IN VIVO ANALYSIS OF HUMAN *LHX3* ENHANCER REGULATION

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DEDICATION

I dedicate this dissertation to my family and my husband Cheolwoong. Without their love and support I would never have reached this point.

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ABSTRACT

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IN VIVO ANALYSIS OF HUMAN *LHX3* ENHANCER REGULATION

The LHX3 transcription factor is essential for pituitary gland and nervous system development in mammals. In humans, mutations in the *LHX3* gene underlie combined pituitary hormone deficiency (CPHD) disease featuring deficits in anterior pituitary hormones and defects in the nervous system. The mechanisms that control temporal and spatial expression of the *LHX3* gene are poorly understood. The proximal promoters of the human *LHX3* gene are insufficient to guide expression *in vivo* and downstream elements including a conserved 7.9 kilobase (kb) enhancer region appear to play a role in tissue-specific expression in the pituitary and nervous system. In this study, I characterized the activity of this downstream enhancer region in regulating gene expression at the cellular level during development. Human *LHX3* enhancer-driven *Cre* reporter transgenic mice were generated to facilitate studies of enhancer actions. The downstream *LHX3* enhancer primarily guides gene transcription in αGSU-expressing cells secreting the TSHβ, LHβ or FSHβ hormones and expressing the GATA2 and SF1 transcription factors. In the developing nervous system, the enhancer serves as a targeting module for expression specifically in V2a interneurons. These results demonstrate that the downstream *LHX3* enhancer is important in specific endocrine and neural cell types but also indicate that additional regulatory elements are likely involved in *LHX3* gene

expression in other cell types. Further, these studies demonstrate significant gonadotrope cell heterogeneity during pituitary development, providing insights into the cellular physiology of this key reproductive regulatory cell. The human *LHX3* enhancer-driven *Cre* reporter transgenic mice provide a valuable tool for further developmental studies of cell determination and differentiation in the pituitary and nervous system. Furthermore understanding the regulation of human *LHX3* gene will help develop tools to better diagnose and treat pituitary CPHD disease.

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Adrenocorticotropic hormone	ACTH
Alpha glycoprotein subunit	αGSU
Alpha melanocyte-stimulating hormone	αMSH
Anterior pituitary	AP
Arginine vasopressin	AVP
Base pair	bp
Basic helix loop helix	bHLH
Bone morphogenetic protein	BMP
Combined pituitary hormone deficiency	CPHD
Diaminobenzidine	DAB
Diencephalon	DIEN
Diphtheria toxin A	DTA
Enhanced green fluorescent	EGFP
Fibroblast growth factor	FGF
Follicle-stimulating hormone	FSH
Follicle-stimulating hormone beta	FSHβ
Gonadotropin-releasing hormone	GnRH
Gonadotropin-releasing hormone receptor	GnRHR
Growth hormone	GH
Growth hormone-releasing hormone	GHRH
Growth hormone-releasing hormone receptor	GHRHR

Heat shock protein 68	HSP68
Homeodomain	HD
Interneuron	IN
Islet1	ISL1
Kilobases	kb
Luria-Bertani broth	LB
LIM homeobox 3	LHX3
LIM homeobox 4	LHX4
LIM homeodomain	LIM-HD
Luteinizing hormone	LH
Luteinizing hormone beta	LHβ
Motor neuron	MN
Nuclear factor 1	NF1
Nuclear factor Y	NFY
Nuclear LIM domain interactor	NLI
Nuclear localization signal	NLS
Oral ectoderm	OE
Oxytocin	OT
Phosphate buffered saline	PBS
Pituitary specific transcription factor 1	PIT1
Polymerase chain reaction	PCR
Pro-opiomelanocortin	POMC
Prolactin	PRL

Prophet of Pit1	PROP1
Rathke's pouch	RP
Sonic hedgehog	SHH
Specificity protein 1	SP1
Steroidogenic factor 1	SF1
T-box transcription factor	TPIT
Thyroid-stimulating hormone	TSH
Thyroid-stimulating hormone beta	TSHβ
Untranslated region	UTR
Wingless/integrate	Wnt
Yellow fluorescent protein	YFP

CHAPTER ONE

INTRODUCTION

1.1 The Pituitary Gland and its Hormones

The pituitary, or hypophysis, is located at the base of the brain and it is situated within the sella turcica which is formed by the sphenoid bone. The pituitary is crucial for the maintenance of various physiological processes including growth, lactation, homeostasis, reproduction, metabolism, and stress response. The mature pituitary is composed of three lobes: the posterior lobe, the intermediate lobe, and the anterior lobe.

The primary physiological role of the pituitary is to secrete hormones. The posterior lobe consists of an extension of magnocellular neurosecretory cells of the hypothalamus that synthesize peptide hormones such as oxytocin (OT) and arginine vasopressin (AVP) (Scully and Rosenfeld, 2002). OT and AVP are transported by axon terminals to the posterior lobe of the pituitary gland from the hypothalamus and released into bloodstream. AVP regulates osmotic balance by acting to increase water absorption in the collecting ducts of the kidney. OT is released after labor to stimulate muscle contraction, and in response to a positive feedback loop to stimulate milk ejection from the mammary glands.

Within the intermediate lobe of the pituitary, melanotropes secrete alpha melanocyte-stimulating hormone (αMSH). αMSH hormone is a 13 amino acid peptide produced by proteolytic processing of its pro-opiomelanocortin pro-hormone encoded by the *pro-opiomelanocortin* (*POMC*) gene. αMSH has functions in skin pigmentation and other functions in lower vertebrates [reviewed in (Roubos et al., 2010)]. The human intermediate lobe is less developed than in many other species, consisting of a thin layer

of cells between the posterior and anterior lobes, and produces less αMSH than amphibians [reviewed in (Catania et al., 2000)].

The anterior pituitary is a critical component of endocrine physiology and it contains five distinct hormone-secreting cell types (Figure 1.1). Somatotropes secrete growth hormone (GH) that regulates linear growth and some metabolic activities. Lactotropes produce prolactin (PRL), regulating milk production and other reproductive functions. Corticotropes secrete adrenocorticotrophic hormone (ACTH): a proteolytic product of the POMC pre-hormone encoded by the *POMC* gene. ACTH regulates metabolism and other activities by stimulating the functions of the hormone-secreting adrenal gland cortex. Thyrotropes produce thyroid-stimulating hormone (TSH), which promotes thyroid follicle development and thyroid hormone secretion which subsequently control multiple homeostatic functions. Gonadotropes produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which act on the gonads to initiate sexual maturation and reproductive functions [reviewed in (Zhu et al., 2007)]. TSH, FSH, LH are heterodimeric glycoproteins composed of a common alpha glycoprotein subunit (αGSU) and a unique beta subunit [e.g. TSHβ; reviewed in (Perez-Castro et al., 2012)]. During early development in mammals, anterior pituitary hormone cell lineages are located in distinct areas of the gland. Corticotropes and gonadotropes are more concentrated in the ventral area; and somatotropes and lactotropes are enriched in the dorsal regions of the anterior pituitary. Thyrotropes are found in two independent areas during development; the rostral tip and the caudomedial region (Lin et al., 1994; Dasen et al., 1999; Kioussi et al., 1999).

The hypothalamus maintains control of anterior and intermediate pituitary hormone production by the secretion of releasing and inhibiting hormones from the median eminence. These regulatory hormones are transported through the hypophyseal portal blood system and bind to specific anterior pituitary cell surface receptors. For example, GH-releasing hormone (GHRH) binds to the GH-releasing hormone receptor located on anterior pituitary somatotropes resulting in GH hormone synthesis and release. Similarly, gonadotropin-releasing hormone (GnRH) is released from the hypothalamus and binds to the GnRH-receptor on the gonadotrope cell surface to promote secretion of LH and FSH.

1.2 Pituitary Development

The mature pituitary derives from two distinct components, the neurohypophysis and the adenohypophysis, which have different embryonic origins. The neurohypophysis is derived from the ventral diencephalon and is therefore neuroectodermal in origin. It includes the posterior pituitary, the infundibular (pituitary) stalk which is connected to the hypothalamus, and the median eminence. The adenohypophysis develops from from an invagination of the oral ectoderm to form a rudimentary Rathke's pouch, the rudimentary structure that will form the anterior pituitary, the intermediate pituitary, and the pars tuberalis (an extension of the anterior pituitary that wraps around the pituitary stalk) (Figure 1.1). (Watanabe, 1982a). The end of the anterior neural ridge in the oral ectoderm becomes the primordium of the anterior and intermediate lobes of the pituitary. The adjacent neural plate becomes the hypothalamus and the posterior lobe of pituitary.

In the mouse, the first sign of pituitary development occurs at e7.5 with the development of the hypophyseal placode. At e8.5, the anterior neural tube bends and

extends rapidly. At e9, the placode forms the rudimentary Rathke's pouch. Ectodermal invagination brings physical contact between the ventral diencephalon and oral ectoderm. By e10.5, a restricted region of ventral diencephalon above Rathke's pouch forms the infundibulum that becomes the posterior pituitary and the pituitary stalk. At e12.5, Rathke's pouch is morphologically developed (Kawamura and Kikuyama, 1998). By e17.5, the hormone-expressing cell types are present (Japón et al., 1994) and are concentrated in distinct regions. In the adult gland the anterior pituitary cells are more mixed.

1.3 Signaling Molecules in Early Pituitary Development

Gradients of protein signaling molecules between the oral ectoderm and ventral diencephalon are important for formation of Rathke's pouch and the subsequent differentiation of the hormone-secreting cells in early pituitary development (Figure 1.1) [e.g. (Daikoku et al., 1982; Watanabe, 1982a; Watanabe, 1982b; Kawamura and Kikuyama, 1995; Gleiberman et al., 1999)]. Signaling molecules such bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), sonic hedgehog (SHH), Notch, and Wnt (wingless/integrated) are important for the development of a diverse array of mammalian organs including the pituitary gland (Figure 1.1).

BMP signaling is critical for the development and patterning of the mouse pituitary from the initial induction of Rathke's pouch to cell specification in the anterior lobe (Ericson et al., 1998; Davis and Camper, 2007). The earliest characterized secreted molecule from the prospective infundibulum is BMP4. The initial step of Rathke's pouch formation requires invagination of the oral ectoderm and this process is BMP4-dependent. The highest expression level of BMP4 is at e9.5 and it stays on until Rathke's

pouch is fully formed (Ericson et al., 1998). *Bmp4* null mice display no formation of the Rathke's pouch placode (Takuma et al., 1998).

Fibroblast growth factors (FGFs) are a family of signaling proteins involved in many aspects of embryonic development such as angiogenesis [reviewed in (Cross and Claesson-Welsh, 2001)]. FGFs are important for organogenesis and cell differentiation in the developing pituitary gland. FGF8, 10, and 18 have been found in the ventral diencephalon and posterior pituitary in mice after initial induction by BMP4 (Treier et al., 1998; Treier et al., 2001). In mice lacking either *Fgf10* or FGF receptor 2, Rathke's pouch forms but undergoes apoptosis at e14.5 (Ohuchi et al., 2000). This suggests that *Fgf10* is essential for pituitary cell survival (Sun et al., 1999; Revest et al., 2001).

SHH has an important role in delineating the area for Rathke's pouch formation and it is expressed in both in the ventral diencephalon and throughout the oral ectoderm (Treier et al., 2001). The effect of loss of *Shh* in mice on pituitary development disrupts diencephalon formation at an early embryonic stage. Pituitary-specific blockage of SHH signaling by the SHH antagonist HIP prevents Rathke's pouch formation, and experimental over-expression of SHH in the developing pituitary of mice resulted in pituitary hyperplasia (Treier et al., 2001).

Wnt/β-catenin pathway signaling is important for controlling proliferation of cell types and stem cell fate during development. Wnts are highly expressed between e11.5 and e15.5 in mouse pituitary development and are required for pituitary cell determination and pituitary gland growth, especially for the pituitary specific transcription factor-1 (PIT-1)-dependent cell lineages (thyrotropes, somatotropes, and lactotropes). Several studies support roles for Wnt/β-catenin signaling in pituitary

development. For example, β-catenin can regulate the activity of two transcription factors with roles in pituitary development: pituitary homeobox 2 (PITX2), and steroidogenic factor 1 (SF1) (Kioussi et al., 2002; Brinkmeier et al., 2003; Gummow et al., 2003). Ten of the 19 *Wnt* genes are detected in the developing pituitary (Olson et al., 2006). *Wnt4* and *Wnt5* have been reported to be specifically associated with the developing events of the anterior pituitary. *Wnt5* is expressed in the ventral diencephalon and deletion of this gene causes extra bifurcations of Rathke's pouch (Cha et al., 2004). *Wnt4*$^{-/-}$ mice have anterior hypoplasia with reduced number of gonadotropes, thyrotropes, and somatotropes (Treier et al., 1998). *Wnt5*$^{-/-}$ mice have an enlarged intermediate lobe and an increased number of POMC cells in the anterior and intermediate lobe (Cha et al., 2004).

1.4 Transcription Factors in Anterior Pituitary Development

During embryogenesis, important signaling molecules (as described above) and transcription factors including PITX1, PITX2, PIT-1, PROP1, TPIT, SF1, GATA2, ISL1, LHX4, and LHX3 and important in guiding the anterior pituitary development (Figure 1.1). These factors have been shown to play important roles in anterior pituitary lineage specification and differentiation (Figure 1.1) (Hashimoto et al., 2005; Kelberman et al., 2009; Davis et al., 2010; Mollard et al., 2012). For example, the PIT-1 homeodomain transcription factor is required for development of the GH-, TSH-, and PRL-expressing cells; and steroidogenic factor 1 (SF1) and GATA2 play roles in gonadotrope cell differentiation (Ingraham et al., 1988; Li et al., 1990; Ingraham et al., 1994; Dasen et al., 1999).

PITX1

The pituitary homeobox (*Pitx*) family contains three bicoid-related homeobox genes (*Pitx1, 2, 3*) and each gene has roles in development (Gage et al., 1996b). The PITX1 and PITX2 proteins are best characterized in pituitary development. PITX1 was discovered as a protein interaction partner of the pituitary specific transcription factor 1, PIT-1 (Szeto et al., 1999). PITX1 was also identified as an activator of the *POMC* gene in corticotropes (Lamonerie et al., 1996). PITX1 and PITX2 expression initiates at e8 in the tissue that gives rise to Rathke's pouch and expression persists through adulthood (Suh et al., 2002). Both factors can bind to the promoters of pituitary-expressed genes such as *αGSU, TSHβ, LHβ, FSHβ, PRL,* and *GH* and may be involved in transcription of these genes in hormone-secreting cells (Tremblay et al., 2000). PITX1 activation is required for the later actions of other transcription factors including PIT-1 (Tremblay et al., 1998). *In vitro* PITX1 knockdown experiments show a lack of *αGSU* and *LHX3* expression in PITX1 knockdown cell lines (Tremblay et al., 1998) and similar results were recapitulated *in vivo* (Charles et al., 2005). These data suggest that PITX1 acts as an upstream regulator of LHX3 in the pituitary development signal cascade (Figure 1.1). *Pitx1*$^{-/-}$ mice form a normal Rathke's pouch, however the number of gonadotrope and thyrotrope cells is reduced. Also there is abnormal high expression of ACTH at e17.5 (Szeto et al., 1999), and hindlimb and cleft plate development defects are reported (Lanctot et al., 1999; Szeto et al., 1999).

PITX2

Pitx2 is also implicated in early pituitary development. With expression in developing pituitary, eye, and brain, PITX2 is proposed to have roles in the proper

development and maintenance of anterior structures. The *PITX2* gene was initially identified as the gene responsible for Rieger syndrome in humans and the symptoms include abnormalities in eye, tooth, brain, heart, and anterior pituitary with some patients displaying GH deficiency (Semina et al., 1996) (Semina et al., 1996; Gage and Camper, 1997). Mice with deficiency of the *Pitx2* gene have abnormal left-right asymmetry; developmental defects in heart, lung, eye, tooth, and abnormalities in the proliferation and differentiation of pituitary cells leading to hypoplastic pituitary structures (Semina et al., 1996; Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Lin et al., 1999; Lu et al., 1999; Suh et al., 2002). In these mice, invagination of Rathke's pouch occurs normally, however, the gland becomes "frozen" at e10.5 and contains few corticotropes. Furthermore, no PIT-1 induction occurs and TSH, GH, PRL cells are missing. There is low expression of the PROP1 homeodomain protein in *Pitx2*$^{-/-}$ mice suggesting that PITX2 is a regulator of pituitary development upstream of PROP1 (Figure 1.1) and necessary for hormone-expressing lineage precursor expansion (Suh et al., 2002).

PIT-1

The POU-homeodomain family of transcription factors are named from the three "founding" transcription factors, the pituitary-specific Pit-1, the octamer-binding proteins Oct-1/2, and the neural *Unc*-86. PIT-1 is a lineage-specific transcription factor and was the first pituitary specific transcription factor to be identified (Bodner et al., 1988; Ingraham et al., 1988). Expression of *Pit-1* is restricted to the caudomedial region of the developing pituitary gland and PIT-1 activates pituitary genes such as the *GH*, *GH-releasing hormone receptor* (*GHRHR*), *PRL*, *TSHβ* and genes and its own gene via

autoregulation [e.g. (Li et al., 1990; Rhodes et al., 1993)]. Mutations in the mouse *Pit-1* gene underlie the phenotypes of the Snell and Jackson dwarf mouse models which lack GH, PRL, and TSH, providing evidence for an essential role of PIT-1 in determination of pituitary somatotropes, lactotropes, and thyrotropes (Li et al., 1990). Studies using the Snell mice reveal that thyrotropes may be derived from two distinct cell populations; PIT-1 dependent lineages are located in the caudomedial portion of the gland and these TSH-expressing cells persist into adulthood. In contrast, PIT-1-independent thyrotropes are located in the rostral tip region, and these cells are only seen in early pituitary development (Lin et al., 1994). Rostral tip thyrotropes start to appear at ~e12.5 and PIT-1 dependent thyrotropes arise at ~e14.5 during pituitary development. Consistent with the *Pit-1*-disrupted mouse phenotypes, humans with mutations in the *PIT-1* gene (also known as the *POU1F1* gene in humans) have hypothyroidism, dwarfism, and deficiency of the PRL, GH, and TSH hormones (Figure 1.1) (Ohta et al., 1992; Pfaffle et al., 1992; Radovick et al., 1992).

