IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF NOVEL DETERMINANTS OF THE SOMATOTROPES AND LACTOTROPE LINEAGES IN THE ANTERIOR PITUITARY

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DEDICATION

To my parents, Thomas and Denise Peel, who sacrificed so much so that my sister and I could always pursue the best education options available to us, culminating in me writing this thesis today. I love you and I hope that I make you proud.

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but enthusiastically offered me support and advice to “try giving them cheese” nonetheless.

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ABSTRACT

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF NOVEL DETERMINANTS OF THE SOMATOTROPE AND LACTOROPE LINEAGES IN THE ANTERIOR PITUITARY

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Stephen A. Liebhaber

The differentiation of the hormone-producing cell lineages of the anterior pituitary represents an informative model of mammalian cell fate determination. The generation and maintenance of two of these lineages, the growth hormone (GH) producing somatotropes and the prolactin (PRL) producing lactotropes, are dependent on the pituitary-specific transcription factor, POU1F1. While POU1F1 is expressed in both cell types, and plays a direct role in the activation of both the *Gh* and *Prl* genes, GH expression is restricted to somatotropes and PRL expression is restricted to lactotropes. These observations imply the existence of additional, cell type-enriched factors, that contribute to the somatotrope and lactotrope cell identities. Here, we use transgenic mouse models to facilitate sorting of somatotrope and lactotrope populations based on the expression of fluorescent markers expressed under *Gh* and *Prl* gene transcriptional controls. The transcriptomic analyses reveal a concordance of gene expression profiles in the two populations. The limited number of mRNAs that are selectively enriched in each of the two populations includes a set of transcription factors. A subset of

these factors may have roles in pituitary lineage divergence, and/or in regulating expression of cell-type specific genes after differentiation. Four of these factors were validated for lineage enrichment at the level of protein expression, two somatotrope-enriched and two lactotrope-enriched. Three of these four factors were shown to have corresponding activities in appropriate enhancement or repression of landmark somatotrope or lactotrope genes in a pituitary-derived cell culture model system. Conditional inactivation of the genes encoding these factors in mice revealed that two of these transcription factors impact the expression of landmark hormone genes and alter cell phenotypes in primary murine pituitary. Subsequent mechanistic studies revealed that these two factors act on their target genes either through a direct binding at the target promoter and release of paused Pol II complexes, or through indirect mechanism(s). The functions of these two factors are further remarkable in how they maintain hormone expression in the lactotrope and somatotrope lineages; one serves to enhance landmark *Prl* gene expression in lactotropes while the second serves to reciprocally repress expression of *the* Prl gene in somatotropes. In conclusion, these studies identify novel regulators of the somatotropes and lactotropes, explore their mechanisms of action, and establish a useful database for further study of these lineages in the anterior pituitary.

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CHAPTER I: INTRODUCTION

The abilities to preserve homeostasis and to react to physiological stimuli are among the most fundamental processes to life. In animals, entire organ systems are dedicated to regulating and maintaining these processes throughout the body, and many of these systems interact with each other dynamically. The endocrine system is a key component of this intricate network of regulation, signaling to a wide array of organs within the body through signaling molecules known as hormones. A central regulator of the endocrine system is the pituitary gland[1]. The pituitary gland is a small endocrine gland located at the base of the brain sometimes referred to as the “master gland” due to its central role in regulating a broad range of downstream endocrine functions. The pituitary is divided into two lobes: a posterior lobe and an intermediate/anterior lobe. Each of these lobes responds to physiological signals received from the hypothalamus, and secretes the appropriate hormone into the bloodstream in response to these signals[1,2]. Despite its small size, the pituitary produces a broad and diverse set of hormones, which regulate processes as varied as somatic growth, carbohydrate metabolism, reproduction, lactation, stress response, and skin pigmentation.

Conventional understanding of the pituitary states that each of the pituitary hormones is produced and secreted by a dedicated cell type within the gland[1,2]. Thus, pituitary cells are not a singular cell type, but rather a set of multiple cell types occupying the same gland, each receiving specific signals from the hypothalamus and secreting their respective hormones in response. Within the anterior lobe of the pituitary, which produces the highest number of hormones of

the lobes, all of the cell types arise from a common developmental origin[1,2] (**Fig. 1**).

The development of the pituitary is most clearly defined in the mouse. At e9.5, the oral ectoderm begins to invaginate in response to BMP4 expressed by the nearby diencephalon, forming an invagination known as Rathke's pouch[2]. Within Rathke's pouch, Gli family transcription factors, namely *Gli1* and *Gli2*, are crucial regulators of early pituitary development, and deletion of *Gli1* and *Gli2* causes a complete failure of the pituitary to develop in mice[3]. The Gli family of transcription factors is a component of the hedgehog (HH) signaling pathway. Pituitary development requires Sonic Hedgehog (*Shh*) expression, as inhibition of *Shh* prevents Rathke's pouch from growing and prevents the development of all lineages of the endocrine cells of the anterior pituitary[4]. At approximately e11, the cells of Rathke's pouch begin to express a cascade of transcription factors such as Lhx3, Pitx1, and Pitx2[1,2,5] , that guide development of endocrine cells within the developing pituitary. Many stages of anterior pituitary development remain incompletely understood, and there is conflicting information about the origin of some cell lineages. However, it is commonly agreed that after e12.5, sets of transcription factors drive development of the various endocrine cell lineages of the anterior pituitary from the common precursor cells of Rathke's pouch[1,2].

The most abundant cells in the anterior pituitary are those that belong to the so-called "*Pou1f1* lineages.[6]" The *Pou1f1* lineages comprise three cell types within the anterior pituitary that all require the expression of the pituitary-specific

POU domain transcription factor, *Pou1f1*, for their development and for their continued function in the adult pituitary. These cell types, the thyrotropes, lactotropes, and somatotropes, produce thyroid stimulating hormone, prolactin, and growth hormone, respectively[7-9]. Both mice and humans with mutations in the *Pou1f1* gene are characterized by a deficiency of growth hormone, prolactin, and thyroid stimulating hormone, highlighting that the differentiation and expansion of all three of these cell lineages share a common reliance on the actions of *Pou1f1*[10,11].

Somatotropes and lactotropes are linked by a particularly close developmental relationship that remains incompletely understood[1]. *Pou1f1* expression is first detected in the mouse at e13.5, following activation via an unknown mechanism by the transcription factor *Prop1* which is initially expressed at e10 [2]. The subsequent activation of growth hormone (GH) expression can be detected in the developing pituitary starting at e15.5 [2]. Prolactin (PRL) becomes detectable at e16.5, and many PRL positive cells also express GH at this stage in development, though the amount of these so called “somatolactotropes” varies across different species[1,12,13]. These somatolactotrope cells are postulated to resolve into somatotropes and lactotropes shortly after their appearance at e16.5, with somatolactotropes constituting only ~1% of all cells in the adult anterior pituitary[14].

Lineage tracing studies have examined the developmental relationship between somatolactotropes, somatotropes, and lactotropes. The findings of

these lineage tracing studies, carried out by a variety of approaches, are complex. Expression of various reporters under the control of *Gh* regulatory elements in mice indicates that a subset of lactotropes originate from cells that at one point in their development expressed *Gh*, though estimates of the percentage of lactotropes with *Gh*$^+$ origins varies widely from 10% to nearly all lactotropes[15-17]. These data suggest that two sub-populations of lactotrope cells may exist: a population that derives from a *Gh* expressing progenitor, and a second population that originates independently of *Gh* expressing cells. Reciprocal lineage tracing studies using reporters under the regulation of *Prl* elements have found that somatotrope cells do not have a *Prl*$^+$ developmental origin. These data thus suggest that lactotropes may be able to arise from somatotropes, but not *vice versa*[18,19]. Studies in which the lineage tracing was performed by targeted ablation of lactotrope cells in adult mice frequently note an increase in GH$^+$/PRL$^+$ somatolactotropes during the recovery period, raising the possibility of somatolactotrope cells being a transitional state in a one-way trans-differentiation between somatotropes and lactotropes rather than a precursor to both somatotropes and lactotropes[19,20]. In conclusion, while the exact role of the somatolactotrope cells remains unclear, it is clear that the somatotrope and lactotrope cells have a complex and close relationship.

As previously noted, the somatotrope and lactotrope cells both require expression of the pituitary-specific transcription factor, *Pou1f1,* for their differentiation and expansion[8]. However, the role of *Pou1f1* in these cells extends

beyond the development of the two lineages. POU1F1 binds directly to the *Gh* and *Prl* promoters in terminally differentiated somatotropes and lactotropes and is the only clearly defined activator of both genes[21]. The paradox between the observation that POU1F1 activates both the *Gh* and the *Prl* genes, and yet the somatotropes and lactotropes exclusively express GH and PRL, respectively, forms the central issue that this thesis project seeks to address. The fundamental hypothesis of this study is that additional transcription factors are present in the somatotrope and lactotrope lineages that in some manner work in conjunction with POU1F1 activities so that GH and PRL are specifically expressed in their respective cell lineages.

Despite an understanding of how the somatotrope and lactotrope cells function at the level of hormone secretion (i.e., knowledge of which physiological cues and signals from the hypothalamus act on these cells), there are only a few additional (non-hormonal) genes that are known to be expressed specifically in the somatotropes or the lactotropes. Several of these genes express key cell surface receptors that control the expression and/or secretion of each hormone[22,23]. Additionally, previous studies have suggested that factors such as the ubiquitously-expressed repressor NCoR, and the transcription (co)factor LSD1 have opposing functions in the somatotropes and lactotrope cells[24,25]. Unfortunately, such identification of potential regulators of each lineage originate primarily from a single laboratory, have not been followed up or confirmed since

publication, fail to investigate functional impacts of their identified factors, are based primarily on cell line models, and lack validation in animal models[24,26,27].

In the following studies, we use a variety of orthogonal methods to gain insight into the crucial differences between the somatotropes and lactotropes at the level of gene expression. These studies focus primarily on the identification of novel regulators of both the somatotrope and lactotrope lineages. The results of these studies are likely to further our understanding of how these two cell types are able to maintain separate cell identities and selectively express their corresponding hormone products.

Figures and legends

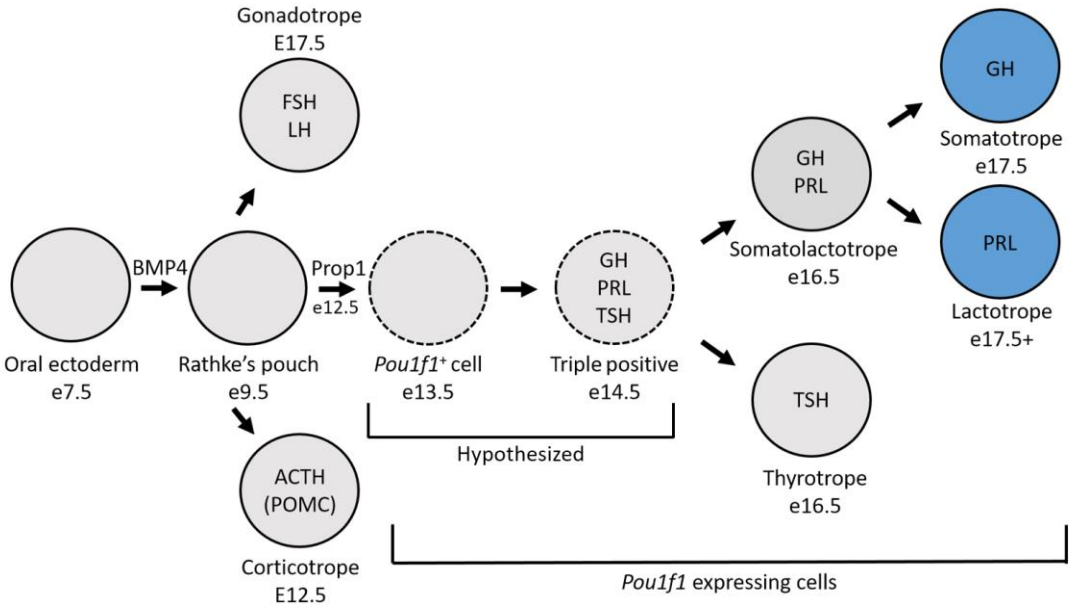


Figure 1. Current standard model of anterior pituitary differentiation in mouse. The cells of the anterior pituitary begin to differentiate from oral ectoderm at e8.5. Expression of the transcription factor *Prop1* is a precursor to *Pou1f1* expression, and *Pou1f1* is expressed in all cells encompassed by the bracket. The somatolactotrope precursor cell arises at e16.5 and differentiates into both somatotropes and lactotropes (highlighted in blue). The existence of the hormone-negative *Pou1f1*$^{+}$ cell and the triple positive cell type is hypothetical (dashed lines) and is based on a Tag-capture study from our lab[28]. This figure is adapted from[2].

CHAPTER II: IDENTIFICATION AND VALIDATION OF NOVEL SOMATOTROPE AND LACTOTROPE ENRICHED TRANSCRIPTION FACTORS

Introduction

The differentiation of multiple cell types from a common precursor cell and the subsequent maintenance of distinct cell identities throughout adult life are processes central to most developmental systems[29,30]. A key question in such processes is how a common precursor cell can give rise to multiple cell types with discrete transcriptomic profiles. Cell types differentiating from a common precursor cell must not only activate transcription of genes necessary for the appropriate cell fate, but also repress/silence expression of genes that drive alternate cell identities. The coordination of these opposing regulatory processes is likely to reflect cell type specific mechanisms of transcriptional control. In addition, terminally differentiated cells must be subsequently maintained in the differentiated state throughout adult life. This necessitates sustained mechanisms and pathways for maintaining cell fate. The establishment and maintenance of cell fate may be regulated by transcription factors that are expressed during development and/or by factors that are induced and sustained during the lifespan of the organism[31,32].

The anterior pituitary is an ideal system for studying mechanisms of lineage establishment and maintenance due to the terminal differentiation and divergence of somatotropes and lactotropes. In mice, the pituitary-specific master regulatory transcription factor, POU1F1, is expressed in a subset of developing pituitary cells beginning at e13.5[2,33,34]. According to a frequently cited model, these POU1F1^{+} cells differentiate into the thyroid stimulating hormone (TSH)

producing thyrotropes and the GH/PRL dual expressing somatolactotropes at e16.5[2,35] (**Fig. 1**). The somatolactotrope precursor then gives rise to the terminally differentiated somatotropes and lactotropes, which produce high levels of GH and PRL protein, respectively[36]. While small populations of somatolactotropes remain present in the pituitaries of adult mice, comprising approximately 1-2% of all cells in the anterior pituitary, their function remains poorly understood (See **Chapter I** for more details on somatolactotropes)[13,37]. Thus, there is a need to better understand the mechanisms that regulate the differentiation, expansion, and functions of the somatotrope and lactotrope populations.

Despite some questions about the pathway of somatotrope and lactotrope divergence and terminal differentiation, all current models of this pathway agree that expression of *Pou1f1* is essential to the establishment of both populations of cells and that the somatotrope and lactotrope cells are closely related. In addition to being a master regulator of the somatotrope and lactotrope lineages, POU1F1 is also a direct activator of both the *Gh* and *Prl* genes, and remains expressed in terminally differentiated somatotropes and lactotropes, driving robust hormone expression throughout adult life[9,21,38,39]. Despite this expression of *Pou1f1* in both cell types, the expression of the GH and PRL proteins remains primarily restricted to the somatotropes and lactotropes, respectively[2,40]. This observation suggests the existence of unknown somatotrope and lactotrope specific factors

that contribute to the divergence and corresponding specificity of gene expression in the two lineages.

Several genes have been previously identified as lineage-enriched and essential to the ability of somatotropes and lactotropes to respond to the appropriate physiological signals to secrete their respective hormones. The growth hormone releasing hormone receptor, GHRHR, and the dopamine receptor DRD2 are examples of known receptors expressed on the surfaces of somatotrope and lactotrope cells, respectively. These cell surface receptors regulate the expression and secretion of GH and PRL in response to physiological cues that trigger signaling from the hypothalamus[23,41,42]. However, the currently known sets of somatotrope and lactotrope specific genes are insufficient to adequately explain the mechanism(s) by which the somatotrope and lactotrope cell identities are established and maintained. Importantly, and central to this thesis, is the fact that POU1F1 remains the only known transcription factor that has been reproducibly demonstrated to directly activate the *Gh* and *Prl* genes[26,43]; some potential activators and repressors have been suggested but not validated or definitively explored (See **Chapter I**)[24]. Taken together, these observations suggest that there exists a set of currently unidentified transcription factors that contribute to the respective somatotrope and/or lactotrope expression profiles. The identification of these factors is essential to a detailed understanding of the function and pathology of these two predominant cell types within the anterior pituitary.

Here we have utilized a set of transgenic mouse lines to facilitate the isolation of primary somatotrope and lactotrope populations from the adult mouse pituitary. Transcriptomic profiling of these two cell populations reveals a noteworthy concordance in their respective transcriptomes. Despite this transcriptomic similarity, we were able to identify from this analysis approximately 300 genes that are significantly enriched (Log_2 fold change >2 and p-value <0.05) in each of the two cell populations. These enriched genes included a subset of transcription factors. Six of these transcription factors were selected for in-depth analysis based on their putative functions. Four of these factors were confirmed for selective enrichment in primary somatotrope and lactotrope cells at the level of protein expression. Our data establish a transcriptomic resource for analysis of somatotrope and lactotrope gene expression and cellular function, and identify a set of novel cell-type enriched transcription factors that have potential roles in regulating the expression of landmark hormone genes.

Results

Establishing the transcriptomes of flow sorted somatotropes and lactotropes.

The goal of this work was to identify novel transcription factors involved in the maintenance of cell identity in the terminally differentiated somatotropes and lactotropes. This goal was approached by marking primary cell populations in the adult mouse pituitary with transgene-encoded fluorescent proteins. Somatotrope cells were tagged with a GFP reporter under the control of the human growth

hormone promoter and its upstream locus control region (LCR)[44], while lactotrope cells were tagged by a DsRed reporter regulated by the rat Prl promoter and upstream sequences (**Fig. 2A**). These regulatory elements have been previously demonstrated as sufficient to define somatotrope and lactotrope-specific expression in mice[45,46]. Modifications were made to the GH-GFP cosmid using conventional sub-cloning techniques to truncate the growth hormone signal peptide (see **Methods**), allowing accumulation of GFP in somatotrope cells rather than secretion, thus improving the GFP signal in this mouse line (**Supplemental Figure 1**). The somatotrope specificity of this modified Gh-GFP line was validated by immunofluorescence microscopy (**Supplemental Figure 2**). 6-8 week old virgin female compound transgenic mice carrying the Gh-GFP and Prl-DsRed transgenes were generated for analysis. The use of compound transgenic mice permits the isolation of somatotropes and lactotropes from the same mouse pituitaries simultaneously, allowing the direct comparison of somatotropes and lactotropes with a minimization of experimental variables. The use of females in these studies ensured our ability to isolate appreciable numbers of lactotropes for subsequent analysis. These females were analyzed at 6-8 weeks; at this point in “adult” life the pituitary has fully matured and established the full array of resident cell lineages[47,48]. 5-6 pituitaries were collected for each RNA-seq analysis, and these pituitaries were pooled and FACS sorted into GFP^+ and $DsRed^+$ populations (**Fig. 2B**).

The study was carried out in biological triplicate, beginning each independent study with a new set of compound transgenic mice. RNA was extracted from each of the two flow sorted cell populations and libraries were generated using low cell input methods (see **Methods** for more details). The RNA-seq data from these three biological replicates were then pooled and the composite transcriptome data set was used to establish the RNA profiles for the GFP and DsRed cell sorts. The use of a composite transcriptomic data set allowed for statistical analyses that aided the process of selecting genes of interest from the data set based on the robustness of their enrichment in each of the replicate samples. The enrichment of somatotropes or lactotropes in the flow sorted cell populations was confirmed by comparing the relative levels of anterior pituitary hormone gene expression in the GFP^+ and $DsRed^+$ populations to the expression of these hormone genes in total pituitaries from virgin female, age-matched controls (**Fig. 2C**). These analyses included all hormone-encoding genes of the anterior pituitary, including pro-opiomelanocortin (*Pomc*), the beta subunits for thyroid stimulating hormone (*Tshb*), luteinizing hormone (*Lhb*), and follicle stimulating hormone (*Fshb*), as well as the shared heterodimerizing alpha subunit (*Cga*). Combined with *Gh* and *Prl*, these genes represent the full set of anterior pituitary hormones and serve as markers for each of the cell lineages of the anterior pituitary. *Gh* expression was enriched in the GFP^+ samples while the expression of all other hormone genes was depleted as compared to total pituitary. Similarly, *Prl* expression was enriched in $DsRed^+$ samples over the *Prl* expression observed in total pituitary. These data demonstrate that the described

FACS procedure yields cell pools that are highly enriched for the somatotrope and lactotrope cell populations.

A second set of analyses of the transcriptomic data assessed the relative expression of sets of well-characterized marker genes that define the somatotrope and lactotrope lineages. A plot of three known markers for each cell type as well as five genes expected to be expressed at similar levels in both cell types **(Fig. 3A)** revealed that each of the somatotrope and lactotrope marker genes was appropriately enriched in its respective GFP^+ or $DsRed^+$ cell pool while genes known to be shared in the somatotrope and lactotrope lineages were expressed at approximately equal levels in both populations. GFP and DsRed reads were also appropriately enriched in each population, confirming proper FACS sorting by fluorescent reporter (**Supplemental Table 1**). Together, these data indicate that the transcriptomic data set is consistent with the assignment of somatotrope and lactotrope cell identities to the GFP^+ and $DsRed^+$ labeled populations, respectively[2,16,22,49-51].

The concordant transcriptomes of the flow sorted somatotropes and lactotropes reveal a limited subset of lineage-enriched transcription factors.

Comparison of the transcriptomes of the flow sorted GFP^+ and $DsRed^+$ cell pools reveals a striking level of concordance in gene expression (**Fig. 3B**). The overall Pearson correlation value for somatotropes vs. lactotropes was calculated as 0.42. However, the expression of *Gh* and *Prl* in these cells is so

robust (note their extreme outlier positions on a $\log_2$ scale in **Fig. 3B**) that these two transcripts markedly skew the Pearson R value and distort the overall transcriptomic comparison. Removing *Gh* and *Prl* from the correlation calculations yields a Pearson R value of 0.94. The significance of these values is two-fold. First, the change from 0.42 to 0.94 observed in the Pearson value when *Gh* and *Prl* are excluded demonstrates the dominance of these two transcripts in the transcriptomes of the somatotropes and lactotropes, with *Gh* and *Prl* reads totaling approximately 25-33% of the mRNA reads in both cell types. Second, the Pearson value of 0.94 after exclusion of *Gh* and *Prl* highlights the overall similarity of the transcriptomes of these two cell types, except for their hormone expression levels. This overall similarity at the transcriptomic level is consistent with a close developmental relationship of these two cell types and suggests that the somatotrope and lactotrope identities are likely to be driven and maintained by a limited number of lineage-enriched factors[2,20,52].

A specific issue that could be directly addressed from these data was whether the structure of *Pou1f1* in the two lineages might differ. The transcriptome data demonstrated that the structure of the *Pou1f1* mRNA is identical in the two pools. These data effectively rule out a contribution by alternative splicing of the *Pou1f1* transcript[53] and generation of lineage-specific *Pou1f1* isoforms to cell type specific functions of *Pou1f1* (**Fig. 3C**). These findings further highlight the importance of additional factors in driving somatotrope and lactotrope specificity.

Genes that surpassed a threshold of a Log_2 fold change of 2 (**Fig. 3B**, highlighted in red) were further filtered by significance (p-value <0.05) and expression level to remove genes that are expressed at low levels and not consistently enriched across all replicates. This differential expression analysis yielded approximately 300 lineage enriched genes for each cell type. Using gene ontology (GO) terms to guide the filtering of these 300 lineage enriched genes produced a list of 24 differentially expressed genes with annotated transcription factor activity (**Fig. 3D**). Thus, these transcriptomic analyses identified a set of lineage-enriched transcription factors that may be important to the distinct attributes of the somatotrope and lactotrope lineages.

Validation of transcription factor differential expression in somatotropes and lactotropes by immunofluorescence analysis of primary pituitary cells.

In order to maximize the effectiveness of downstream studies on the identified lineage-enriched transcription factors, we prioritized for further study a subset of six lineage-enriched transcription factors -- 3 enriched in somatotropes and 3 enriched in lactotropes-- that have functions reported in the literature that are consistent with a role that could be related to pituitary gene regulation. We next attempted to validate that each of the six transcription factors identified enriched at the level of mRNA expression was also enriched at the level of protein expression with the understanding that mRNA expression is not always paralleled by protein production. We also wanted to determine the site of accumulation of each of these proteins, as functional transcription factors would

be expected to accumulate in the nucleus in order to impart transcriptional controls.

Wild type adult female mouse pituitaries were disaggregated and assessed by immunofluorescence microscopy (IF) for each of the six factors. This was done in parallel with immunostaining for GH and PRL proteins in order to mark somatotropes and lactotropes. The first factor to be studied was NUPR1, a somatotrope-enriched factor chosen based on its role in a defined pituitary cell differentiation process centered on the gonadotrope lineage[43,54]. NUPR1 was enriched in the nuclei of somatotrope (GH^+) cells, consistent with the enrichment of the respective mRNA in the GFP^+ cells (**Fig. 4A**). Of note, NUPR1, while enriched in somatotropes vs. lactotropes, was also detected in additional pituitary cells that are both GH and PRL negative. This observation is consistent with reports that NUPR1 plays a role in the development gonadotropes[54].

A second somatotrope-enriched factor, RXRG, was selected for study due to the stimulating effects of its ligand, retinoic acid, on growth hormone production, as well as on its reported heterodimerization with the thyroid hormone receptor, which is reported to bind at the *Gh* promoter[55-58]. RXRG protein was also observed to be enriched in the nuclei of somatotrope cells, identifying it as a gene of interest for further study (**Fig. 4B**). The third somatotrope candidate factor, PPARG, has been shown in previous studies to be frequently expressed in GH secreting pituitary adenomas, suggesting a role in regulating the somatotrope lineage[59]. Immunofluorescence microscopy of

PPARG protein revealed that it was enriched in somatotropes as expected (**Fig. 4C**), but was also present in 48% of lactotropes (detailed cell counts for all immunofluorescence studies are available in **Table 1**). Therefore, while *Pparg* may have a role in the regulation of these lineages, we excluded *Pparg* from further study and focused on *Nupr1* and *Rxrg* due to their more clearly defined somatotrope enrichment. These studies thus confirmed that 2 of the 3 identified somatotrope-enriched factors are appropriately expressed in primary somatotrope cells, and thus these factors were selected for further studies.

