream of a noncoding exon [57]. The more distal promoter is located ~5 kb upstream of the more proximal promoter, and it drives expression in Sca-1+/c-kit+ hematopoietic progenitor cells, while the downstream promoter drives Gata2 expression in most other tissues. The RNA-seq data revealed that the downstream promoter is the only one utilized in TaT1 and GHF-T1 cells. Gata2 expression is five-fold higher in TaT1 cells than in GHF-T1 cells and there is a larger area of accessible chromatin upstream of Gata2 in the TaT1 cells (~2.8 kb vs 0.9 kb, Fig. 6a). There is no difference in activity between the 0.2 kb and 0.9 kb promoter-proximal region in TaT1 cells, but the larger, 2.8 kb promoter-proximal region of Gata2 stimulated luciferase activity 5-fold (p value = 0.009). This indicates the presence of enhancer elements between 0.9 and 2.8 kb of the common TSS for Gata2. We tested three distal elements, fusing them with the smallest, 0.2 kb Gata2 promoter construct. An element ~100 kb 3′ of the common Gata2 promoter had significant POU1F1 binding and drove the highest levels of luciferase expression (3-fold increase, p value = 0.005). Thus, we identified two enhancer elements for Gata2 expression in thyrotopes,
one in the proximal promoter region, within 2.8 kb of the TSS, and a more distal one, approximately 110 kb downstream.

\[ \text{Cga} \] is the alpha-subunit of TSH, and the gonadotropins, FSH and LH, and it is expressed in both thyrotropes and gonadotropes. We tested three \text{Cga} enhancer
elements, and two appeared to have some activity, although they did not reach statistical significance. The element \(-7 \text{ kb upstream of } Cga\) increased luciferase activity 1.5-fold, and it has not been previously described. The element located 4.6 kb upstream of the \(Cga\) gene increased luciferase activity 1.6-fold and approached statistical significance (\(p = 0.07\)). This element is sufficient for developmental activation, cell type-specific expression, and hormonal regulation in transgenic mice (Fig. 6b) [16, 58, 59].

\(Pitx1\) has a role in \(Pomc\) expression and hindlimb formation in mice and humans [60, 61]. \(Pitx1\) and the related \(Pitx2\) gene drive early pituitary development and are expressed in thyrotropes and gonadotropes [24]. Mice with a pituitary-specific disruption of \(Pitx2\) exhibit elevated \(Pitx1\) expression in response to induced hypothyroidism, suggesting functional compensation [22]. The \(Pitx1\) regulatory landscape extends over 400 kb and includes a pituitary enhancer 110 kb upstream [62]. ATAC-seq signatures revealed two previously undescribed thyrotrope-specific regions of open chromatin 9 and 19 kb upstream of the \(Pitx1\) transcription start site (Fig. 6c). The proximal element did not have statistically significant enhancer activity. The distal element, however, increased luciferase activity 3-fold (\(p = 0.009\)).

**Discovery of a novel TSH \(\beta\)-subunit enhancer**

A bacterial artificial chromosome clone containing \(Tshb\) and 150 kb of surrounding DNA sequence was sufficient to drive expression in thyrotropes of transgenic mice [22], but there is no information about the location of key regulatory elements within this region. In fact, multiple efforts to drive expression in transgenic mice with smaller constructs were unsuccessful [63]. We sought to leverage the information we have about the chromatin states in the \(T\alpha T1\) cells to identify elements sufficient for \(Tshb\) expression in mice. Knowing that the 150 kb \(BAC\) was sufficient to drive expression in thyrotropes, we limited our search to this region and found five areas with high ATAC-seq signal in \(T\alpha T1\) cells (Fig. 7a). We tested each of these elements both in the forward and reverse orientation fused to a 438 bp \(Tshb\) promoter-proximal region driving luciferase expression (Fig. 7b, genomic coordinates of the cloned promoter and putative enhancer elements are in Supplemental Table 4). We discovered that a 1.4 kb element located 7.7–6.6 kb upstream of the \(Tshb\) transcription start site drove significant levels of luciferase (hereafter named element 4). Element 4 had extensive ATAC-seq signal and H3K4Me1 and POU1F1 binding, consistent with the observation that POU1F1 is important for \(Tshb\) expression. We tested the ability of GATA2 and POU1F1 to activate \(Tshb\) promoter alone and in conjunction with element 4 in heterologous CV1 cells. GATA2 and POU1F1 independently cause modest increases in \(Tshb\)-luc reporter gene expression (2.7-fold and 1.6-fold respectively), and together they drive higher expression (5-fold, Fig. 7c), similar to previous reports [13]. In the presence of element 4, POU1F1 has very modest effects on reporter gene expression (1.3-fold increase), but GATA2 has a strong effect (4.3-fold increase). GATA2 and POU1F1 do not have an obvious additive effect on element 4 reporter activity (4.4-fold increase) in contrast to the promoter-proximal region. This suggests that GATA2 is a powerful regulator of \(Tshb\) expression through interaction with element 4. To determine which other factors may be binding Element 4, we checked for the presence of over 1000 JASPAR motifs within element 4 at an 80% threshold. We found extensive predicted GATA2 and PITX1 binding (Fig. 7d). A more complete list of predicted binding factors is presented in Supplemental Table 5.