PROP1

A paired-class homeodomain gene, the *Prophet of Pit-1* (*Prop1*) gene, is expressed in the developing anterior pituitary gland at ~e10.5 until ~e16.5 and is required for pituitary organogenesis (Nasonkin et al., 2004; Ward et al., 2005). The mutant Ames dwarf mice ($Prop1^{df/df}$) have a missense mutation causing a serine to proline substitution in the homeodomain of murine PROP1 and have notable decreases of thyrotropes, somatotropes, and lactotropes, similar to the Snell and Jackson mice (Andersen et al., 1995; Sornson et al., 1996). *Pit-1* is effectively absent in Ames mice suggesting that *Pit-1* lies downstream of *Prop1* in pituitary development (Tang et al., 1993; Andersen et al.,

1995; Gage et al., 1995; Gage et al., 1996a; Sornson et al., 1996). Mutations in the *PROP1* gene are the most common known cause of combined pituitary hormone deficiency (CPHD; meaning loss of GH plus one or more other pituitary hormones) (the majority of CPHD diseases remain uncharacterized). The clinical features of these patients include dwarfism and deficiency of GH, TSH, PRL, LH hormones with common onset of ACTH deficiency (Böttner et al., 2004).

TPIT

TPIT is T-box class pituitary transcription factor that is expressed in *POMC*-producing corticotrope cells of the intermediate and anterior pituitary (Lamolet et al., 2001). TPIT synergizes with PITX1 to activate the *POMC* gene in specification of the corticotropes of the anterior pituitary (Lamolet et al., 2001; Liu et al., 2001). Mice lacking the *Tpit* gene and pediatric patients with mutations in the human *TPIT* gene display a reduced corticotrope cells in the anterior pituitary (Lamolet et al., 2001; Pulichino et al., 2003). Together, these studies suggest that TPIT is important for *POMC* gene transcription and expansion or survival of corticotrope cells in the pituitary gland.

SF1

SF1 (steroidogenic factor 1), a nuclear receptor, is necessary for adrenal and gonadal development and also is expressed in the ventral diencephalon and in the gonadotropes of the developing anterior pituitary gland [reviewed in (Schimmer and White, 2010)]. SF1 regulates the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axes (Ikeda et al., 1995). SF1 is first detected at ~e13.5 in the developing pituitary and *Sf1* gene knockout mice are not viable and have no expression of FSH, LH, and the gonadotropin-releasing hormone receptor (GnRHR) (Ingraham et al.,

1994; Ikeda et al., 1995). Pituitary-specific *Sf1* knockout mice are viable, but sexually immature with no expression of FSH and LH. The male mice exhibit hypoplasia of the gonads and reduction of Leydig cells. In female mice, development of ovaries occurs normally but pre-ovulatory follicles are not produced (Zhao et al., 2001). Mouse model studies suggest that SF1 is involved in the differentiation of the gonadotrope lineages and directly regulates LH and FSH expression.

GATA transcription factors

Normal reproduction depends on proper development and function of the hypothalamic-gonadotrope-gonadal axis. The GATA transcription factor family is well characterized as a regulator of gene expression in hematopoietic cells (Orkin, 1992), and they also play important roles in reproduction (LaVoie, 2003; Viger et al., 2008). In vertebrates, the GATA family is composed of six members. GATA1/2/3 play roles in hematopoietic and nervous system development (Ohneda and Yamamoto, 2002; Patient and McGhee, 2002), and GATA4/5/6 factors are involved in development of organs including the heart, gut, and vasculature (Morrisey et al., 1996; Molkentin, 2000; Molkentin et al., 2000). GATA members have highly conserved protein sequences in their zinc finger DNA binding domains and GATA factors bind to a consensus binding motif (A/T)GATA(A/G) in DNA (Merika and Orkin, 1993; Lowry and Atchley, 2000).

GATA2 and GATA3 play roles in blood and nervous system development but also are expressed in the developing anterior pituitary (Dasen et al., 1999; Suh et al., 2002). Both of mRNAs are found at ~e10.5 and GATA2 expression persists into the adult (Dasen et al., 1999). GATA2 has been shown to be involved in the differentiation of gonadotrope and thyrotrope cell lineage through the interactions with PIT-1 in the

developing pituitary (Dasen et al., 1999; Suh et al., 2002). GATA2-expressing cells include gonadotropes and PIT-1 dependent thyrotropes (Dasen et al., 1999). GATA2 also has been proposed to activate the expression of SF1 (Steger et al., 1994), which in turn stimulates *αGSU* and *LHβ* gene expression, however, GATA2 may not influence *FSHβ* expression (Brown and McNeilly, 1999). *In vitro* analysis reveals that GATA2 can activate transcriptional activity of the *LHβ* gene promoter by interacting with SF1 (Lo et al., 2011). Studies have shown that GATA2 colocalizes with αGSU pituitary lineages, the thyrotropes and gonadotropes. *Gata2* null homozygous embryos die at e11.5 due to severe anemia (Tsai et al., 1992). Pituitary-specific *Gata2* knockout mice display reduction of gonadotropes and thyrotropes in neonates (Charles et al., 2006). Recently, mutations were identified within the human *GATA2* gene and the majority of these patients have primary lymphedema (Ishida et al., 2012; Kazenwadel et al., 2012).

1.5 LIM-Homeodomain Transcription Factors, ISL1, LHX4 and LHX3

The LIM domain nomenclature is derived from the first letters of the names of the founding members: LIN11, ISL1, and MEC3 (Bach, 2000; Hunter and Rhodes, 2005). The LIM domain is a zinc-coordinated protein-protein interaction domain. The LIM family includes transcription factors, cytoskeletal proteins, signaling proteins, and transcriptional co-activators [reviewed in(Bach, 2000)]. LIM-homeodomain (LIM-HD) transcription factors have two LIM domains located in the amino terminus that are involved in protein-protein interactions, and a central homeodomain involved in DNA binding [reviewed in (Hunter and Rhodes, 2005; Prince et al., 2011)]. Members of the LIM-HD transcription factor family, including ISL1, LHX3, and LHX4, have important roles in pituitary and nervous system development [reviewed in (Prince et al., 2011)].

ISL1

LIM-HD transcription factor Islet1 (ISL1) was first found insulin-producing cells in the pancreas and it is able to bind to the *insulin* gene enhancer (Karlsson et al., 1990). *Isl1* is expressed in multiple organs including pancreas, pituitary, thyroid, kidney, spinal cord, diencephalon, telencephalon, inner ear, and heart (Karlsson et al., 1990; Dong et al., 1991; Thor et al., 1991; Cai et al., 2003; Mitsiadis et al., 2003; Radde-Gallwitz et al., 2004). *Isl1* is an early expressed transcription factor during pituitary development and can be detected at ~e8.5 in the oral ectoderm (Pfaff et al., 1996; Ericson et al., 1998). Dorsal-ventral patterning involving gradients of signaling molecules including BMP2 and FGF8/FGF2 appear to control the expression of *Isl1* during pituitary development (Ericson et al., 1998). Recently, studies have shown that ISL1 activates *FSHβ* and *LHβ* gene expression in response to leptin (Wu et al., 2010).

Lack of the *Isl1* gene in the mouse is embryonic lethal at approximately at ~e10.5 due to developmental defects in the pancreas, heart, pituitary, and nervous system. *Isl1*$^{-/-}$ mice form a primitive pouch at e9.5 with a thin epithelium layer, but then development arrests (Takuma et al., 1998). *Isl1* knockout mice have no LHX3 expression in the pituitary but ISL1 expression is present in *Lhx3* null mice at e9.5. However, ISL1 expression in *Lhx3* null mice is transient in that there is no ISL1 expression at e12.5 and after this time point ISL1 expression returns (Ellsworth et al., 2008). These results suggest that ISL1 is upstream of LHX3 expression and that *Lhx3* is required for maintaining ISL1 expression at certain period of time in the developing pituitary.

ISL1 is a motor neuron (MN) marker in MN specification in the developing nervous system. Using the chicken models system, ISL1 has been shown to work with

other LIM-HD factors and signaling molecules to specify MN subtypes in the developing nervous system [reviewed in (Liang et al., 2011)]. For example, the signaling molecule SHH activates ISL1 and LHX3 expression, and ISL1 and LHX3 expression are required for proper MN differentiation and specification (Lee and Pfaff, 2001). In order to specify MNs, ISL1 and LHX3 form a MN hexamer complex which includes NLI (nuclear LIM domain interactor), and the NeuroM and E47 basic-helix-loop-helix (bHLH) transcription factors (Thaler et al., 2002; Lee et al., 2008). MNs can be defined by expression of ISL1 and the homeobox 9 (Hb9) transcription factor, and these are the some of the first detected MN genes expressed in the ventral area of post-mitotic neurons in spinal cord (Pfaff et al., 1996; Saha et al., 1997; Tanabe et al., 1998). *Isl1*$^{-/-}$ embryos lack MNs and a subpopulation of interneurons (Pfaff et al., 1996).

LHX4

LHX4 is another member of the LIM-HD transcription factor family. LHX3 and LHX4 are structurally similar, sharing 63% overall amino acid identity, with 75~95% homology in the LIM domains and the homeodomain [reviewed in (Mullen et al., 2007)]. LHX4 and LHX3 have overlapping functions in pituitary development and MN specification (Sheng et al., 1996; Sheng et al., 1997; Sharma et al., 1998). LHX4 can bind to pituitary target gene promoters and enhancers including the *αGSU*, *GH*, *PRL*, *FSHβ*, and *PIT-1* genes, and activate their expression [e.g. (Sloop et al., 2001; Kawamata et al., 2002; West et al., 2004; Machinis and Amselem, 2005; Castinetti et al., 2008)].

In mice, *Lhx4* is expressed in the developing pituitary gland, hindbrain, cerebral cortex, and spinal cord (Li et al., 1994; Liu et al., 2002). *Lhx4* expression in the pituitary begins at e9.5 and its expression is restricted in the primordium of anterior lobe and by

e12.5 coincides with *Lhx3* (Sheng et al., 1997). Mice lacking *Lhx4* form Rathke's pouch normally but fail to expand and fully differentiate hormone-secreting cells. These mice die shortly after birth due to a defect in lung development (Li et al., 1994). However, heterozygous *Lhx4* mutant mice are apparently normal. The development of the pituitary in the *Lhx3* and *Lhx4* double knockout mice arrests at a rudimentary pouch development stage, whereas single mutant mice form a definitive pouch (Sheng et al., 1997). Further studies suggest that *Lhx4* action with *Prop1* is necessary for proper function of *Lhx3* and pituitary hormone-secreting cell expansion (Raetzman et al., 2002). Although *LHX4* is structurally and functionally similar to the *LHX3* gene, the clinical features of patients with *LHX4* gene mutations are somewhat different than those of *LHX3* patients (Prince et al., 2011). All *LHX4* patients characterized to date have heterozygous mutations and CPHD. They commonly lack GH and TSH, but lack of LH, FSH, PRL, and ACTH is variable (Machinis et al., 2001; Tajima et al., 2007; Castinetti et al., 2008; Pfaeffle et al., 2008; Dateki et al., 2010). Other clinical symptoms of mutations in *LHX4* can include hypoplasia or hyperplasia of the anterior pituitary, ectopic posterior pituitary glands, sella turcica abnormalities, and brain malformations (Machinis et al., 2001; Tajima et al., 2007; Castinetti et al., 2008; Pfaeffle et al., 2008; Dateki et al., 2010).

LHX3

The human *LHX3* gene contains seven exons and six introns and is located on chromosome 9q34 (Sloop et al., 2000a; Sloop et al., 2000b). Mammalian *LHX3* genes produce two mRNAs known as *LHX3a* and *LHX3b* from alternative splicing of exon Ia and exon Ib, and these mRNAs encode three protein isoforms, LHX3a, LHX3b, and M2-LHX3. LHX3a and LHX3b both contain the two LIM domains and DNA-binding

homeodomain but have alternate amino termini (Figure 1.2) (Sloop et al., 1999; Sloop et al., 2001; Yaden et al., 2006). M2-LHX3, a truncated form of the protein, has no LIM domains in the amino terminus, but has a homeodomain and a *trans*-activation domain (Sloop et al., 2001). The human *LHX3* gene has two proximal promoters (*LHX3a* and *LHX3b)* that are located upstream of exon Ia and exon Ib respectively: these promoters guide production of the *LHX3a* and *LHX3b* mRNAs. Both promoters have GC-rich regions and lack a true TATA box. Transcriptional regulation at the human *LHX3* promoters involves binding of specificity protein 1 (SP1) and nuclear factor 1 (NF1) (Yaden et al., 2006). Recently, we demonstrated that the two promoters are not sufficient to guide *LHX3* gene expression *in vivo* (Mullen et al., 2012). In the human *LHX3* gene, a 7.9 kilobase (kb) 3'-located enhancer containing potential binding sites for PITX1 and ISL1 can guide pituitary and spinal cord gene expression (Mullen et al., 2012).

The LHX3 protein is expressed in the developing and mature pituitary gland, the neurons of the developing spinal cord and hindbrain, the retina, and the pineal gland (Zhadanov et al., 1995; Sheng et al., 1996; Sharma et al., 1998; Sloop et al., 2000a; Sloop et al., 2000b). The importance of LHX3 during pituitary development was elucidated in *Lhx3* knockout mice models. In mice, *Lhx3* expression begins at about e9.5 in Rathke's pouch and persists in the anterior and intermediate lobes of the adult pituitary. *Lhx3* null mice are born but die within ~24 hours of birth (Sheng et al., 1996). In these mice, the pituitary gland is arrested after formation of a definitive Rathke's pouch, however the posterior and intermediate lobes develop normally. Heterozygous *Lhx3*$^{+/-}$ mice are viable and all of the hormone-producing cells are present in the anterior pituitary (Sheng et al., 1996). In the *Lhx3* null mice, the somatotropes, thyrotropes, lactotropes, and

gonadotropes fail to differentiate and proliferate, and only a few corticotropes are present (Sheng et al., 1996; Sheng et al., 1997). Neither the early pituitary-expressing *Hesx1* gene or the pituitary cell lineage-specific *Pit-1* gene are detectable in *Lhx3* null mice (Sheng et al., 1996). A study using mice ($Lhx3^{Cre/Cre}$) generated with an insertion of 3' untranslated region (UTR) into the *Lhx3* gene reveals that reduced levels of LHX3 cause a similar phenotype to *Lhx3* null mice. In these mice with reduced LHX3 level there is increased cell apoptosis (Zhao et al., 2006); similarly, high apoptosis is noted in *Pitx1* null mice which lack LHX3 expression (Charles et al., 2005). These data suggest a threshold level of LHX3 is important for proper pituitary development.

LHX3 has additional roles in differentiation of MNs and V2 interneurons (INs) (Pfaff et al., 1996; Glasgow et al., 1997; Sharma et al., 1998; Thor et al., 1999; Thaler et al., 2002). The V2 INs are derived from a common progenitor population expressing LHX3 (Peng et al., 2007), and are subdivided into two classes V2a INs: those that express the CHX10 homeodomain transcription factor, and V2b INs that express GATA2 and GATA3 (Ericson et al., 1997; Zhou et al., 2000; Karunaratne et al., 2002; Smith et al., 2002). For V2 IN specification, LHX3 is proposed to form a tetrameric complex comprised of two LHX3 proteins and 2 copies of NLI. A hexameric MN protein complex is needed for MN specification as described above (Thaler et al., 2002; Lee et al., 2008).

Coding region mutations in pituitary transcription factor genes including *PROP1, PIT-1*, *PITX2*, *LHX3* and *LHX4* have been indicated in cases of CPHD [e.g. (Pfaffle et al., 1992; Dattani et al., 1998; Wu et al., 1998; Netchine et al., 2000; Brickman et al., 2001; Lamolet et al., 2001; Machinis et al., 2001; Thomas et al., 2001; Pfaeffle et al., 2007)]. However genetic defects have not been characterized in most CPHD patients yet.

Twelve homozygous mutations have been found in the coding regions of the human *LHX3* gene that result in CPHD (Table 1.1). Most of the patients carrying *LHX3* mutations have a rigid cervical spine and this may due to the role of LHX3 in specification of neuronal cells in the developing spinal cord. One family has a W224Ter *LHX3* mutation and CPHD but not the limited neck rotation (Pfaeffle et al., 2007). This mutation is found in four siblings from a consanguineous Lebanese couple. This substitution introduces a premature stop codon predicted to cause loss of the carboxyl terminus of the LHX3 protein (W224Ter) (Pfaeffle et al., 2007). An animal model mimicking the human *LHX3* W224Ter mutation reveals that these mice are viable, but have reduced levels of PRL and TSH. Female homozygous mice display infertility but there are no obvious nervous system defects (Colvin et al., 2011).