IF studies were next carried out on the three transcription factors whose mRNAs were enriched in lactotropes. Among the candidate transcription factors selected from the lactotrope cell population, NR4A2 has been demonstrated to positively regulate transcription of the prolactin gene in extra-pituitary (lymphoid) tissue, making it a gene of interest for its potential role in the regulation of the lactotrope lineage[60]. The NR4A2 protein was enriched in the nuclei of lactotrope (PRL^+) cells (**Fig. 4D**), consistent with transcriptomic data. POU4F1, a member of the POU family of transcription factors, was selected for study due to its role in stimulating aggressive proliferation of pituitary adenomas[61], and on its multiple regulatory roles in development throughout the neuroendocrine system[7,62]. We observed that the POU4F1 protein was also enriched in the nuclei of lactotrope cells, suggesting a potential for regulating the lactotrope lineage (**Fig. 4E**). Lastly, TBX19 was selected for study because it is known to be crucial for pituitary development and establishment of cellular differentiation in the anterior lobe and

intermediate lobe in pituitary, with its primary known function being in regulating the corticotrope lineage where it has been reported to be specifically expressed[63-65]. Immunofluorescence microscopy revealed TBX19 protein in both somatotropes and lactotropes, as well as in additional cell types that were not further characterized (**Fig. 4F**). Thus, while *Tbx19* might be of interest to the regulation of lactotropes, we chose to drop it from our initial studies.

The IF studies identified four factors that were enriched in the nuclei of the appropriate cell types: *Nupr1*, *Rxrg*, *Nr4a2*, and *Pou4f1*. Each of these four factors demonstrated robust cell type enrichment at the mRNA and protein levels, and demonstrated restriction to the nucleus. All of these observations are consistent with their identification as lineage-enriched transcription factors in our transcriptomic analyses. In conclusion, the immunofluorescence microscopy studies of these four transcription factors validated the RNA-seq data generated from primary somatotropes and lactotropes, and suggested that they may play a role in somatotrope vs. lactotrope differentiation and/or specification of cellular function.

Discussion

The establishment and maintenance of cell lineages within the anterior pituitary represents a robust model of cell type specification[66,67]. Delineating the controls of the relevant pathways is critical to understanding normal and pathologic pathways of pituitary functions as they relate to mammalian growth, reproduction, stress response and metabolic functions. Identification of these

pathways and their driving factors has the potential to provide novel targets for diagnosis and therapy of abnormalities in hormone expression and function. As an illustration of the potential benefits of improving understanding of these lineages, the majority of prolactin secreting adenomas (prolactinomas) can be treated medically rather than surgically by administration of dopamine agonists, which bind to the DRD2 receptor and selectively inhibit lactotrope proliferation and prolactin expression[68]. While this medical approach is widely employed in clinical practice, a subset of prolactinomas do not respond to such therapy, necessitating more invasive treatment due to the lack of known treatment targets beyond the dopamine receptor. This gap in therapeutic efficacy highlights the unmet need for better understanding these lineages and their underlying control pathways[69,70].

Extensive genetic studies in humans and mice have identified a number of transcription factors that are essential for pituitary development and function in the mammalian embryo. However, the identification and functional characterization of factors that drive specification of the two primary lineages in the anterior pituitary, the somatotropes and lactotropes, remains remarkably limited[2,43]. According to current models, these lineages are hypothesized to diverge from a common “somatolactotrope” precursor late in embryogenesis (**Fig. 1**)[2], although there is some evidence that a subset of lactotropes emerge directly from somatotrope cells[15]. What is clear is that the pathways of cell expansion and terminal differentiation that generate and maintain both

somatotropes and lactotropes are dependent on the functions of the pituitary master regulatory transcription factor, POU1F1. Although both of these lineages depend on POU1F1 for their expansion and hormonal expression, it is evident that additional factors must be involved in driving their functional divergence. Factors identified in limited previous studies (see **Chapter I**) failed to exhibit evidence of differential expression in our transcriptome analyses, highlighting the challenges in selecting targets for further study without transcriptomic data for isolated cell populations. The identification of such factors has also been hampered in most studies due to their focus on the limited number of known marker proteins or their encoding mRNAs. Thus, substantial advances will depend on the use of more broad based discovery approaches.

In the initial phase of this thesis project, we established and compared the transcriptomes of the somatotrope and lactotrope lineages isolated from the adult mouse pituitary. These transcriptomic databases were derived by RNA-seq analysis of cell populations isolated on the basis of fluorescent markers (GFP and DsRed) driven by the *Gh* or the *Prl* transcription regulatory elements. The validity of this approach was supported by a high level of enrichment of the expected hormone mRNAs in each RNA-seq data set (**Fig. 2C**) and by the enrichment of other known marker genes (**Fig. 3A**). Analysis of these data sets allowed us to arrive at several fundamental observations. First, the direct comparison of the transcriptomes of the two cell types, after removing *Gh* and *Prl* from the analysis, revealed a striking overall correlation (Pearson = 0.94; **Fig.**

3B). This high level of concordance affirms the close developmental and functional relationships of these two lineages. The second observation is that the two cell populations partially overlap in the expression of their key hormone genes, *Gh* and *Prl*. While we find that *Gh* mRNA is appropriately enriched in the GFP^+ cell pool and *Prl* mRNA is appropriately enriched in the $DsRed^+$ cell pool (**Fig. 3A**), there are appreciable levels of *Gh* mRNA in the lactotrope ($DsRed^+$) population and a reciprocal representation of *Prl* mRNA in the somatotrope (GFP^+) population (**Fig. 2C**). While it is likely that small amounts of cross-contamination inherent in techniques such as FACS contribute in part to this result, these data are consistent with the established observations that both genes are activated by *Pou1f1*, which is expressed in both somatotropes and lactotropes, raising the possibility of a basal level of transcription of the reciprocal hormone in each lineage. This observation that a detectable amount of *Gh* and *Prl* mRNAs are produced in the reciprocal lineages supports a model in which somatotropes and lactotropes repress but do not entirely silence the *Prl* and *Gh* genes, respectively. The third observation is that, despite the overall similarities in the transcriptomes of the somatotropes and lactotropes noted above, a set of approximately 300 genes, including multiple transcription factors, is significantly enriched in each lineage. Taken together, these observations suggest that the transcriptomic differences between the somatotrope and lactotrope lineages are quite limited, yet clearly crucial; differential expression of a small set of genes is able to drive noteworthy differences in cell identity and function.

The transcriptome data generated in the present study provide insights into aspects of the somatotrope and lactotrope lineages beyond lineage-enriched transcription factors. While the scope of the present study centers on these transcription factors, other aspects of the transcriptomes of the somatotropes and lactotropes may be of interest for future studies. One post-transcriptional mechanism that has been hypothesized to contribute to differential regulation in the somatotrope and lactotrope lineages is alternative splicing of *Pou1f1*. There are two known isoforms of *Pou1f1*: the major isoform, known simply as *Pou1f1*, and the alternative isoform *Pou1f1β*, formed by alternative splicing extending the second exon, which has been previously suggested to have a repressive function in regulating gene expression in the POU1F1-dependent lineages of the pituitary[53,71,72]. Our data reveals that the levels of *Pou1f1* mRNA and its exonic structure (**Fig. 3C**) are equivalent in the somatotrope and lactotrope populations. This observation supports the hypothesis that the divergence of lactotropes and somatotropes is based on the functions of a set of somatotrope and lactotrope specific factors that may work independently, or in conjunction with POU1F1, rather than differences in the levels or structure of POU1F1 itself.

Further analysis of the genes that are enriched in somatotropes and lactotropes was performed. Enriched Gene Ontology (GO) terms were determined by running the Gene Ontology Consortium's enrichment analysis tool on the approximately 300 statistically enriched genes in both somatotropes and lactotropes (**Supplemental Tables 1 and 2**, respectively). While many of the

enriched GO terms do not have obvious links to somatotrope/lactotrope function, “hormone activity” is a significantly enriched GO term, consistent with the primary function of these cell types. However, many of the genes included in the enrichment of this term are previously known genes such as *Gh* itself and *Ghrhr*. Thus, the utility of GO term analysis in identifying novel genes involved in the somatotrope and lactotrope lineages is limited due to the reliance of GO term analysis on existing knowledge about the roles of many genes, and the narrow set of genes known to be crucial to the regulation of each cell type. We thus focused our later analyses of this transcriptomic data on more targeted approaches rather than broad GO term analyses.

Among the sets of differentially expressed genes, we identified a set of lineage-enriched transcription factors. Of note, most of these factors have not been previously linked to somatotrope or lactotrope identity and function (**Fig. 3D**). Six differentially expressed transcription factors were chosen for detailed study. This selection was based on reports of these six factors having mechanisms of action or functions that would be consistent with their suspected roles in the somatotrope and lactotrope lineages. In an initial screen, immunofluorescence analysis confirmed that four of these six factors were robustly enriched in the nuclei of the cell type predicted by their differential mRNA expression (**Fig. 4**). The remaining two factors were not similarly enriched and were therefore eliminated from further study. The functional impacts of the four validated transcription factors are explored in **Chapter III**. Together, these

transcriptomic analyses not only identify candidate regulators of both the somatotrope and lactotrope lineages, but provide a robust data set for further probing the transcriptomic differences between these two cell lineages.

Figures and legends

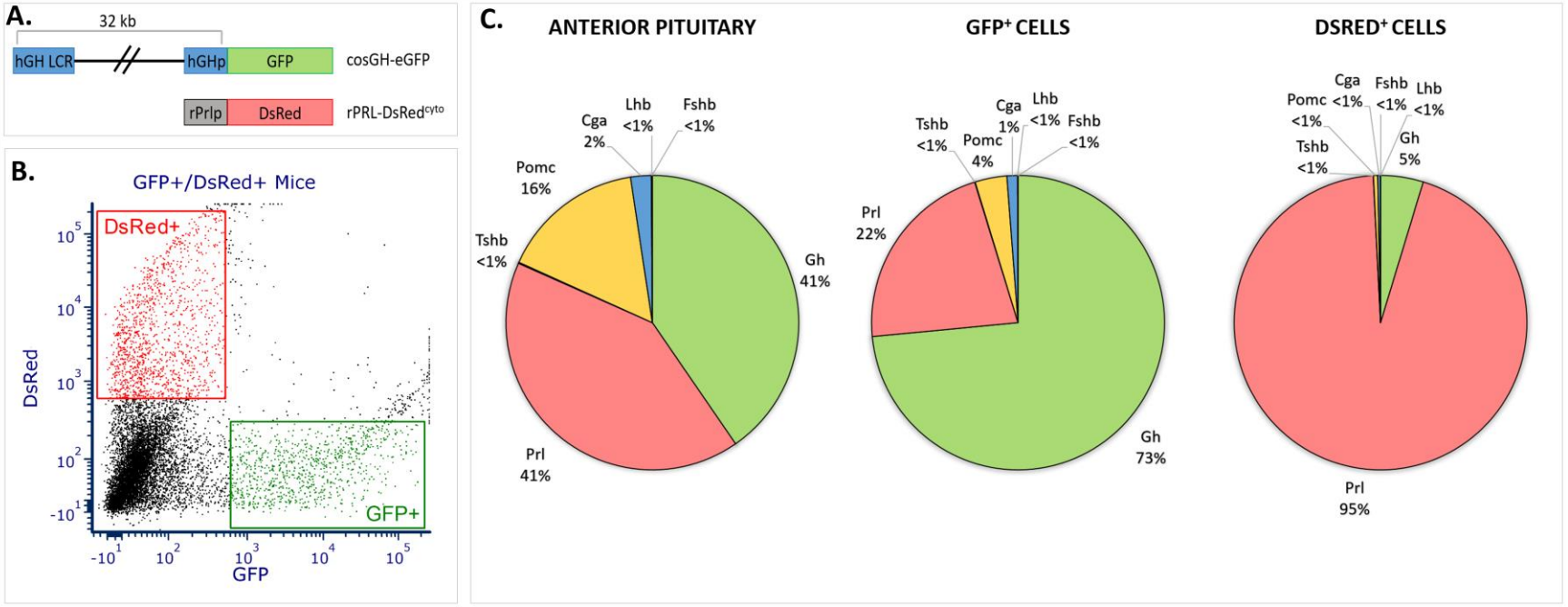
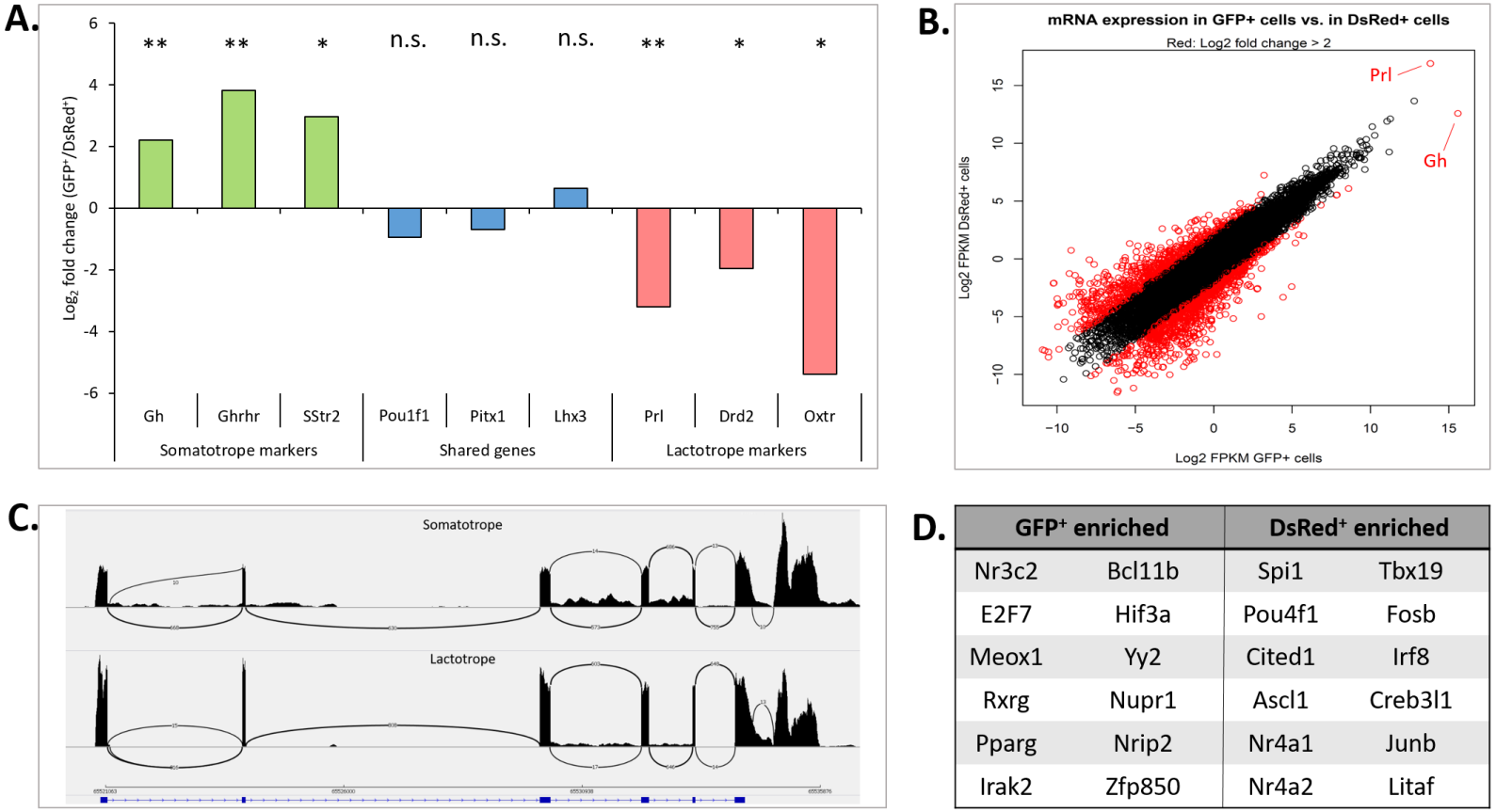


Figure 2. Fluorescence activated cell sorting (FACS) of primary somatotropes and lactotropes from the mouse pituitary. **A.** Schematic of the two transgenes used for selective isolation of somatotropes and lactotropes. **B.** Representative FACS of pituitary cells harvested from cosGH-eGFP/rPrl-DsRed^{cyto} compound transgenic mice. The X

axis represents GFP expression and the Y axis represents DsRed expression. Gating used for sorting is represented by rectangles encompassing the sorted populations. **C.** Relative expression levels of mRNAs encoding the full array of anterior pituitary hormones in the whole pituitary, and from the two FACS populations. Normalized reads (FPKM) are shown for the anterior pituitary hormone genes in total pituitary (left), FACS sorted GFP^+ cells (middle), and FACS sorted $DsRed^+$ cells (right). All samples used for transcriptomic analyses were generated from 6-8-week old virgin female mice.



D.

GFP+ enriched		DsRed+ enriched	
Nr3c2	Bcl11b	Spi1	Tbx19
E2F7	Hif3a	Pou4f1	Fosb
Meox1	Yy2	Cited1	Irf8
Rxrg	Nupr1	Ascl1	Creb3l1
Pparg	Nrip2	Nr4a1	Junb
Irak2	Zfp850	Nr4a2	Litaf

Figure 3. RNA-seq analyses of flow sorted GFP+ and DsRed+ cell populations reveal subsets of lineage-enriched transcription factors. A. Enrichment (Log_2 fold change) of mRNAs in the GFP+ and DsRed+ populations encoding

defined markers for somatotropes (green), lactotropes (red), and for markers shared between somatotropes and lactotropes (blue). The designation of each respective marker gene is noted at the bottom of the figure. A positive value on the Y-axis indicates enrichment in the GFP^+ population while a negative value indicates enrichment in the $DsRed^+$ population. Significance values relate to the enrichment in mRNA expression between the two flow sorted populations. * Indicates p-value of less than 0.05, ** indicates p-value of less than 0.01, n.s. indicates not significant. **B.** Correlation plot of the RNA-seq analysis GFP^+ cells vs. $DsRed^+$ cells. The Log_2 FPKM values for all mRNAs in the GFP^+ (x-axis) and $DsRed^+$ (y-axis) samples were plotted, and mRNAs surpassing a threshold of 4-fold enrichment or greater were highlighted in red. These enriched mRNAs were then further filtered to remove low expressers, leaving only genes that were robustly expressed and significantly enriched ($p \leq 0.05$). The positions of *Gh* and *Prl* are noted on this plot. **C.** Representative Sashimi plot track showing raw mapped mRNA reads at the *Pou1f1* locus in GFP^+ and $DsRed^+$ cell populations. The identity of the two Sashimi plots indicates identical splicing patterns of the *Pou1f1* transcript in somatotropes and lactotropes, in accordance with analysis performed using TopHat2 (see **Methods**). **D.** List of transcription factors enriched in GFP^+ vs. $DsRed^+$ cell populations were identified by taking the GFP/DsRed specific genes identified by differential expression analysis and using PANTHER to identify genes with known GO terms for transcription factor activity.

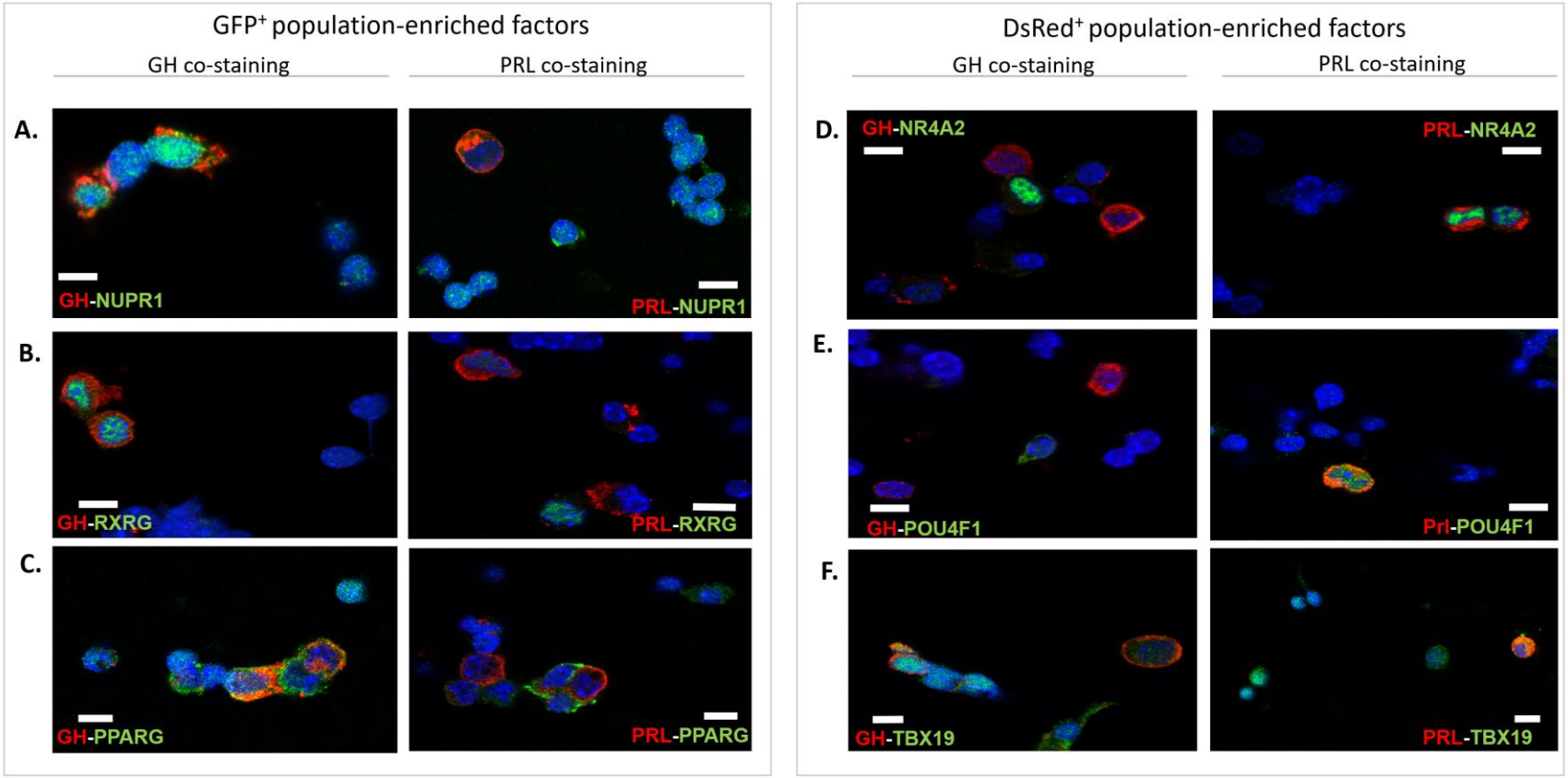


Figure 4. Immunofluorescence microscopy validated cell-type specificity of expression for a subset of lineage enriched transcription factors. Cells disaggregated from wild type pituitaries (female) were stained for each of six

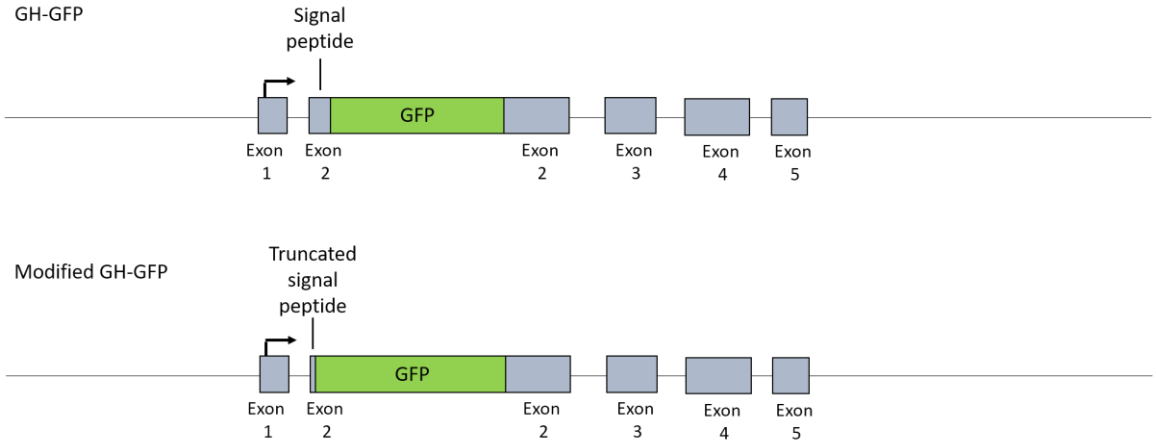
transcription factors whose mRNAs were enriched in the GFP^+ cell population (**A-C**) and the $DsRed^+$ cell population (**D-F**) (as in **Fig. 3D**). Each transcription factor is stained in green, and cells were co-stained red with antibodies to identify GH or PRL as a means of identifying somatotropes and lactotropes, respectively. **A.** NUPR1, **B.** RXRG, **C.** PPARG. **D.** NR4A2. **E.** POU4F1. **F.** TBX19. All cells were stained with DAPI (blue) to mark nuclei. Scale bars = 5 μm.

Tables

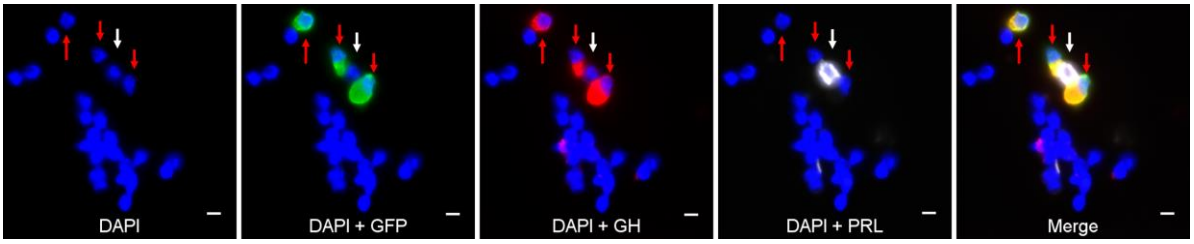
Transcription factor	# Positive somatotropes (% of all somatotropes)	# Positive lactotropes (% of all lactotropes)
Nupr1	176 (88%)	18 (9%)
Rxrg	183 (91.5%)	11 (5.5%)
Pparg	148 (74%)	96 (48%)
Nr4a2	4 (2%)	186 (93%)
Pou4f1	9 (4.5%)	193 (96.5%)
Tbx19	174 (87%)	188 (94%)

Table 1. Cell counts for immunofluorescence assays. Cell counts for immunofluorescence microscopy analyses of selected transcription factors. Columns list the number of GH^+ or PRL^+ cells examined (200 GH^+ and 200 PRL^+ cells counted per study) expressing the given transcription factor, as well as the percentage of total somatotropes/lactotropes observed expressing the protein in parentheses.