We tested whether element 4 was sufficient to drive expression in transgenic mice by placing it in front of the \(Tshb\) promoter and a YFP reporter gene. This construct was injected into fertilized eggs that were subsequently transferred to pseudopregnant foster mothers. We dissected the pituitaries of eleven founder transgenic mice at 4 weeks of age and examined the expression of YFP using immunohistochemistry (Fig. 7e, f). Forty-five percent (5/11) exhibited transgene expression in the pituitary gland: three founders had low levels of YFP expression, and two founder mice had higher levels of YFP activity. This contrasts with our previous transgenic analyses using –1.1 kb to +620 bp of \(Tshb\) that yielded no expression (0/3 lines), and a larger construct containing –6 kb to +43 bp that yielded no expression (0/4 lines), even after 10 days of treatment with propothiouracil, which would be expected to increase \(Tshb\) transcription at least ten-fold [63]. We quantified expression in two element 4 transgenics. 87% of YFP-positive cells \((N = 63/76)\) were also positive for \(Tshb\) in founder 399, indicating high specificity for thyrotropes, and 6% of the transgenic thyrotropes were also positive for YFP \((63/872)\), signifying low penetrance of expression. In total, 31% of YFP-positive cells \((8/26)\) were positive for \(Tshb\) in founder 423, and 3% of the transgenic thyrotropes were also positive for YFP \((8/270)\). This suggests that element 4 contributes to \(Tshb\) expression, although other elements are required for higher penetrance expression. This serves as a proof of the principle that combined transcriptome and epigenome data can be valuable for identifying enhancer elements that function in developmentally specific cell lines and intact animals.

**Discussion**

Our work builds on the ENCODE effort to discover regulatory elements in diverse tissues. This represents...
the first systematic characterization of the epigenome and transcriptome of a thyrotrope-like cell line, providing insight into the changes that are associated with the differentiation of committed POU1F1 pituitary cells into thyrotopes. POU1F1 has a similar binding profile at promoters in the two cell lines, but there are novel binding sites at enhancers in each cell line that are associated with striking shifts in chromatin states. Global analysis of positive histone marks (H3K27Ac and H3K4Me1) revealed putative active enhancers in the two cell lines.
We demonstrated that many of the enhancers surrounding thyrotrope-signature genes drive expression in a thyrotrope cell line. Furthermore, an enhancer element upstream of Tshb was sufficient to drive expression in some thyrotropes in transgenic mice. The transcriptomic, epigenomic, and POU1F1 binding data here contributes significantly to our understanding of thyrotripe cell specification, which are the key cells for regulation of thyroid gland development and growth and metabolism.

This is the first deep expression profiling reported for GHF-T1 and TaT1 cells. The TaT1 cells have been used extensively as a thyrotrope-like cell to study the molecular mechanisms of hypothalamic input and thyroid hormone feedback on Tshb expression, and remarkably, they also respond to retinoids and secrete TSH in response to diurnal cues [28–30]. In total, 92% of the genes we predicted would be expressed in a thyrotrope-like cell were detected in TaT1 cells at > 1 FPKM (Supplemental Table 3). Recent single-cell gene expression studies of pituitary glands from adult rodents and developing human fetuses have revealed expected and novel genes enriched in thyrotropes, including Tshb, Trhr, Dio2, Psk2, Dpp10, Sox11, Nrg6, and others [64–66]. The majority (77%, N = 35) of thyrotrope-enriched genes identified by single-cell sequencing were expressed at levels > 1 FPKM in TaT1 cells, and 96% of those genes exhibited elevated expression in TaT1 cells relative to GHFT1 (N = 27) (Supplemental Figure 9, Supplemental Table 6). Thus, the gene expression data confirm the strong relationship between TaT1 cells and thyrotropes in vivo.