Most *LHX3* patients are deficient in GH, TSH, LH, FSH, PRL and some cases are reported to be ACTH-deficient. These *LHX3* patients survive, but have short stature, hypothyroidism and hypogonadism (Netchine et al., 2000; Machinis et al., 2001; Bhangoo et al., 2006; Rajab et al., 2008; Kristrom et al., 2009). Recently, studies from our group and a collaborator have shown that a novel homozygous mutation causing a threonine to arginine substitution at position 194 (T194R) in the LHX3 homeodomain causes CPHD (Bechtold-Dalla Pozza et al., 2012). This patient has a hypoplastic anterior pituitary and a rigid cervical spine (Figure 1.3). Sequence alignment of human the LHX3 protein amino acid sequence with multiple other species reveals that the threonine at position 194 is conserved in all LHX3/LIM3 proteins. In a 3-dimensional computational model predicted for the wild type LHX3 protein, T194 is located at the end of the second alpha-helix of the homeodomain (Figure 1.3), and it likely contributes to stabilize the

tertiary structure by establishing a predicted hydrogen bond with a glutamine (Q170) residue (Figure 1.3. B). When T194 is changed to arginine, this mutation may cause steric hindrance with leucine (L172) (Figure 1.3. C). This steric hindrance may destabilize the helix-turn-helix tertiary structure. To test whether this might affect DNA binding, I performed electrophoretic mobility shift analyses (EMSA) using wild type LHX3 protein and T194R LHX3 protein. The T194R "mutant" protein could not bind to a LHX3 consensus-binding site (Figure 1.5. A). Further, to examine the ability of the T194R LHX3 protein to activate pituitary genes, LHX3 expression vectors were co-transfected with a *prolactin (PRL)* promoter reporter gene into pituitary GHFT1 cells. Wild type LHX3 activated the *PRL* promoter, but T194R LHX3 was not activated (Figure 1.5. B). These clinical and molecular studies suggest that a novel homozygous T194R LHX3 mutation alters the molecular properties of *LHX3* homeodomain and this mutation causes CPHD.

1.7 Central Hypothesis and Aims

Our lab previously identified two promoters/intronic elements of the human *LHX3* gene that are important for basal gene expression *in vitro* (Yaden et al., 2006), but the mechanisms by which the *LHX3* gene is regulated *in vivo* were not known. *Beta galactosidase* reporter transgenic mouse models were used to examine whether the *LHX3* promoters are able to drive a reporter transgene *in vivo*. However, the *LHX3* promoters were not able to drive beta galactosidase expression in any tissues, including the pituitary and spinal cord (Figure 1.6.) (Mullen et al., 2012). This suggested to us that elements outside of the promoter region are necessary to guide *LHX3* gene expression. Using the bioinformatics VISTA program (Bray et al., 2003; Couronne et al., 2003), we found

highly conserved non-coding elements, which are often associated with regulatory function, within the 7.9 kb region 3′ of the human *LHX3* gene (Figure 1.7. A). The addition of this 7.9 kb region 3′ from the *LHX3* gene to the native promoter *beta galactosidase* transgenic reporter gene resulted in tissue-specific beta galactosidase expression in the developing pituitary and spinal cord of the transgenic mice (Figure 1.7. B) (Mullen et al., 2012). This result demonstrated that the 7.9 kb region 3′ of the *LHX3* gene can mediate reporter gene expression *in vivo* and suggests that this element is important for human *LHX3* gene expression. To further test enhancer activity, we used a heterologous *HSP68* minimal promoter which does not alone direct gene expression without additional enhancers (Kothary et al., 1989; Pennacchio et al., 2006). One of the characteristics of an enhancer is the ability to act independent of position to direct expression from a basal promoter. In the *HSP68* minimal promoter system, the 7.9 kb conserved enhancer region directed tissue-specific expression to the pituitary gland and spinal cord of transgenic mice independent of its "normal" position (Figure 1.7. B) (Mullen et al., 2012). The observed enhancer-directed transgene expression (beta galactosidase activity) matched endogenous LHX3 expression in the developing pituitary and spinal cord (Figure 1.8).

Within the enhancer, several transferable *cis* elements can individually guide nervous system expression *in vivo*; however, a 180 base pair (bp) minimal enhancer is alone sufficient to confer specific expression in the developing pituitary (Mullen et al., 2012). Within this sequence, ISL1 protein binds to A/T rich homeodomain binding sites and these sites are necessary for 3′ enhancer activity in the developing pituitary gland and the spinal cord. Also a putative PITX1 homeodomain element may be required for spatial

patterning of *LHX3* expression in the developing pituitary. This study suggested a mechanism for ISL1 transcriptional regulation of the human *LHX3* gene and described a potential mechanism for regulation by PITX1 (Mullen et al., 2012).

Our previous study (Mullen et al., 2012) did not characterize human *LHX3* gene 3′ enhancer-directed transgene expression on a cellular level. The hypothesis for my study was that the 3′ enhancer has roles in LHX3 expression during the development of the hormone-expressing cell types of the anterior pituitary. This hypothesis was based on the following observations. LHX3 has critical roles in the development of the anterior pituitary hormone-secreting cell types includes somatotropes, lactotropes, thyrotropes, and gonadotropes. Human patients with *LHX3* mutations (Table 1.1) and *Lhx3* knockout mice have TSH, LH, FSH, GH deficiencies [reviewed in (Prince et al., 2011)]. In this study, we investigated the role of this distal regulatory region in the control of regulating *LHX3* gene expression at a cellular level during development.

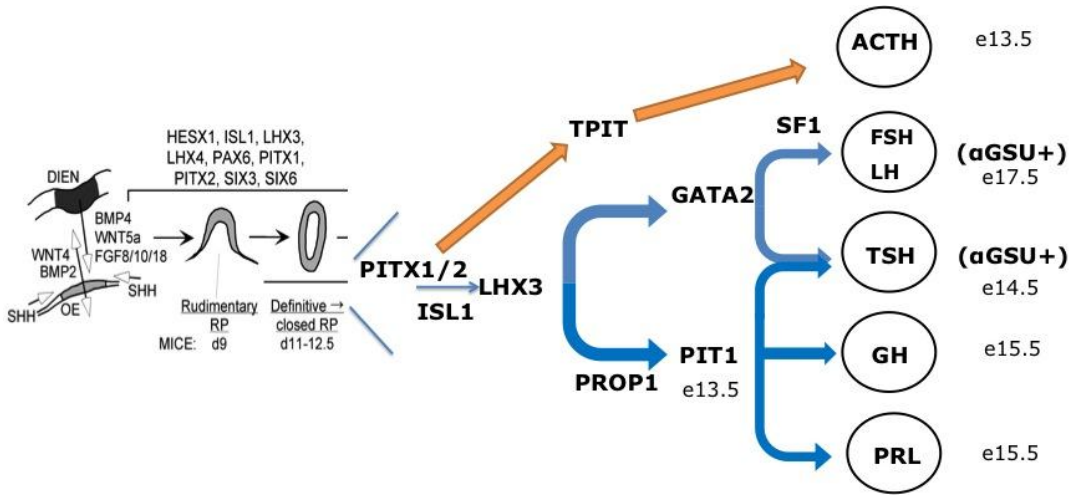

Figure 1.1. Regulation of the development of anterior pituitary gland hormone-expressing cells by signaling molecules and transcription factors. Inductive signaling events between the ventral diencephalon (DIEN) and the oral ectoderm (OE) precede the formation of a rudimentary Rathke's pouch that eventually forms a definitive closed Rathke's pouch, and then the mature anterior pituitary. Multiple transcription factors are involved in the determination and differentiation of the five different hormone-secreting cell types. The time points of appearances of the hormone-secreting cells during mouse embryogenesis are noted. FSH, LH, and TSH are heterodimers sharing a common alpha glycoprotein subunit (αGSU).

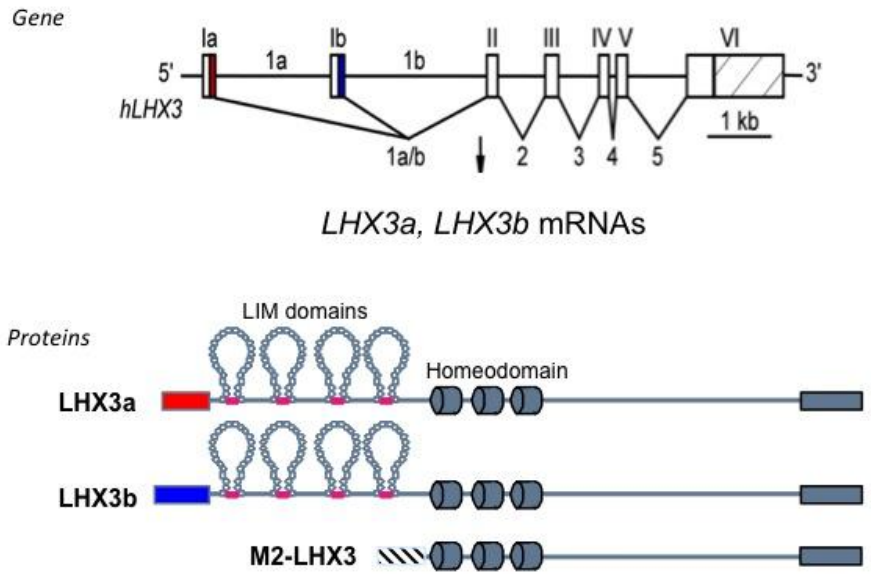

Figure 1.2. The human *LHX3* gene spans approximately 8.7 kb of sequence and contains seven exons (roman numerals) and six introns (arabic numerals). Exons are depicted by boxes and introns are indicated by lines. This gene produces two major mRNAs (*LHX3a* and *LHX3b*) encodes three LHX3 protein isoforms (LHX3a, LHX3b, M2-LHX3). The M2-LHX3 isoform has the homeodomain region but lacks the LIM domains. Modified from (Sloop et al., 1999).

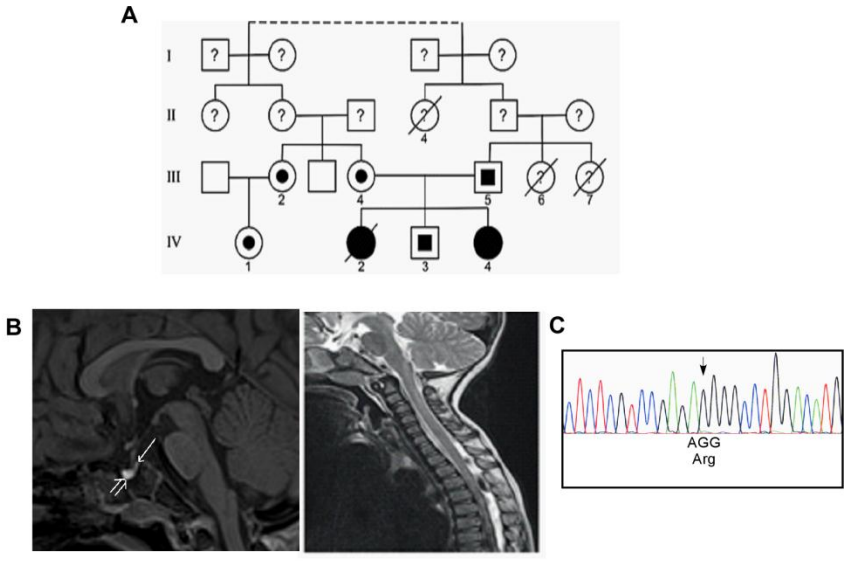

Figure 1.3. DNA analysis reveals a novel homozygous mutation (T194R) in the *LHX3* gene in patients with CPHD. A, pedigree of the affected family. Filled symbols indicate patients with homozygous genotype. Half-filled symbols denote heterozygous individuals. A line through a symbol indicates that the subject is deceased. The dotted line indicates a family connection four or five generations back from the index patient (IV4). B, MRI pictures show patient IV4 has a hypoplastic anterior pituitary gland and rigid cervical spine. The anterior pituitary is hypoplastic (open arrow) and unaffected the posterior pituitary gland is found in the expected location (arrow). C, DNA sequencing analysis of patient IV4 reveals a C to G mutation in the *LHX3* gene altering a threonine (Thr) to an arginine (Arg) in the predicted homeodomain amino acid sequence. Adapted from (Bechtold-Dalla Pozza et al., 2012).

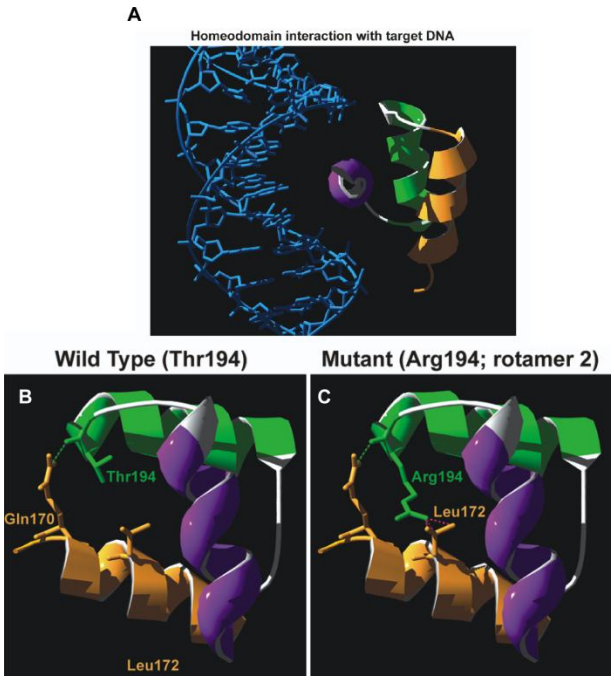

Figure 1.4. Structural prediction of LHX3 homeodomain/DNA interaction. A, Ribbon model of WT LHX3 homeodomain/DNA showing the three α-helices in orange (helix 1), green (helix 2) and purple (helix 3). B, Predicted WT LHX3 homeodomain with a threonine at position 194 (Thr194). C, Predicted T194R LHX3 homeodomain with an arginine at position 194 (Arg194). The figure shows R194 position(green side chains) that may destabilize the tertiary structure by causing steric hindrance (purple dotted lines) with glutamine 170 and leucine 192 (orange side chains). Adapted from (Bechtold-Dalla Pozza et al., 2012).

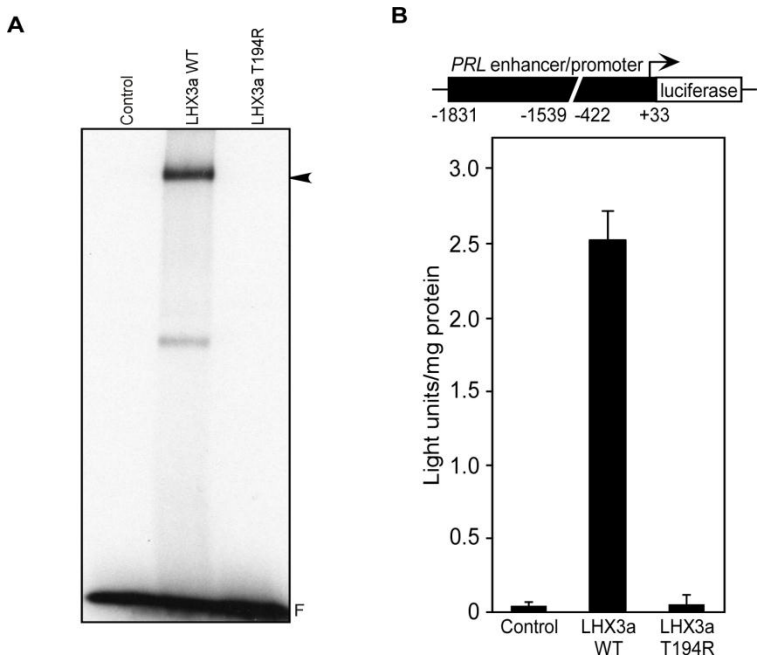

Figure 1.5. The LHX3 T194R protein does not bind to LHX3 DNA recognition elements and is not able to activate a pituitary target gene. A, EMSA experiments were performed using the WT LHX3 or T194R LHX3 proteins translated *in vitro* in rabbit reticulocyte lysates and radiolabeled probes representing the LHX3 consensus-binding site (Bridwell et al., 2001). Unprogrammed lysates were used as negative controls (Control). The LHX3/DNA complexes are denoted by an arrowhead. B, Expression vectors for WT LHX3 and T194R LHX3 proteins were transiently cotransfected into mouse pituitary GHFT1 cells with a *luciferase* reporter gene under the control of the *prolactin (PRL)* promoter. Promoter activity was assayed by measuring luciferase activity 48 h after transfection. Negative controls (Control) received equivalent amounts of empty expression vector plasmid. Activities are mean (light units/10 s/μg total protein) of triplicate assays ± SEM. A representative experiment of at least three experiments is depicted. F, Free probe. Modified from (Bechtold-Dalla Pozza et al., 2012).

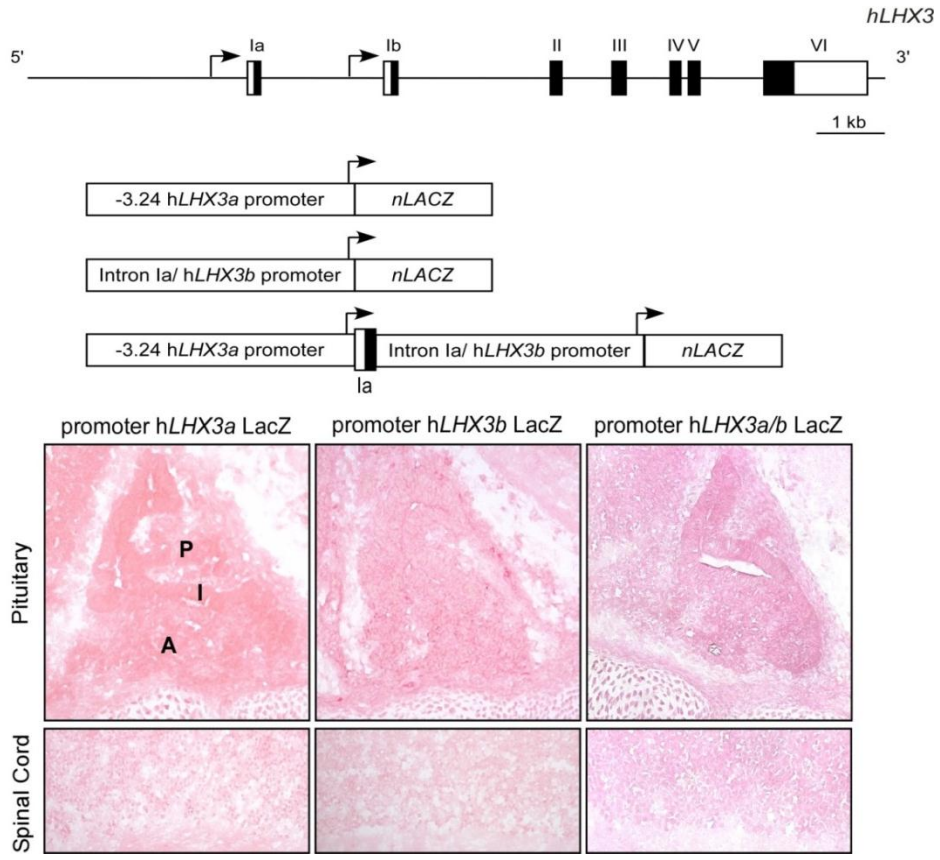

Figure 1.6. Two basal promoters of the human *LHX3* gene are not sufficient to drive transgene (LacZ) activity *in vivo*. Reporter gene constructs with h*LHX3a* promoter, intron Ia/h*LHX3b* promoter, or the h*LHX3a* promoter and intron Ia/h*LHX3b* promoter together used to generate transgenic mice. Sagittal cryosections of e14.5 embryos stained for beta galactosidase activity. There is no beta galactosidase expression in the pituitary or spinal cord. P, posterior lobe; I, intermediate lobe; A, anterior lobe. Adapted from (Mullen et al., 2012).