Supplemental Data



Supplemental Figure 1. Schematic of GH-GFP transgene and modified version of transgene without the growth hormone signal peptide. The previously described GH-GFP transgene (top) produces somatotrope-specific GFP expression, but the presence of the growth hormone signal peptide (first 24 amino acids of the GH protein) causes GFP to be secreted as growth hormone is, leading to some somatotropes having relatively low GFP levels. By truncating the beginning of exon 2 (bottom panel), the signal peptide was reduced from its full 24 amino acids to only 8 amino acids, ablating its function and causing the GFP protein produced from this transgene to accumulate in the cytoplasm of somatotropes. No other modifications were made to the transgene.



Supplemental Figure 2. Immunofluorescence microscopy of GH-eGFP pituitary cells demonstrating that GFP is selectively expressed in somatotropes. Pituitaries from GH-eGFP mice were disaggregated and co-stained for DAPI, GFP, GH, and PRL, as indicated in each frame. The red arrows indicate three GFP^+ cells which are also GH^+. The white arrow indicates a PRL^+ cell which is negative for GFP and GH. The merge frame combines DAPI along with GFP (green), GH (red), and PRL (white). A total of 200 somatotropes and 200 lactotropes (identified by the expression of GH or PRL, respectively) were assayed for GFP expression. Out of 200 somatotropes, 184 (92%) were GFP positive. Out of 200 lactotropes, 2 (1%) were GFP positive. These data demonstrate robust somatotrope specificity for the GH-eGFP line. Scale bar, 5 um.

	Total GFP reads	Total DsRed reads
GFP+ sample #1	328159	699
GFP+ sample #2	1140	260
GFP+ sample #3	3260	776
DsRed+ sample #1	3235	7339
DsRed+ sample #2	158	2056
DsRed+ sample #3	120	5440

Supplemental Table 1. Total read counts for GFP and DsRed mRNAs in each FACS-sorted sample used for RNA-seq analysis.

GO molecular function complete	Raw P-value	FDR
carbohydrate:cation symporter activity (GO:0005402)	3.18E-04	4.66E-02
lipopolysaccharide binding (GO:0001530)	5.92E-05	1.04E-02
carbohydrate transmembrane transporter activity (GO:0015144)	1.87E-04	2.93E-02
nuclear receptor activity (GO:0004879)	4.14E-05	7.85E-03
transcription factor activity, direct ligand regulated sequence-specific DNA binding (GO:0098531)	4.14E-05	7.54E-03
voltage-gated potassium channel activity (GO:0005249)	3.44E-07	5.21E-04
steroid hormone receptor activity (GO:0003707)	6.92E-05	1.16E-02
potassium channel activity (GO:0005267)	4.03E-07	3.06E-04
voltage-gated cation channel activity (GO:0022843)	9.57E-07	3.96E-04
potassium ion transmembrane transporter activity (GO:0015079)	3.69E-07	3.36E-04
voltage-gated channel activity (GO:0022832)	9.35E-06	2.50E-03
voltage-gated ion channel activity (GO:0005244)	9.35E-06	2.36E-03
heparin binding (GO:0008201)	1.22E-04	1.98E-02
glycosaminoglycan binding (GO:0005539)	1.35E-05	2.93E-03
monovalent inorganic cation transmembrane transporter activity (GO:0015077)	3.49E-07	3.97E-04
ion gated channel activity (GO:0022839)	3.19E-06	9.67E-04
gated channel activity (GO:0022836)	4.25E-06	1.21E-03
secondary active transmembrane transporter activity (GO:0015291)	2.26E-04	3.43E-02
cation channel activity (GO:0005261)	1.57E-05	3.24E-03
ion channel activity (GO:0005216)	1.82E-06	6.88E-04
passive transmembrane transporter activity (GO:0022803)	8.62E-07	4.90E-04
channel activity (GO:0015267)	8.62E-07	4.36E-04
substrate-specific channel activity (GO:0022838)	2.53E-06	8.85E-04
metal ion transmembrane transporter activity (GO:0046873)	1.23E-05	2.79E-03
cation transmembrane transporter activity (GO:0008324)	4.53E-07	2.94E-04
inorganic cation transmembrane transporter activity (GO:0022890)	2.93E-06	9.51E-04
ion transmembrane transporter activity (GO:0015075)	4.41E-08	2.01E-04
inorganic molecular entity transmembrane transporter activity (GO:0015318)	2.64E-07	6.01E-04
lipid binding (GO:0008289)	2.46E-05	4.87E-03
transmembrane transporter activity (GO:0022857)	8.99E-07	4.09E-04
transporter activity (GO:0005215)	9.46E-06	2.27E-03

Supplemental Table 2. Molecular function (GO ontology) terms enriched in somatotrope genes.

GO molecular function complete	Raw P-value	FDR
G-protein coupled nucleotide receptor activity (GO:0001608)	7.82E-06	1.78E-03
G-protein coupled purinergic nucleotide receptor activity (GO:0045028)	7.82E-06	1.69E-03
G-protein coupled photoreceptor activity (GO:0008020)	5.48E-04	4.98E-02
chemokine receptor activity (GO:0004950)	3.64E-07	2.36E-04
G-protein coupled chemoattractant receptor activity (GO:0001637)	3.64E-07	2.07E-04
C-C chemokine binding (GO:0019957)	3.64E-07	1.84E-04
purinergic nucleotide receptor activity (GO:0001614)	8.71E-06	1.80E-03
nucleotide receptor activity (GO:0016502)	8.71E-06	1.72E-03
C-C chemokine receptor activity (GO:0016493)	2.64E-06	9.24E-04
chemokine activity (GO:0008009)	4.64E-10	7.03E-07
purinergic receptor activity (GO:0035586)	3.40E-06	1.10E-03
chemokine binding (GO:0019956)	2.03E-06	7.68E-04
transmitter-gated ion channel activity involved in regulation of postsynaptic membrane potential (GO:1904315)	2.30E-05	3.48E-03
chemokine receptor binding (GO:0042379)	6.74E-09	6.13E-06
neurotransmitter receptor activity involved in regulation of postsynaptic membrane potential (GO:0099529)	2.75E-05	3.79E-03
postsynaptic neurotransmitter receptor activity (GO:0098960)	3.89E-05	4.78E-03
transmitter-gated channel activity (GO:0022835)	1.05E-05	1.90E-03
transmitter-gated ion channel activity (GO:0022824)	1.05E-05	1.83E-03
CCR chemokine receptor binding (GO:0048020)	1.14E-04	1.26E-02
G-protein coupled peptide receptor activity (GO:0008528)	2.40E-10	1.09E-06
peptide receptor activity (GO:0001653)	3.96E-10	9.00E-07
extracellular ligand-gated ion channel activity (GO:0005230)	1.12E-04	1.27E-02
neurotransmitter receptor activity (GO:0030594)	1.43E-05	2.32E-03
ligand-gated channel activity (GO:0022834)	6.24E-06	1.77E-03
ligand-gated ion channel activity (GO:0015276)	6.24E-06	1.67E-03
ligand-gated cation channel activity (GO:0099094)	3.17E-04	3.07E-02
hormone binding (GO:0042562)	2.36E-04	2.39E-02
cytokine activity (GO:0005125)	4.39E-07	2.00E-04
carbohydrate binding (GO:0030246)	5.92E-07	2.45E-04
G-protein coupled receptor binding (GO:0001664)	2.84E-05	3.80E-03
cytokine receptor binding (GO:0005126)	9.83E-06	1.86E-03
ion gated channel activity (GO:0022839)	3.63E-05	4.59E-03
gated channel activity (GO:0022836)	5.57E-05	6.49E-03
G-protein coupled receptor activity (GO:0004930)	8.10E-10	9.21E-07
ion channel activity (GO:0005216)	1.27E-05	2.15E-03
passive transmembrane transporter activity (GO:0022803)	7.63E-06	1.93E-03
channel activity (GO:0015267)	7.63E-06	1.83E-03
substrate-specific channel activity (GO:0022838)	1.87E-05	2.94E-03

cation channel activity (GO:0005261)	5.55E-04	4.95E-02
receptor ligand activity (GO:0048018)	4.14E-05	4.95E-03
receptor regulator activity (GO:0030545)	2.47E-05	3.51E-03
signaling receptor activity (GO:0038023)	2.42E-05	3.54E-03
molecular transducer activity (GO:0060089)	3.19E-05	4.15E-03
transmembrane signaling receptor activity (GO:0004888)	1.27E-04	1.37E-02
protein binding (GO:0005515)	4.59E-04	4.26E-02
binding (GO:0005488)	4.80E-06	1.46E-03
molecular_function (GO:0003674)	2.12E-04	2.19E-02
Unclassified (UNCLASSIFIED)	2.12E-04	2.25E-02
RNA binding (GO:0003723)	2.61E-04	2.58E-02
olfactory receptor activity (GO:0004984)	1.02E-07	7.70E-05
odorant binding (GO:0005549)	3.27E-04	3.10E-02

Supplemental Table 3. Molecular function (GO ontology) terms enriched in lactotrope genes

CHAPTER III: A SET OF CELL TYPE-ENRICHED TRANSCRIPTION FACTORS IMPACTS EXPRESSION OF LANDMARK PITUITARY GENES

Introduction

Transcription factors may act through a variety of mechanisms and can exert either activating or repressing effects. Transcription factors enriched in either the somatotrope or lactotrope cell populations may have roles in enhancing the expression of landmark genes within their cell lineage or they may play a role in repressing genes of the reciprocal lineage. For example, a transcription factor enriched in somatotropes may have a role in enhancing *Gh* expression or in repressing *Prl* expression. Elucidating these functional impacts as well as their mechanistic bases is crucial to understanding the role that an identified transcription factor has in regulating the somatotrope or lactotrope lineages.

Having identified and validated a set of four candidate transcriptions—two expressed in somatotropes and two expressed in lactotropes—we next wanted to assess their roles in regulating their respective cell types. Here, we utilize a set of murine pituitary-derived cell line models to assay the functional impacts of each of the candidate transcription factors on known landmark genes in both the somatotrope and lactotrope lineages. These transcription factors were also assessed for occupancy at chromatin sites encompassing the *Gh* and *Prl* gene promoters. Mechanistic studies reveal that one of the lactotrope-enriched factors binds adjacent to POU1F1 at the *Prl* gene promoter and synergizes with POU1F1 in stimulation of *Prl* gene transcription by enhancing the release of Pol II from the *Prl* promoter. In contrast, a second factor was found to repress *Prl*

expression in somatotropes through an indirect mechanism. These studies highlight and begin to define the variety of mechanisms that underlie lineage specificity in the mammalian pituitary.

Results

Functional assessment of candidate transcription factors: impacts on somatotrope and lactotrope gene expression in cell culture

To determine the functional impact of the four candidate factors on somatotrope and lactotrope identity, we expressed each factor in a murine pituitary-derived cell line, Pit-1/Triple[28]. Pit-1/Triple cells, isolated by T-antigen immortalization of mouse pituitary cells, express POU1F1 (also referred to as PIT-1[6]) as well as the three POU1F1 dependent hormones: GH, PRL, and TSH. The Pit-1/Triple cells express robust levels of *Pou1f1* and express each of the *Gh*, *Prl*, and *Tshb* mRNAs at levels substantially lower than those detected in whole adult mouse pituitary[28]. On the basis of this profile of gene expression, we have proposed that these Pit-1/Triple cells may represent cells at an early stage in pituitary development, immediately following the activation of *Pou1f1* but prior to terminal differentiation of the individual hormone expressing lineages[28] (corresponding to the “Triple positive, e14.5” cells noted in **Fig. 1**). Transcriptomic analyses of the Pit-1/Triple cells (unpublished data) revealed that each of the four candidate transcription factors identified and validated in **Chapter II** is either not expressed or expressed at low levels. We hypothesized that forced expression of each of the factors would have a specific impact on

genes that in some manner define lactotrope or somatotrope cell identity. To test this hypothesis, each candidate factor was ectopically expressed in Pit-1/Triple cells from a bi-cistronic expression vector containing the transcription factor ORF and a GFP ORF behind an internal ribosome entry site (IRES) (see details in **Methods**). 48 hours post transfection, GFP^+ Pit-1/Triple cells were isolated by FACS. RNA was isolated and assayed by qRT-PCR to assess impacts of each factor on the expression of somatotrope and lactotrope marker genes. The expression of each target gene was compared to the corresponding mRNA levels in GFP^+ cells isolated from a control transfection with an empty (GFP-only) vector.

A panel of five lineage marker genes was assayed in each qRT-PCR assay. *Gh* and the growth hormone releasing hormone receptor (*Ghrhr*) were assayed as markers of the somatotrope lineage while *Prl* and the dopamine receptor (*Drd2*) were assayed as markers of the lactotrope lineage. *Pou1f1* was included in the panel of assayed genes as a gene that is common to both lineages. This panel of genes, while not encompassing a comprehensive set of somatotrope/lactotrope markers, includes the most prominent known markers and thus serves as a useful metric for assaying changes in the expression of genes crucial to these two lineages.

Ectopic expression of the somatotrope candidate factor, *Nupr1*, in the Pit-1/Triple cells caused a significant repression of both *Prl* and *Drd2* while failing to impact on expression of *Gh* or *Ghrhr* (**Fig. 5A**). This impact of *Nupr1* would be

consistent with a role for this somatotrope-enriched factor in selectively repressing the expression of lactotrope genes in the somatotrope lineage. Expression of *Rxrg* failed to impact the expression of any of the assayed genes (**Fig. 5B**), even in the presence of its ligand, retinoic acid (**Supplemental Figure 3**)[73,74], and so *Rxrg* was excluded from further study. Expression of the lactotrope candidate factors, *Nr4a2* and *Pou4f1*, both resulted in a significant increase in *Prl* expression and while leaving the expression of the other assayed genes unchanged (**Fig. 5C, D**). These data are consistent with these two lactotrope-enriched factors serving to enhance the expression of *Prl* in the lactotrope lineage. In sum, the data from these cell transfection studies support potential reciprocal roles for three lineage-enriched factors; activating lactotrope specific genes linked to lactotrope identity (*Nr4a2* and *Pou4f1*), and repressing lactotrope specific genes to maintain somatotrope identity (*Nupr1*).

Characterization of transcriptional regulatory mechanisms at the *Prl* promoter of the lineage-enriched transcription factors

The mechanism of action by which a regulatory factor affects the transcription of a gene can be direct or indirect. One indication of direct action on transcription is the occupancy of the factor at regulatory elements linked to the gene of interest. *Nr4a2* and *Pou4f1* both enhance *Prl* expression when expressed in Pit-1/Triple cells (**Fig. 5C, D**), raising the possibility of direct binding at the *Prl* promoter. Two binding sites for both NR4A2 and POU4F1 are predicted within a 250 bp region of the *Prl* promoter by JASPAR analysis[75], and these sites

are intermingled with 4 known POU1F1 binding sites[21,75]. Of particular interest was the observation that one of the NR4A2 predicted binding sites is located immediately adjacent to the POU1F1 binding site most proximal to the *Prl* start codon (**Fig. 6A**). This close positioning of the POU1F1 and NR4A2 binding sites raises the possibility that NR4A2 and POU1F1 interact at the *Prl* promoter. Pit-1/Triple cells expressing recombinant NR4A2 or POU4F1 were generated and enriched by FACS (as above), and chromatin was assayed for factor occupancy at the *Prl* promoter. The *MyoD* promoter was assayed as a control for non-specific binding and the *Gh* promoter was assayed as a specificity control. Additional sites 500 bp upstream and downstream of the *Prl* promoter were also assayed to confirm that any binding at the promoter was promoter-specific. The NR4A2 ChIP revealed significant and specific binding at the *Prl* promoter (**Fig. 6B**), while a parallel ChIP assay for POU4F1 failed to reveal evidence of significant binding in this region. POU4F1 binding to its known autoregulatory element[76] confirmed the efficacy of the POU4F1 ChIP study. (**Fig. 6C, D**). In order to confirm that NR4A2 binds to the *Prl* promoter *in vivo*, NR4A2 ChIP was performed on chromatin isolated from FACS-sorted mouse lactotropes (**Fig. 6E**). The data from the analysis of these primary mouse cells demonstrated selective *in vivo* binding of NR4A2 at the *Prl* promoter. Thus, these ChIP studies along with the preceding gene expression assays reveal that NR4A2 is recruited to the *Prl* promoter and enhances *Prl* gene expression. In contrast, POU4F1 does not bind the *Prl* promoter and thus the enhancement of *Prl* expression observed in the Pit-1/Triple cell transfection study may reflect its binding to an as yet

uncharacterized regulatory element beyond the proximal *Prl* promoter, or through an indirect mechanism.

The lactotrope-enriched transcription factor, NR4A2, acts in conjunction with POU1F1 to enhance Prl gene expression.

The binding of NR4A2 adjacent to a POU1F1 binding site within the *Prl* promoter suggested a model in which NR4A2 works in concert with POU1F1 to support *Prl* gene expression. This model was tested by transfections of the two factors either alone or in combination into the Pit-1/0 cell line[28]. Pit-1/0 cells, generated by SV40 T-antigen mediated transformation of mouse pituitary cells (as with the Pit-1/Triple cells), model an early stage of anterior pituitary development in which *Pou1f1* is activated, but not yet expressed at significant levels, and in which none of the three POU1F1-dependent hormone genes (*Gh*, *Prl*, and *Tshb*) have yet been activated (corresponding to the "*Pou1f1*$^{+}$ cell, e13.5"; **Fig. 1**)[28]. Pit-1/0 cells were transfected with plasmids encoding either *Pou1f1* or *Nr4a2* alone, or with a 1:1 mix of the two expression vectors. Cells transfected with the *Pou1f1* expression vector alone exhibited a 4-fold increase in *Prl* expression over a vector control (**Fig. 6F**). Although a parallel transfection with *Nr4a2* plasmid alone displayed no activation of *Prl* expression, co-transfection of *Nr4a2* plasmid with the *Pou1f1* plasmid stimulated *Prl* expression 8-fold over the vector control. There was no apparent impact of the factor transfections, either alone or in combination, on the other marker genes in the

assay panel. These data suggest that NR4A2 requires the presence of POU1F1 to enhance expression of the *Prl* gene.

NR4A2 fails to enhance POU1F1 occupancy at the *Prl* promoter or enhance the presence of activating chromatin marks

What is the mechanism by which NR4A2 enhances POU1F1 actions at the *Prl* promoter? One potential mechanism is by facilitating recruitment of POU1F1 to the *Prl* promoter. To test this hypothesis, POU1F1 occupancy was assayed by ChIP in Pit-1/Triple cells expressing exogenous NR4A2 and compared to POU1F1 occupancy in Pit-1/Triple cells not expressing NR4A2 (**Fig. 7**). The ChIP was performed using a POU1F1 antibody generated in the lab and validated by demonstrating specific detection of POU1F1 (**Supplemental Figure 4**). This experiment revealed no enhancement of POU1F1 occupancy at the *Prl* promoter in the presence of NR4A2. Thus, the functional interaction of NR4A2 with POU1F1 at the *Prl* promoter does not rely on increasing POU1F1 recruitment to the *Prl* promoter.

Having observed that NR4A2 binds the *Prl* promoter adjacent to a known POU1F1 binding site and that NR4A2 does not enhance the binding of POU1F1, we next hypothesized that NR4A2 may play a role in histone modification at the *Prl* promoter prior to transcription. Histone acetylation constitutes an early step towards initiating transcription[77,78], and histone 3 lysine 27 (H3K27) acetylation as well as histone 3 lysine 4 (H3K4) tri-methylation have been identified as

promoter marks that strongly correlate with active transcription[79-81]. To test our hypothesis, we assayed changes in H3K27 acetylation and H3K4 tri-methylation at both the *Gh* and *Prl* promoters in Pit-1/0 cells transfected with *Pou1f1* or *Nr4a2* alone, or transfected with a 1:1 mix of both factors. Pit-1/0 cells were used in this assay because the *Gh, Prl,* and *Pou1f1* genes are inactive, making these cells a useful model for detecting epigenetic modifications at these loci that track with the process of transcriptional activation. Transfection with *Pou1f1* alone caused a significant increase in H3K27 acetylation at both the *Gh* and *Prl* promoters, consistent with the known roles of POU1F1 in activating both of these genes (**Fig. 8A**). In contrast, transfection with *Nr4a2* alone did not cause an increase in H3K27 acetylation over an empty vector control, and cells transfected with both *Pou1f1* and *Nr4a2* had levels of H3K27 acetylation similar to those of cells transfected with *Pou1f1* alone. A similar effect was observed when assaying changes in H3K4me3 levels (**Fig. 8B**) with expression of *Pou1f1* alone causing an increase in H3K4me3 levels at both the *Gh* and *Prl* promoters while the addition of *Nr4a2* caused no significant change in H3K4 tri-methylation over the level observed in the presence of *Pou1f1* alone. These data indicate that POU1F1 is sufficient to recruit histone acetylases and methylases to impart activating modifications at both the *Gh* and *Prl* promoters. The data further suggest that these activating modifications imparted by POU1F1 are not sufficient for selective transcriptional activation of *Gh* vs. *Prl*, as *Pou1f1* selectively increases levels of *Prl* but not *Gh* mRNA in the *Pou1f1* transfected Pit-1/0 cells (**Fig. 6F**). Finally, the data indicate that NR4A2 appears to enhance *Prl*

expression *via* a mechanism unrelated to any augmentation in H3K27 acetylation or H3K4 tri-methylation over that imparted by POU1F1. Taken together, these data lead us to conclude that the lactotrope-enriched factor, NR4A2, binds adjacent to POU1F1 at the *Prl* promoter and synergizes with POU1F1 to enhance *Prl* gene expression through a mechanism that is unrelated to POU1F1 recruitment or to the augmentation of defined histone modifications.

NR4A2 binding at the Prl promoter is POU1F1 dependent

In our preceding studies we determined that NR4A2 and POU1F1 bind at adjacent sites in the *Prl* gene promoter, that NR4A2 does not impact on POU1F1 occupancy at the *Prl* promoter, nor does it augment the levels of activating modifications at the *Prl* promoter. We also demonstrated that *Nr4a2* has no impact on *Prl* gene expression in the absence of *Pou1f1*. These data could be unified in a model in which POU1F1 initially binds to the *Prl* promoter and makes the adjacent chromatin of the promoter accessible for NR4A2 binding. To determine if NR4A2 binding at the *Prl* promoter is in fact POU1F1 dependent, we transfected Pit-1/0 cells (which express neither *Nr4a2* nor appreciable levels of *Pou1f1*) with combinations of plasmids expressing *Nr4a2* and *Pou1f1*. These cells were harvested, chromatin isolated and the occupancy of NR4A2 at the *Prl* promoter was assessed by NR4A2 ChIP. NR4A2 protein binding to the *Prl* promoter was only observed in cells that co-expressed *Nr4a2* and *Pou1f1*. There was no evidence of binding of NR4A2 in cells containing *Nr4a2* but lacking *Pou1f1* (**Fig. 9**). Thus POU1F1 plays an essential role in facilitating the binding of

NR4A2 at the *Prl* promoter. These data, in conjunction with the prior observation that *Nr4a2* can only enhance *Prl* expression in the presence of *Pou1f1* (**Fig. 6F**), suggest a model in which NR4A2 binds at the *Prl* promoter in a POU1F1-dependent manner and enhances *Prl* expression in conjunction with, and fully dependent on the co-binding of POU1F1. This conclusion raises the mechanistic question of how NR4A2 acts to enhance the action of POU1F1 at the *Prl* promoter.

NR4A2 enhances the release of RNA polymerase II from the *Prl* promoter, increasing transcription

The observation that NR4A2 binding was selective to the *Prl* promoter vs. the *Gh* promoter and that its functions were dependent on *Pou1f1* raised the possibility that it served to in some way potentiate *Pou1f1* actions on *Prl*. Based on the preceding data we hypothesized that NR4A2 may enhance *Prl* transcriptional activity by augmenting in some manner the recruitment of RNA polymerase II (Pol II) to the *Prl* promoter or by enhancing the actions of Pol II once recruited. To initially test these models, we transfected Pit-1/0 cells with plasmids that express *Pou1f1* and *Nr4a2* (see above and **Methods**) and assayed Pol II occupancy at key regulatory elements of the *Prl* locus as well as regulatory elements at the *Gh* and *Pou1f1* loci (**Fig. 10**). This Pol II ChIP study produced the noteworthy observation that Pol II recruitment to the *Prl* promoter is dependent on the actions of *Pou1f1* and that this recruitment to the Prl promoter is not impacted to any extent by the co-expression of *Nr4a2*. Surprisingly,

however, the ChIP analysis revealed that *Nr4a2* robustly enhanced the presence of Pol II within the *Prl* gene body (**Fig. 10**, “Prl downstream”). Together, these data suggest that NR4A2 has an impact on *Prl* promoter function that is distal to POU1F1 binding and distal to Pol II recruitment. One possible mechanistic model that fits these findings is that NR4A2 plays a role in the release of Pol II from the *Prl* promoter into the gene body.

The observation that NR4A2 may be involved in the release of Pol II from the *Prl* promoter prompted us to hypothesize that NR4A2 plays a role in recruiting Pol II release factors to the *Prl* promoter. The major release factor that permits the release of Pol II from promoters is P-TEFb, composed of Cdk9 and cyclin T1[82,83]. To determine if NR4A2 enhances recruitment of P-TEFb to the *Prl* promoter, we transfected Pit-1/0 cells with plasmids expressing *Pou1f1* and *Nr4a2* and performed ChIP for the Cdk9 component of P-TEFb. The presence of POU1F1 stimulated significant increases in Cdk9 occupancy at both the *Gh* and *Prl* promoters, as well as a robust recruitment of Cdk9 to the auto-regulatory *Pou1f1* promoter[9] (**Fig. 11**). Remarkably, however, there was no significant increase in Cdk9 occupancy at the *Prl* promoter in cells co-expressing POU1F1 and NR4A2 as compared with POU1F1 alone. We conclude from these studies that the selective stimulatory impact of NR4A2 on Pol II release from the *Prl* promoter appears to act by a P-TEFb independent pathway. These data establish a foundation for future studies that focus on identifying the components and mechanism of Nr4a2-mediated Pol II release at the Prl promoter.