As hormone-producing cells mature, they ramp up translational machinery for robust hormone production, and CREB3L2 is a master regulator of this process in the pituitary corticotropes [67]. While Creb3l2 is not highly expressed in TaT1 cells, Creb3l1 is expressed nearly 30-fold higher in TaT1 cells than in GHFT1 cells (172.5 FPKM vs. 5.9 FPKM). It is possible thyrotropes use a similar mechanism of increasing translation to meet the demand for thyrotropin. Consistent with this, Creb3l1 is upregulated in a model of thyrotrpie adenoma [68].

Pituitary endocrine cells are electrically excitable, and voltage-gated calcium influx is the major trigger for hormone secretion [37]. G protein-coupled receptors, ion channels, and hormones all are considered components of cellular identity. For example, thyrotropes have unique electrical activity relative to other pituitary hormone-producing cell types. TaT1 cells exhibit a bursting pattern of action potentials that are affected by exposure to TRH and thyroid hormone, but the nature of the ion channels regulating TSH secretion is not understood [69, 70]. The involvement of ion channels in excitation-secretion coupling is an area of active study. The hypothalamic factors CRH, TRH, GHRH, and somatostatin have an effect on electrical activity in corticotropes, lactotropes, and somatotropes. Thrytropes have not been well-studied in this regard. Our study provides evidence for the acquisition of ion channel gene expression as progenitors adopt the thyrotrope fate. Voltage-gated potassium channels Kcnq2, Kcnq4, Kcnf1, and Kcns2 were highly expressed in TaT1 cells. Interestingly, KCNQ1 missense mutations cause growth hormone deficiency [71]. We also found that sodium channel genes are more highly expressed in two cell types that express hormones, Pit1-triple and TaT1, than in lines that do not express hormones. The calcium channel CACNA1G, a low-voltage activated, T-type channel, was highly elevated in TaT1 cells. These observations suggest that ion channel expression may be acquired as cells begin to differentiate. Additional studies may be necessary to determine whether they are pruned during maturation [37]. Knowing which ion channels are expressed in thyrotrope cells is the first step in understanding the mechanism whereby TRH stimulates TSH release in a pulsatile manner and according to the appropriate diurnal rhythm.

Several transcription factors had higher levels of expression in TaT1 cells relative to GHFT1, including ISL1, RXRG, and LH6X3. Increased expression of Isl1 and Rxrg was expected because pituitary-specific deletion of Isl1 causes reduced thyrotrope differentiation [19], and several lines of evidence support a role for Rxrg. RXRG suppresses serum TSH levels and Tshb transcription, Rxrg-deficient mice have central resistance to thyroid hormone, and loss of retinoic acid signaling suppresses thyrotrope differentiation [46, 47]. Isl1 had extensive POU1F1 binding across the 1 MB region surrounding it. Lhx3 expression is detectable at e9.5 in the mouse pituitary placode and expression persists through adulthood [72]. Thus, we expected to detect Lhx3 transcripts in all pituitary cell lines. Lhx3 transcripts were nearly undetectable in Pit1-zero cells and in the precursor GHFT1 lineage, but transcripts were higher in Pit1-triple (1.6 FPKM) and highest in TaT1 cells (44.2 FPKM). Recently, an SV40-transformed pituitary precursor cell line was developed that expresses the stem cell marker SOX2 but not LHX3 [73]. There may be dynamic changes in Lhx3 expression during development that has not been documented.