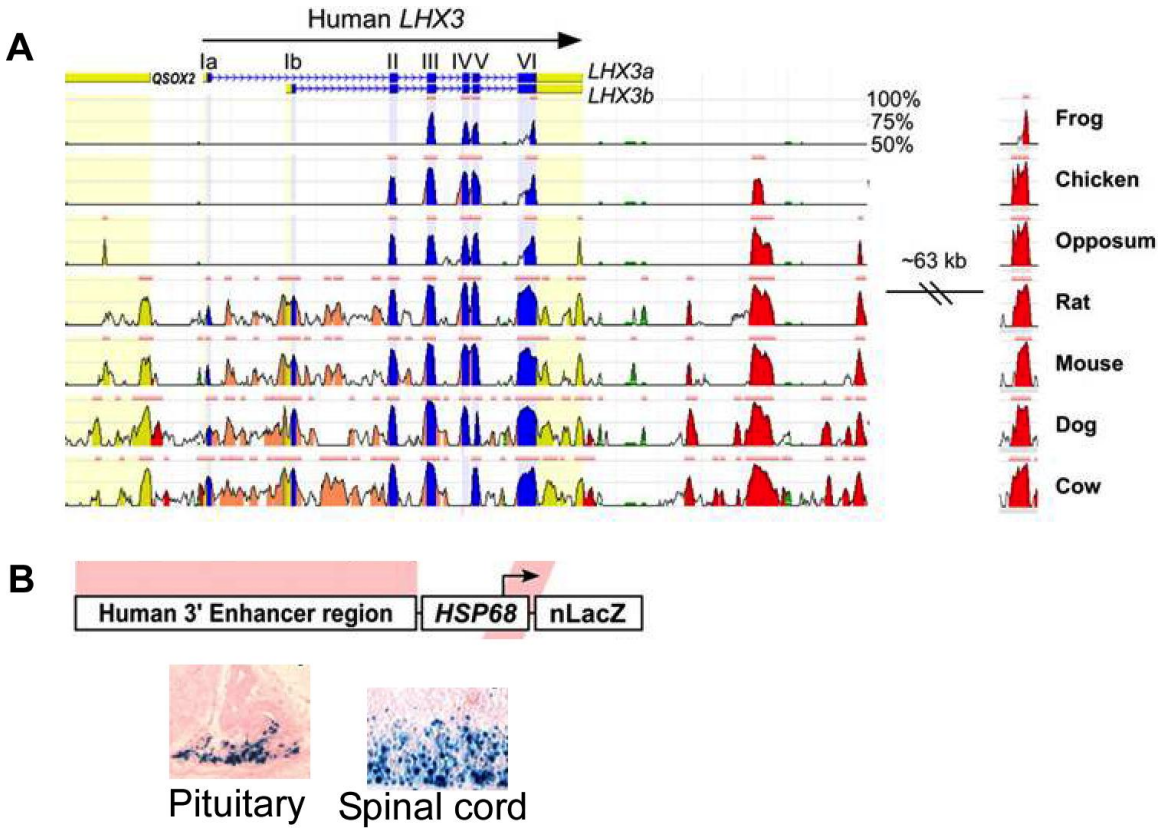

Figure 1.7. A conserved distal downstream region (~7.9 kb) of the human *LHX3* gene can direct expression to the developing pituitary and spinal cord *in vivo*. A. Comparative genomic analysis of the human *LHX3* gene using ECR browser. *LHX3* gene sequence alignments in multiple species revealed that non-coding elements (CNE) in the 3′ region of the human *LHX3* gene. CNE are shown in red, coding exons in blue, conserved intronic regions in salmon, UTR in yellow, and simple repeats in green. B, reporter gene containing the 7.9 kb 3′ region guide transgene expression in the developing pituitary and spinal cord. Sagittal frozen mouse pituitary and spinal cord sections of e14.5 founder embryos stained for beta galactosidase activity. Modified from (Mullen et al., 2012).

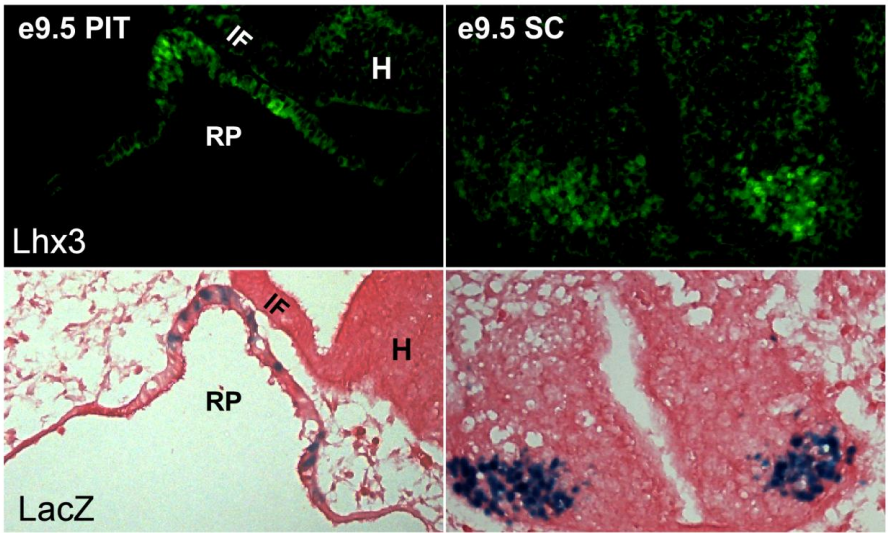

Figure 1.8. 7.9 kb 3′ enhancer-directed transgene expression coincides with native LHX3 expression. Frozen pituitaries and spinal cord sections from the human *LHX3* 7.9 kb enhancer-LacZ mice at e9.5 were stained for LHX3 expression using antibodies (green) or beta galactosidase transgene activity (blue). RP, Rathke's pouch; SC, spinal cord. Adapted from (Mullen et al., 2012).

Table 1.1 Mutations within the *LHX3* gene associated with combined pituitary hormone deficiency disease. Adapted from (Bechtold-Dalla Pozza et al., 2012).

	p.Y111C (Netchine et al., 2000; Rajab et al., 2008)	**23 bp deletion** (Netchine et al., 2000; Rajab et al., 2008)	**g.159delT** (Bhangoo et al., 2006)	**p.A210V** (Pfäffle et al., 2007)	**p.E173Ter** (Pfäffle et al., 2007)	**p.W224Ter** (Pfäffle et al., 2007)	***LHX3* gene deletion** (Pfäffle et al., 2007)	**p.K50Ter** (Rajab et al., 2008)	**Intragenic deletion of 3088 bp** (Rajab et al., 2008)	**A→G splice mutation in intron 3** (Kristrom et al., 2009)	**p.R77Ter** (Bonfig et al., 2011)	**p.T194R** (Bechtold-Dalla Pozza et al.,2012)
Hormone deficiencies reported	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH, (ACTH sometimes low)	GH, TSH, PRL, LH, FSH, (ACTH low)	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH, ACTH	GH, TSH, PRL, LH, FSH, ACTH	GH, TSH, PRL, LH, FSH, ACTH	GH, TSH, PRL, LH, FSH, ACTH	GH, TSH, PRL, LH, FSH, ACTH
Anterior pituitary morphology	Hypoplastic	Enlarged	Hypointense pituitary lesion	Enlarged	Hypoplastic	Normal	Severely hypoplastic/ aplastic	Hypoplastic	Hypoplastic	Aplastic/hypoplastic (sometimes cysts)	Hypoplastic	Hypoplastic (possible cyst)
Neck rotation	Limited	Limited	Limited	Limited	Limited	Normal	Limited	Limited	Limited	Limited	Limited	Limited
Hearing deficiencies	Moderate to mild sensorineural hearing defect	Completely deaf	Not reported	Not reported	Not reported	Not reported	Not reported	Sensorineural deafness	Sensorineural hearing defect	Sensorineural hearing defect	Sensorineural hearing defect	Sensorineural hearing defect
Mental/learning deficiencies	Not reported	Extreme mental retardation	Some mental retardation	Not reported	Not reported	Not reported	Psychomotor development is severely retarded	Not reported	Some learning difficulties	Not reported	Not reported	Not reported
Location of mutation	Missense mutation within exon 3	Deletion affecting exon 3 and splice-donor site	Deletion in exon 2	Missense mutation in exon 5	Complex mutation in exon 3	Nonsense mutation in exon 5	Complete gene deletion	Nonsense mutation in exon 2	Deletion of exons 2-5	A→G splice acceptor mutation in intron 3	Nonsense mutation in exon 2	Missense mutation in exon 4
Protein domain(s) affected	2nd LIM domain	Multiple	Multiple	HD	Multiple	Loss of carboxyl terminus	Multiple	Multiple	Multiple	Multiple	Multiple	HD

CHAPTER TWO

MATERIALS AND METHODS

2.1 DNA Cloning and Vector Construction

3′ Enhancer pSC-B Construct

To construct the 7.9 kb 3′ enhancer pSC-B cloning vector, two sets of primers were used to amplify 7.9 kb 3′ human *LHX3* enhancer from 700 ng of BAC clone RP11-83B9/ALI38781 using *Pfu Ultra II HS* DNA polymerase (Stratagene, La Jolla, CA). The following primers: 5′-cgggatccgacccagttcgacctatcc-3′ (S) and 5′-gaacagtcggcacttattaaccacctgtcagc-3′ (AS) for fragment I; 5′-ccaggtcgaaggcggaattagggag-3′ (S) and 5′-acgcgtcgaccactggcgacatcatctctg-3′ (AS) for fragment II were used. PCR parameters were 95 ℃ 2min; 95 ℃ 20 sec, 65 ℃ 20 sec, 72 ℃ 1 min 15 sec for 25 cycles, 72 ℃ 5 min. PCR products were sub-cloned into pSC-B vector (Stratagene). Inserted fragment I and II-pSC-B were digested with *NotI* and ligated together to form the 3′ enhancer pSC-B vector.

3′ Enhancer-*HSP68* promoter pSC-B transgene

The *HSP68-Hand2-LacZ* pSK-Bluescript (a gift from Dr. Simon Conway, Indiana School of Medicine, Indianapolis, IN) vector was modified to delete the *Hand2* enhancer. Next, the *HSP68-LacZ* sequence was excised from *HSP68-LacZ* pSK-Bluescript and ligated into the 3′ enhancer pSC-B plasmid to make the 3′ enhancer- *HSP68-LacZ* pSC-B construct (construction by Rachel Mullen).

3′ Enhancer-*HSP68-Cre* transgene (*ELHX3*^{Cre})

To create the human *LHX3* gene 3′ enhancer-*HSP68-Cre* (*ELHX3*^{Cre}) transgene, the 7.9 kb enhancer fragment, and the 920 bp *HSP68* minimal promoter were removed

from the 3′ *LHX3* enhancer-*HSP68-LacZ* construct and were subcloned into the pBS594-*EGFP-Cre* expression vector (Le et al., 1999) using *BamHI* and *NcoI* restriction sites (Figure 2.1). The pBS594-*EGFP-Cre* vector was obtained as a generous gift from Dr. Simon Conway, Indiana School of Medicine, Indianapolis, IN.

DNA sequencing

DNA sequencing was performed at the Biochemistry Biotechnology facility at the Indiana School of Medicine using a Perkin Elmer DNA sequencer. The templates for sample DNA were submitted using the recommended guidelines from the sequencing facility. The sequence analyses and alignments were completed with the DNASIS (Hitachi Software Engineering, San Francisco, CA) software.

2.2 Generation, Genotyping and Breeding of Transgenic Mice

Generation of transgenic mice

One hundred micrograms of 3′ enhancer-*HSP68-Cre* construct was linearized with *PacI* digestion. Digested transgene DNA was submitted to the Indiana University Cancer Center Transgenic and Knock-out Mouse Core (Indianapolis, IN) for gel purification and microinjection. The linearized transgenes were microinjected into F2 zygotes from C3He/FeJ parents at a concentration of approximately 2-3 ng/µl. After microinjection, two-cell stage embryos were transferred to 0.5 day pseudopregnant females. C3He/FeJ embryos and transferred into pseudopregnant foster mothers. Founder transgenic mice were used for breeding as adults for the generation of stable transgenic lines.

Genotyping of transgenic mice

Mouse genomic DNA was obtained from mouse tail snips taken between postnatal days 14 and 21 using the genomic DNA solution set kit (Gerard Biotech, Oxford, OH) to prepare DNA according to the manufacturer's instructions. Genotyping for Cre-expressing transgenic mouse lines was performed using a multiplex PCR amplification of the *Cre* recombinase region and a wild type control region from the *receptor-associated protein of the synapse* (*Rapsn)* gene (to check for genomic DNA integrity). Oligonucleotides primer sequences used for detection of the *Cre* recombinase region and the wild type gene were as follows; 5′-caatgctgtttcactggttatgc-3′ (S) and 5′-gtacgtgagatatctttaaccctgat-3′ (AS) for *Cre* detection and 5′-aggactgggtggcttccaactcccagacac-3′ (S), 5′-agcttctcattgctgcgcgccaggttcagg-3′ (AS) for the *Rapsn* gene. PCR parameters used for genotyping experiments were 2 min at 94 ℃; 30 sec at 94 ℃, 30 sec at 56 ℃, 30 sec at 72 ℃ for 30 cycles, and 7 min at 72 ℃. expected *Cre* and wild type *Rapsn* gene size were approximately 500 bp and 350 bp.

Genotyping of reporter lines $R26R^{LacZ}$, $R26R^{YFP}$ and $R26^{DTA}$ was determined by PCR analysis using mutant and wild type region-specific sequences with the following oligonucleotides: 5′-gcgaagagtttgtcctcaacc-3′ (mutant reverse), 5′-aaagtcgctctgagttgttat-3′ (common) and 5′-ggagcgggagaaatggatatg-3′ (wild type reverse). The expected mutant and wild type PCR product size were 340 bp and 650 bp, respectively. PCR parameters used for genotyping experiments were 3 min at 94 ℃, (30 sec at 94 ℃, 1 min at 65 ℃, 1 min at 72 ℃) x 35 cycles, and 2 min at 72 ℃. Homozygous $R26^{DTA/DTA}$ mice were generated from male heterozygous and female homozygous mice.

Breeding of transgenic mice

To maintain *ELHX3*^{Cre} stable transgenic lines, transgenic founder animals and their progenies were bred to C3H female mice (Harlan Laboratories, Indianapolis, IN) to generate heterozygotes. The morning after copulation, indicated by the presence of a vaginal plug, was considered embryonic day 0.5 (e0.5) and the day of birth was postnatal day 0 (P0).

Reporter strains, *ROSA26R*$^{\text{LacZ}}$ (Soriano, 1999) (*R26R*^{LacZ}; 129S-*Gt(ROSA)26Sor*^{tm1sor}/J, stock number: 003310) and *ROSA26*$^{eGFP\text{-}DTA}$ (Ivanova et al., 2005) *(R26*^{DTA}; C.129P2(B6)-*Gt(ROSA)26Sor*$^{tm1(DTA)Lky}$/J, stock number: 009670) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). *ROSA26R*^{EYFP} (Srinivas et al., 2001) (*R26R*^{YFP}; B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J, stock number:006148) were generously provided by Dr. Paul Herring, Indiana School of Medicine, Indianapolis, IN. Mice were housed in a specific pathogen-free environment under controlled conditions of temperature and light and were provided free access to tap water and commercial mouse chow. The Indiana University Committee on Use and Care of Animals approved all procedures using mice, and all experiments were performed in agreement with the principles and procedures outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

2.3 Histology and Immunohistochemistry

Preparation and fixation of embryos and tissue

Embryos and mouse heads from P0 were collected and fixed on ice in 4% paraformaldehyde in 1X PBS (pH 7.2) for 1 to 24 hr at room temperature. Embryos were fixed for 2 hr to overnight. All harvested tissues were washed three times in 1X PBS (pH 7.2 for quick rinse, 30 min and 1 hr) and placed in 20% sucrose overnight. On the next

morning embryos and tissues were embedded in optimal cutting temperature compound (O.C.T.) (Sakura Finetek, Torrance, CA) on dry ice and stored at -80 ℃ until cryosectioning. Tissue cryosections of 7 μm were prepared for immunohistochemistry and beta galactosidase staining. Embryos and tissues used for beta galactosidase staining were fixed on ice for 1 hr in 2% paraformaldehyde and 0.2% glutaraldehyde in 1X PBS (pH 7.2). Fixed embryos and tissues were washed three times in 1X PBS (pH 7.2; quick rinse, 1 hr, and 30 min) on ice and placed in 20% sucrose at 4 ℃ overnight.