Discussion

We have previously identified a set of transcription factors enriched in either somatotropes or lactotropes (**Chapter II**). The four transcription factors with appropriate localization and enrichment by immunofluorescence analysis were subsequently assessed for functions relevant to lineage identity by expression of the recombinant proteins in the Pit-1/Triple cell line. Our studies of the somatotrope-enriched transcription factor, *Nupr1*, were highly informative. Ectopic expression of *Nupr1* in the Pit-1/Triple cells, caused a decrease in the two most prominent lactotrope gene markers, *Prl* and *Drd2*. This action suggests that the somatotrope factor *Nupr1* exerts a repressive action on lactotrope specific genes, an activity that could contribute to the maintenance of somatotrope lineage identity (**Fig. 5A**). It is of note that *Nupr1*$^{-/-}$ mice have been reported to be fertile and of normal size[54]. The normal size of these *Nupr1*$^{-/-}$ mice is consistent with our observations that *Nupr1* expression does not impact *Gh* levels. Based on the repressive effect of *Nupr1* on *Prl* expression in the Pit-1/Triple cells, we would predict that *Nupr1*$^{-/-}$ mice will display elevated levels of *Prl* expression. The observation that *Nupr1*$^{-/-}$ mice are fertile suggests that the loss of *Prl* repression in somatotropes is not to an extent sufficient to result in fertility problems. Such conclusions can be best addressed by *in vivo* condition gene modification studies, as described in **Chapter IV**. Of further interest is our observation that NUPR1 is expressed in approximately half (54%) of somatolactotropes (**Supplemental figure 5**). These data suggest that NUPR1 is not fully sufficient to repress *Prl* expression and that there are likely other factors

involved in repressing *Prl* expression in the somatotrope lineage, either additively or synergistically with NUPR1. Transcription factor binding site analyses did not identify a putative binding site for NUPR1 within the *Prl* promoter, suggesting that the mechanism of action may be indirect or may be dependent upon NUPR1 binding at a currently unknown *Prl* regulatory element. While the current study did not identify a reciprocally-acting transcription factor in lactotropes that represses somatotrope genes in lactotropes, it would be reasonable to hypothesize that such factors exist and future studies will aim to identify them.

Expression of the second somatotrope-enriched factor, *Rxrg*, in Pit-1/Triple cells did not significantly alter the expression of the assayed genes (**Fig. 5B**) even after the addition of retinoic acid, a known ligand of RXRG, to the tissue culture medium during transfection (**Supplemental Figure 3**)[73]. While this result does not preclude a function for *Rxrg* in regulating the expression of somatotrope genes that were not represented in our experimental approach, *Rxrg* was excluded from further study in order to focus on transcription factors that impact the expression of the landmark somatotrope and lactotrope genes.

Both of the lactotrope-enriched factors, *Pou4f1* or *Nr4a2*, were notable for a significant enhancement in *Prl* expression when expressed in Pit-1/Triple cells (**Fig. 5C, D**). The lack of a significant change in *Drd2* expression when these factors were ectopically expressed suggested that they target expression of the *Prl* gene, rather than impacting on multiple genes in the lactotrope transcriptional program. Taken together, our transcription factor data reveal transcription factor

actions that either enhance expression of a lineage-defining gene or repress expression of genes from a competing lineage. These observations further support a model in which these cells must actively repress the transcriptional program of the reciprocal lineage to maintain their cell identities and functions.

The function of the transcription factor NR4A2 was further explored by a combination of ChIP studies and cell transfection assays. NR4A2 ChIP analysis revealed direct binding to the *Prl* promoter in Pit-1/Triple cells (**Fig. 6B**). A parallel ChIP assay conducted using a primary cell pool enriched for lactotropes (**Fig. 6E**) confirmed that this same binding to the *Prl* promoter occurs *in vivo*. This suggests a mechanistic basis for NR4A2 activation of *Prl* gene expression *via* a direct interaction with the *Prl* promoter, and the concordance of the NR4A2 ChIP assays in both primary lactotropes and the Pit-1/Triple cell line supports the validity of using the Pit-1/Triple cell line as an experimental model. qRT-PCR assays performed on Pit-1/0 cells expressing *Pou1f1*, *Nr4a2*, or both factors together provided further insight into the mechanism of action for *Nr4a2* (**Fig. 6F**). Expression of *Pou1f1* alone activated *Prl* while the expression of *Nr4a2* alone had no effect. However, co-expression of *Nr4a2* with *Pou1f1* resulted in a significant enhancement of *Prl* expression over that seen with *Pou1f1* alone. These data suggest that NR4A2 is not sufficient to activate *Prl* expression on its own, but that it acts synergistically when POU1F1 is present at the *Prl* promoter to enhance *Prl* expression.

The positioning of an NR4A2 binding site in the *Prl* promoter adjacent to a functionally-defined POU1F1 binding site suggested that its function(s) may involve enhancement of POU1F1 and occupancy. However, a POU1F1 ChIP analysis failed to reveal a significant impact of NR4A2 on POU1F1 occupancy at the *Prl* promoter (**Fig. 7**). Additionally, a ChIP analysis of the *Gh* and *Prl* promoters in Pit-1/0 cells transfected with *Pou1f1, Nr4a2,* or both factors revealed that POU1F1 establishes H3K27 acetylation and H3K4 tri-methylation while NR4A2 has no apparent effect on its own, nor does it enhance the modifications seen with *Pou1f1* alone (**Fig. 8**). Based on these observations we conclude that NR4A2 enhancement of *Prl* expression relates to a synergistic interaction between the two bound factors at the *Prl* promoter rather than reflecting enhancements of POU1F1 occupancy or a role in promoting histone H3K27 acetylation or H3K4 tri-methylation at the target promoter. Reports from other systems indicate that the family of nuclear orphan receptors to which *Nr4a2* belongs is able to heterodimerize with a wide range of transcription factors and drive transcription[84-86], suggesting that NR4A2 might heterodimerize with POU1F1 or with other, currently unknown, transcription factors at the *Prl* promoter to exert its actions on productive transcription.

We next sought to elucidate the mechanism by which the NR4A2 protein exerts this enhancer effect. Knowing that POU1F1 binds the *Prl* promoter independent of NR4A2 occupancy (**Fig. 7**), we next performed a reciprocal experiment, performing NR4A2 ChIP using chromatin isolated from Pit-1/0 cells

to determine whether NR4A2 occupancy at the *Prl* promoter requires POU1F1 (**Fig. 9**). These Pit-1/0 cells do not express *Pou1f1* or *Nr4a2* on their own. After transfection with either *Nr4a2* alone or *Nr4a2* and *Pou1f1*, this ChIP assay revealed that NR4A2 requires POU1F1 in order to bind the *Prl* promoter. This observation is consistent with previous data demonstrating that the enhancement of *Prl* gene expression by *Nr4a2* is dependent on *Pou1f1* actions (**Fig. 6F**). These data lead us to propose a model in which POU1F1 binds the *Prl* promoter and activates *Prl* expression, followed by NR4A2 binding at the *Prl* promoter which further enhances *Prl* expression.

Next, we hypothesized that the enhancing activity of NR4A2 reflects its ability to enhance the recruitment of Pol II to the *Prl* promoter in the presence of POU1F1. To test this hypothesis, we performed ChIP for serine 5 phosphorylated RNA polymerase II (Pol II) at the *Prl* promoter as well as regions 500 bp upstream ("Prl upstream") and 500 bp downstream within the *Prl* gene body ("Prl downstream") (**Fig. 10**). The serine 5 phosphorylated form of Pol II is associated with initiation of transcription and active transcription[87,88]. Serine 5 phosphorylated Pol II occupancy was detected in equal levels at the *Prl* promoter in cells that express *Pou1f1* alone and in cells that express both *Pou1f1* and *Nr4a2*, suggesting that *Pou1f1* expression is sufficient to recruit Pol II and assemble a pre-initiation complex (PIC) at the *Prl* promoter. The Pol II ChIP studies revealed that Pol II occupancy downstream in the *Prl* gene body was markedly enhanced by co-expression of *Nr4a2* with *Pou1f1*. Pol II occupancy at

the region approximately 500 bp upstream of the *Prl* promoter is also enhanced by expression of *Pou1f1* and further enhanced by the co-expression of *Pou1f1* and *Nr4a2*. This increase in Pol II occupancy at regions both upstream and downstream of the *Prl* promoter may be indicative of bidirectional transcription, which is a previously described phenomenon at many active promoters[89-91]. As was expected, given the lack of NR4A2 in somatotropes and the lack of binding sites at the *Gh* promoter, the expression of *Nr4a2* in the Pit-1/0 cell line failed to impact on Pol II occupancy at the promoter or within the body of the *Gh* gene (see also **Supplemental Figure 6**). These data suggest that NR4A2 enhances the release of Pol II from the *Prl* promoter and into the *Prl* gene body for active transcription.

The basis for the NR4A2-enhanced release of Pol II from the Prl promoter was further investigated by asking if it reflected an enhanced recruitment of known release co-factors. While we observed no evidence that NR4A2 enhances recruitment of the prominent Pol II release factor, P-TEFb (**Fig. 11**), there are other mechanisms by which NR4A2 may trigger the release of Pol II such as by recruiting a different release factor, ejecting a negative release factor, or by stabilizing the Pol II transcriptional machinery at the *Prl* promoter. These data lead us to hypothesize a model in which POU1F1 binds at the *Prl* promoter and activates *Prl* expression. This POU1F1 occupancy is followed by NR4A2 binding in a POU1F1-dependent manner at the *Prl* promoter and further increasing the rate of transcription of *Prl* by triggering the release of Pol II from the *Prl* promoter

(outlined in **Fig. 12**). The identity of factor(s) involved in the NR4A2 enhancement of Pol II release can now be directly addressed as summarized in **Chapter V**.

The observation of a novel, cell-type enriched transcription factor binding to the promoter of the *Prl* gene and acting in conjunction with POU1F1 offers a potential explanation to the dilemma of *Pou1f1* being expressed in both somatotropes and lactotropes, yet selectively driving the production of one hormone protein *per* cell type. While POU1F1 binding at the *Gh* and *Prl* promoters may be ubiquitous in somatotropes and lactotropes, and prime the respective promoters *via* targeted histone acetylation, the presence or absence of additional, lineage-enriched factors such as NR4A2 may be the crucial determinant of which hormone gene is activated by POU1F1 in a cell. It is also noteworthy that some factors, such as POU4F1, have effects on the expression of lineage markers such as *Prl*, yet are not observed to directly bind the promoters of either hormone gene (**Fig. 6D**). These data suggest that POU4F1 acts on other, currently unidentified, regulatory elements to alter the expression of genes critical to cell identity such as *Prl*.

In conclusion, these analyses support a model in which cell type-enriched transcription factors are likely induced during the transition from precursor cells into a somatotrope or lactotrope. These factors then enhance the expression of genes crucial to cell identity or suppress the genes that drive the reciprocal lineage. These factors continue to be expressed in adult pituitary cells, and are likely needed for the maintenance of cell fate. Mechanistically, these transcription

factors can act directly at the promoters of their target genes, or indirectly *via* additional mediators. The newly identified transcription factors presented here are likely to represent only a subset of the factors that play important roles in the maintenance of somatotrope and lactotrope cell identities. The identification of additional lineage defining determinants is now possible through the analysis of the transcriptomic profiles of both cell types.

Figures and legends

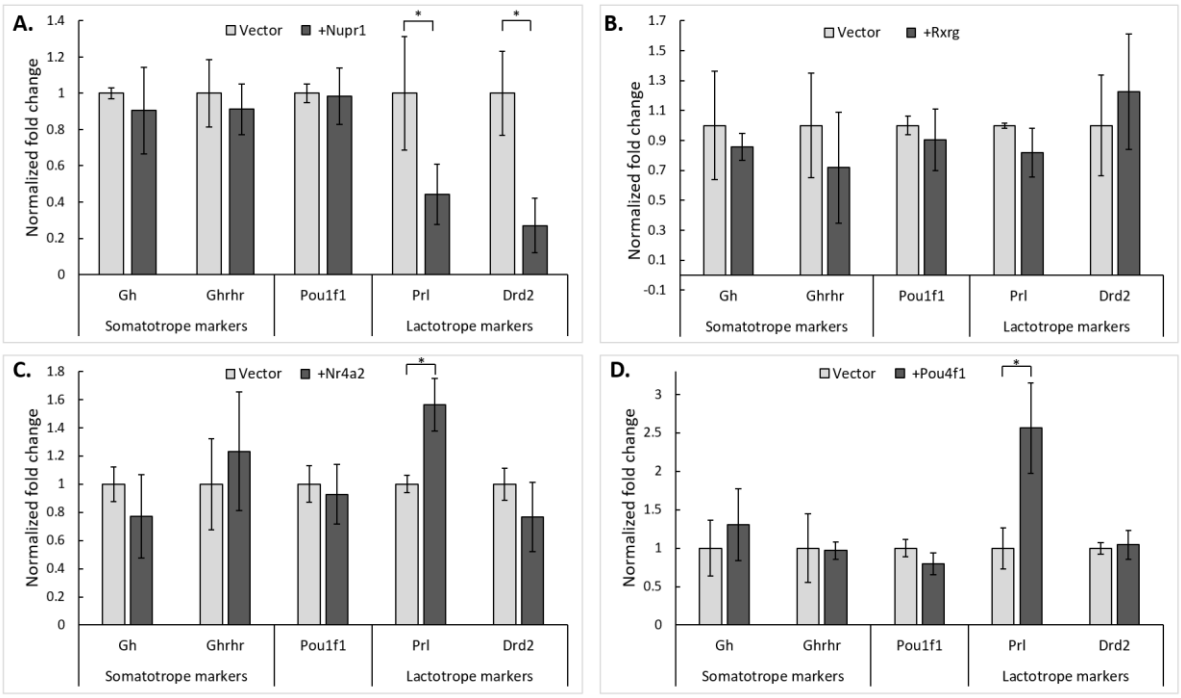


Figure 5. Impact of four validated lineage-enriched transcription factors on expression of somatotrope and lactotrope marker genes in Pit-1/Triple cells. Pit-1/Triple cells[28] were transfected with expression constructs encoding each noted transcription factor linked by an IRES to a GFP reporter ORF (See **Methods**). Transfected cells were isolated by GFP FACS and their RNA content was assayed for specific marker gene expression by qRT-PCR. **A.** *Nupr1*. **B.** *Rxrg*. **C.** *Nr4a2*. **D.** *Pou4f1*. In all assays, expression of the recombinant transcription factor was confirmed by targeted qRT-PCR (data not shown). Gene expression was normalized to an internal *Gapdh* control and the fold change over empty vector controls was calculated using the ΔΔCt method (see **Methods**).

Gapdh expression levels were stable across all assayed conditions, with no significant changes in *Gapdh* expression in any of the transfected samples. Error bars indicate one standard deviation. Significance calculated by T-test, * indicates a p-value of <0.05. n = 5 for all experiments.

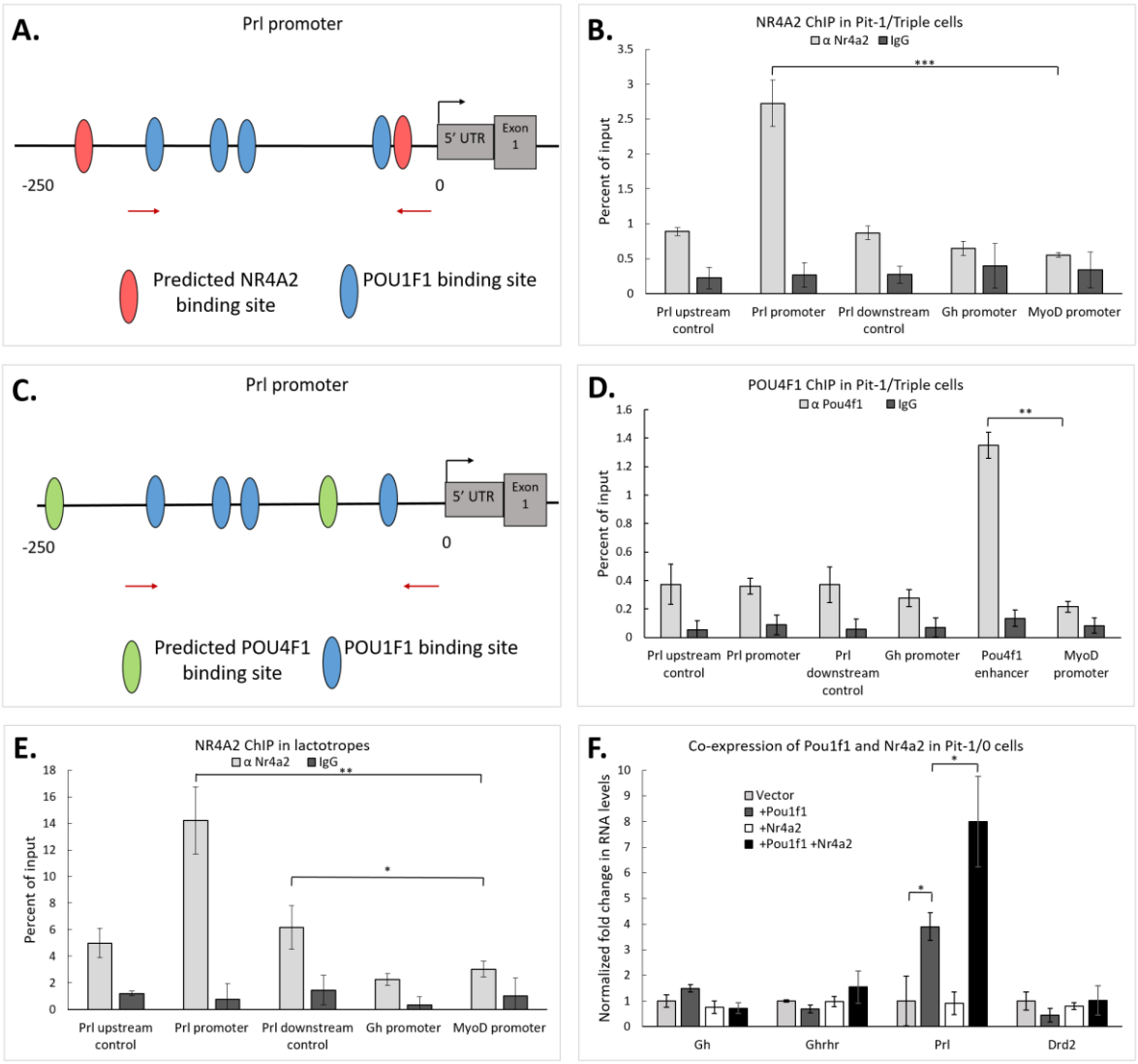


Figure 6. The lactotrope-enriched transcription factor, NR4A2, acts in conjunction with POU1F1 at the *Prl* promoter to enhance *Prl* expression. A. Schematic of the m*Prl* gene promoter indicating the relative positions of known POU1F1 binding sites (blue ovals,[21]) as well as predicted binding sites for NR4A2 (red ovals) (identified by JASPAR), along with primer positions used for amplification in ChIP assays (red arrows). **B.** NR4A2 ChIP. Pit-1/Triple cells were transfected with an expression vector encoding NR4A2 and chromatin isolated

from GFP$^+$ cells (as in **Fig. 5**) was assayed by NR4A2 ChIP. Levels of transcription factor occupancy at specific sites within the *Prl* promoter were quantified by qRT-PCR of immunoprecipitated samples. Parallel controls included quantitation of occupancy at the *Gh* promoter and assessment of binding in regions 500 bp upstream and downstream of Prl promoter. The *MyoD* promoter served as a negative control in all studies. **C.** Schematic of the m*Prl* gene promoter indicating the relative positioning of known POU1F1 binding sites (blue ovals) as well as predicted binding sites for POU4F1 (green ovals) (identified by JASPAR). **D.** POU4F1 ChIP. Pit-1/Triple cells were transfected with an expression vector encoding POU4F1 and chromatin isolated from GFP$^+$ cells (as in **B.**) was assayed by ChIP**.** The *Pou4f1* enhancer, which contains an autoregulatory POU4F1 binding site, was assayed in this study as a positive control for the POU4F1 ChIP[76]. **E.** NR4A2 ChIP on FACS sorted mouse lactotropes revealed a similar binding profile as that observed in Pit-1/Triple cells, confirming the validity of the cell line model. **F.** NR4A2 enhances *Pou1f1*-dependent activation of the Prl gene in Pit-1/0 cells. Pit-1/0 cells were transfected with expression vectors encoding *Pou1f1*, *Nr4a2*, or both linked by IRES to GFP, and GFP$^+$ cells were collected by FACS. qRT-PCR analysis was performed to measure changes in mRNA expression of somatotrope and lactotrope marker genes. *: p-value <0.05. **: p-value <0.01. ***: p-value <0.001. n = 3 for all experiments.

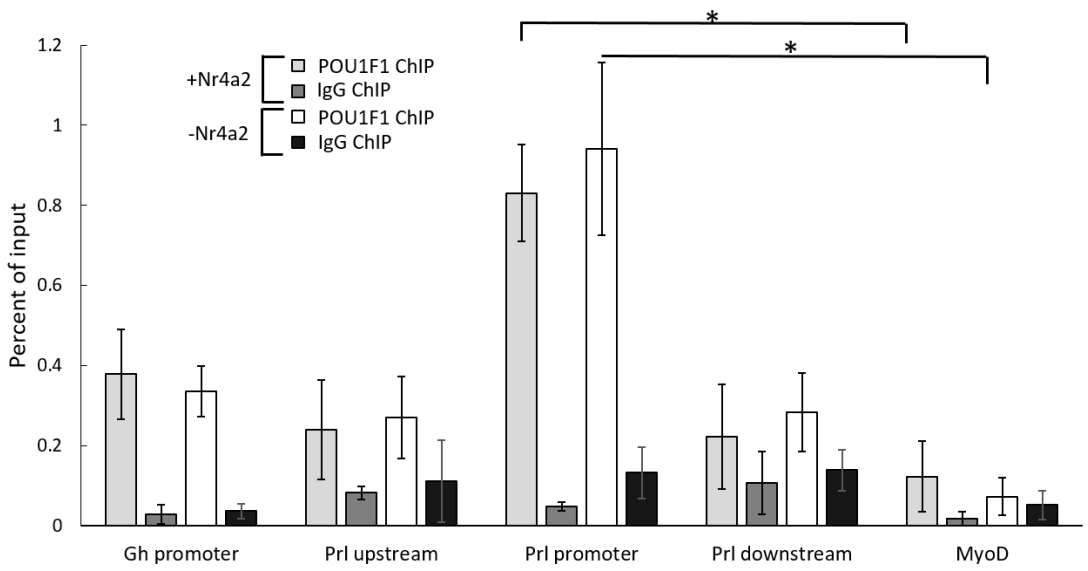


Figure 7. NR4A2 has no significant impact on POU1F1 occupancy at the *Prl* promoter. POU1F1 chromatin immunoprecipitations were carried out on Pit-1/Triple cells +/- Nr4a2. Pit-1/Triple cells, which do not express *Nr4a2* on their own, were transfected with an *Nr4a2* expression vector (the same *Nr4a2*-IRES-GFP plasmid used in assays presented in **Fig. 5C)**, and control cells were transfected with the empty IRES-GFP vector. GFP positive cells were sorted by FACS, chromatin was isolated, and ChIP was performed using an antibody that recognizes POU1F1. Following immunoprecipitation, qRT-PCR was used to measure the relative levels of POU1F1 binding in Pit-1/Triple cells that had been transfected with *Nr4a2* plasmid (+Nr4a2), and those transfected with empty vector. Immunoprecipitations using IgG served as a negative control. * indicates p-value <0.05. N = 3 biological replicates.

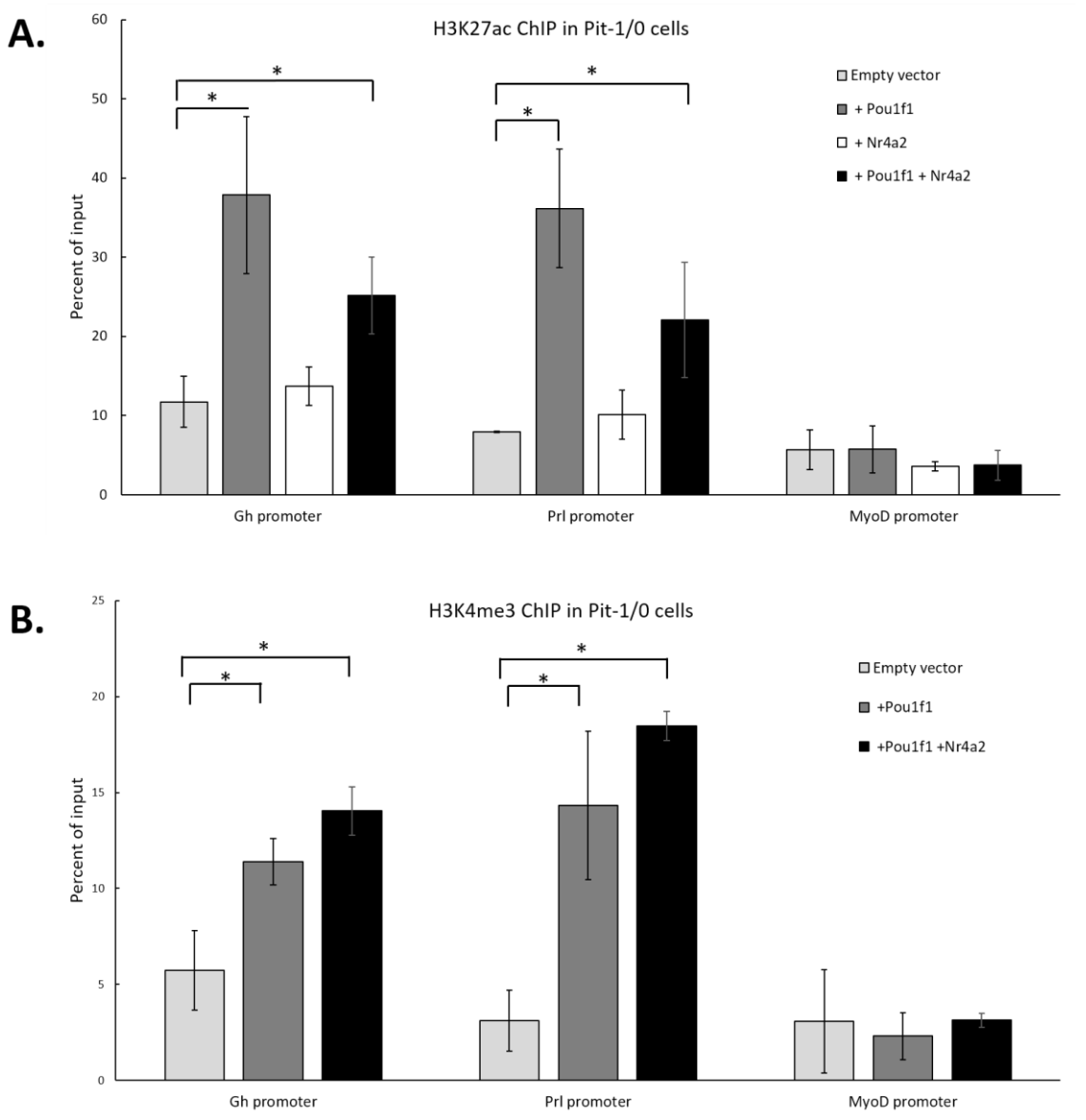


Figure 8. Expression of *Nr4a2* does not impact levels of histone 3 lysine 27 acetylation or histone 3 lysine 4 tri-methylation. Pit-1/0 cells, which express neither *Nr4a2* nor appreciable levels of *Pou1f1*, were transfected with *Nr4a2*-IRES-GFP, *Pou1f1*-IRES-GFP, or a 1:1 mixture of both expression vectors, and control cells were transfected with an empty IRES-GFP vector. Cells transfected with the 1:1 mixture of the *Pou1f1* and *Nr4a2* expression vectors express lower

levels of each protein due to the concentration of each expression vector being halved during transfection in order to keep the total amount of DNA constant in each transfection. GFP positive cells were isolated by FACS and chromatin was isolated. ChIP was performed using an antibody that recognizes histone 3 lysine 27 acetylation (H3K27ac) (**A.**), or an antibody that recognizes histone 3 lysine 4 tri-methylation (H3K4me3) (**B.**). Following chromatin immunoprecipitation, qRT-PCR was performed to assay the levels of each marker at the *Gh* promoter, the *Prl* promoter, and the *MyoD* promoter as a negative control. IgG controls (data not shown) were also included in this assay for all samples. In all cases, ChIP performed with IgG yielded <1% of input. * indicates a p-value <0.05. N = 3 biological replicates.