SHH signaling is critical for establishing the pituitary placode and induction of Lhx3 expression [34]. The GHFT1 precursor lineage expressed Gli2 and Gli3, which are downstream targets of SHH, at higher levels than TaT1 cells, suggestive of active SHH signaling. Gli2 and Gli3 promoters are associated with extensive H3K4Me1 and H3K27Ac, and active enhancers can be found upstream, downstream, and within their introns. By contrast, Gli2 and Gli3 have broad stretches of
development has not been studied, but they may be both of pituitary cell lines are the repressive bHLH factor with MYT1L and POU1F1 to drive thyrotrope fate. Thus, it is possible that ASCL1 acts in concert with other factors that specify the three different cell fates. Motif analysis near unique POU1F1 binding sites suggests which other factors may be involved. POU1F1 binding is associated with the homeodomain consensus binding motif, and sites of TaT1-specific POU1F1 binding that are repressed in GHF-T1 cells and active in TaT1 cells are heavily enriched for bHLH and HTH motifs. This raises the possibility that bHLH and HTH factors pioneer the binding of POU1F1 which then activates thyrotrope-specific expression.

Members of the RFX family of transcription factors are attractive candidates for interaction with POU1F1 to drive thyrotrope fate. These HTH factors contain DNA binding and heterodimerization domains and regulate cell fate in many organ systems, including the pancreatic islets and the sensory cells of the inner ear [74, 75]. They interact with other POU and SIX factors to direct fate. Several members of the gene family are expressed in both GHF-T1 and TaT1 cells and in pituitary development between e12.5 and e14.5, a time when progenitors leave the cell cycle and initiate differentiation [76]. Future studies will be necessary to define the role of these genes in pituitary development.

The expression of bHLH factor genes Ascl1 (Mash1) and NeuroD4 (Math3) are elevated in TaT1 cells. However, thyrotrope commitment is normal in Ascl1 knockout and in triple knockout mice deficient in Ascl1, Neurod4, and Neurod1. These bHLH activating factors have overlapping functions in promoting somatotrope, gonadotrope, and corticotrope development [39, 40, 77, 78]. They promote pituitary stem cell exit from the cell cycle and act as selectors of cell fate, as triple knockout mice have more SOX2-positive cells and more lactotropes. bHLH factors play important roles in neuronal differentiation. For example, the BAM factors ASCL1, BRN2, and MYT1L are sufficient to transdifferentiate mouse embryonic fibroblasts (MEFs) into neurons. The Zn finger transcription factor MYT1L is highly upregulated in TaT1 cells, and although the POU factor Brn2 is barely expressed in TaT1 cells, POU1F1 is highly expressed. Thus, it is possible that ASCL1 acts in concert with MYT1L and POU1F1 to drive thyrotrope fate.

The top three most highly expressed bHLH factors in both of pituitary cell lines are the repressive bHLH factors in the ID family. The role of these genes in pituitary development has not been studied, but they may be important in regulating progenitor differentiation and cell fate selection. For example, a proper balance of activating and repressive bHLH factors is critical for cortical development [79]. Repressive ID and Hes factors are expressed in cortical progenitors, and induction of key activating bHLH factors drives these progenitors to differentiate. Astrocytes, however, require continued repressive bHLH factor expression. The interplay of active and repressive bHLH factors in pituitary development is likely complex.

**Conclusion**

This work represents the first thorough characterization of the epigenome and transcriptome in Pou1f1 lineage progenitors and thyrotropes. We used this genome-wide catalog and tested enhancer function of elements in genes encoding thyrotropin, Cga and Tshb, the receptor for the hypothalamic releasing hormone that regulates thyrotropin, Trhr, and two crucial transcription factors, Gata2 and Pitx1. This provides proof of the principle that the catalog is valuable for dissecting gene regulation. In addition, we demonstrate that the Tshb enhancer element is sufficient for expression in thyrotropes in transgenic mice and is directly regulated by GATA2. We discovered that unique POU1F1 binding sites are associated with bZIP factor binding motifs in Pou1f1 lineage progenitors and bHLH or bHTH binding motifs in thyrotropes. This suggests candidate gene families for regulating thyrotrope differentiation. Of the more than 30 known genes that are mutated in patients with hypopituitarism, 18 are transcription factors that regulate pituitary development and cell specification, indicating the clinical importance of this field of study [80]. The overwhelming majority of patients with hypopituitarism have no molecular diagnosis, suggesting additional genes remain to be discovered [17]. We provide a rich selection of candidate transcription factors that are differentially expressed in progenitors and thyrotropes. Amongst the top 40 of these, 9 are already implicated in pituitary development and disease. Future analysis of the remaining 31 candidates may uncover additional disease genes.