Beta galactosidase activity detection

After fixation and washing as described above, pre-fixed embryos were incubated at room temperature overnight with gentle agitation in X-gal solution (in 1X PBS (pH 7.2)), 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.2% each of Triton X-100, Nonidet P-40 and Tween 20, and 0.5 mg/ml X-gal diluted in dimethylformamide). After X-gal staining, embryos were washed in 1X PBS (pH 7.2) at room temperature 2 hr to overnight with gentle agitation. Washed embryos were imaged for surface staining. To remove background X-gal staining, embryos were transferred to polypropylene tube and dehydrated at room temperature in 70% (30 min), 95% (30 min), and 100% ethanol (20 min or overnight). Dehydrated embryos were incubated in 100% methyl salicylate at room temperature for 1 hr. Imaging was done rapidly after the clearing steps. Single heterozygous littermates served as negative controls.

Frozen sections for beta galactosidase activity staining were air dried for 5 min and fixed for 10 min with 0.5% glutaraldehyde in 1X PBS (pH 7.2) then washed three times in 1X PBS (pH 7.2; quick rinse, 10 min, and 5 min) followed by staining in X-gal solution (described above) at room temperature in the dark for 2 hr or overnight with

gentle agitation. Following staining, the slides were washed three times in 1X PBS (pH 7.2; quick rinse, 1 hr, 1 hr or overnight). After PBS washing, X-gal stained slides were dehydrated in ethanol washes (70%, 80%, 95%, and100%; two times each for 30 sec) and then eosin counterstained (Sigma-Aldrich; 1 time 3 min) and then washed in 100% ethanol and allowed to dry. After drying, slides were cover-slipped using Permount (Fisher Scientific, Pittsburg, PA).

Immunohistochemistry

Frozen pituitary and embryo sections were air dried, rehydrated in 1X PBS (pH 7.2). Antigen retrieval was performed by citrate boil [10 min boiling in10 mM citric acid (pH 6.0)] methods. Slides were washed in 1X PBS (pH 7.2) and blocked with 10% normal goat serum, 1% BSA and 0.1% Triton X-100 in PBS (pH 7.2) for 20 min at room temperature and then incubated with the primary antibodies: rabbit anti-ACTH (1:500 Fluor) (1:1000 DAB) (AFP-39032082), rabbit anti-αGSU (1:100 Fluor) (1:500 DAB) (AFP-66P9986), rabbit anti-LHβ (1:500 Fluor) (1:800 DAB) (AFP-571292393), guinea pig anti-LHβ (1:400 Fluor) (AFP-22238790), rabbit anti-FSHβ (1:500 Fluor) (AFP-77981289), rabbit anti-PRL (1:100 Fluor) (AFP-131078), rabbit anti-TSHβ (1:500 Fluor) (1:1000 DAB) (AFP-1274789), rabbit anti-GH (1:500 Fluor) (1:1000 DAB) (AFP-5672099) (all antibodies from the National Hormone and Pituitary Program, Torrance, CA), mouse anti-ISL1 (1:50 Fluor, Developmental Studies Hybridoma Bank (DSHB), University of Iowa 39.4D5), rabbit anti-PIT-1 (1:500 Fluor) (Voss et al., 1991), rabbit anti-SF1 (1:1000 Fluor) from Dr. Gary Hammer, University of Michigan, guinea pig anti-GATA2 (1:1000 Fluor) (Peng et al., 2007) from Dr. Kamal Sharma, University of Chicago, mouse anti-HB9 (1:100 Fluor, DSHB 81.5C10), guinea pig anti-CHX10

(1:1000 Fluor) (Thaler et al., 1999) from Dr. Samuel Pfaff, Salk Institute, rabbit anti-TPIT (1:200 Fluor) (Lamolet et al., 2001) from Dr. Jacques Drouin, Institut de Recherches Cliniques de Montréal, Canada, rabbit anti-β-galactosidase (1:500 Fluor, Cappel) and rabbit anti-LIM3/LHX3 (1:100 Flour, Chemicon) as described (Savage et al., 2007).

Species-specific biotinylated secondary antibodies and fluorescence avidin kits (Texas Red Avidin DCS, Fluorescein Avidin DCS, and AMCA Avidin D) were purchased from Vector Labs and used as recommended by the manufacturers. The Vectastain Elite ABC kit (Vector Labs) was used for Avidin/Biotin complex detection and the Mouse-on-Mouse kit was used (Vector Labs) for detection of mouse primary antibodies. For immunofluorescent staining, VECTA shield hard-set mounting medium (Vector Labs) was used.

3, 3′-diaminobenzidine (DAB) detection was prepared as follow methods: frozen sections were rehydrated in 1X PBS (pH 7.2), incubated with 0.3% H_2O_2 in 100% methanol for 20 minutes at room temperature with gentle agitation, then applied followed by application of the desired primary antibodies. Peroxidase was visualized by incubation with 3, 3′-diaminobenzidine (DAB) (Sigma). Following DAB immunostaining, slides were counterstained with Harris modified hematoxylin (Fisher Scientific) and mounted using Permount (Fisher Scientific).

2.4 Microscopy

Digital light and fluorescent images of embryo and spinal cord sections were captured with a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, NY) with DAPI, FITC, and TRITC filter cubes. Canvas (ACD Systems of America, Inc.,

Miami, FL) and NIS Elements Vol. 3.2 (Nikon Instruments, Inc.) were used to process the images. Light images of whole embryos were obtained using a Leica MZ 6 microscope and a CCD camera (PL A662, PixeLINK, Ottawa, Ontario, Canada) with PixeLINK Capture software.

2.5 General Molecular Techniques

Ligations and transformations

DNA fragments to be ligated were analyzed for size and concentration by agarose gel electrophoresis. DNA fragments were joined using 1 μL T4 DNA ligase (Roche) in a total volume of 20 μL buffered solution. Ligation mixtures were incubated at 4°C overnight. Ten μL of the ligation reaction or 2-5 ng of plasmid preparation were transformed into bacterial cells to generate clones. Plasmid DNA was added to 100 μL of chemically competent *E. coli* DH5α cells (Invitrogen) on ice. Transformation of plasmid DNA into *E. coli* using heat shock methods was performed. This mixture was heat shocked at 42°C for 1min and placed on ice for 2 min. The addition of 800 μL of LB broth supplemented with 0.2% glucose and 10 mM $MgCl_2$ preceded incubation at 37 ℃ for 45-60 min with shaking at 220 rpm. After incubation, mixtures were centrifuged at 1000 rpm for 1 min to pellet the cells. The supernatants were discarded about 700 μL and resuspended remaining cells using left supernatants. Suspended cells were plated on LB agar plates containing the appropriate selective antibiotic.

Small scale alkaline lysis plasmid preparation

E. coli DH5α transformants were incubated at 37 ℃ with shaking overnight in LB broth with appropriate antibiotics. To pellet cells, 2 mL of liquid culture was centrifuged at 16,000 x g for 1 min. The supernatant was aspirated and the pellet was resuspended in

100 µL hypertonic solution 1 (50 mM Tris-HCl, pH 8.0, 0.9% glucose, 10 mM EDTA) and allowed to incubate on ice for 5 min. The addition of 200 µL lysis buffer, solution 2, (0.2 M NaOH, 0.5% SDS) lysed the cells by gentle mixing and incubation on ice for 5 min. 175 µL solution 3 (3 M KOAc, 11.5% (v/v) glacial acetic acid) was added followed by an additional incubation on ice for 5 min. The solution was centrifuged at 16,000 x g for 10 min followed by the addition of 300 µL of phenol/chloroform (1:1) solution and centrifugation at 16,000 x g for 10 min. The upper aqueous phase was removed and 1 volume of room temperature isopropanol was added to precipitate nucleic acids. The sample was centrifuged at 16,000 x g for 10 min and the pellet washed with 75% ethanol. The pellet was dried at 37 ℃ for 2 min and resuspended in 20 µL TER (100 mg/mL RNaseA in 1X TE buffer [10 mM Tris-Cl, pH 7.4, 1 mM EDTA]).

One to two microliters of extracted plasmid DNA were digested with appropriate restriction endonucleases using the recommended buffer and conditions (New England Biolabs). Restriction enzyme digest products were visualized on agarose/1X Tris-borate gels to select properly constructed plasmids.

Gel purification of DNA fragments

DNA fragments to be gel purified were separated on 0.7%, 1.0%, or 1.5% agarose/Tris-borate gels. Gel slices containing the fragments of interest were excised as quickly as possible using razor blades and long wavelength ultraviolet light. Excision of agarose/Tris-borate gel slices was performed by razor blades and done as quickly as possible. Gel purification was performed with Qiaquick or MinElute gel extraction columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. Elution from the column was performed with either 10 µL (MinElute) or 30 µL (Qiaquick) of the

supplied buffer.

2.6 Quantification and Statistical Analyses

To quantify $LacZ^+$, YFP^+, and antibody-stained cells in the anterior pituitary and spinal cord, at least three comparable sections of each staining were counted for positively-labeled cells on cryosections. Individual cells identified by the antibody staining were counted and normalized to the number of nuclei observed using Image J software. Differences in mean values for each experiment were analyzed with a two-tailed Student's t-test. The data were considered significantly different when $P<0.05$.

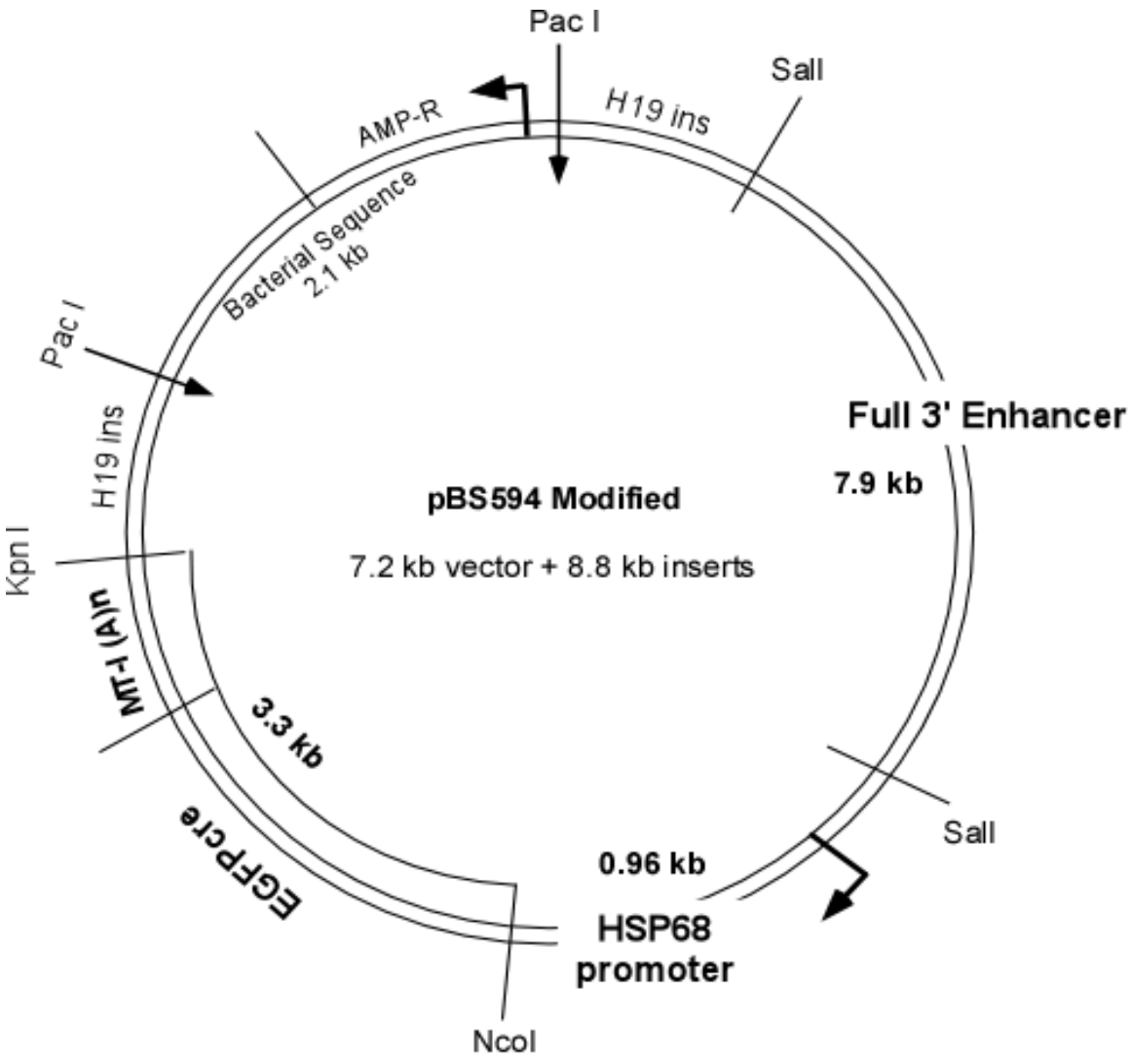

Figure 2.1. The 3′ human *LHX3* enhancer region and the *heat shock protein 68* (*HSP68*) promoter were inserted into the modified pBS594-*EGFP*-*Cre* vector as shown. EGFPcre recombinase expresses depend on enhancer; mouse metallothionein MT-I region including the polyadenylation site and several introns (MT-I (A)n); murine H19 insulator regions (H19 ins).

CHAPTER THREE

IN VIVO ANALYSIS OF HUMAN *LHX3* ENHANCER REGULATION

3.1 Results

The 3′ Human *LHX3* enhancer reporter gene and the endogenous mouse LHX3 protein are co-expressed in anterior pituitary αGSU cell lineages, but not in GH cells

Our lab previously characterized a 7.9 kb region of 3′ of the human *LHX3* gene that directs tissue-specific expression to the pituitary gland and spinal cord in a pattern consistent with endogenous gene expression. To characterize the activity guided by the 3′ 7.9 kb enhancer of the human *LHX3* gene at the cellular level, enhancer-directed *beta galactosidase* transgene expression was determined in tandem with immunohistochemistry for pituitary hormone proteins and the endogenous mouse LHX3 protein (Mullen et al., 2012). Serial frozen sections were stained for either beta galactosidase activity and mouse LHX3 protein or double immunohistochemistry was performed using antibodies against hormone subunits (GH, ACTH, αGSU, TSHβ, and LHβ) and the mouse LHX3 protein (Figure 3.1 A). The immunohistochemistry experiments showed that enhancer transgene-guided LacZ expression was co-localized with αGSU-positive cells but not GH somatotropes in the pituitary glands at e17.5 and postnatal day 0 (P0) (Figure 3.1 A; h, i, j, m, n, and o). Endogenous LHX3 protein was found to co-localize with αGSU-, TSHβ-, and LHβ- expressing cells, but not GH-positive cells (Figure 3.1 A; a-e).

The observation that the enhancer transgene activity and the endogenous LHX3 protein were not detected in GH cells was intriguing considering that both *Lhx3* knockout mice and human patients with *LHX3* mutations exhibit GH deficiencies.

Generation of 3′ *LHX3* enhancer-*HSP68-Cre* (*ELHX3*^{Cre}) transgenic mice

One possible explanation for the lack of reporter activity in the GH cells is that LHX3 may be only transiently expressed in these cells and thus at the time points we examined the endogenous LHX3 and enhancer directed reporter activity may have been silent. In order to determine which pituitary cell types exhibited enhancer-directed expression in the developing pituitary, I generated 3′ *LHX3 Enhancer*^{Cre} (*ELHX3*^{Cre}) transgenic mice to characterize enhancer-directed pituitary and neuronal cell lineages. A schematic diagram shows generation of *ELHX3*^{Cre} (human *LHX3* gene 3' enhancer-*HSP68-Cre*) transgenic mice and crossing to *ROSA26* reporter strains to reveal enhancer-driven activities (Figure 3.1 B). *ELHX3*^{Cre} mice were crossed to reporter mice for conditional activation of reporter gene expression including *ROSA26R*^{LacZ} (Soriano, 1999), *ROSA26R*^{YFP} (Srinivas et al., 2001), or *ROSA26*$^{eGFP\text{-}DTA}$ mice (Ivanova et al., 2005). These animals carry LacZ, yellow fluorescent protein (YFP), or diphtheria toxin A (DTA) "reporter genes" that are activated in the presence of Cre recombinase activity thus marking Cre-expressing cells from that time point onward (Figure 3.1 B). The *ELHX3*^{Cre} transgenic mouse model therefore provides a valuable tool for studies of LHX3-associated pituitary hormone cell lineage determination during development.

The 3′ enhancer from the human *LHX3* gene directs Cre recombinase expression in the developing and neonatal anterior pituitary and spinal cord

Several enhancer-*Cre* founder animals were crossed to Cre-dependent reporter mice and analyzed for LacZ, YFP or DTA activity in the developing pituitary and spinal cord. In preliminary LacZ reporter experiments, I identified two enhancer-*Cre* founder lines with anterior pituitary and spinal cord expression patterns similar to those of the

original enhancer-LacZ transgenic mice patterns (Mullen et al., 2012). Frozen sagittal sections from single heterozygous *ELHX3*$^{+/+}$/*R26*$^{LacZ/+}$ mice pituitaries (as negative controls) and double heterozygous *ELHX3*$^{Cre/+}$/*R26*$^{LacZ/+}$ (Cre-expressing) pituitary glands were analyzed at e14.5 (Figure 3.2 A, B) and P0 (Figure 3.2 C-F) in mice carrying either *LacZ* (Figure 3.2 A, B, C, D) or *YFP* (Figure 3.2 E, F) reporter genes. Beta galactosidase and YFP were expressed specifically in the developing anterior pituitary in *ELHX3*$^{cre/+}$/*R26R*$^{LacZ/+}$ and *ELHX3*$^{cre/+}$/*R26R*$^{YFP/+}$ mice but not controls.