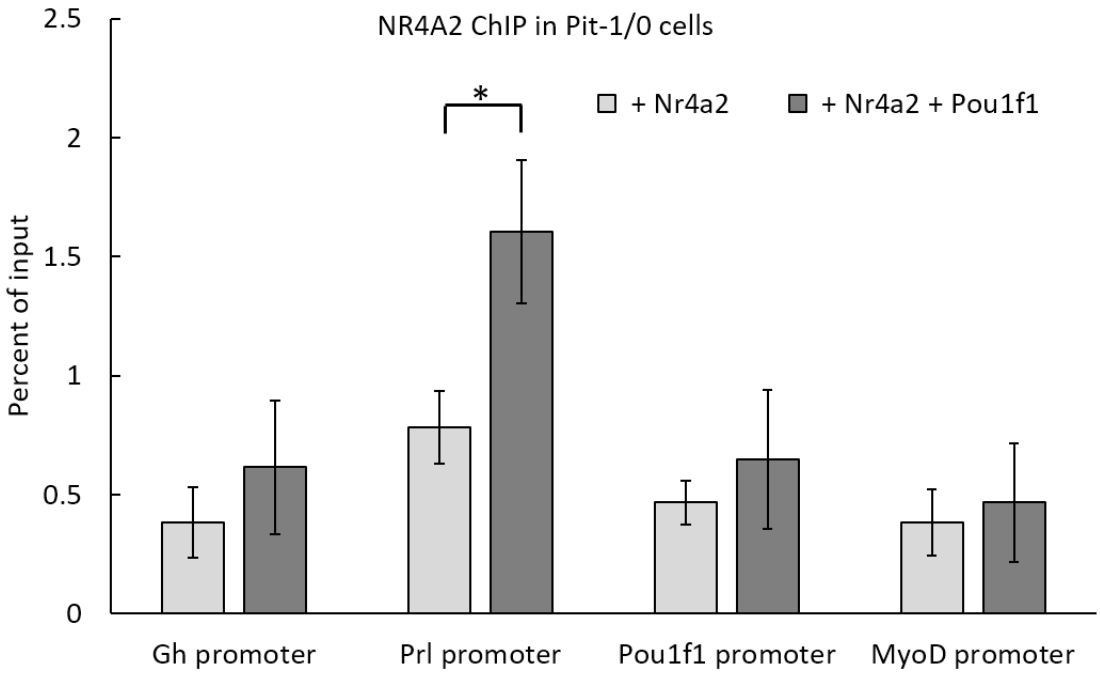


Figure 9. NR4A2 binding at the *Prl* promoter is POU1F1 dependent. Pit-1/0 cells were transfected with plasmids expressing either *Nr4a2* or *Pou1f1* linked by IRES to a GFP reporter as in **Figure 6**. GFP positive transfected cells were collected and chromatin was isolated before performing ChIP for the NR4A2 protein. NR4A2 occupancy was assayed at the promoters of *Gh*, *Prl*, *Pou1f1*, and *Myod* as a control. * indicates p-value <0.05 as determined by Student's T-test. N = 3 biological replicates.

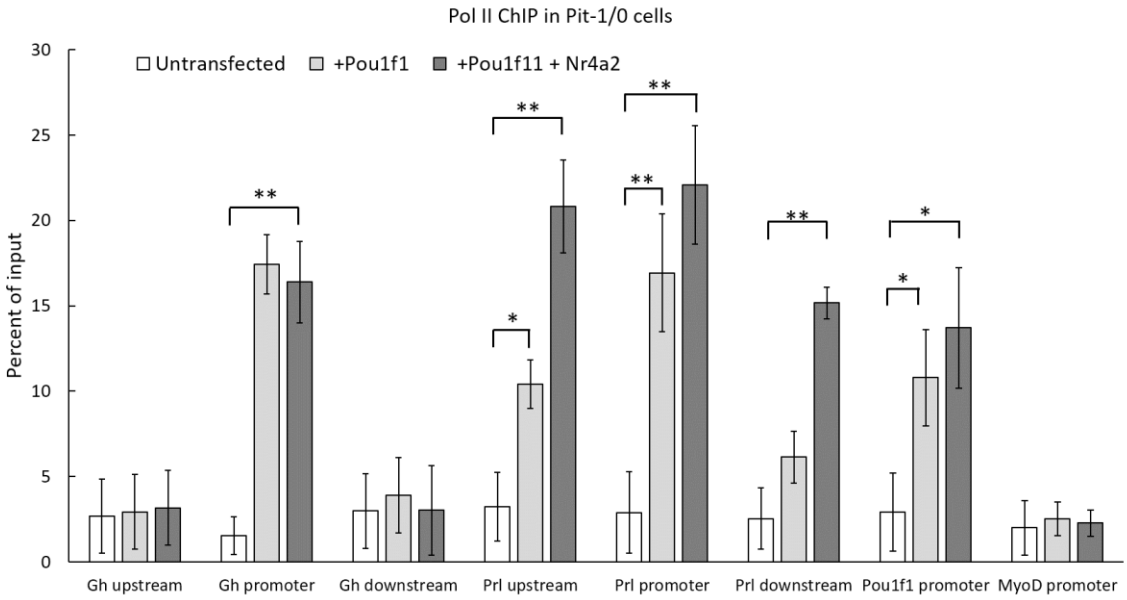


Figure 10. NR4A2 enhances the release of Pol II from the *Prl* promoter into the gene body. Pit-1/0 cells were transfected with either *Pou1f1* alone, or with a combination of *Pou1f1* and *Nr4a2*, with untransfected cells serving as a negative control. ChIP was performed for serine 5 phosphorylated Poll II and binding was quantified by qPCR. The *Gh*, *Pou1f1*, and *Prl* promoters were assayed along with upstream and downstream controls for specificity. Here, “upstream” refers to regions 500 bp upstream of the canonical promoters and “downstream” refers to 500 bp downstream of the promoters, inside the gene body. * indicates p-value <0.05, ** indicates p-value <0.01 as determined by Student’s T-test. N = 3 biological replicates.

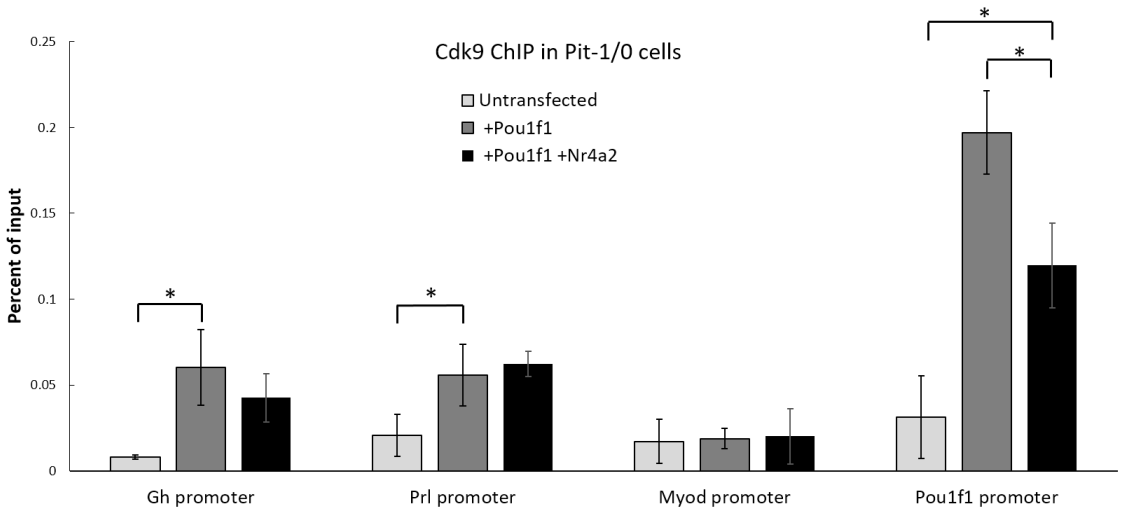


Figure 11. NR4A2 fails to enhance Cdk9 recruitment at the *Gh* and *Prl* promoters. Pit-1/0 cells were transfected with plasmids expressing *Pou1f1* alone or co-expressed with expression constructs for both *Pou1f1* and *Nr4a2* and chromatin immunoprecipitation was performed using an antibody against the Cdk9 subunit of the release factor P-TEFb. RT-PCR was performed to assay Cdk9 occupancy at the *Gh* and *Prl* promoters, with the *Pou1f1* and *Myod* promoters serving as positive and negative controls, respectively. N = 3 biological replicates.

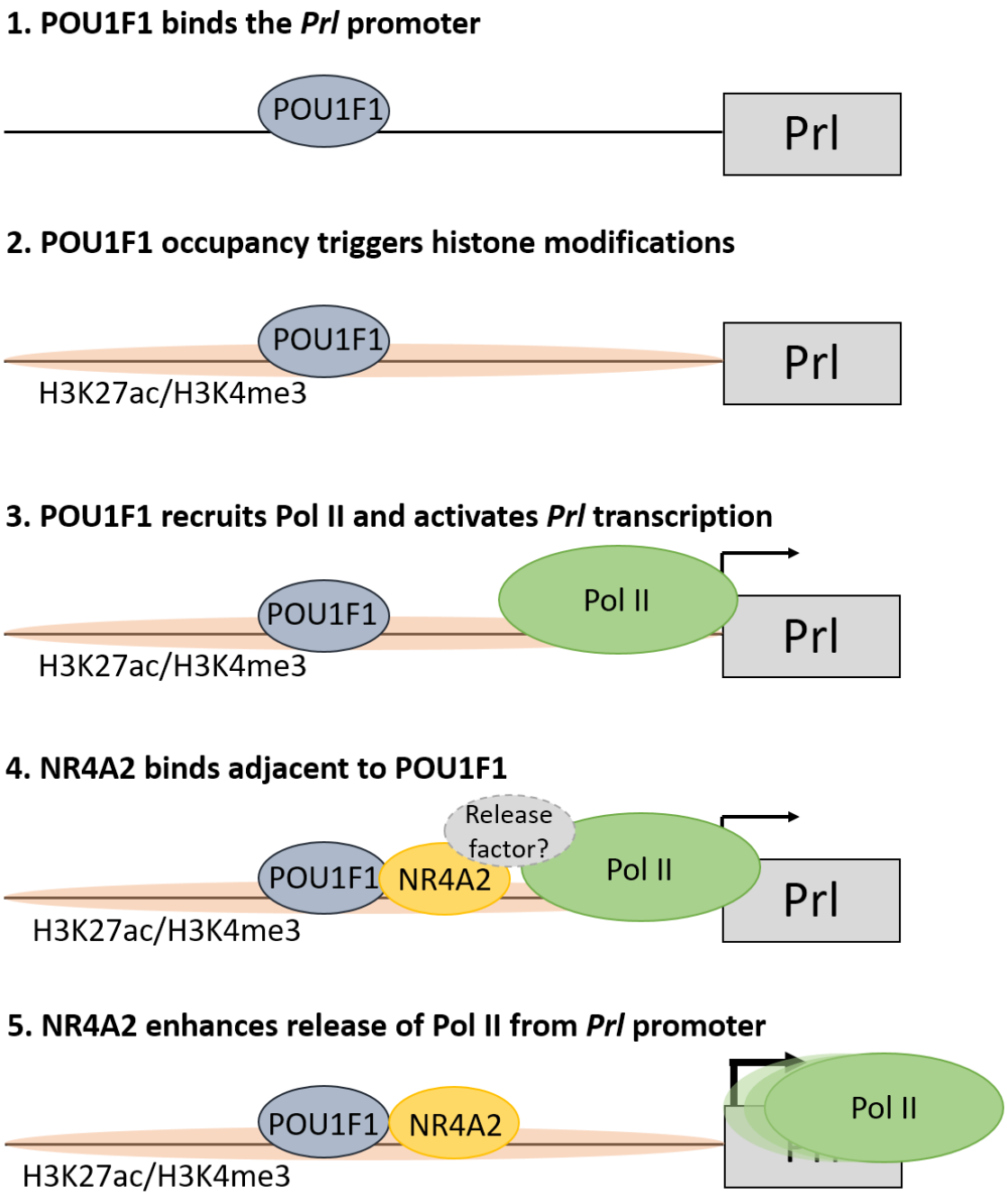
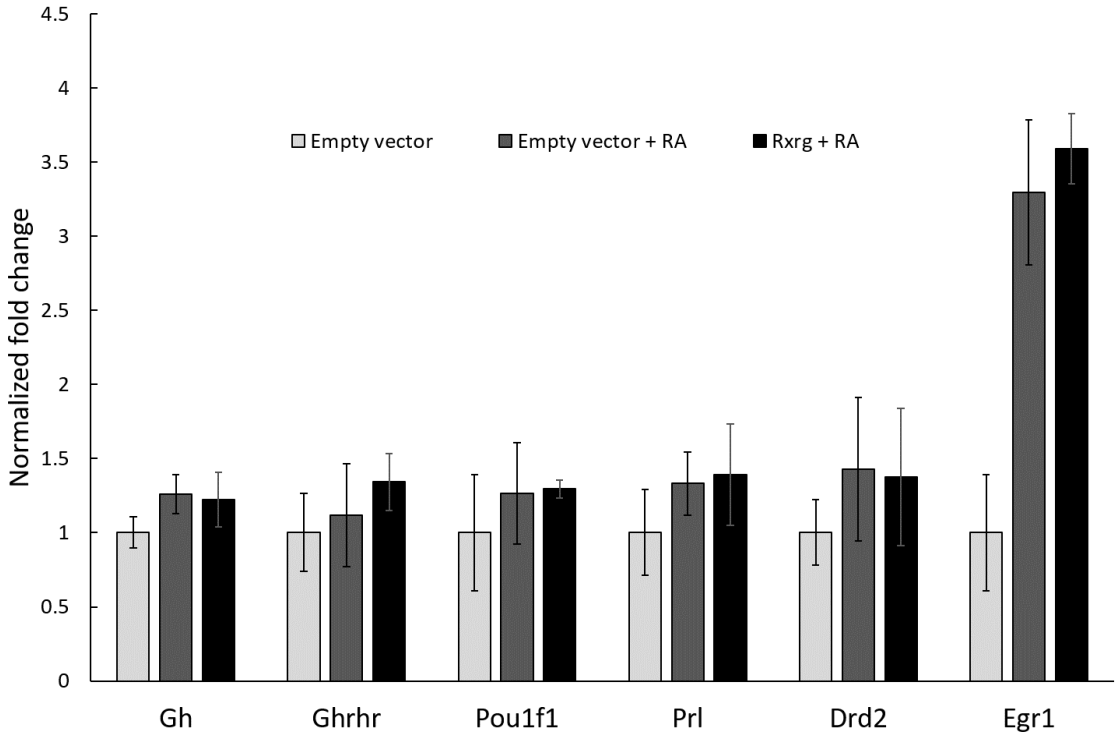


Figure 12. Model of the role of NR4A2 in enhancing the expression of Prl.

POU1F1 binds the *Prl* promoter independently of NR4A2 (**1**) and triggers histone acetylation and tri-methylation throughout the *Prl* promoter by recruiting histone

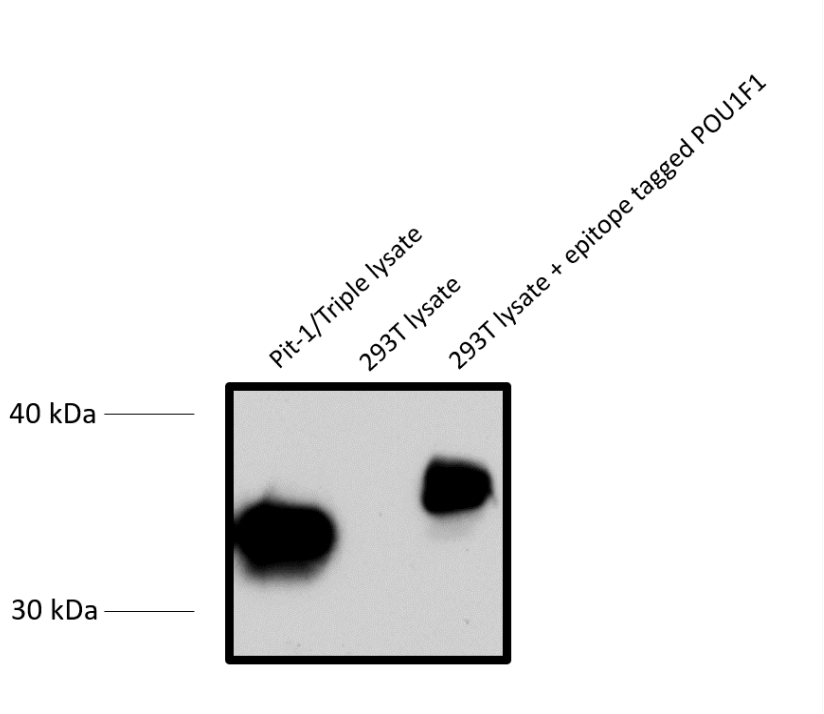
acetyltransferases and methyltransferases, respectively (**2**). POU1F1 is able to recruit Pol II to the *Prl* promoter, which activates transcription and leads to some measure of *Prl* expression (**3**). NR4A2 binds the *Prl* promoter only in the presence of POU1F1, potentially recruiting a release factor to the complex (**4**), and further enhances the rate of *Prl* transcription by triggering an increase in the rate of Pol II release from the *Prl* promoter (**5**).

Supplemental Data

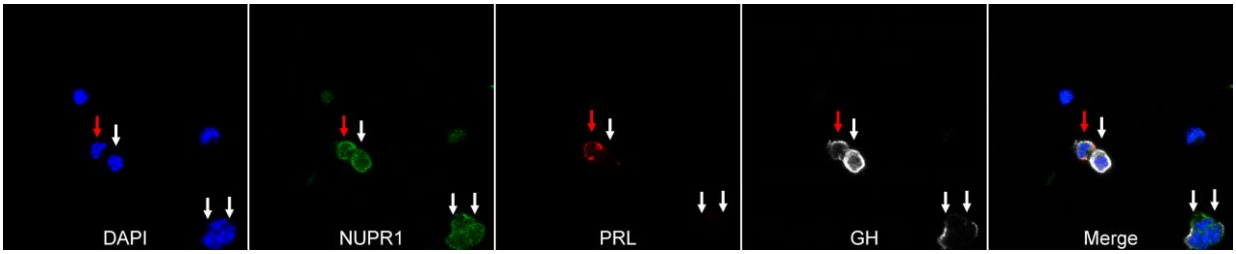


Supplemental Figure 3. **RXRG has no impact on the expression of signature somatotrope or lactotrope genes in the presence of retinoic acid**. Pit-1/Triple cells were transfected with an Rxrg expression vector as in **Fig. 5** and grown in culture medium supplemented with 10 mM retinoic acid (RA). Cells transfected with an empty vector control were grown in both with and without RA added to the culture medium. Two days after transfection, RNA was collected, cDNA generated, and qRT-PCR for each of the indicated mRNAs was performed as in **Fig. 5**. *Egr1* was included as a positive control for RA effect based on a previous report that it is transcriptionally activated by retinoic acid[74] and is expressed in Pit-1/Triple cells (data not shown). All data was normalized to the *Gapdh* mRNA signal. *Rxrg* expression from the Rxrg expression vector was

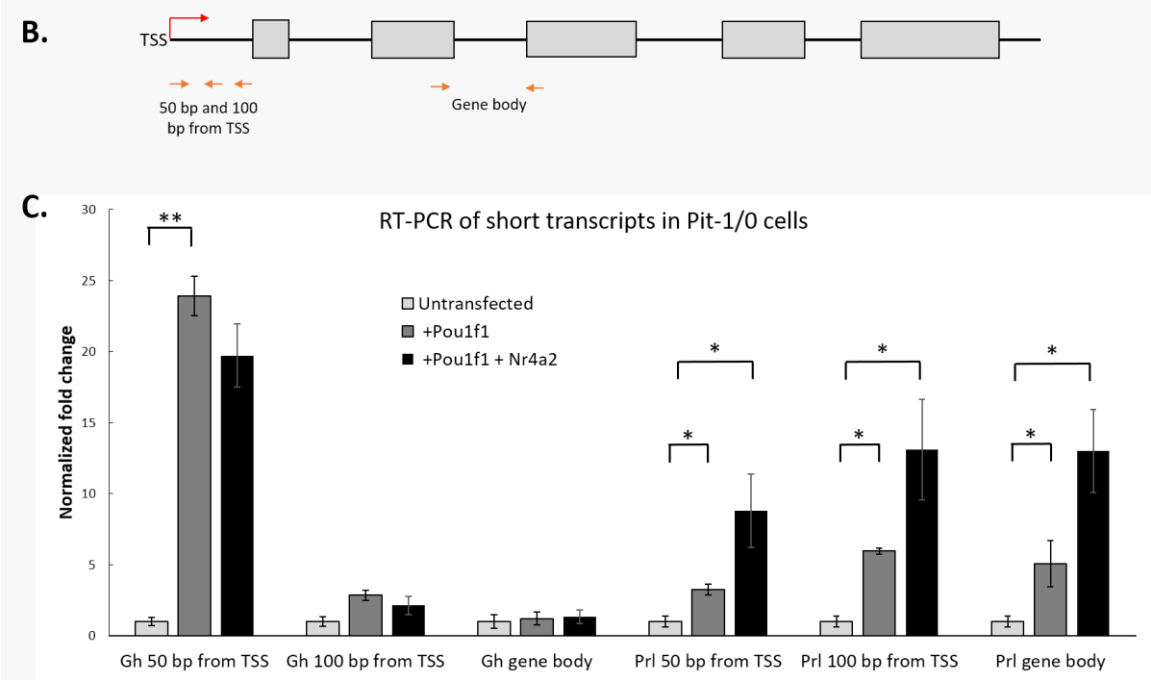
confirmed in transfected cells by PCR (data not shown). N=3, * = p-value <0.05 as determined by Student's T-test.



Supplemental Figure 4. **Western blot validation of POU1F1 antibody specificity**. POU1F1 antibody was used to perform a western blot on lysate from the murine Pit-1/Triple cells (lane 1), producing a band of 33 kDa corresponding to endogenous POU1F1. Lysate from human 293T cells (lane 2) produce no bands (293T cells do not express POU1F1). 293T cells transfected with a biotin ligase receptor peptide (2.5 kDa) epitope-tagged version of POU1F1 (lane 3) produces a band with a corresponding shift in size in accordance with the additional epitope added to POU1F1 (33 kDa to 36 kDa).



Supplemental Figure 5. **NUPR1 is present in a majority of somatolactotropes**. A representative immunofluorescence microscopy image of wild-type female mouse pituitary cells that were disaggregated and immuno-stained for NUPR1, GH, and PRL. Multiple somatotropes (white arrows) can be seen which stain strongly for NUPR1, along with a single somatolactotrope (GH+/PRL+) (red arrow) that also is positive for NUPR1. Two GH^-/PRL^- cells are also visible which stain much more weakly for NUPR1. In this experiment, a total of 50 somatolactotropes (GH+/PRL+) were identified (out of approximately 2000 pituitary cells imaged) and assayed for NUPR1 expression. Of these 50 somatolactotropes, 27 (54%) were positive for NUPR1.



Supplemental Figure 6. Expression of *Pou1f1* in Pit-1/0 cells drives productive transcription of *Prl* as compared with the production of short, abortive transcripts from the *Gh* promoter. A. Schematic of PCR amplification strategy to detect short, abortive transcripts. The intron/exon structure of the *Gh* and *Prl* genes is remarkably similar, and so this five exon schematic represents both the *Gh* and *Prl* genes as the primer design (orange arrows) approach was similar for both genes. A shared forward primer was placed at the transcription start site (TSS) and two reverse primers, one 50 bp downstream of the TSS and one 100 bp downstream of the TSS were combined with the forward primer to analyze abortive transcripts. Exon-spanning primers were designed further into the gene body ("gene body" primers) to assay productive, elongated transcripts. **B.** Quantitative RT-PCR was performed using these primer sets in Pit-1/0 cells

that had been transfected with plasmids expressing *Pou1f1* +/- *Nr4a2*. RNA was isolated and treated with DNase to eliminate genomic DNA contamination. N = 3 biological replicates. * indicates a p-value <0.05 while ** indicates a p-value < 0.01.