**Methods**

**Cell culture and transfection**

GHF-T1 and TaT1 cells were provided by Dr. Pamela Mellon at University of California San Diego and grown on uncoated 100-mm dishes and Matrigel-coated 60-mm dishes, respectively. They were grown in DMEM (Gibco, 11995-065) with 10% fetal bovine serum (Corning, 35016CV) and 1% penicillin streptomycin (Sigma-Aldrich P4333). Cells were split 1:10 once they achieved 80% confluence. Six replicates of TaT1 cells were transfected using FuGENE 6 with a 3:1 transfection reagent/DNA ratio. Cells were collected 48 h post-transfection.
for collection and luciferase measurement was performed using Promega Dual-Glo (Promega #E2920), and a GloMax 96 microplate luminometer.

Pit1-zero and Pit1-triple cells were developed by Dr. Stephen Liebhaber and grown as described [31].

Cloning

The DNA prepared for the plasmids used in the transfection experiments and transgenic mice was amplified from TaT1 DNA. Primers were designed to work with Phusion Green Hot Start II High-Fidelity PCR Master Mix (catalog #F566S). Two methods were employed to clone plasmids containing the regulatory element, the respective promoter, and the YFP reporter. The first method involved adding 10–15 nucleotides onto the insert that overlapped with the pCDNA3-YFP Basic plasmid that had been cut with Kpn1 and Xho1. The amplicon and linearized plasmid backbone were combined using the NEB HiFi DNA Assembly Master Mix (catalog #E2621L) with 1:2 vector:insert ratios, and 60 min incubation time at 50 °C. The subsequent plasmid was transformed into DH5α cells. The second method was to insert the amplified construct into a Zero Blunt TOPO vector (Thermo Fisher #450245), select clones that are in the forward and reverse orientation, then cut the TOPO vector containing the insert with Kpn1 and Xho1. The resulting fragment is then ligated into a digested pCDNA3-YFP Basic plasmid that had been cut with Kpn1 and Xho1 using a standard T4 DNA Ligase protocol (NEB #M0202). Once the insert, plasmid, and breakpoint sequences were confirmed by Sanger sequencing, the plasmids were extracted from overnight 1 L cultures of DH5α cells using Qiagen Plasmid Maxi Kits (catalog #12163). Endotoxins were removed from the plasmids using the Endotoxin removal Solution (Sigma, E4274-25ML).

RNA-seq

One million GHF-T1 and TaT1 cells were collected for each of the three replicates for each cell line. Once collected, the RNA was extracted using the RNAqueous™ Total RNA Isolation Kit (catalog #AM1912). The RNA was prepared by the University of Michigan Advanced Genomic Core for mRNA enrichment followed by 50-cycle, paired-end sequencing on the Illumina HiSeq-4000. The RNA was checked for quality using FastQC and mapped and analyzed using the VIPER Snakemake pipeline [81]. Briefly, VIPER aligns the files to the mm9 transcriptome using STAR, followed by differential expression analysis using DESeq2 and cell type clustering and expression quantification using QoRTs [82]. The quality of the alignment was also analyzed using QoRTs.

We measured the significance of the increase in expression of sodium, potassium, and calcium using a one-way ANOVA. This demonstrated the significance of the increase in expression of the sodium and potassium, but not the calcium channel genes.

GO term and KEGG pathway enrichment

We performed GO term enrichment on the top 5% of most differentially expressed genes (by log-2 fold-change) in both lines [32, 33]. This represented 453 genes in GHFT1 cells, and 490 genes in TaT1 cells. We used the default settings on the web-based Gene Ontology Resource (geneontology.org), using the biological process and Mus musculus options. The resulting GO terms were plotted by their log-2 fold enrichment, and their p values.

We also performed a directional Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on all of the genes using RNA-enrich [83]. We set the maximum number of genes per concept to 500 and the minimum number of genes per concept to 5, and otherwise used the default settings. The resulting KEGG pathways were plotted by their coefficients and p values.

ATAC-Seq

The Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) was performed as previously described [45, 84]. Briefly, 50,000 nuclei were extracted from collected GHF-T1 and TaT1 cells. The cells were transposed with Illumina transposase (Illumina #FC-121-1030) for 30 min at 37 °C while shaking at 250 RPM. The resulting fragmented DNA was amplified using ¼ of the cycles required to reach saturation in the described qPCR QC. The final amplified DNA library was purified using the Qiagen PCR purification kit (catalog #28104) and sequenced on the Illumina HiSeq platform. The quality of the reads was checked using FastQC, aligned to the mm9 genome, and had its peaks called using the Parker lab’s Snakemake pipeline [85].