Whole mount staining at e12.5 displayed beta galactosidase activity in the developing spinal cord and pituitary (Figure 3.2 H). Some beta galactosidase activity also was detected in the retina, hindbrain, and forebrain consistent with the known locations of LHX3s (Sheng et al., 1996; Sharma et al., 1998; Sloop et al., 2000a; Sloop et al., 2000b). Some additional likely ectopic expression was seen in the facial/vestibuloacoustic ganglion complex of the nervous system. In subsequent experiments, one line of transgenic *ELHX3*$^{Cre/+}$ mice was used.

<u>αGSU hormone cell lineages are reduced in *ELHX3*$^{cre/+}$/*R26R*$^{DTA/+}$ mice</u>

Double immunohistochemistry of the pituitary gland showed that, similar to the data described above from enhancer-*LacZ* transgenic mice, beta galactosidase expressing cells are co-localized with αGSU cells but not GH-positive cells in enhancer Cre transgenic mice (Figure 3.1 A). To further test the role of the 3′ enhancer progenitor cells, a cell ablation method was performed using the *ROSA26*$^{eGFP\text{-}DTA}$ (*R26*^{DTA}) mice (Ivanova et al., 2005). *R26*^{DTA} mice encode an attenuated form of diphtheria toxin fragment A (DTA). When Cre is present, the DTA kills the cells. This approach is useful to investigate gene expression-cell lineage relationships. We crossed *ELHX3*^{Cre} to the

R26^{DTA} strain so that enhancer-guided Cre activity would result in DTA expression causing cell death in the absence of Cre. Expression of DTA is prevented by a strong transcriptional stop signal but when bred to *ELHX3*$^{cre/+}$ mice, Cre-mediated excision of the floxed stop activates DTA expression to ablate 3′ enhancer-expressing cells. Frozen pituitary sections from *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ (as negative controls; Figure 3.3 A, C, E, G, I) and *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ (Figure 3.3 B, D, F, H, J) mice at e14.5 were stained with antibodies specific for αGSU, TSHβ, ACTH, hormones; or the PIT-1 and TPIT transcription factor proteins; and protein expression was revealed using with DAB chemistry (Figure 3.3 A-D) and immunofluorescence methods (Figure 3.3 E-J). In these experiments, immunohistochemistry of e14.5 embryo pituitary glands revealed a notable reduction in αGSU-positive cells in the caudomedial portion of the developing anterior pituitary (AP) compared to controls, but αGSU-expressing cells in the rostral tip region of the AP were largely retained (Figure 3.3 A, B). Similar patterns were observed using antibodies recognizing TSHβ (Figure 3.3 C, D). Anti-TSHβ staining at e14.5 in the caudomedial region revealed weak TSHβ expression in *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ mice. Caudomedial TSHβ cells start to appear approximately at e14.5, and the tested e14.5 time point may perhaps be a little early to fully observe caudomedial TSHβ cells. However, TSHβ expressing cells are notably reduced in the caudomedial region, but not in the rostral tip area, in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. These data suggest that the 3′ enhancer expressing αGSU cell lineages in the caudomedial region are ablated in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice.

The PIT-1 transcription factor is critical for establishment of the thyrotrope, lactotrope and somatotropes cell types in the caudomedial area (Dasen et al., 1999; Scully

et al., 2000); by contrast, an earlier thyrotrope population in the rostral tip region is PIT-1-independent (Lin et al., 1994). PIT-1 expression levels were not notably reduced in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice at e14.5 (Figure 3.3 E, F). I also examined whether 3′ *LHX3* enhancer-guided DTA activity is target in corticotropes, which secrete ACTH. ACTH hormone levels were unaffected (Figure 3.3 C, D). In accord with this observation, levels of the TPIT T-box transcription factor that is necessary for corticotrope differentiation (Pulichino et al., 2003) were similar in e14.5 *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ embryos and controls (Figure 3.4 G, H).

Thyrotrope and gonadotrope cells are reduced in neonatal *LHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice

To examine the expression patterns of pituitary hormone-expressing cells in neonatal (P0) mice, transverse pituitary sections were used for immunohistochemistry analysis. The numbers of ACTH, TPIT, PIT-1, and GH-positive AP cells were not notably changed in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice compared to *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ controls (Figure 3.4 J-R). However, a majority of αGSU- and TSHβ-expressing cells were absent in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice (Figure 3.4 A-D). FSHβ-positive gonadotrope cells were moderately reduced (Figure 3.4 E, F) and LHβ-expressing gonadotrope cells were strikingly lower in number (Figure 3.4 G, H) with some animals having no detectable LHβ protein expression (Figure 3.4 I). Overall, these experiments indicate that the 3′ human *LHX3* enhancer is most active in the mouse thyrotrope and gonadotrope AP cell lineages that also express the αGSU subunit hormone component.

The majority of ablated gonadotropes in *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice are LHβ-expressing cells that also express FSHβ and SF1

Because the LH-expressing gonadotropes were the cell type most strikingly affected by human *LHX3* enhancer-guided DTA activity, I next focused on a closer examination of pituitary gonadotropes in the transgenic and control mice. LHβ-expressing gonadotropes are strikingly ablated in *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice with ~90% of the cells missing compared to controls (Figure 3.5 A, C). Similarly, a large fraction of the αGSU-expressing cells are missing (Figure 3.4 A, B) but a majority (~80% of total FSHβ) of FSHβ cells remain (Figure 3.5 A, C).

Immunofluorescence staining experiments also were performed using anti-LHβ and anti-FSHβ antibodies at P0 to characterize bi-hormonal (FSHβ- and LHβ-expressing) gonadotropes in the developing mouse pituitary. Most LHβ cells are FSHβ-expressing bi-hormonal cells (Figure 3.5 A, C). Overall, the FSHβ-expressing, LHβ-negative ("single positive") cells form a majority of the gonadotrope cell population compared to LHβ cell numbers. In *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice, most (~90%) of the bi-hormonal cells are removed by DTA action. These data suggest that the human *LHX3* enhancer-DTA actions preferentially target the bi-hormonal gonadotrope cells (Figure 3.5 C). When single FSHβ staining was performed in transgenic animals, ~20% of FSHβ cells were reduced (Figure 3.4 E, F) and these affected FSHβ cells are bi-hormonal cells. Taken together, our results suggest that the 3′ *LHX3* enhancer is active in LHβ-expressing bi-hormonal gonadotropes.

The SF1 transcription factor is expressed in LHβ-expressing gonadotropes and is notably reduced in *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice

SF1 is an essential transcription factor for gonadotrope function in mice and *Sf1* knockout mice are infertile and lack gonadotropes (Ingraham et al., 1994; Parker, 1998; Zhao et al., 2001). Using anti-SF1 antibodies, double immunofluorescence staining with SF1 and FSHβ or LHβ was performed. I observed that SF1 is co-expressed in LHβ cells and FSHβ-positive bi-hormonal gonadotropes and SF1-expressing cells are notably reduced in *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice (Figure 3.5 B; b and d). In wild type mice, virtually all LHβ-positive cells were also positive for SF1. By contrast, FSHβ cells were not always SF1-positive. We also detected a few SF1-positive cells that do not co-express either LHβ or FSHβ (Figure 3.5 B; c and d). The majority of double SF1 and LHβ positive cells are absent in *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$mice (Figure 3.5 B; c and d). This result again is consistent with the hypothesis that the 3′ human *LHX3* enhancer is especially active in LHβ-expressing gonadotropes. Similarly, SF1 expression is co-localized in LHβ cells in wild type animals and therefore a significant fraction of SF1 expression is reduced in *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice.

LHX3-expressing interneurons are deleted in the mediolateral region of the developing spinal cord in *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice

V2 INs in the ventral spinal cord are derived from a common progenitor population expressing LHX3 (Peng et al., 2007), and are subdivided into two classes: V2a INs, which are characterized by expression of the CHX10 transcription factor marker, and V2b INs, which express GATA2 and GATA3 (Ericson et al., 1997; Zhou et al., 2000; Karunaratne et al., 2002; Smith et al., 2002). During V2 IN specification,

LHX3 forms the V2 IN "tetramer complex" consisting of LHX3 and the nuclear LIM domain interactor, NLI (Al-Mosawie et al., 2007; Lundfald et al., 2007; Peng et al., 2007). In order to specify MNs, LHX3 participates in a multiprotein gene regulatory complex involving ISL1, NLI, and the NeuroM and E47 transcription factors. MNs are characterized by expression of the ISL1 and HB9 homeodomain proteins (Pfaff et al., 1996; Saha et al., 1997; Tanabe et al., 1998; Thaler et al., 1999). ISL1 is found in MNs, dorsal INs, and dorsal root ganglion (DRG) sensory neurons (Liem Jr et al., 1997); whereas HB9 is a restricted MN marker (Thaler et al., 1999). To examine whether the human 3′ *LHX3* enhancer is active during specification of MNs and V2 INs, we characterized expression of the ISL1 and HB9 protein for MNs and the CHX10 and GATA2 for protein V2 INs in $ELHX3^{+/+}/R26^{DTA/+}$ controls and $ELHX3^{Cre/+}/R26^{DTA/+}$mice. For nervous system analysis, transverse sections of e14.5 embryos were used to examine the neural tube and sagittal sections were used to visualize the spinal cord in the other axis. First, I tested LHX3 protein expression levels in $ELHX3^{Cre/+}/R26^{DTA/+}$ in the developing nervous system. LHX3 expression was reduced in the mediolateral in mid cervical region of the spinal cord of $ELHX3^{Cre/+}/R26^{DTA/+}$ mice (Figure 3.7 A, B). Similar data were collected in transverse sections of the spinal cord withLHX3-positive cells diminished in the presumptive V2 IN specific area, but not in the presumptive MNs in the spinal cord (Figure 3.6 C, D). Next, I tested ISL1 and HB9 expression levels in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice. HB9 and ISL1-expressing cells in the ventral MN area were not notably affected but ISL1-expressing INs were reduced in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice (Figure 3.7 E-H). These data suggest that INs, not MNs, are targeted by 3′ *LHX3* enhancer-DTA activity in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice.

LHX3 and CHX10 co-expressing V2a IN cells are reduced in the spinal cord of *ELHX3$^{Cre/+}$/R26$^{DTA/+}$* mice

To further investigate the loss of LHX3-expressing cells in the V2 IN area, LHX3 and CHX10 double immunofluorescent staining was performed. V2a and V2b INs can be identified by expression of CHX10 or GATA2/3, respectively. These markers are expressed in the intermediate part of neural tube (Al-Mosawie et al., 2007; Lundfald et al., 2007; Peng et al., 2007). I observed that ablated LHX3 cells are located in the region of V2 INs (Figure 3.7 B, E). LHX3 and CHX10 co-localize in the V2 INs area in *ELHX3$^{+/+}$/R26$^{DTA/+}$* controls, but V2a IN CHX10-expressing cells were reduced in *ELHX3$^{Cre/+}$/R26$^{DTA/+}$* mice (Figure 3.7 A, D). This data suggests that LHX3 and CHX10 co-expressing cells are targeted in *ELHX3$^{Cre/+}$/R26$^{DTA/+}$*. Together, these observations suggest that the human 3′ *LHX3* enhancer is active in mouse V2a INs but not in MNs. Similar V2a IN data have been described by another laboratory in transgenic animals expressing a *Chx10* promoter-DTA transgene; these mice exhibited defects in left-right coordination and died shortly after birth due to respiratory defects (Crone et al., 2008; Crone et al., 2009). Our *ELHX3$^{Cre/+}$/R26$^{DTA/+}$* embryos typically contained pituitary glands that were morphologically normal, although these embryos were found at lower frequencies than predicted, indicating some prenatal loss of viability (Table 3.1). Furthermore, the very few surviving *ELHX3$^{Cre/+}$/R26$^{DTA/+}$* mice were smaller (n=1) than littermates (Table 3.1).

In *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice, GATA2-expressing cells are reduced in the caudomedial region of the developing pituitary gland but not in the spinal cord

The GATA2 transcription factor is expressed in several tissues including the developing pituitary (Dasen et al., 1999; Suh et al., 2002), and in the developing nervous system where it is known as a V2b IN marker (Zhou et al., 2000). In the developing pituitary, GATA2 has been shown to be involved in the differentiation of gonadotrope and thyrotrope cell types in a mechanism suggested to involve interactions with the PIT-1 transcription factor (Dasen et al., 1999; Suh et al., 2002). GATA2 expression therefore is observed in developing gonadotropes and in the PIT-1-dependent thyrotropes in the caudomedial region of the developing AP (Dasen et al., 1999).

To determine whether GATA2 expression is affected in the developing pituitary and spinal cord of *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$mice, e14.5 embryo frozen sections were probed with an anti-GATA2 antibody. Compared to controls, GATA2-positive cells were reduced in the caudomedial portion of the developing pituitary, but not in the rostral tip area (Figure 3.8 A, B), a similar pattern to that observed for αGSU staining (Figure 3.3 A, B). In the developing nervous system, no notable differences in GATA2 expression were detected in DTA-expressing or control mice (Figure 3.8 C, D). This data suggests that the human 3´ *LHX3* enhancer is active in GATA2-positive/αGSU-positive cell lineages in the developing pituitary and in CHX10-positive V2a INs in the developing nervous system.

ELHX3$^{Cre/+}$/*R26*$^{DTA/+}$mice have low viability

"Double heterozygous" *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice were produced from enhancer-*HSP68-Cre* (*ELHX3*^{Cre}) transgenic and homozygous *R26*$^{DTA/DTA}$ mice. These double heterozygous mice have low viability both prenatally and postnatally (Table 3.1). To

date, there only three double heterozygous mice have survived. One of these died before weaning (postnatal day 21) due to unknown reasons. This mouse was much smaller than control littermates. All of the surviving mice were male. One of the two remaining mice was dwarfed (Figure 3.9), showed no detectable hearing impairment (could respond to noises), and slow moving and had an apparent balance impairment based on observation of movement. Whole pituitaries, seminal vesicles, and testes were isolated from control and this surviving dwarf *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mouse and the isolated organ sizes were proportionally reduced in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ (Figure 3.9 and data not shown). The other surviving male mouse (n=1) was of normal size and fertile.

Immunohistochemistry performed at 12 weeks of age on the pituitary from the surviving *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ dwarf mouse revealed that there were no significant hormone deficiencies including FSHβ and GH compared to control littermates. However, it is important to note that this was one, rare surviving animal that may not be representative of a typical outcome.

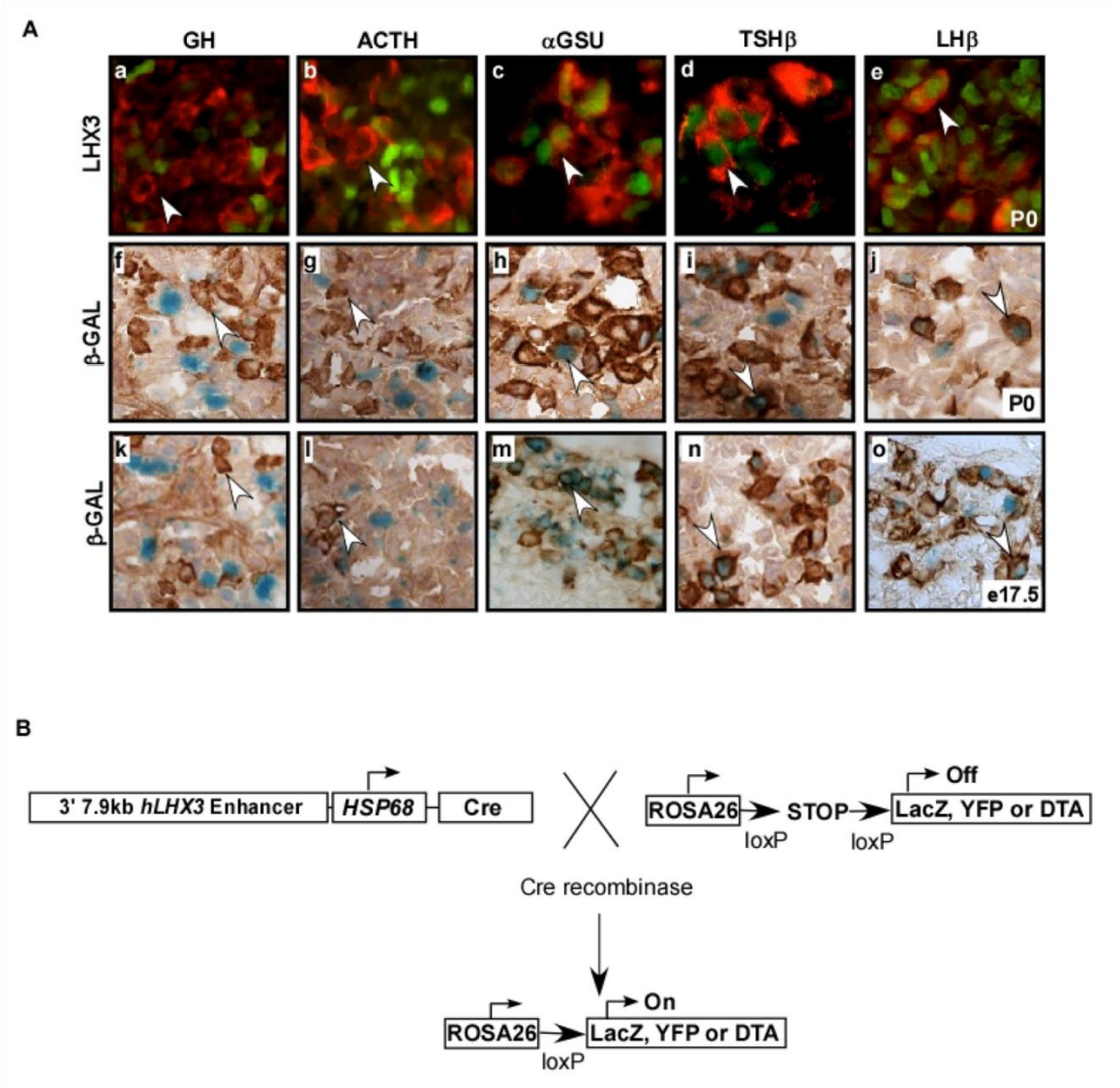
A
GH
ACTH
αGSU
TSHβ
LHβ
LHX3
β-GAL
β-GAL
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
P0
P0
e17.5
B
3' 7.9kb hLHX3 Enhancer
HSP68
Cre
ROSA26
STOP
LacZ, YFP or DTA
Off
loxP
loxP
Cre recombinase
ROSA26
LacZ, YFP or DTA
On
loxP

Figure 3.1. The endogenous mouse LHX3 protein and a human *LHX3* gene 3′ enhancer-guided reporter gene are expressed in anterior pituitary αGSU-expressing thyrotrope and gonadotrope cells, but not in somatotropes and corticotropes. A. Frozen transverse pituitary sections from newborn (P0) (a-j) and e17.5 (k-o) 3' human *LHX3* gene enhancer-*HSP68-nLacZ* transgenic mice were stained by immunohistochemistry for LHX3 (green) (a-e) or stained for transgene-derived β-galactosidase activity (blue) (f-o) and co-labeled for hormone subunits (red or brown): GH (a, f, k), ACTH (b, g, l), αGSU (c, h, m), TSHβ (d, i, n), and LHβ (e, j, o). Co-localization was highest (arrowheads) for gonadotropes and thyrotropes (c, d, e, h, i, j, m, n, o) and nearly absent (arrowheads) for corticotropes and somatotropes (a, b, f, g, k, l). All experiments were performed a minimum of three times. B. Schematic diagram showing generation of *ELHX3*^{Cre} (human *LHX3* gene 3' enhancer-*HSP68-Cre*) transgenic mice and crossing to *ROSA26* reporter strains to reveal enhancer-driven activities. *ELHX3*^{Cre} mice were crossed to reporter mice for conditional activation of LacZ, YFP, or DTA "reporter" gene expression.