CHAPTER IV*: IN VIVO* FUNCTIONAL CHARACTERIZATION OF NEWLY IDENTIFIED TRANSCRIPTION FACTORS BY CONDITIONAL GENE INACTIVATION IN TRANSGENIC MOUSE MODELS

Introduction

Having identified a set of transcription factors that are enriched in somatotropes and lactotropes and appear to have functional impacts on the expression of crucial somatotrope and lactotrope genes, a key challenge was to validate these function(s) in the *in vivo*. *Nr4a2*, *Nupr1*, and *Pou4f1* were selected for further study based on their functional impacts on landmark somatotrope and lactotrope genes in the context of our cell line model system (**Chapter III**). These transcription factors may be important regulators of the maintenance of somatotrope/lactotrope cell fate in terminally differentiated cells, by enhancing landmark genes of a cell lineage, or in reciprocally repressing the landmark genes of a divergent lineage. It is crucial to understand the effect these transcription factors have on the cell identity of somatotropes and lactotropes in the context of primary pituitary cells in order to gain a more complete understanding of their mechanisms of action and contribution(s) to the cellular composition and hormonal functions of the intact pituitary.

Given the effects of the two lactotrope transcription factors *Nr4a2* and *Pou4f1* on *Prl* expression in our cell-based assays, we hypothesized that their ablation in the lactotropes of mice might result in a decrease in *Prl* expression and/or a decrease in lactotrope representation in the pituitary. We anticipated that impacts of the deletions of these factors might be detected at the cellular level by decreases in the number or intensity of PRL^{+} cells, by decreases in *Prl* mRNA in total pituitary RNA, and/or lactation or maternal activity phenotypes. Our cell-based data would predict that deletion of either of these transcription

factors would only have a partial impact on *Prl* expression because *Pou1f1* is sufficient in the absence of either factor to drive *Prl* expression to appreciable levels (**Fig. 6F**)[92], an observation consistent with studies that have identified *Pou1f1* as a major activator of *Prl* expression[8,9,21]. Thus, mice lacking either *Nr4a2* or *Pou4f1* in lactotrope cells are predicted to maintain an appreciable level of *Prl* expression, albeit at levels lower than those observed in wild-type mice.

A third transcription factor of interest based on our prior cell-based studies is *Nupr1*. This factor is enriched in somatotropes and appears to be involved in the repression of two lactotrope-specific proteins; *Prl* and *Drd2*. These data suggest that lactotrope-defining genes are under active repression in somatotropes and that the deletion of *Nupr1* in primary mouse somatotropes might therefore result in a de-repression of *Prl* in the somatotropes. Thus, we would predict that mice with a selective ablation of *Nupr1* in somatotropes might exhibit conversion of a subset of somatotropes to a dual positive state (GH^+/PRL^+).

We describe in this Chapter a series of studies that were designed to validate the roles of the three identified transcription factors in primary somatotropes and lactotropes. This was accomplished by obtaining and/or generating lines in which the genes encoding each of the three transcription factors could be inactivated in a cell-type specific manner. To achieve this goal, we crossed mouse lines that selectively express Cre recombinase in either somatotropes or in lactotropes with lines in which each of the three target loci were flanked by LoxP elements (“floxed” mouse lines). Compound transgenic

progeny (Cre x Flox/Flox), were studied to probe the function of each transcription factor in the respective cell lineage. Here, we present data from these cell type specific knockout mouse lines to demonstrate that two of the newly identified somatotrope/lactotrope enriched transcription factors, *Nupr1* and *Nr4a2*, have respective repressing and enhancing actions on *Prl* expression in somatotropes and lactotropes within the adult murine pituitary. These observations in an *in vivo* setting are consistent with the activities observed in our cell culture-based studies (**Chapter III**) and provide an expanded understanding of somatotrope and lactotrope regulation.

Results

Generation and validation of mouse lines that support conditional inactivation of targeted transcription factors in the somatotrope or lactotrope lineages

To generate inactivating mutations of selected transcription factors specifically in somatotropes or lactotropes, we needed mouse lines that express Cre recombinase under the control of either the *Gh* or *Prl* regulatory elements. An existing Gh-Cre mouse line that has been validated for expression of Cre in somatotropes[16] was provided by the lab of Dr. Sally Camper. A corresponding Prl-Cre mouse line was not available, so we generated this line by inserting the *Cre* ORF into the second exon of the *Prl* gene within the context of a 200 kb bacterial artificial chromosome (BAC) containing the mouse *Prl* locus. The BAC clone used for this insertion was selected based on its extensive (>50 kb)

sequences both 5' and 3' of the *Prl* gene. In the absence of evidence that the *mPrl* gene is under remote regulatory control[93], the presence of these extensive flanking sequence was assumed to be sufficient to drive lactotrope-specific expression (**Fig. 13A**). The recombinant BAC was generated by standard BAC recombineering (see **Methods**) and was microinjected into B6SJLF1/J mouse embryos by the Penn Transgenic Mouse facility[94]. After establishing a line of transgenic mice carrying this Prl-Cre transgene, the mice were crossed with mice (provided by Dr. Douglas Epstein) carrying a ZsGreen reporter gene with an upstream lox-stop-lox to identify sites of Cre recombinase expression[95].

In order to validate the accuracy of Cre expression from the Prl-Cre transgene, we collected pituitaries from mice carrying both the Prl-Cre and ZsGreen transgenes (Prl-Cre/ZsGreen). These pituitaries were disaggregated, and immunostained for GH, PRL, and ZsGreen using an anti-GFP antibody (**Fig. 13B**). 91% of PRL positive lactotropes were also positive for the ZsGreen reporter protein, while only 1.1% of all GH positive somatotropes expressed ZsGreen (**Fig. 13C**). These data indicated robust lactotrope specificity for the expression of Cre recombinase. This lactotrope-specific Cre line was used along with the previously established somatotrope-specific Cre line for the subsequent conditional knockout studies[16]. The specificity of the Gh-Cre mouse line, previously described[16], was similarly assayed and validated (**Supplemental Figure 7**).

Having established mouse lines expressing somatotrope/lactotrope specific Cre recombinase, we next focused on obtaining mouse lines carrying

floxed alleles of each of the three transcription factors of interest: *Nupr1*, *Pou4f1*, and *Nr4a2*. Existing lines carrying floxed alleles of *Pou4f1* and *Nr4a2* were obtained from cooperating labs (the labs of Dr. Tudor Badea[96] and Dr. Thomas Perlmann[97], respectively). We generated the remaining mouse line carrying a floxed *Nupr1* allele in conjunction with the Penn CRISPR/Cas9 Mouse Targeting Core. LoxP elements were inserted flanking exon 2 of endogenous *Nupr1* locus. The positioning of these LoxP elements was chosen based on previous reports that germline deletion of exon 2 is sufficient to abolish NUPR1 activity in the mouse[98]. Together with the somatotrope and lactotrope specific Cre lines, the three floxed mouse lines allowed us to selectively delete each transcription factor specifically in the somatotrope or lactotrope lineages.

Deletion of Nr4a2 in lactotropes causes a decrease in prolactin expression in mouse pituitary

In our preceding studies we demonstrated in a cell line model and in primary pituitary cells that the orphan nuclear receptor NR4A2 binds within the *Prl* promoter at a site that is adjacent a known POU1F1 binding site. We further demonstrated by transfection studies in the Pit-1/0 cell line that forced expression of *Nr4a2* enhanced *Prl* expression in a POU1F1 dependent manner (**Fig. 6F**)[92]. To validate and extend these findings *in vivo*, we generated *Nr4a2$^{flox/flox}$; Prl-Cre* mice and performed immunofluorescence microscopy on disaggregated cells from their pituitaries. Control mice that were homozygous for the floxed allele of *Nr4a2* but negative for Cre recombinase (*Nr4a2$^{flox/flox}$)* expressed NR4A2 in

nearly all lactotropes (90%, N = 371 PRL^+ cells) (**Fig. 14A**). This percentage of lactotropes expressing the NR4A2 protein is consistent with that seen in wild type mice (**Fig. 4D**), confirming that the inserted loxP sites do not affect expression of the *Nr4a2* gene. In clear contrast, we observed a dramatic decrease in the number of cells that expressed NR4A2 in *Nr4a2*$^{flox/flox}$*; Prl-Cre* mice, where NR4A2 protein was expressed at detectable levels in only 31% of the PRL^+ cells (N = 393 PRL^+ cells.). These data lead us to conclude that *Nr4a2*$^{flox/flox}$; Prl-Cre mice exhibit greatly reduced NR4A2 expression in lactotrope cells, confirming successful ablation of *Nr4a2* in the majority of lactotrope cells.

Next, an immunofluorescence study was performed to determine the impact of *Nr4a2* ablation on PRL expression in mouse lactotropes. De-identified slides produced from pituitary cells isolated from littermates with *Nr4a2*$^{flox/flox}$ or *Nr4a2*$^{flox/flox}$; Prl-Cre genotypes were stained for GH and PRL. We observed that the pituitaries isolated from *Nr4a2*$^{flox/flox}$; Prl-Cre mice had approximately half as many PRL^+ cells as their *Nr4a2*$^{flox/flox}$ littermates (**Fig. 14B**) while the numbers of GH^+ cells and dual GH^+/PRL^+ cells were not significantly different between these two groups. This reduction in PRL expression was paralleled by RT-PCR assays of mRNA levels, demonstrating an approximately 40% decrease in *Prl* mRNA in the pituitaries of *Nr4a2*$^{flox/flox}$; Prl-Cre mice as compared to littermate controls (**Fig. 14C**). These data suggest that *Nr4a2* expression is required for robust *Prl* expression in mice. The marked depletion of NR4A2 in 70% of lactotrope cells (**Fig. 14A**) corresponded to a loss of approximately 50% of PRL positive cells

(**Fig. 14C**), supporting a correlation between the amount NR4A2 ablation and the loss of PRL expression.

Deletion of *Nr4a2* in lactotropes leads to the presence of "silent" lactotropes in the anterior pituitary.

The approximately 50% decrease in PRL positive cells observed in the pituitaries of mice lacking *Nr4a2* in lactotropes may be the result of a loss of lactotrope cells in the absence of *Nr4a2*, leading to a decrease in the number of lactotropes in the pituitaries of *Nr4a2$^{flox/flox}$; Prl-Cre* mice. However, an alternate explanation is that Nr4a2$^{flox/flox}$; Prl-Cre mice have the same number of lactotropes as their Nr4a2$^{flox/flox}$ littermates, but only ~50% of these "lactotropes" actively express PRL. In this second model, the deletion of *Nr4a2* in lactotropes leads to the presence of "PRL-silent" lactotropes, i.e., lactotropes that are negative for PRL staining. In an effort to distinguish between these two models, we stained the pituitaries of *Nr4a2$^{flox/flox}$*; Prl-Cre mice and *Nr4a2$^{flox/flox}$* littermates for PRL and POU1F1. POU1F1 is expressed in the three *Pou1f1* lineages: the somatotropes, the lactotropes, and the TSH-producing thyrotropes. Since thyrotropes constitute <5% of anterior pituitary cells, while somatotropes and lactotropes can range from 30-50% of all anterior pituitary cells[1,2], nearly all POU1F1 positive cells in the anterior pituitary are either GH-positive somatotropes or PRL-positive lactotropes. By staining for these two proteins, we would predict that the presence of "PRL-silent" lactotropes could be detected by

observing an increase in the amount of POU1F1^{+}/PRL^{-} cells in *Nr4a2*$^{flox/flox}$; Prl-Cre as compared to *Nr4a2*$^{flox/flox}$ littermates.

Consistent with this prediction, immunofluorescence microscopy revealed an increase in the amount of POU1F1^{+} cells that do not express PRL in *Nr4a2*$^{flox/flox}$; Prl-Cre mice, as indicated by a significantly higher ratio of POU1F1^{+}/PRL^{-} to POU1F1^{+}/PRL^{+} cells in their pituitaries (ratio of 4.72) as compared to *Nr4a2*$^{flox/flox}$ littermate controls (ratio of 1.64) (**Fig. 15 A and B**). This observation suggests a reduction of *Prl*-expressing cells within the pituitaries of *Nr4a2*$^{flox/flox}$; Prl-Cre mice, but not a loss in the total number of POU1F1^{+} pituitary cells. Notably, a subset of cells that co-express both POU1F1 and PRL (**Fig. 15B**, white arrow) can be detected in *Nr4a2*$^{flox/flox}$; Prl-Cre mice, suggesting that while the number of PRL^{+} lactotropes has decreased, there is not a complete loss of this cell population. This increase in POU1F1^{+}/PRL^{-} cells (**Fig. 15C**) in the pituitaries of mice that have ablated expression of *Nr4a2* in their lactotrope cells suggests that loss of *Nr4a2* does not cause complete loss of the cell population, but rather causes a loss of *Prl* expression in adult lactotropes, rendering them PRL-silent lactotropes (POU1F1^{+}/PRL^{-} cells).

Deletion of the lactotrope-enriched transcription factor *Pou4f1* in adult mouse lactotropes fails to alter *Prl* expression

Pou4f1 was identified as a second lactotrope-enriched transcription factor that had a positive impact on *Prl* expression when tested in our Pit-1/Triple cell line model system (**Chapter III**)[92]. These results predict that inactivation of

Pou4f1 in the lactotropes of the mouse pituitary would have a negative impact on the expression of *Prl* and/or the number of PRL positive lactotropes. Such findings would parallel those observed after conditional deletion of *Nr4a2* in lactotropes. A comparison of pituitaries from *Pou4f1*$^{flox/flox;}$ Prl-Cre mice and their control *Pou4f1*$^{flox/flox}$ littermates by targeted RT-PCR confirmed a significant reduction in the level of *Pou4f1* expression in the pituitaries of *Pou4f1*$^{flox/flox}$; Prl-Cre mice (*Pou4f1* mRNA levels at 30% those observed in *Pou4f1*$^{flox/flox}$ control mice). However, the study failed to reveal a corresponding decrease in *Prl* mRNA expression or an impact on any of the assayed landmark somatotrope or lactotrope specific genes. Furthermore, a set of parallel IF studies failed to reveal any significant differences in the representations of somatotropes and lactotropes within the pituitary cell populations (**Fig. 16**). These *in vivo* data suggest that *Pou4f1* does not play a significant role maintaining the expression of *Prl* in the lactotropes or in maintaining the cellular composition of the pituitary when studied in adult mice. What remains a possibility is that the impact of *Pou4f1* on *Prl* expression in the Pit-1/Triple cell model reflects a role in lactotrope development at an earlier developmental stage of the pituitary rather than in terminally differentiated lactotropes as previously hypothesized. Such a model can be tested in future studies (see **Chapter V**).

Deletion of the somatotrope-enriched transcription factor *Nupr1* in mouse somatotropes causes ectopic expression of PRL in somatotropes

While the lactotrope-enriched transcription factors *Nr4a2* and *Pou4f1* were selected for study due to their observed positive effects in enhancing *Prl* expression, the somatotrope-enriched transcription factor *Nupr1* was chosen for further study based on our data pointing to a role in repressing two landmark lactotrope markers, *Prl* and *Drd2*, in the Pit-1/Triple cell line[92] (**Fig. 5A**). Such a role would predict that the ablation of this locus in the somatotrope lineage might result in a de-repression of *Prl* and *Drd2* in the somatotropes, with an increase in the number of dual positive (GH^+/Prl^+) cells in the pituitary. To test this model that *Prl* gene expression is actively repressed in somatotropes by *Nupr1*, we first assayed the impact of a conditional inactivation of *Nupr1* in somatotropes of adult mice carrying the *Nupr1* floxed allele. Pituitaries of *Nupr1*$^{flox/flox}$; Gh-Cre mice and their *Nupr1*$^{flox/flox}$ littermates were assayed for *Prl* mRNA expression by RT-PCR (**Fig. 17A**). We observed that expression of *Nupr1* was significantly reduced in the pituitaries of *Nupr1*$^{flox/flox}$; Gh-Cre mice as compared to their *Nupr1*$^{flox/flox}$ littermates, thus confirming the conditional inactivation of the *Nupr1* allele. This ablation of *Nupr1* resulted in a significant (2.7-fold) increase in *Prl* levels in the pituitary. While this increase in *Prl* mRNA was consistent with our previous observations (**Fig. 5A**), the parallel impact on the lactotrope-specific gene *Drd2* was not observed. These data obtained from analysis of the whole pituitary are

consistent with a repressive function for *Nupr1* on the expression of *Prl* in the somatotrope cells.

To further investigate the impact of *Nupr1* in the adult pituitary, we assayed the pituitaries of *Nupr1*$^{flox/flox}$; Gh-Cre and *Nupr1*$^{flox/flox}$ mice by immunofluorescence microscopy (**Fig. 17B**) for alterations in the representations of the *Pou1f1* dependent lineages. Dispersed pituitary cells were stained with antibodies against GH and PRL and cells were counted in a blinded manner as previously described. These IF studies revealed a significant decrease in the number of somatotropes (GH^+/PRL^- cells) in the pituitaries of *Nupr1*$^{flox/flox}$; Gh-Cre mice, from 22% of all pituitary cells in *Nupr1*$^{flox/flox}$ control mice to 14% of all pituitary cells in *Nupr1*$^{flox/flox}$; Gh-Cre mice (**Fig. 17C**). Importantly, the decrease in the number of somatotropes was matched by a reciprocal increase in the number of dual positive GH^+/PRL^+ cells from 2% in *Nupr1*$^{flox/flox}$ mice to 9% in *Nupr1*$^{flox/flox}$; Gh-Cre mice (**Fig. 17D**). GH^+/PRL^+ cells, typically classified as "somatolactotrope" cells, are rare within wild-type adult mouse pituitaries, constituting approximately 1-2% of total cells[1,12,13]. The increase in the number of such cells in *Nupr1*$^{flox/flox}$; Gh-Cre to 9% suggests that inactivation of the *Nupr1* gene in somatotropes resulted in de-repression of the *Prl* gene. This de-repression led to the appearance of somatotropes co-expressing both GH and PRL, a phenotype that is typically categorized as a "somatolactotrope" cell. Thus, our cell culture studies and the conditional gene inactivation studies are concordant in assigning a repressive function to *Nupr1* at the *Prl* locus in

somatotropes. These findings lead us to conclude that landmark gene(s) of the lactotrope cell identity (i.e., *Prl*) must be actively repressed in somatotropes in order to maintain somatotrope cell identity in the adult pituitary (summarized in **Fig. 18**).

Discussion

Despite recent advances in understanding the regulation of the somatotrope and lactotrope lineages in the anterior pituitary[92], many questions remain. The three transcription factors that we focus on in these studies, *Nr4a2*, *Pou4f1*, and *Nupr1* were determined, on the basis of cell culture assays, to be regulators of *Prl*. Two of these factors enhance *Prl* expression in the context of a murine pituitary cell line model (*Nr4a2* and *Pou4f1*), while the third factor (*Nupr1*) suppresses expression of both *Prl* and *Drd2*. Thus all three of these transcription factors are observed to act on *Prl* gene expression, but are likely to be acting through a variety of mechanisms. These data suggest a previously unrecognized complexity in the regulation of hormone genes in the somatotrope and lactotrope lineages in the mouse pituitary. Knowledge of the mechanisms of action for transcription factors involved in enhancing or repressing expression of hormones may provide future studies with potential targets for therapeutics in treating disorders of the somatotrope/lactotrope lineages.

We began these studies by developing transgenic mouse lines that would allow us to probe the effect(s) of each transcription factor in the context of primary somatotropes and lactotropes in the adult pituitary. While some of the

required mouse lines were already available and were generously provided by their respective labs (Gh-Cre[16,99], Nr4a2^{flox} [97], and Pou4f1^{flox} [96]), lines for a lactotrope-specific Cre and a Nupr1^{flox} allele had to be generated *de novo*. A Prl-Cre line was established using BAC recombineering techniques (see **Methods**) to insert the Cre ORF into the second exon of the *Prl* gene in the context of a BAC containing the region of the mouse genome (~200 kb) containing the *Prl* locus. Mice carrying this transgene were validated for lactotrope specific Cre expression (**Fig. 13**), establishing a lactotrope-specific Cre mouse line to complement the existing Gh-Cre mouse line. A *Nupr1*^{flox} line was generated by the CRISPR/Cas9 Mouse Targeting Facility (see **Methods**) and validated for insertion of the LoxP sites *via* PCR and sequencing. Together, this set of mouse lines with floxed alleles and with lineage-specific Cre drivers allowed us to explore the *in vivo* functions of the selected transcription factors and to determine their roles in maintaining the somatotrope and lactotrope lineages in the adult pituitary.

Ablation of the lactotrope-enriched factor, *Nr4a2*, reduces *Prl* expression in a subset of lactotropes

We focused our initial studies on the transcription factor *Nr4a2*. This factor enhances *Prl* expression in the context of a cell line model system by binding to the *Prl* promoter **(Fig. 5C)**[92]. The majority of lactotropes (69%, N = 393 PRL^{+} cells) in *Nr4a2*$^{flox/flox}$; Prl-Cre mice did not express substantial amounts of NR4A2 protein as assayed by immunofluorescence microscopy (**Fig. 14A**). The

remaining 31% of lactotropes did express NR4A2, indicating that ablation of NR4A2 in these mice is not total, but is substantially reduced in comparison to *Nr4a2*$^{flox/flox}$ littermate controls, where 90% (N = 371 PRL^{+} cells) express NR4A2. Interestingly, we observe a subset of lactotropes in *Nr4a2*$^{flox/flox}$; Prl-Cre mouse pituitary that is depleted of NR4A2 but still produces PRL (**Fig. 14A**, right panel). The reduction in the number of lactotropes was quantified *via* blinded immunofluorescence microscopy assays to ensure that there were no biases in the cell counting (**Fig. 14B**). Notably, approximately half of the lactotrope population persists in the pituitaries of *Nr4a2*$^{flox/flox}$; Prl-Cre mice, suggesting that although *Nr4a2* is an important regulator of the lactotrope lineage, it is not the sole determinant of this cell type.

It is noteworthy that a subset of lactotropes show a complete loss of PRL protein expression as assayed by immunofluorescence microscopy, rather than a partial loss of PRL expression as initially hypothesized. While further study is necessary to determine the cause of the complete loss of PRL expression from some lactotropes, one interesting possibility is that there are multiple sub-types of lactotrope cells. It is possible that there exists a lactotrope sub-type that cannot express *Prl* in the absence of *Nr4a2* while there exists one or more sub-type(s) that can continue to express *Prl* in the presence of *Pou1f1* alone. The existence of such lactotrope sub-types would be consistent with single-cell RNA sequencing analysis that shows evidence of multiple sub-populations of lactotrope cells within the murine pituitary (Ho et al., In review). Consistent with

the detection of a 45% loss of cells expressing the PRL protein, *Prl* mRNA levels are also reduced by approximately 40% in *Nr4a2*$^{flox/flox}$; Prl-Cre mice (**Fig. 14C**). Together, these data support the hypothesis that *Nr4a2* represents a non-redundant enhancer of *Prl* gene expression.

To further characterize the loss of PRL expressing lactotropes in *Nr4a2*$^{flox/flox}$; Prl-Cre mice, we performed immunofluorescence microscopy by co-staining for POU1F1 and NR4A2. POU1F1 is the primary known activator of both *Gh* and *Prl* expression and is essential to the development of the somatotrope and lactotrope populations[21,39]. Based on our previous studies, ablation of *Nr4a2* is not expected to impact the expression of *Pou1f1* and therefore, even if *Prl* expression is lost, *Pou1f1* will remain expressed in lactotrope cells and can be used as a marker. The majority of POU1F1 positive cells in the pituitary are either somatotropes or lactotropes, with thyrotropes comprising only 5% of the total cells[1]. Thus, co-staining for POU1F1 and NR4A2 in the pituitaries of *Nr4a2*$^{flox/flox}$; Prl-Cre mice and *Nr4a2*$^{flox/flox}$ littermate controls can be used to detect the presence of “silent” lactotropes: lactotrope cells that express POU1F1 and other lactotrope proteins, but no longer express PRL due to deletion of a transcription factor such as *Nr4a2*.

Using this method, ablation of *Nr4a2* in lactotropes was observed to cause a significant shift in the ratio of POU1F1 positive, PRL negative cells to POU1F1 positive, PRL positive lactotropes in comparison to the ratio observed in littermate controls (**Fig. 15**). This result suggests that “lactotrope” cells are still

present in the pituitaries of *Nr4a2*$^{flox/flox}$; Prl-Cre mice, but that they no longer express prolactin. Further study is ongoing to confirm that these POU1F1^{+}/PRL^{-} cells are also negative for GH. These data suggest that ablation of *Nr4a2* causes a loss of prolactin expression in lactotrope cells, rather than an outright loss of lactotropes, as evidenced by the increase in POU1F1 single positive cells in *Nr4a2*$^{flox/flox}$; Prl-Cre mice. Taken together with previous data, this study indicates that *Nr4a2* likely plays a role in maintaining the expression of *Prl* in the lactotropes of adult mice.

The lactotrope-enriched transcription factor, *Pou4f1*, failed to impact *Prl* expression in mice

In addition to our studies on the role of *Nr4a2* in lactotrope cells, we also assayed the effect of *Pou4f1* in lactotropes with our hypothesis being that *Pou4f1* plays a similar role to *Nr4a2* in these cells due to its similar effect on *Prl* expression in previous cell line studies (**Fig. 5D**). However, both immunofluorescence microscopy (**Fig. 16A and B**) and RT-PCR (**Fig. 16C**) failed to reveal any evidence that *Pou4f1*$^{flox/flox}$; Prl-Cre mice have lower levels of *Prl* expression or fewer lactotropes than their *Pou4f1*$^{flox/flox}$ littermates, although a reduction of *Pou4f1* mRNA was confirmed, indicating successful ablation of *Pou4f1*. These data contrast with previous data from mouse pituitary cell lines (**Fig. 5D**) and may suggest that *Pou4f1* does not play a role in the regulation of *Prl* expression in the lactotropes of adult mice. Conversely, it is possible that *Pou4f1* plays a role in the regulation of *Prl*, but there is another gene or genes

that are functionally redundant with *Pou4f1*, such that deletion of *Pou4f1* alone is not sufficient to impact *Prl* expression in mice. Alternatively, *Pou4f1* may play a role in an earlier stage of lactotrope development, prior to *Prl* expression. Testing this possibility would require producing mice that carry both the *Pou4f1*$^{flox/flox}$ allele as well as a Cre recombinase transgene that is expressed at an earlier stage of pituitary development than the Prl-Cre used in these studies. Development of such mice falls outside the scope of the present study, but is an interesting topic for future studies to assay and is discussed further in **Chapter V**.