CUT&RUN

CUT&RUN was performed under high-digitonin conditions as described with few exceptions, namely all steps with < 1 ml of liquid requiring mixing were done by 500 RPM shaking instead of inversion [41]. Briefly, 250,000 cells per sample, and one sample per cell line-antibody pair were collected, washed, and bound to Concanavalin A beads (Bangs Laboratories, BP531). The cells attached to beads were incubated at 4°C overnight with the respective antibodies: POU1F1, Batch#1603-F, RRID=AB_2722652, provided by Dr. Simon Rhodes, University of North Florida, Jacksonville, FL [86]; H3K27Me3 – Cell Signal #9733, Batch #14, RRID=AB_2616029; H3K27Ac = Abcam ab4729, Batch GR321673-1, RRID = AB_2118291; H3K4Me1 – Abcam ab8895, Batch GR323554-4, RRID=
AB_306847; Rabbit IGG – R&D AB-105-C, RRID= AB_354266). The antibodies were washed, and no secondary antibody was used. The protein A/MNase fusion protein was added, followed by Ca\(^{2+}\)-induced digestion at 0 °C for 30 min. The fragmented chromatin was then collected and purified using Macherey-Nagel NucleoSpin Gel and cleanup columns (catalog #740609). Libraries of this DNA was prepared using the Kapa Biosystems library prep kit (catalog #KK8702) at a 100:1 adapter to sample ratio. The libraries were paired-end sequenced on a single lane of the Illumina HiSeq-4000 for 50 cycles.

The resulting data was checked for quality using FastQC, aligned to the mm9 genome using Bowtie2 using the flags recommended for CUT&T&RUN (--local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700), and peaks were called using MACS2. We used ENCODE standards for evaluating the quality of our data. One of the metrics is the fraction of reads in peaks, or FRIP, which should correlate with the number of called regions, and it is generally accepted that values should be greater than 1% [87]. The FRIP values for GHFT1 and TaT1 cells, respectively, are as follows (in %): H3K27Ac = 21, 15; H3K4Me1 = 76, 78; POU1F1 = 32, 23; H3K27Me3 = 4, 6.

ChromHMM

ChromHMM was performed on both cell lines using H3K4Me1, H3K27Ac, ATAC-seq, and H3K27Me3 as input. The number of states was iteratively increased to find the number of states that resulted in the fewest number of states with the best log-likelihood. An eleven-state model was selected as a result. Association of each state with the various marks and genomic features can be seen in Supplementary Figure 5. Contiguous states of value two and three were stitched together as enhancers using bedtool’s mergeBed function with a -d 1 flag [88].

Association enrichment test

Enrichment analysis of disease SNPs at stretch enhancers in GHFT1, TaT1, and heterologous cell lines was performed using GARFIELD and stretch enhancers published previously [53, 55]. To find the human sequences orthologous to GHFT1 and TaT1 stretch enhancers, we used a conversion file generated using bnMapper and an mm9 to hg19 chain file [89]. We selected associations that had association counts of at least 50, had a full complement of summary statistics, had more than three million tested SNPs, and were in the harmonized data format were chosen from the GWAS catalog [90]. The resulting heatmap of odds ratios and \( p \) values for each association, tissue-type pair is shown in Supplementary Figure 7.

Transgenic mice

All mice were housed in a 12-h light–12-h dark cycle in ventilated cages with unlimited access to tap water and Purina 5020 chow. All procedures were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines on the Care and Use of Experimental Animals and approved by our Institutional Animal Care and Use Committee.

Recombinant DNA was generated by amplifying genomic mouse DNA regions in Supplemental Table 4, and the previously described Phusion polymerase. The putative regulatory element was then combined with 438 base pairs of the TSHβ promoter (chr3:102,586,594-102,587,032) and a YFP reporter. These elements were combined using the DNA HiFi reaction into a pGEM-T Easy plasmid. The putative regulatory element, the promoter, the YFP, and the breakpoints were checked for accuracy with Sanger sequencing. Once the plasmid was confirmed, larger quantities of the plasmid were generated from overnight 1 L cultures of DH5α cells using Qiagen Plasmid Maxi Kits (catalog #12163). To reduce the effect of the plasmid backbone on the viability of injected eggs, the enhancer, promoter, and YFP were amplified from the plasmid. The resulting amplicon was gel purified and injected into fertilized eggs of mice on a C57BL/6 and SJL mixed background. The resulting mice were genotyped for the YFP allele according to the Jackson Laboratory recommended primers and conditions [91]. We dissected the pituitaries from mice that were positive for the YFP transgene (and four age-matched, negative, control littermates) at 3 weeks of age.