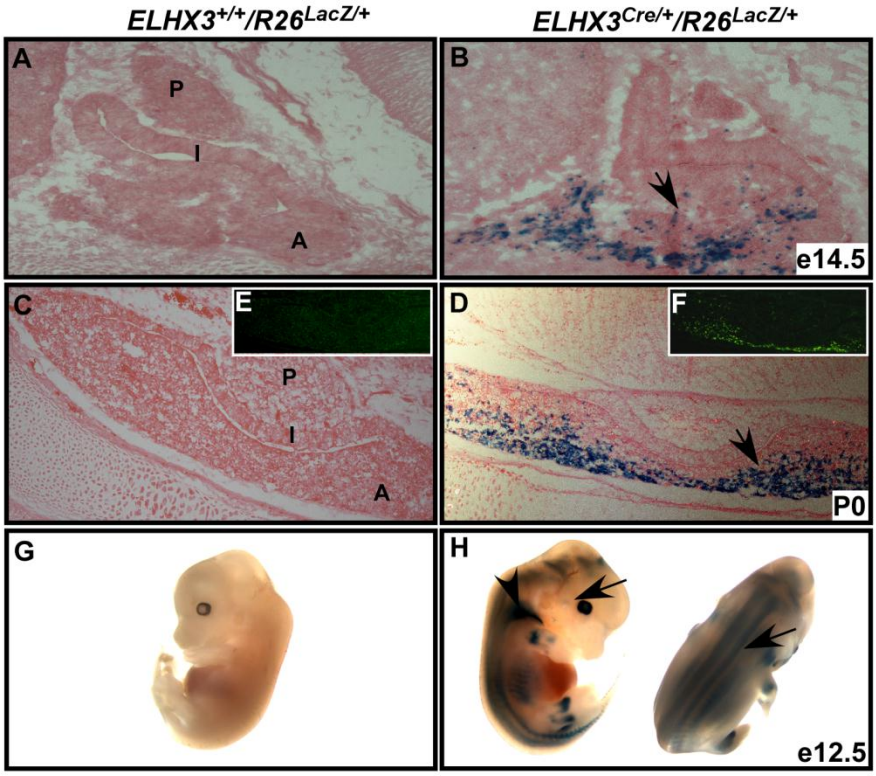

Figure 3.2. The 3′ enhancer from the human *LHX3* gene directs Cre recombinase expression in the developing and neonatal anterior pituitary and spinal cord. Frozen sagittal sections from *ELHX3*$^{+/+}$/*R26*$^{LacZ/+}$ (negative control) and *ELHX3*$^{Cre/+}$/*R26*$^{LacZ/+}$ (Cre-expressing) pituitary glands were analyzed at e14.5 (A, B) and P0 (C-F) in mice carrying either LacZ (A-D) or YFP (E, F) reporter genes. G and H, X-gal stained whole embryos at e12.5 show enhancer-driven LacZ activity in the developing pituitary and spinal cord (arrows). Ectopic expression is also seen in some other tissue such as the facial/vestibuloacoustic ganglion complex (arrowhead); G, single heterozygous *ELHX3*$^{+/+}$/*R26*$^{LacZ/+}$ mice as controls (n = 3), H, double heterozygous *ELHX3*$^{Cre/+}$/*R26*$^{LacZ/+}$ mice (n = 5). P, posterior lobe of the pituitary gland; I, intermediate lobe; A, anterior lobe.

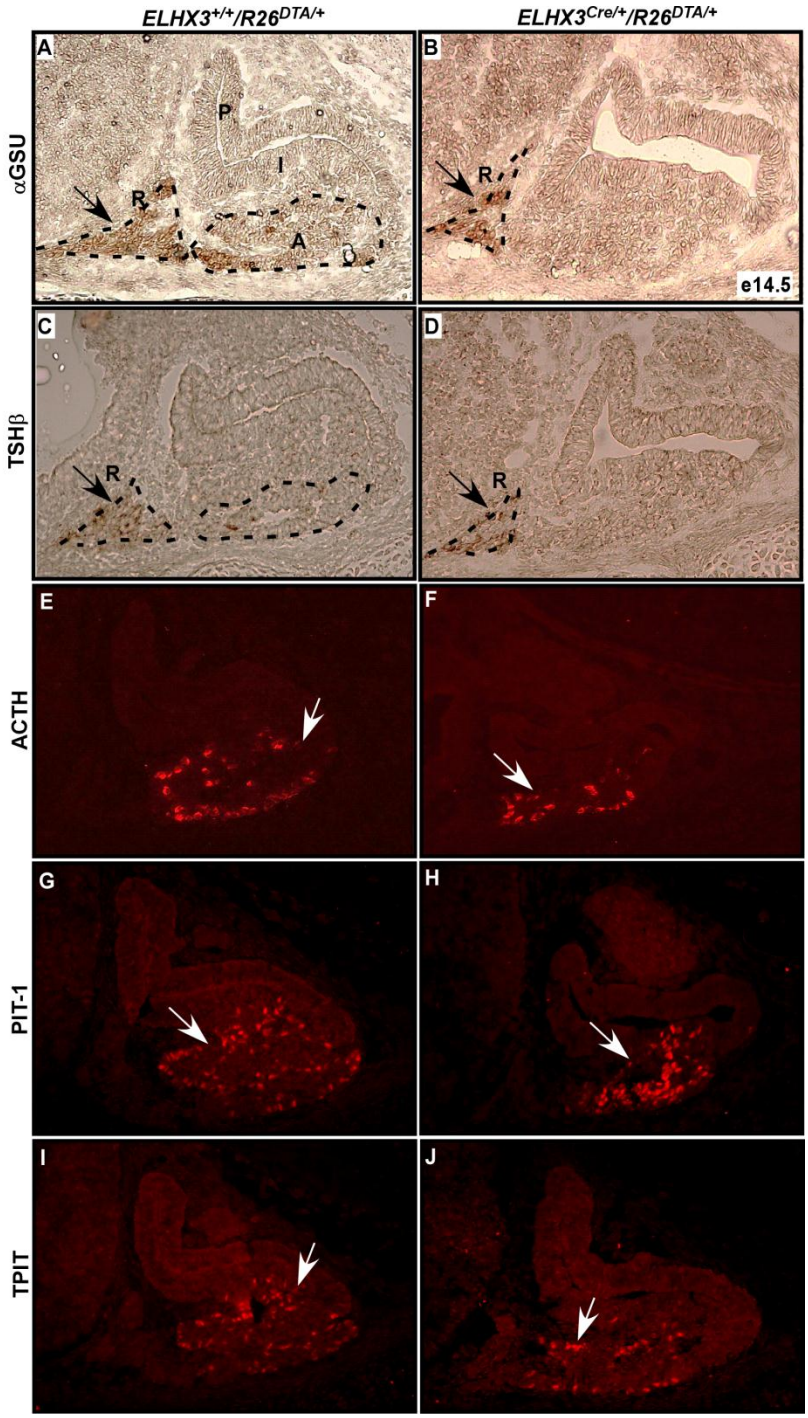
ELHX3+/+/R26DTA/+
ELHX3Cre/+/R26DTA/+
αGSU
TSHβ
ACTH
PIT-1
TPIT
A
B
C
D
E
F
G
H
I
J
P
I
R
A
e14.5

Figure 3.3. The human 3′ *LHX3* enhancer is active in many αGSU-expressing anterior pituitary cells: αGSU cells are notably reduced in the developing pituitary glands of *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. Frozen pituitary sections from e14.5 *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ (as negative controls; A, C, E, G) and *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ (B, D, F, H) mice were stained with antibodies specific for αGSU (A, B), TSHβ (C, D), ACTH (E, F), PIT-1 (G, H), and T-PIT (I, J) proteins and expression was revealed using either DAB chemistry (A, B) or immunofluorescence (C-H). Arrows indicate positively-stained cells. P, posterior lobe, I, intermediate lobe, A, caudomedial region of the anterior lobe; R, rostral tip region of the anterior lobe.

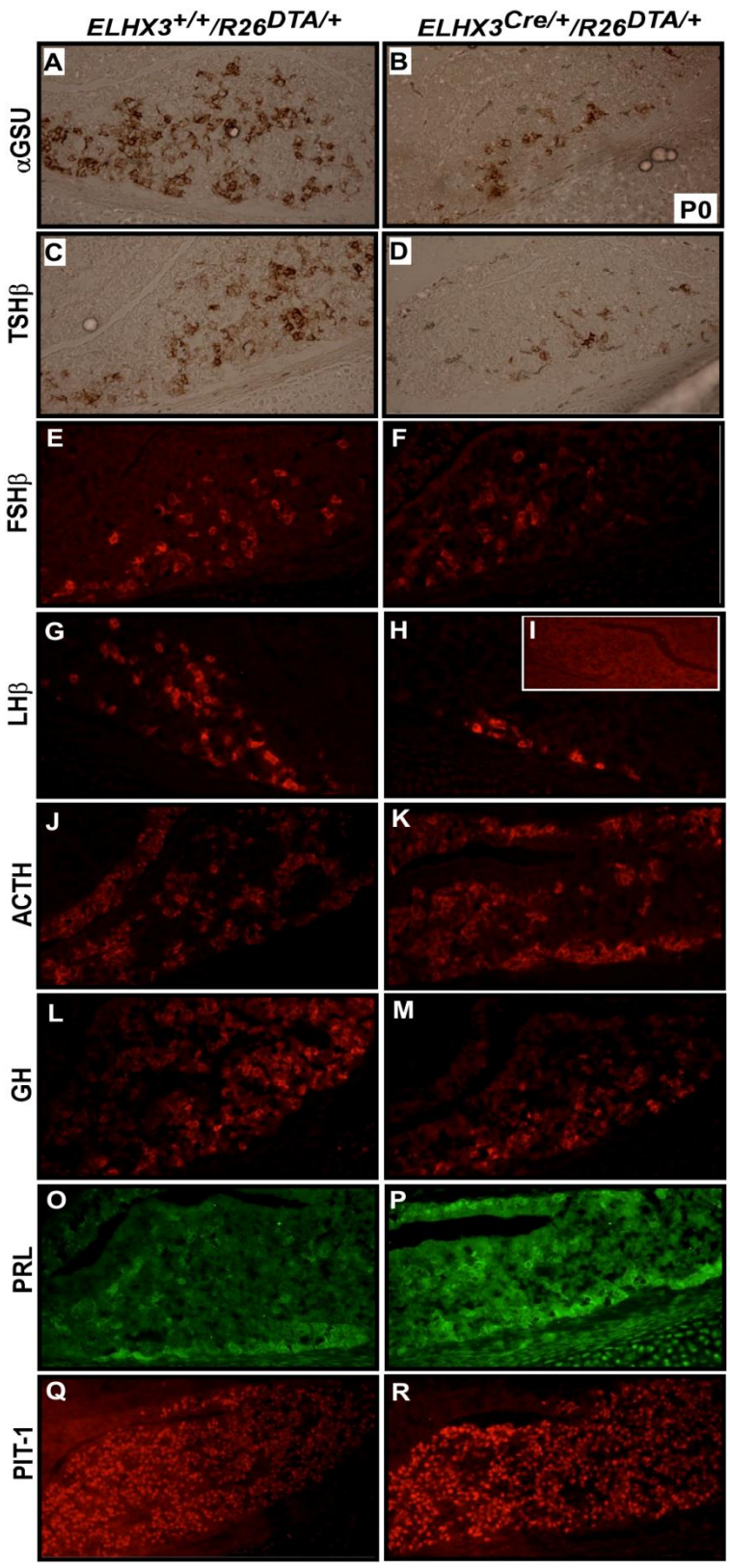
ELHX3+/+/R26DTA/+
ELHX3Cre/+/R26DTA/+
αGSU
TSHβ
FSHβ
LHβ
ACTH
GH
PRL
PIT-1
A
B
P0
C
D
E
F
G
H
I
J
K
L
M
O
P
Q
R

Figure 3.4. Thyrotrope and gonadotrope cell lineages are reduced in neonatal *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. Immunostaining of transverse sections from P0 pituitaries reveals reduction of αGSU cells (A, B); TSHβ-positive thyrotropes (C, D) and gonadotropes (E-I) in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. FSHβ-staining gonadotropes are reduced (E, F) and LHβ-expressing gonadotropes are either highly reduced (G, H) or in some cases completely ablated (I). Control and enhancer-expressing *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice did not have different levels of ACTH (J, K), GH (L, M), or PRL (O, P) hormone-positive cells in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice at P0. Similarly, levels of the PIT-1 transcription factor were not notably altered (Q, R).

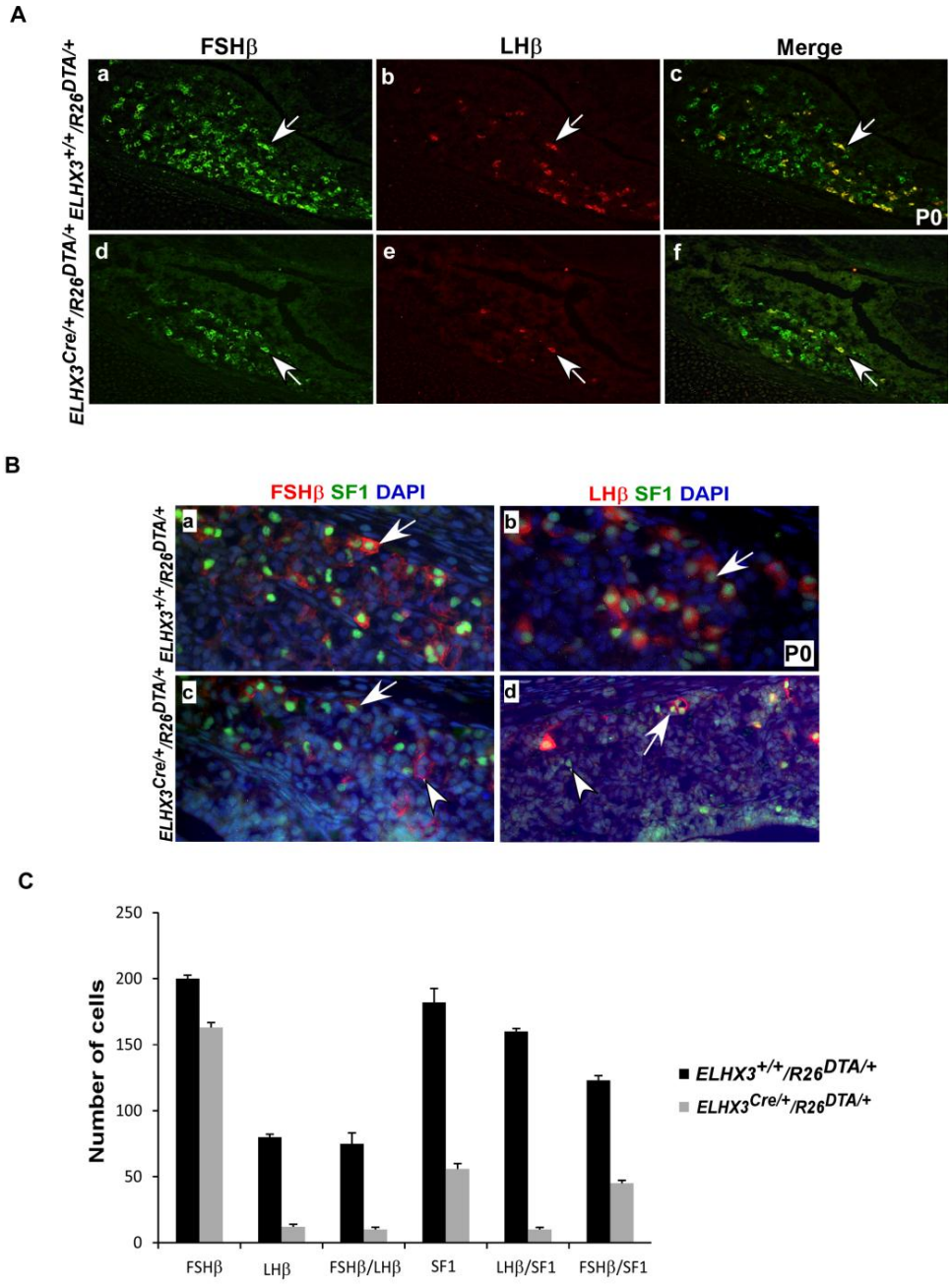
A
FSHβ
LHβ
Merge
a
b
c
d
e
f
P0
ELHX3+/+/R26DTA/+
ELHX3Cre/+/R26DTA/+
B
FSHβ SF1 DAPI
LHβ SF1 DAPI
a
b
c
d
P0
ELHX3+/+/R26DTA/+
ELHX3Cre/+/R26DTA/+
C
Number of cells
250
200
150
100
50
0
FSHβ
LHβ
FSHβ/LHβ
SF1
LHβ/SF1
FSHβ/SF1
ELHX3+/+/R26DTA/+
ELHX3Cre/+/R26DTA/+

Figure 3.5. LHβ-expressing gonadotropes are strikingly ablated in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice and LHβ cells co-localize with FSHβ and SF1. A, Frozen transverse pituitary sections of P0 animals were examined with antibodies to LHβ and FSHβ to determine the FSH^{+} and LH^{+} populations present in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. FSHβ and LHβ cells are predominantly located in ventral area of the pituitary. Most LHβ cells and some FSHβ cells are ablated in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice (a, b, d, e). B, LHβ cells are predominantly FSHβ bi-hormonal cells, and are co-express with SF1 cells (c, f). The SF1 transcription factor is expressed in LHβ-expressing gonadotropes and is notably reduced in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. Frozen transverse pituitary sections at P0 were co-immunostained with antibodies recognizing gonadotrope hormones FSHβ (a, c) or LHβ (b, d) (red), the SF1 transcription factor (green), and DAPI stain to reveal nuclei (blue). SF1^{+} cells co-localize with FSHβ and LHβ cells, and are reduced in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice (c, d). Bi-hormonal (FSHβ/LHβ) cells are indicated by white arrows and single hormone (FSHβ or LHβ) cells are indicated by white arrowheads. C, LHβ-expressing cells are strikingly diminished in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. The graph shows the total number of αGSU-, FSHβ-, LHβ-, FSHβ-/LHβ-, SF1-, LHβ-/SF1-, and FSHβ-/SF1-expressing cells compared to controls. Experiments were carried out in control *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ littermates (n = 3) and *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ (n = 3). Data are expressed as means ± SEM. Differences are significant at the following levels as determined by a Student's t test: αGSU ($p < 0.002$), FSHβ ($p < 0.05$), LHβ ($p < 0.002$), FSHβ/LHβ ($p < 0.02$), SF1 ($p < 0.02$), LHβ/SF1 ($p < 0.001$), and FSHβ/SF1 ($p < 0.01$).