The somatotrope-enriched transcription factor, *Nupr1*, represses *Prl* expression in somatotrope cells

In contrast to lactotrope-enriched transcription factors such as *Nr4a2* which enhances *Prl* expression, we also assayed a somatotrope-enriched transcription factor, *Nupr1*, that our cell based assay suggested has a repressing effect on *Prl* expression (**Fig. 5A**). *Nupr1*$^{flox/flox}$; Gh-Cre mice have fewer somatotropes (GH^+/PRL^-) in their pituitaries than *Nupr1*$^{flox/flox}$ littermate control mice, and demonstrate a robust increase in the representation of GH/PRL dual positive cells (**Fig. 17**). Conventionally, such dual positive cells are identified as somatolactotropes and constitute 1-2% of the adult pituitary population[1]. As noted earlier (see **Chapter I**), the origin of the somatolactotrope cells remains ambiguous, with some reports identifying them as precursors to both the somatotropes and the lactotropes[2] while others hypothesize that they are a transitional state between somatotropes and lactotropes[13]. While a conventional

somatolactotrope and a GH/PRL dual positive cell that arises from de-repression of *Prl* expression in *Nupr1*$^{flox/flox}$*;* Gh-Cre mice may appear indistinguishable by immunofluorescence microscopy with GH/PRL staining, it should be noted that they are not necessarily identical nor are they necessarily generated by similar mechanisms. Somatolactotropes may have currently unknown traits or transcriptomic differences which distinguish them from the somatotropes and lactotropes beyond simply co-expressing GH and PRL. In contrast, the GH/PRL dual positive cells observed after deletion of *Nupr1* in mouse somatotropes may simply be somatotropes that are ectopically expressing *Prl* with no further changes in cell identity, a distinction that will require a better understanding of the somatolactotrope cells to resolve.

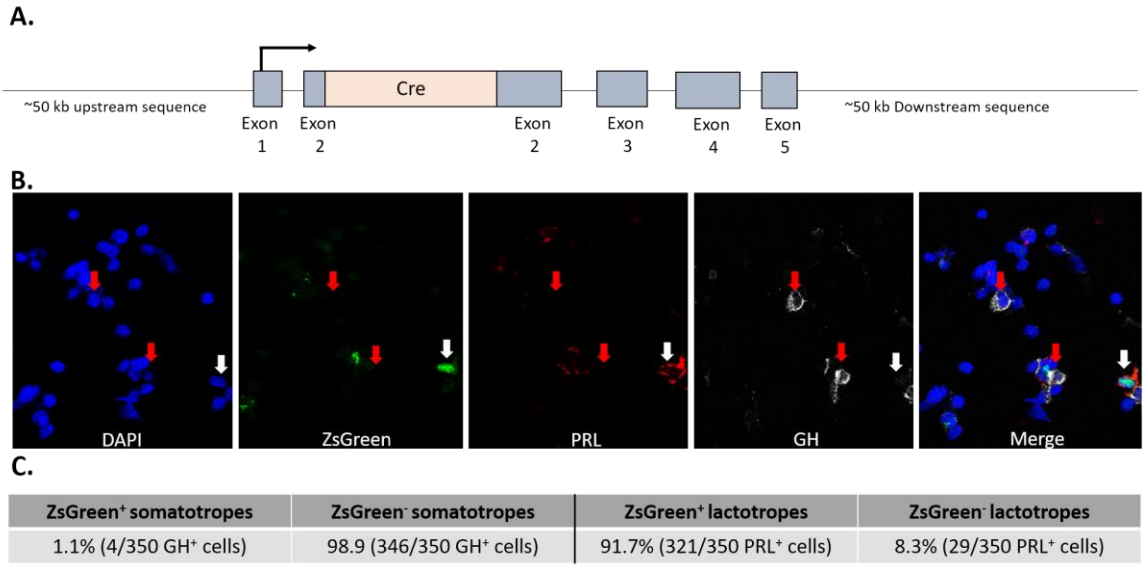
While this distinction between somatolactotropes and somatotropes that ectopically express PRL may appear minor, it raises a question that has been central in the study of anterior pituitary cell types, yet remains incompletely answered: how does one define a somatotrope or a lactotrope? Conventionally, cells that express GH alone are defined as somatotropes while cells that express PRL alone are categorized as lactotropes. This classification paradigm has been useful in the study of these cells, but it obfuscates the lack of a deep understanding of somatotrope/lactotrope cell identity. As previously discussed (**Chapter I**), there are few genes that can be classified as crucial to the somatotrope or lactotrope cell identities beyond the hormone genes themselves, *Gh* and *Prl*, and receptors for hypothalamic signals such as *Ghrhr* and *Drd2*,

respectively. Without knowledge of more genes that are essential to the somatotrope/lactotrope identities, it is a challenge to classify these cells in a truly meaningful way. Classifying somatotropes/lactotropes purely by their expression of the GH or PRL proteins may be sufficient when describing the normal, adult pituitary, but it is less useful for studies in which perturbations of transcription factors such as *Nr4a2* or *Nupr1* occur.

Defining a cell type by its expression of a hormone alone may be problematic. For example, if a lactotrope ceases to express PRL, as seen when *Nr4a2* is deleted (**Fig. 13**), should it still be classified as a lactotrope? *Drd2*, while enriched in lactotropes, is also expressed in other pituitary cells[100], and thus cannot be used to classify lactotropes without other identifying marks to supplement it. The only method of addressing this problem is to identify more genes crucial to the somatotrope/lactotrope cell identities, which has been the goal of this work. Only with a more complete understanding of the set of genes that make a somatotrope a somatotrope can one deal with classifying cells in which the hormones-- currently the only landmarks used to determine cell identity-- are perturbed. Thus, while GH/PRL dual positive cells that arise from the deletion of *Nupr1* may appear identical to the somatolactotropes that are observed at low frequencies in wild type mouse pituitaries insofar as they both co-express GH and PRL, they may in reality represent two different cell types. Further study of the somatotrope, lactotrope, and somatolactotrope cell identities will be needed to resolve this dilemma. The work presented here in

characterizing the transcriptomes of the somatotropes and lactotropes provides a critical resource that will be essential in reaching that goal.

Figures and legends



C.

ZsGreen⁺ somatotropes	ZsGreen⁻ somatotropes	ZsGreen⁺ lactotropes	ZsGreen⁻ lactotropes
1.1% (4/350 GH⁺ cells)	98.9 (346/350 GH⁺ cells)	91.7% (321/350 PRL⁺ cells)	8.3% (29/350 PRL⁺ cells)

Figure 13. Prl-Cre mouse line produces lactotrope-specific expression of Cre recombinase. A. Schematic of Prl-Cre transgene. The Cre ORF was inserted into the second exon of the *Prl* gene in the R23-441I3 BAC via BAC recombineering. The transgene preserves approximately 50 kb of upstream and downstream sequence flanking the *Prl* locus to include any previously undescribed regulatory elements. **B.** Immunofluorescence microscopy of dispersed pituitary cells from Prl-Cre/ZsGreen mice carrying transgenes for both the Prl-Cre and a ZsGreen fluorescent reporter preceded by a lox-stop-lox element such that ZsGreen is only expressed in cells that also express Cre recombinase. Somatotropes are indicated by red arrows, while lactotropes are indicated by white arrows. **C.** Quantification of somatotropes and lactotropes expressing ZsGreen reporter detected in **B.**

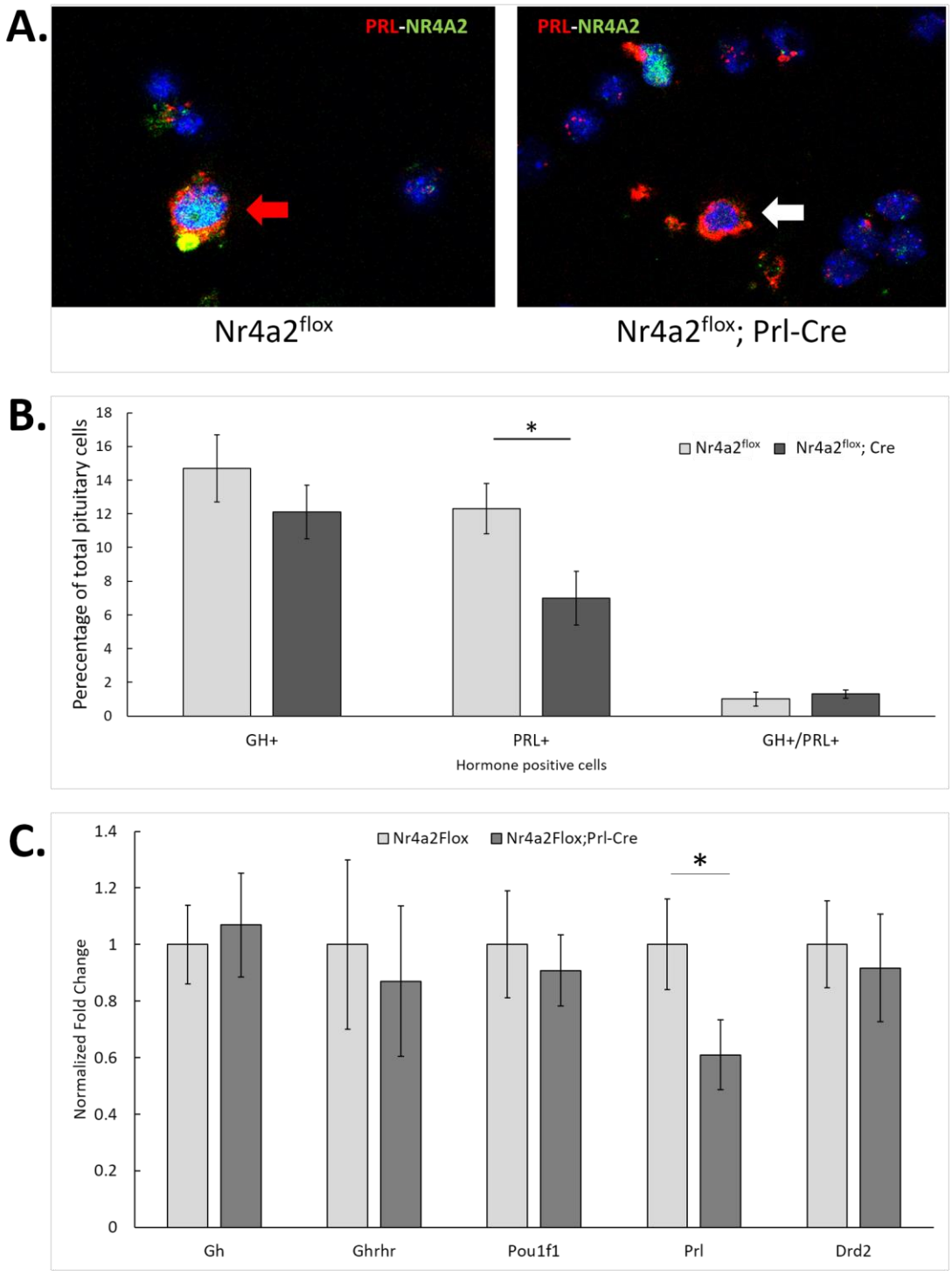


Figure 14. Deletion of Nr4a2 in mouse lactotropes causes a decrease in prolactin positive cells. A. Representative immunofluorescence microscopy of

dispersed mouse pituitary cells from control (left panel) and conditional *Nr4a2* knockout (right panel) mice showing $NR4A2^{+}$ lactotropes (red arrow) in control mice and $NR4A2^{-}$ lactotropes (white arrow) in knockout mice. Staining for PRL is shown in red, highlighting lactotrope cells while NR4A2 staining is shown in green. **B.** Quantification of cells counted (N = 1500 cells) in blind study of the number of GH, PRL, and GH/PRL positive cells in control and Nr4a2 conditional knockout mice. **C.** Quantitative reverse transcription PCR of RNA isolated from whole pituitaries of control and *Nr4a2* knockout mice. mRNA levels for 5 landmark genes for the somatotrope and lactotrope lineages were assayed for changes (N=2).

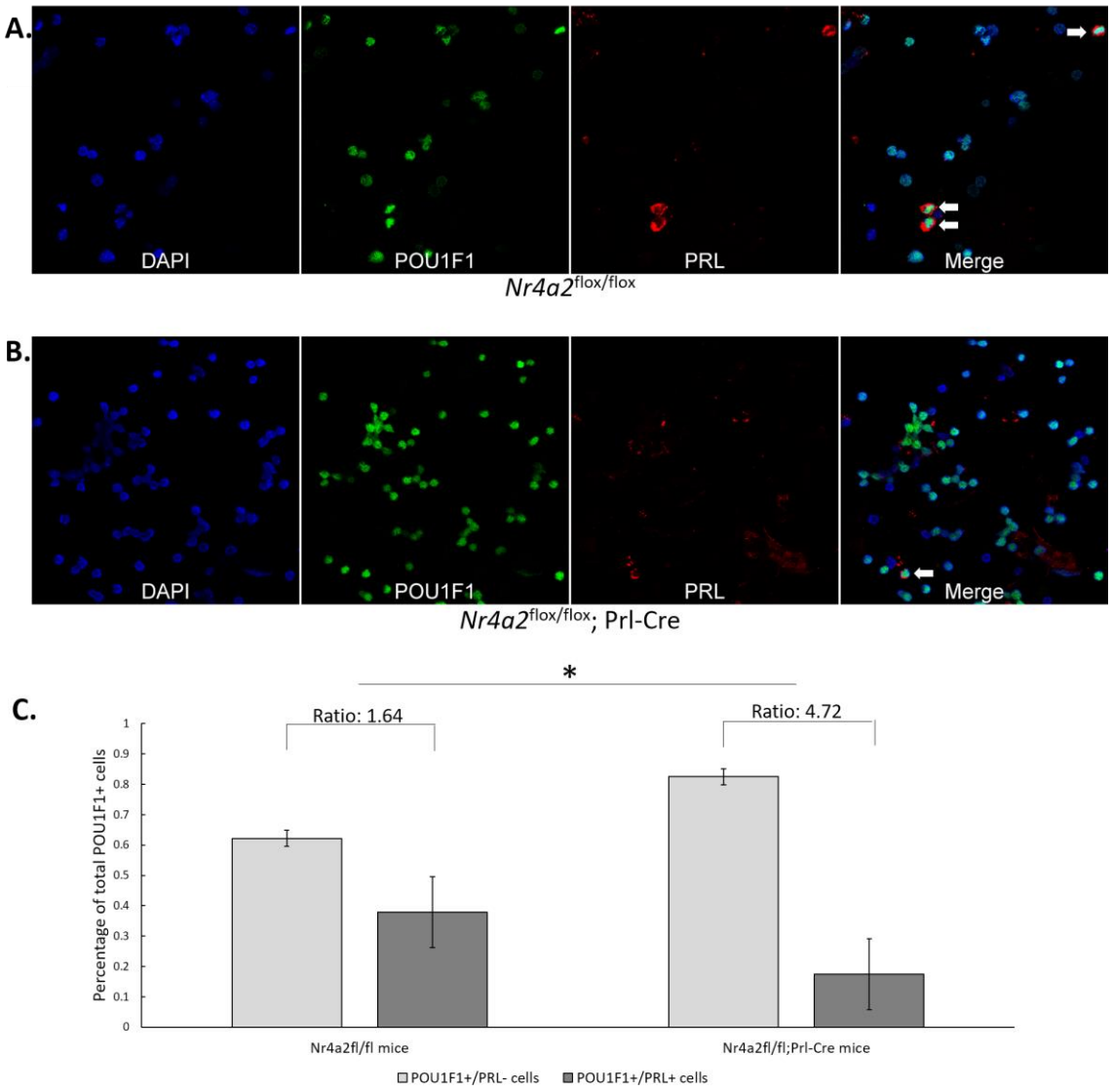


Figure 15. Ablation of Nr4a2 causes and increase in POU1F1^{+}/PRL^{-} cells and a corresponding decrease in POU1F1^{+}/PRL^{+} cells. **A**. Representative immunofluorescence microscopy images of dispersed pituitary cells from *Nr4a2*$^{flox/flox}$ control mice. POU1F1, the primary known driver of both GH and PRL expression, co-stains with PRL in lactotrope cells (white arrows). **B**. Representative immunofluorescence microscopy images from *Nr4a2*$^{flox/flox}$; Prl-

Cre mice. While POU1F1^{+}/PRL^{+} are detected, their numbers relative to the POU1F1^{+}/PRL^{-} cells (ratios) are significantly lower than in *Nr4a2*$^{flox/flox}$ littermate control mice (* indicates significance of difference of ratios between the two mouse genotypes). **C**. Quantification of immunofluorescence studies from **B**. N = 2 mice per experimental group.

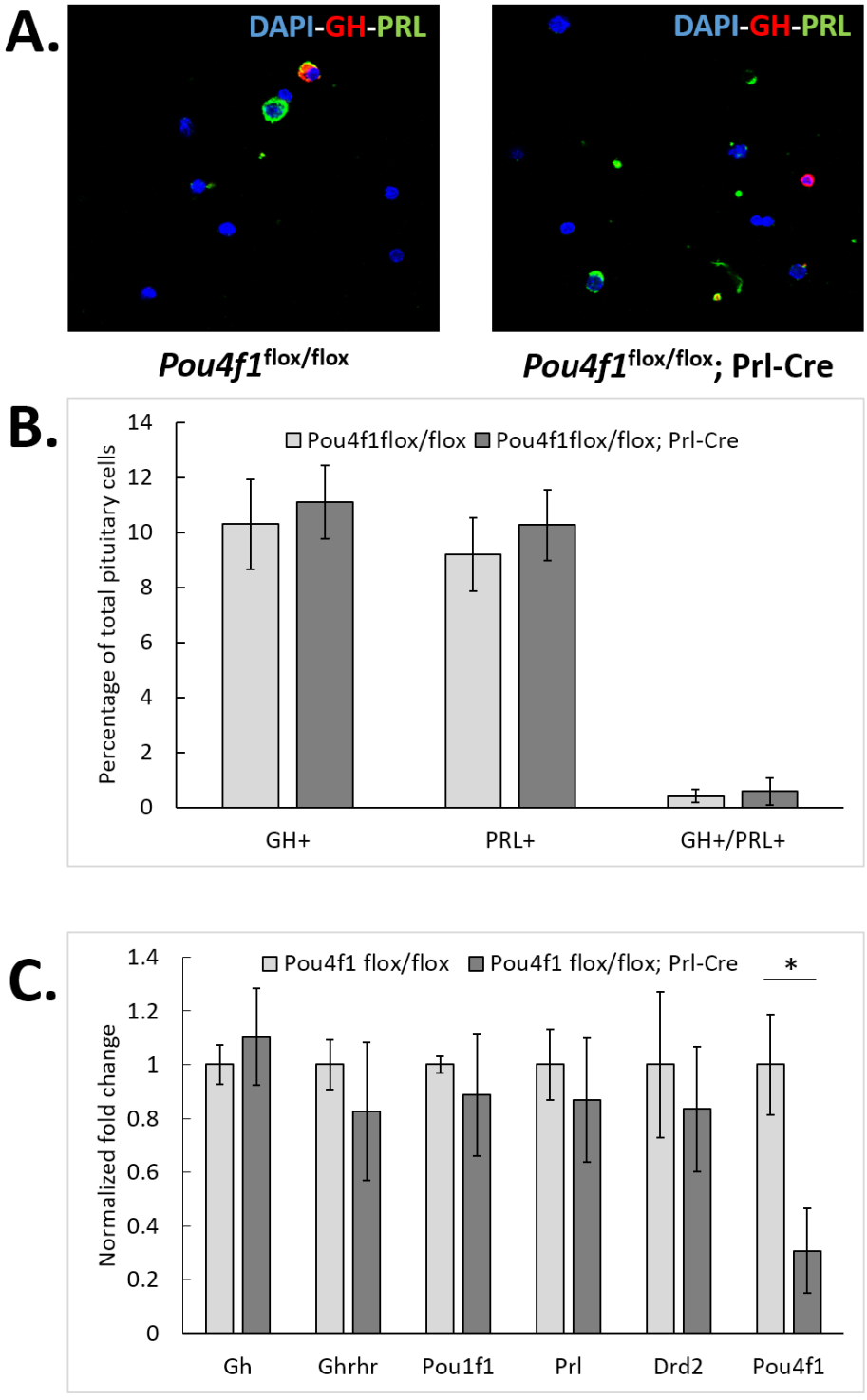


Figure 16. Conditional depletion of the lactotrope-enriched transcription factor Pou4f1 fails to impact on *Prl* expression in adult mouse lactotrope

cells. A. Representative immunofluorescence microscopy images of dispersed pituitary cells from *Pou4f1*$^{flox/flox}$ control mice (left panel) and *Pou4f1*$^{flox/flox}$; Prl-Cre mice (right panel) exhibiting similar distributions of somatotrope and lactotrope cells. Cells were stained with antibodies against prolactin (green) and growth hormone (red), with DAPI staining (blue) marking nuclei. **B.** Representation of somatotropes (GH^+), lactotropes (PRL^+), and GH/PRL dual positive somatolactotropes in the pituitaries of *Pou4f1*$^{flox/flox}$; Prl-Cre mice and control littermates. These data are derived from the IF studies, as shown in **A**. **C.** RNA expression of hallmark genes in total RNA of whole pituitaries from *Pou4f1*$^{flox/flox}$; Prl-Cre mice and control littermates. These data were generated by RT-PCR and normalized to *Gapdh* expression. N=2. * indicates p-value <0.05.

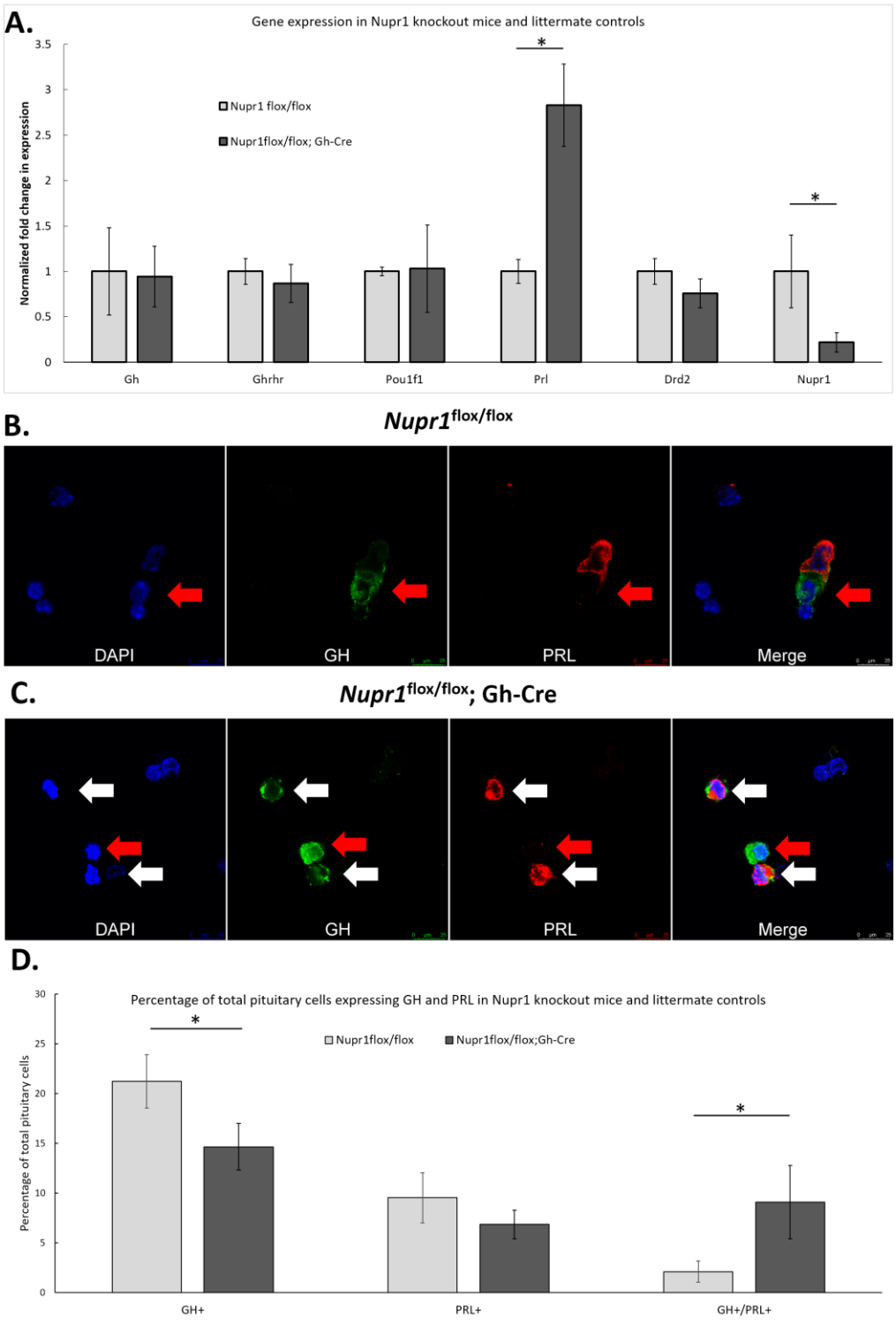


Figure 17. Conditional depletion of Nupr1 in somatotropes results in an increase in Prl expression in the mouse pituitary with a corresponding

increase in GH/PRL dual positive cells. A. qRT-PCR of total RNA isolated from whole pituitary samples from *Nupr1*$^{flox/flox}$; Gh-Cre and *Nupr1*$^{flox/flox}$ mice. N = 3 mice per genotype. **B**. Representative immunofluorescence microscopy images of dispersed pituitary cells from *Nupr1*$^{flox/flox}$ mice, showing a typical distribution of GH and PRL single positive cells (somatotropes and lactotropes). Images are separated into nuclear DAPI staining (blue), growth hormone (green), prolactin (red) as well as a merged image. Somatotropes are indicated by red arrows. **C**. Representative immunofluorescence microscopy images of dispersed pituitary cells from *Nupr1*$^{flox/flox}$; Gh-Cre mice, showing multiple GH/PRL dual positive cells (white arrows). **D**. Cell counts for GH positive somatotropes, PRL positive lactotropes, and GH/PRL dual positive cells observed by immunofluorescence microscopy presented as a percentage of total pituitary cells. N = 3 mice per genotype, 300 cells counted per mouse.