Tissue preparation and immunohistochemistry

Mouse pituitaries were fixed in 4% formaldehyde in PBS overnight at 4 °C. The tissue was washed three times in PBS and put in 10% EDTA for 3 h. They were then dehydrated by putting them in 25%, then 50%, and then 70% ethanol for 1 h each. The pituitaries were embedded in paraffin with 4-h cycles using a Tissue Tek VIP Paraffin tissue-embedding machine (Miles Scientific). The embedded pituitary was cut into coronal, six-micron sections, and was analyzed by immunohistochemical markers as previously described [22, 92]. Anti-YFP antibody (1:100) was from Abcam ab6556, Batch GR3216572-1, RRID= AB_305564, and anti-Tshb (1:1000) was from the National Hormone and Peptide Program, Batch AFP967793.

Antibodies were detected using either the tyramide signal amplification (TSA) (33002 CFF488A Streptavidin HRP, Biotium, Fremont, CA) and streptavidin-conjugated Alexa-fluor 488 (1:200, S11223, Invitrogen). DAPI (1:200) was incubated on the slides for 5 min to stain nuclei. DABCO-containing permount was used to mount the slides, which were then imaged using a Leica DMRB fluorescent microscope. To quantify gene expression, a set of...
8–12 slides with pituitary sections were analyzed from each founder mouse. Total TSH positive cells/set were quantified, as well as total YFP-positive, and total double-positive cells expressing TSH and YFP. A total of 270–872 thyrotropes were counted for two founders.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12915-021-01009-0.

Additional file 1: Figure S1. Loss of ASCL1 has minimal impact on thyrotrope number. Figure S2. Multi-omics tracks for loci with similar levels of expression and chromatin landscapes in both cell types. Figure S3. Multi-omics tracks for selected genes with higher levels of expression in GHF-T1 cells. Figure S4. Multi-omics tracks for selected loci with higher levels of expression in TaT1 cells. Figure S5. ChromHMM summary data. Figure S6. Motif density at POU1F1 binding sites in GHF-T1 and TaT1 cells. Figure S7. Heatmap of associations with each cell type. Figure S8. Functional enhancer testing of elements of open chromatin in and around Trh. Figure S9. Thyrotop-specific genes identified by single cell sequencing are elevated in TaT1 cells relative to GHF-T1.

Additional file 2: Table S1. Genes Associated with SV40 Immortalized Pituitary Cell Lines. Table S2. bHLH genes expressed in GHF-T1 and TaT1 cells. Table S3. Thyrotop signature genes. Table S4. Genomic coordinates for promoter and enhancer elements tested in transfection (Mm9).

Table S5. Factors with binding motifs in Trh-E4 Element. Table S6. Expression of reporter thyrotop-enriched genes in GHF-T1 and TaT1 cells.

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Disclosure declaration
The authors have nothing to disclose.

Authors’ contributions
AZD performed the RNA-seq, ATAC-seq, and CUT&RUN (for POU1F1, H3K27Ac, H3K4Me1, and H3K27Me3) library preparation and data analysis. He analyzed the founder reporter mice. LAD performed immunohistochemistry generated the plasmids and performed the transfections for the luciferase assay. AZD performed the RNA-seq, ATAC-seq, and CUT&RUN (for POU1F1, Tshb, Tshα) under accession number PRJNA643917, SUB7682741. All raw sequencing data generated in this study have been submitted to the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA643917, SUB7682741.

All data generated or analyzed during this study are included in this published article, its supplementary information files and publicly available repositories. All raw sequencing data generated in this study have been submitted to the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA643917, SUB7682741.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article, its supplementary information files and publicly available repositories. All raw sequencing data generated in this study have been submitted to the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA643917, SUB7682741.

Competing interests
The authors declare that they have no competing interests.

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