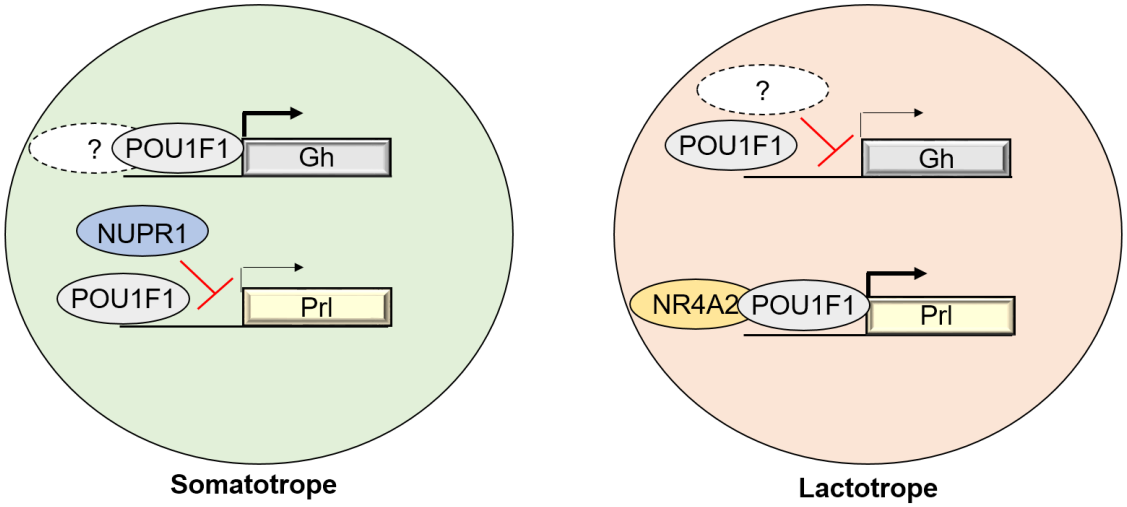
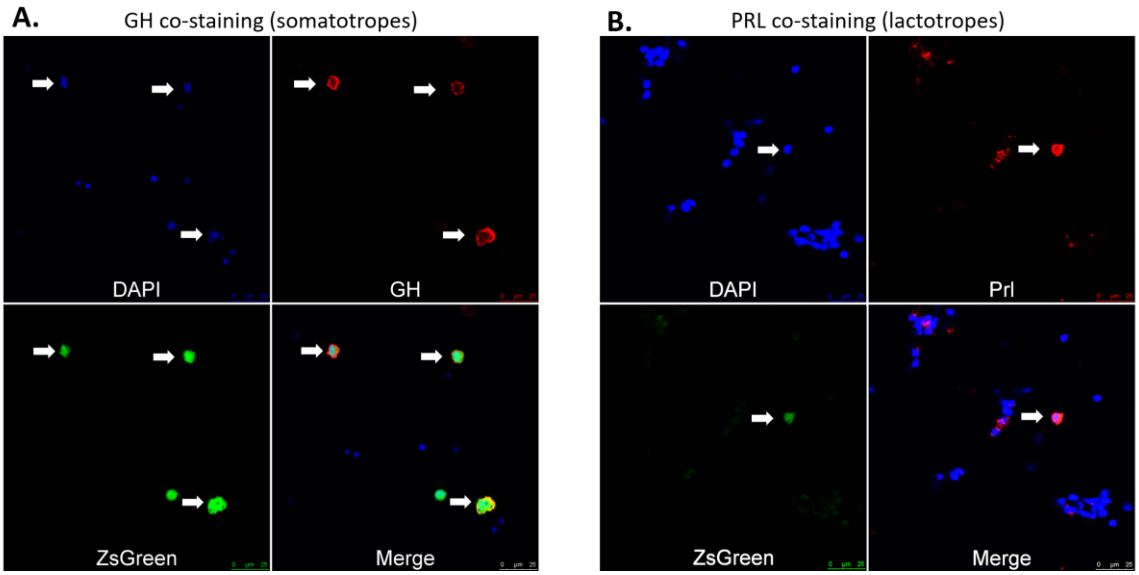


Figure 18. Modified model for selective expression of *Gh* and *Prl* in somatotropes and lactotropes (based in work in the current thesis). The master regulatory transcription factor POU1F1 is present in both somatotropes and lactotropes and occupies the promoters of the *Gh* and *Prl* genes. The actions of POU1F1 at these sites is responsible for base-line expression of both hormone genes in both lineages. The selective enrichment of *Gh* and *Prl* in their respective somatotrope and lactotrope lineages reflects the reciprocal actions of transcriptional enhancers and repressors. In somatotropes (left), the robust expression of *Gh*, most likely stimulated *via* interactions of yet unidentified factor(s) ("?", dashed oval) with POU1F1, is complemented by a reciprocal repression of *Prl* transcription by NUPR1. NUPR1 does not appear to act directly on the *Prl* promoter; whether its repressive activity is linked to direct actions at a site in *cis* to the *Prl* gene, or *via* indirect mechanism(s) is yet to be determined. In lactotropes (right), *Gh* expression is postulated to be actively repressed ("?",

dashed oval), while *Prl* expression is selectively activated by the actions of NR4A2. NR4A2 occupancy and activity is dependent on the actions of POU1F1. NR4A2 acts in conjunction with an adjacently situated POU1F1 to enhance the release of Pol II from the *Prl* promoter. This release activity allows the extension of Pol II into the *Prl* gene body with consequent production of *Prl* mRNA.

Supplemental Data



Supplemental Figure 7. Validation of Gh-Cre mouse line. **A.** Gh-Cre mice, previously reported[16], were obtained from the lab of Dr. Sally Camper. To validate the mouse line, Gh-Cre mice were crossed with mice carrying a ZsGreen transgene in which the expression of ZsGreen is controlled by a Lox-Stop-Lox system such that cells which express Cre will also express ZsGreen. The pituitaries of Gh-Cre/ZsGreen mice were disaggregated and stained with antibodies against GH to mark somatotropes and against ZsGreen to mark Cre-expressing cells. Of 200 somatotropes (GH^+ cells) assayed, 182 (91%) were also positive for ZsGreen, indicating robust Cre expression in somatotrope cells. **B.** The same study was performed co-staining cells for both ZsGreen and PRL to mark lactotrope cells. Of 200 lactotropes (PRL^+ cells) assayed, 64 (32%) were also positive for ZsGreen, consistent with previous reports[16].

Chapter V: Summary and Future Directions

Summary

The anterior pituitary is composed of multiple cell types that are each responsible for the production and regulated secretion of a given hormone. The *Pou1f1*-dependent lineages-- the somatotropes, lactotropes, and thyrotropes-- have been shown to be primarily regulated by *Pou1f1* with few other factors having been identified as part of this process. Here, we generated the first transcriptome-wide view of gene expression in murine somatotropes and lactotropes, giving an unprecedented view into their differential gene expression as well as their many similarities. While our transcriptomic data revealed that the somatotropes and lactotropes are overall quite similar cell types—an observation that differs from the conventional model of these cells as well-distinguished, discrete cell types—there also exist small, but crucial subsets of somatotrope and lactotrope enriched genes that contribute to the distinct cell identities of these populations.

Among the sets of differentially expressed genes that we identified in somatotrope and lactotrope cells, we selected six transcription factors for further study, and narrowed down this list of transcription factors. Using a cell line model system, we found that the somatotrope enriched transcription factor, *Nupr1*, and two lactotrope enriched factors, *Nr4a2* and *Pou4f1*, had significant functional impacts on the expression of genes that are known to be crucial to the somatotrope and lactotrope cell identities. While *Nr4a2* and *Pou4f1* were observed to have a positive regulatory effect on the expression of *Prl*, *Nupr1*

exerted a repressive effect on the expression of both *Prl* and another crucial lactotrope gene, *Drd2*, in our cell line model system. These observations suggested the existence of transcription factors that are expressed in either the somatotrope or lactotrope cells that can influence the expression of genes central to cell identity through a variety of mechanisms, both enhancing and repressive.

We next sought to test the functions of these transcription factors in mouse models. After generating or acquiring the required mouse lines carrying floxed alleles of each transcription factor as well as transgenes that express Cre recombinase in either a somatotrope or a lactotrope specific manner, we assayed the impact of ablating these transcription factors in primary mouse pituitary. Assays of the ablation of *Pou4f1* failed to reveal a functional impact on *Prl* expression as predicted by our cell line model studies, and thus it was excluded from further study. Ablation of the somatotrope enriched factor, *Nupr1*, led to ectopic expression of PRL in cells that co-express GH, consistent with the prediction that *Nupr1* is a repressor of *Prl* in somatotrope cells. Lastly, ablation of the lactotrope transcription factor *Nr4a2* caused a loss of approximately half of all PRL^+ cells in the pituitaries of knock-out mice. This loss was further characterized and found to be the result of lactotrope cells ceasing expression of *Prl* rather than a failure of lactotrope cells to develop in earlier stages of pituitary development. We focused on *Nr4a2* to elucidate the mechanism by which it enhances *Prl* expression. These studies revealed that NR4A2 directly binds to the *Prl* promoter and enhances the release of RNA polymerase II from the *Prl*

promoter. Our *in vivo* mouse studies, focusing on the subset of three transcription factors that were identified by our transcriptomic analyses and validated in the cell culture studies, reveal a diversity of actions carried out by transcription factors in the somatotrope and lactotrope cells that are essential to maintaining cell identity.

Taken together, the data presented in this dissertation reconfigure and expand upon the previous standard model of pituitary cell identity. *Pou1f1* acts in conjunction with other transcription factors that are enriched in either cell type. These transcription factors can exert either enhancing or repressive effects on the expression of the *Prl* gene, and the set of transcription factors highlighted in this dissertation is likely to represent only a subset of the factors involved in this process. These data suggest that the somatotrope and lactotrope cells are cells with highly similar transcriptomes, but the expression of a small set of somatotrope and lactotrope genes is sufficient to maintain separate cell identities.

Future directions

Determination of the mechanism of action of *Nr4a2*

Although progress has been made in determining the mechanism by which *Nr4a2* increases *Prl* expression, some questions remain open. An outstanding question regarding *Nr4a2* is what co-factor(s) are recruited to the *Prl* promoter in order to enhance the release of Pol II. To resolve this question,

future studies will utilize co-immunoprecipitation of NR4A2 in transfected Pit-1/Triple cells (which express endogenous *Pou1f1*) coupled with mass spectrometry. A reciprocal co-immunoprecipitation of POU1F1 will allow analysis of the complex at the *Prl* promoter in the absence of NR4A2 (untransfected Pit-1/Triple cells). By precipitating NR4A2 along with nearby and/or complexed proteins, we will be able to identify additional factors that are recruited in the presence of NR4A2, and the use of mass spectrometry will provide an unbiased view of these factors. Identified factors will then be the subject of mechanistic studies to determine their role in enhancing *Prl* expression.

Another question is whether *Nr4a2* impacts other genes beyond *Prl*. While *Prl* was the only gene affected by the presence of *Nr4a2* in our cell based studies, these studies assayed a small selection of known somatotrope and lactotrope genes in order to establish an initial screening of *Nr4a2* functions. It is possible that other genes are impacted by *Nr4a2* beyond the genes assayed in our studies, but their identification would require a broad approach. To identify other genes that are impacted by *Nr4a2*, RNA-seq will be performed on *Nr4a2*$^{flox/flox}$; Prl-Cre and *Nr4a2*$^{flox/flox}$ mice. Differential gene expression analyses will permit the identification of genes that are impacted by the loss of *Nr4a2* specifically in lactotrope cells, generating a data set that will highlight genes for further study and synergize with the above Co-IP experiments to provide a better understanding of the role of *Nr4a2* in lactotropes and the full impact of the loss of *Nr4a2* on the lactotrope population.

Determination of the mechanism of action of *Nupr1* in somatotropes

Unlike *Nr4a2*, the potential mechanism of action of the somatotrope-enriched factor *Nupr1* is not currently understood. ChIP of NUPR1 has unfortunately not been feasible due to a lack of ChIP grade antibodies and known NUPR1 binding sites to serve as positive controls, and thus it is not known at present if NUPR1 binds the *Prl* promoter. Future studies will take a genome-wide approach to identifying targets of *Nupr1* regulation by performing RNA-seq experiments comparing gene expression in somatotrope-specific *Nupr1* knockout mice to floxed control mice in order to identify genes impacted by *Nupr1* expression in somatotropes. Genes that are identified as being differentially expressed in the absence of *Nupr1* will be assayed for a role in repression of *Prl* using cell line models.

Although ChIP of NUPR1 is not currently feasible without knocking an epitope tag into the endogenous mouse *Nupr1* locus, changes in chromatin modifications at the *Prl* promoter can be assayed in the presence and absence of *Nupr1*. Pit-1/Triple cells expressing *Nupr1* exhibit a repression of both *Prl* and *Drd2* expression, which may be the result of repressive chromatin marks such as H3K27me and/or H3K9me at the promoters of these genes. This possibility will be assayed by comparing the H3K27me profiles at the promoters of *Prl* and *Drd2* in Pit-1/Triple cells in the presence and absence of *Nupr1* expression. An increase in repressive chromatin marks when *Nupr1* is expressed would indicate that *Nupr1* operates by in some manner recruiting these histone marks to the

promoters of the genes it represses, giving an insight into the mechanism by which *Nupr1* represses *Prl* expression in mouse somatotropes.

Characterization of impact(s) of identified transcription factors in earlier stages of pituitary cell development

As previously discussed, conditional deletion of *Pou4f1* using a Prl-Cre mouse line did not yield any impact on *Prl* expression in mice (**Chapter IV**) despite an observed impact on *Prl* expression in Pit-1/Triple cells (**Chapter III**). One possible explanation for this discrepancy is that *Pou4f1* acts on the lactotrope lineage at a developmental stage prior to *Prl* expression, and thus the Prl-Cre is expressed too late to trigger an effect in *Pou4f1*$^{flox/flox}$; Prl-Cre mice. This problem could be further investigated in future studies through the use of a Cre recombinase under the regulatory control of a gene expressed prior to *Prl*, such as *Pou1f1*. As the required mice carrying floxed alleles for *Pou4f1*, *Nupr1*, and *Nr4a2* are all established in the lab, this study can be expanded to all three transcription factors of interest. Conditional deletion of *Pou4f1* in mice carrying a *Pou1f1*-Cre recombinase will be assayed for a reduction in either the number of lactotropes, or the level of *Prl* expression in the lactotropes. Conditional deletions of the remaining transcription factors, *Nr4a2* and *Nupr1* in *Pou1f1*-Cre mice will be assayed for any changes in their established impacts on lactotrope and somatotrope cells, respectively, when deleted at an earlier stage in pituitary development. These studies will provide a better understanding of how the transcription factors identified in this thesis function in the anterior pituitary.

Materials and Methods

Cell and transgenic mouse lines. Mice carrying the previously described Gh-GFP and Prl-DsRed transgenes were mated to generate compound transgenic mice carrying both reporter constructs ($GFP^{+}/DsRed^{+}$)[45,46]. Mice carrying the previously described Gh-Cre[16] or the newly generated Prl-Cre alleles were crossed with either the existing $Nr4a2^{flox}$ [97], existing $Pou4f1^{flox}$ [96], or the newly generated $Nupr1^{flox}$ alleles to generate the appropriate conditional knockout mice for the described studies. All mice were of a hybrid CD1 x B6SJLF1/J background, and were aged to 6-8 weeks before use in experiments to allow for the complete maturation of the pituitary gland. All mice used in these studies were virgin females. All mouse studies were reviewed and approved by the University of Pennsylvania Laboratory Animal Use and Care Committee. The Pit-1/Triple and Pit-1/0 cell lines, previously described[28], were cultured in Dulbecco's modified eagle medium (DMEM) (Life Technologies) plus 10% fetal bovine serum (FBS) along with 1× antibiotic-antimycotic solution (Invitrogen). Cells were removed from plates for the purposes of passaging and for harvesting using a 0.05% Trypsin-EDTA solution (Life Technologies).

Generation of transgenic mouse lines. The existing GH-GFP mouse line[45], provided to our lab as a cosmid containing the GH-GFP transgene, was modified with sub-cloning techniques. Briefly, an MluI digestion of the GH-GFP cosmid released a fragment containing the GFP ORF as well as upstream and downstream *Gh* sequence. This fragment was sub-cloned into a vector (pGEM-4)

and inverse PCR was performed using primers that flanked the sequence corresponding to the first 24 amino acids of GH such that only the first 8 amino acids would be preserved in the PCR product. This PCR product was then ligated to form a plasmid containing a truncated version of the GH-GFP sequence that contains only 8 amino acids of GH prior to the beginning of the GFP sequence. Such a truncation has been previously demonstrated to ablate the secretion of the linked protein[45]. Lastly, the plasmid was again digested with MluI to release the modified Gh-GFP and ligated back into the full length cosmid from which it was extracted. This cosmid was then linearized and microinjected into mouse embryos by the Penn Transgenic Mouse Facility and Gh-GFP lines were established.

The Prl-Cre transgenic mouse line was generated via a previously described BAC recombineering method[94]. Briefly, a shuttle vector containing the Cre ORF flanked by 500 bp of sequence homologous to the desired insertion site (the beginning of exon 2 of the *Prl* ORF) in the mouse *Prl* locus was generated with conventional sub-cloning techniques. Then, bacterial cells were co-transformed with the shuttle vector as well as the RP23-441I3 BAC, obtained from the BACPAC repository. This BAC contains the mouse *Prl* locus as well as over 50 kb of upstream and downstream sequence. Co-transformed cells were grown in selective media and screened for co-integrates. After obtaining shuttle vector/BAC co-integrates, a second round of selection was performed to select for resolution of the co-integrate. Resolved BACs containing the Cre ORF in the

proper site within the BAC were confirmed by PCR and pulse field gel analysis. Prl-Cre BAC DNA was linearized by restriction enzyme digestion, purified by gel extraction, and provided to the Penn Transgenic Mouse facility for microinjection into mouse embryos. Prl-Cre mouse lines were confirmed by genotyping after birth and proper function of the Prl-Cre transgene was validated by crossing the Prl-Cre mouse line with a previously described reporter line. The $Nupr1^{flox}$ mouse line was generated by the Penn CRISPR/Cas9 Mouse Targeting facility using conventional CRISPR/Cas9 methodologies to target LoxP sites flanking the second exon of the *Nupr1* locus, as deletion of this exon has been previously reported to successfully ablate NUPR1 activity[98].

Fluorescence activated cell sorting. Pituitaries from female GFP^{+}/$DsRed^{+}$ mice were harvested at 6-8 weeks and dissociated mechanically in enzyme free cell dissociation buffer (Life Technologies) before being passed through a 40 μm filter into DMEM + 10% FBS culture media and centrifuged at 1000 g for 5 minutes. After centrifugation, cells were resuspended in PBS + 0.1% BSA and layered onto a PBS + 4% BSA cushion and centrifuged a second time at 100 g for 5 minutes to remove cell debris. Cell pellets were resuspended in PBS + 0.1% BSA, 1 μg of DAPI was added directly to the samples, and the cells were sorted on a FACS Aria II platform (BD Biosciences) directly into Trizol LS reagent (Thermo Fisher Scientific). The cell sorting was performed by the Flow Cytometry & Cell Sorting Facility at University of Pennsylvania.

RNA isolation and RNA-sequencing. RNA from FACS sorted cells was isolated (Trizol LS protocol; Thermo Fisher Scientific) using 10 µg of GlycoBlue coprecipitant (Thermo Fisher Scientific) as a carrier. RNA quality and concentration were assayed using the RNA 6000 Pico kit (Agilent) with a 2100 BioAnalyzer (Agilent) platform. High quality RNA samples, defined by RIN scores of >7, were submitted to the University of Pennsylvania Next Generation Sequencing Core (NGSC) for analysis. Each sample was amplified using the Ovation ultralow RNA-seq library system (Nugen) and libraries were sequenced on the HiSeq 2500 platform (Illumina). Sequencing data was mapped and analyzed by the Penn Next-Generation Sequencing Core (NGSC). GEO accession number for sequencing data: GSE118863.

Cell transfection assays. The cDNA of *Nupr1* was amplified from mouse pituitary cDNA, and cDNAs for the other studied transcription factors were obtained from OriGene or Addgene and sub-cloned into an IRES GFP vector (Addgene plasmid #51406). The expression of the cDNA and GFP in this vector is controlled by the CMV promoter. Pit-1/Triple and Pit-1/0 cells were transfected with 10 µg of plasmid using the TransIT-293 transfection reagent (Mirus Bio). In co-transfection assays, cells were transfected with 5 µg of each plasmid to maintain the DNA: TransIT-293 ratio as recommended by the manufacturer. Two days after transfection, GFP^+ cells were FACS sorted using the method and platform described above. RNA was isolated from the GFP^+ cells as described above and cDNA was generated using the SuperScript III reverse transcription kit

(Life Technologies). Quantitative real time PCR was performed using the Fast SYBR Green Master Mix kit (Applied Biosystems) on a QuantStudio 7 Flex (Applied Biosystems) platform. qRT-PCR assays were done in biological triplicate, and all samples were normalized to the housekeeping gene Gapdh, and compared to cells transfected with an empty vector by the ΔΔCt method [101]. All transfections were performed using approximately 1.6 million cells per sample, grown to 80% confluence in 10 cm plates. Amplification primer sequences: Gh: 5'-gcccaggctgctttctgc-3' and 5'-caattccatgtcggttctctgc-3'. Prl: 5'-aggggtcagcccagaaagc-3' and 5'-tcaccagcggaacagattgg-3'. Pou1f1: 5'-aggtgggagcaaacgaaagg-3' and 5'-gctccccgaagtgtctctcc-3'. Ghrhr: 5'-tcacttcggctcagcacagg-3' and 5'-ggcaagccacagggtatgg-3'. Drd2: 5'-tgaacctgtgtgccatcagc-3' and 5'-gacagtaactcggcgcttgg-3'. Gapdh: 5'-agcttaggttcatcaggtaaactcagg-3' and 5'-cgttcacaccgaccttcacc-3'. Nr4a2: 5'-gatcagtgccctcgtcagagc-3' and 5'-gtcagggtttgcctggaacc-3'. Nupr1: 5'-gcgggcatgagaggaagc-3' and 5'-gctgggtgtggtgtctgtgg-3'. Rxrg: 5'-aagtttcccaccggctttgg-3' and 5'-gtggctgtccattggcttcc-3'. Pou4f1: 5'-agtacccgtcgctgcactcc-3' and 5'-gtccaggctggcgaagagg-3'.

Immunofluorescence microscopy. Pituitaries were disaggregated and prepared using the same process as noted above for FACS. The immunofluorescent staining was performed as previously described[102]. Briefly, the disaggregated cells were placed on Poly-L-lysine coated slides and incubated at room temperature for 10 minutes to allow the cells to adhere to the

slide. After attaching to the slides, cells were fixed in 4% formaldehyde for 10 minutes, then washed three times with PBS for 5 minutes per wash. Cells were permeabilized in a solution of 0.5% triton X-100 and 0.5% saponin for 10 minutes. The cells were washed with PBS before being incubated in a blocking buffer of 4X SSC, 2% BSA, 0.1% Tween 20, and 15% donkey serum for 20 minutes at room temperature. After blocking, slides were incubated with antibodies specific to proteins of interest for 1 hour at room temperature, washed three times in PBS, then incubated for one hour at room temperature with secondary antibodies conjugated to fluorophores for detection. After incubation with secondary antibodies, the slides were washed a final time, incubated for 1 minute in 1mg/ml DAPI, and cover slips were attached. Slides were imaged with a Leica SP8 confocal microscope platform. Images were analyzed using Fiji software[103]. Antibodies used for each transcription factor are as follows: NUPR1 (Thermo-Fisher PA5-65826, RRID: AB_2662159[104]), RXRG (Santa Cruz SC-514134, RRID: AB_2737293[105]), TBX19 (Santa Cruz SC-22656, RRID: AB_2200381[106]), PPARG (Abcam 55296, RRID: AB_944767[107]), NR4A2 (Abcam ab41917, RRID: AB_776887[108]) and POU4F1 (Abcam ab81213, RRID: AB_1640222[109]).

Chromatin Immunoprecipitation (ChIP). ChIP assays were performed as previously described[110]. Chromatin was isolated from approximately 200,000 FACS sorted transfected cells per sample and chromatin from 100,000 cells was used per ChIP reaction. For the purposes of performing ChIP assays, Pit-1/0

cells were transfected with 20 µg of plasmid DNA using Lipofectamine 3000 (Thermo Scientific) *per* the manufacturer's directions to achieve higher transfection efficiency. Antibodies specific for NR4A2 (Abcam ab41917, RRID: AB_776887) and POU4F1 (Abcam ab81213, RRID: AB_1640222) were used in the respective assays. H3K27ac ChIP was performed using antibody ab4279 (RRID: AB_2118291[111]) and H3K4me4 ChIP was performed using antibody ab8580 (RRID:AB_306649). A rabbit antibody specific for POU1F1 was generated by Alpha Diagnostics (RRID: AB_2732812[112]) using the antigen sequence: PLLAEDPAASEFKQELRRKSKL. This affinity purified antibody was demonstrated to be specific for POU1F1 by Western blot analysis (**Supplemental Figure 2**). Normal rabbit IgG (Millipore) was used as a control in all ChIP assays. Input and bound DNAs were amplified by qRT-PCR using the same QuantStudio 7 Flex platform noted above. A serial dilution of the input chromatin was used to determine the linear range of the amplification and the enrichment of the bound DNA was calculated as a percentage of the input. ChIP of primary mouse pituitary cells was performed using the same protocol, with cells being collected by FACS as above prior to isolation of chromatin. Amplification primer sequences: Gh promoter: 5'-gccttggggtcgaggaaaac-3' and 5'-gggatttgcgcatgcttacc-3'. Prl upstream control: 5'-gaactggaagctgttgaacttgc-3' and 5'-ggtcttgggaactgaacttgg-3'. Prl promoter: 5'-tggccaatgtcttcctgaatatg-3' and 5'-cactggctttataaacctttgaca-3'. Prl downstream control: 5'-catgcattctaccaaaagggtagg-3' and 5'-tgcatctctatagcacttggtttatcc-3'. MyoD

promoter: 5’-gcgtatggctgccagtctct-3’ and 5’-tgtagtagggcggagcttgg-3’. Pou4f1 enhancer: 5’-cgcgaaaccaacaaagagttttc-3’ and 5’-ctgtagcgcgtttaaataaatgaagtg-3’.

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