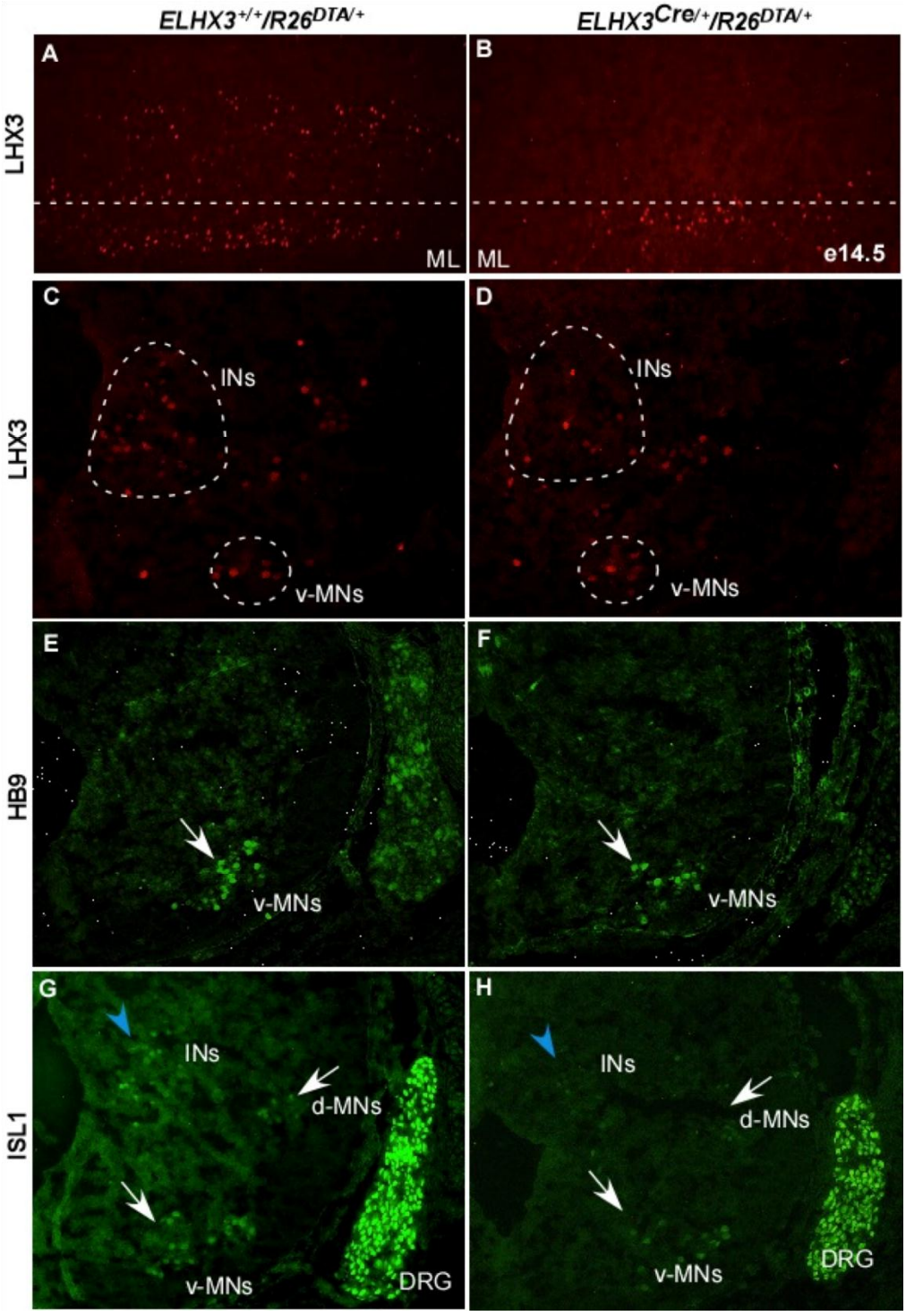
ELHX3+/+/R26DTA/+
ELHX3Cre/+/R26DTA/+
A
B
LHX3
ML
ML
e14.5
C
D
LHX3
INs
INs
v-MNs
v-MNs
E
F
HB9
v-MNs
v-MNs
G
H
ISL1
INs
INs
d-MNs
d-MNs
v-MNs
v-MNs
DRG
DRG

Figure 3.6. LHX3-expressing IN cells are reduced in the V2 IN region of the developing spinal cord in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice but MN populations are unaffected. Sagittal (A, B) and transverse sections (C-H) of e14.5 spinal cords from *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ (as controls) and *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice were examined with antibodies against to LHX3 (A-D), or the motor neuron markers HB9 (E, F) and ISL1 (G, H). The arbitrary dotted line in panels A and B indicates the mediolateral region of the spinal cord. Large white dotted circles in panels C and D indicate the interneuron area and small white dotted circles denote the ventral motor neuron area. White arrows show HB9- or ISL1-positive cells. Blue arrows indicate the interneuron reduction in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. ML, marginal layer of spinal cord; INs, interneurons; v-MNs, ventral-motor neurons; d-MNs, dorsal-motor neurons; DRG, dorsal root ganglion.

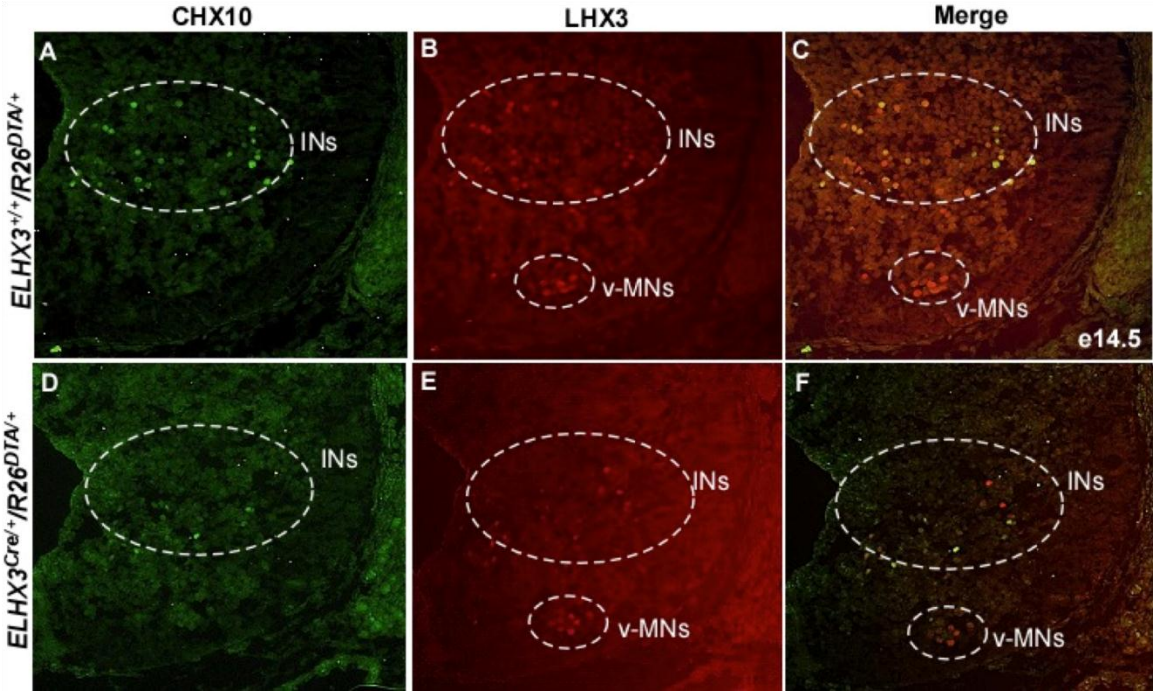

Figure 3.7. LHX3- and CHX10-expressing V2a interneuron cells are reduced in the spinal cord of *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. Transverse sections from e14.5 *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ (A, B, C) and *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ (D, E, F) mouse spinal cords were co-labeled with antibodies to CHX10 (A, D) and LHX3 (B, E). C, F = merged channels. CHX10 and LHX3 co-labeled cells are located in the intermediate ventral area. V2a interneuron CHX10- and LHX3-positive cells are reduced in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ transgenic mice (F). INs, interneurons; v-MNs, ventral-motor neurons.

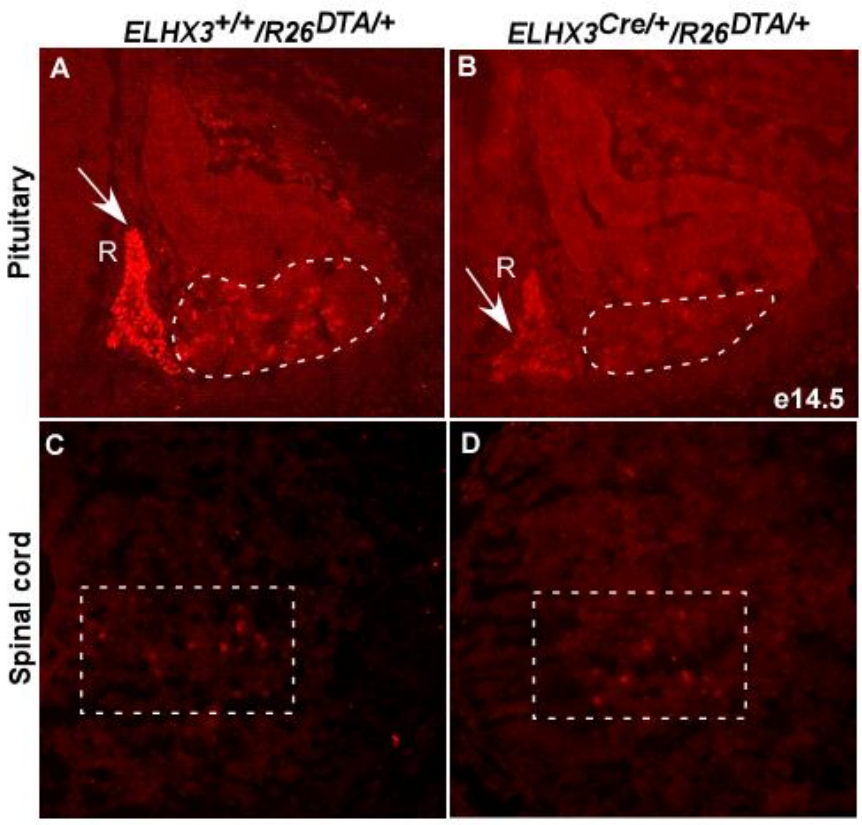

Figure 3.8. GATA2-expressing cells are reduced in the caudomedial region of the developing pituitary gland but GATA2 expression is normal in the embryonic spinal cord. Frozen sagittal sections of the pituitary gland (A, B) and transverse sections of the spinal cord (C, D) from e14.5 *ELHX3*$^{+/+}$*/R26*$^{DTA/+}$ (controls) or *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice were stained with an antibody recognizing the GATA2 transcription factor. GATA2 expression is present in rostral tip area in both types of animal but is lost in the caudomedial region in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice (B). White dotted "circles" indicate the caudomedial area of the pituitary. Arrows point to GATA2-expressing cells in the rostral tip area (R). White dotted rectangles indicate GATA2-positive cells in the spinal cord.

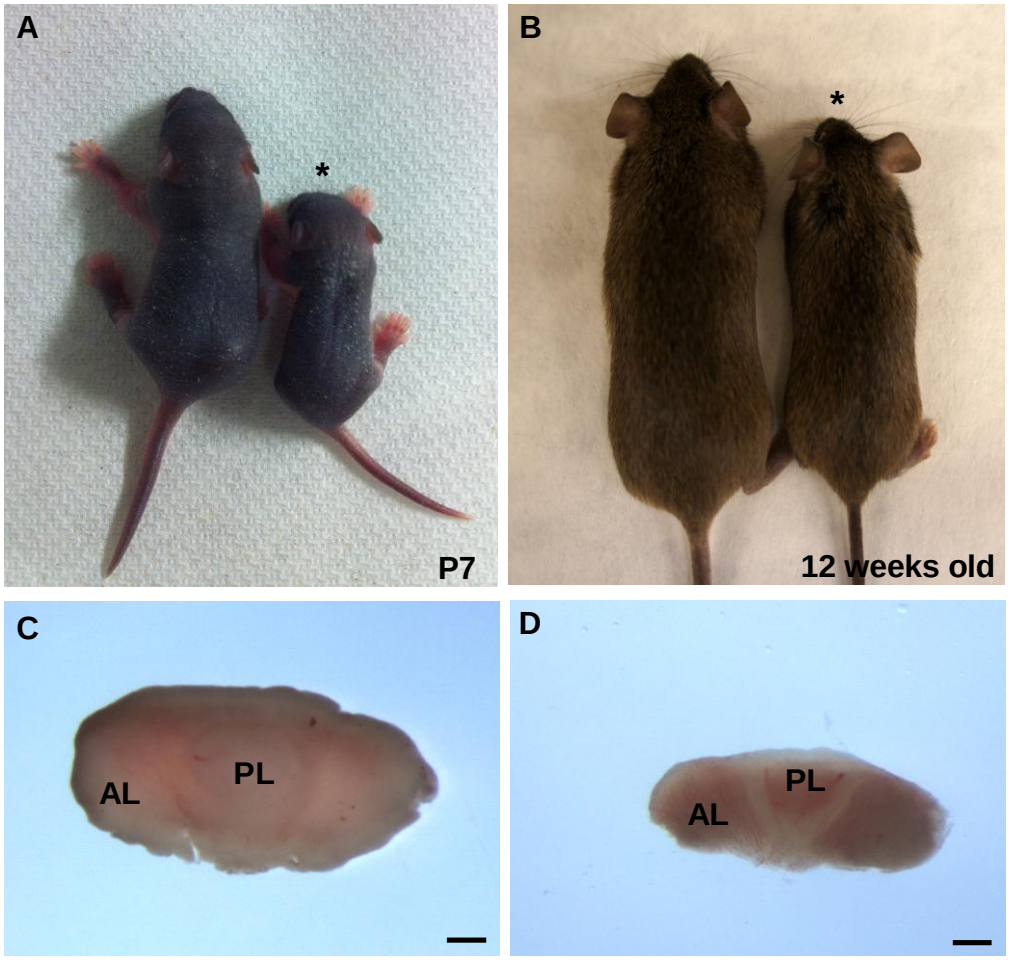

Figure 3.9. Dwarfism in rare surviving *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ mice. A, In comparison to "single heterozygous" control mice, "double heterozygous" *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ present with smaller size at P7 and 12 weeks old (n=1). B, Whole pituitaries were isolated from 12 weeks old mice. Left panel, control; right panel, *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$. Anterior pituitary (AL) and posterior lobe (PL) of 12 weeks old adult *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ pituitary gland is smaller than controls. Asterisks (*) indicate double heterozygous *ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$mice. Scale bar = 1 mm.

Age (days)	*ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$ genotype: live (dead)	
	ELHX3$^{+/+}$/*R26*$^{DTA/+}$	*ELHX3*$^{Cre/+}$/*R26*$^{DTA/+}$
e14.5	29	5
e17.5	19	3
P0	47	4
P21	65	2[a] (1[b])

Table 3.1. Genotypes of progeny from *ELHX3*$^{Cre/+}$ and *R26*$^{DTA/+}$ crosses. For P0 and P21 mice, breeding pairs were maintained a minimum of four months. The numbers of embryos for each genotype at the indicated stages of development are shown. [a]Two male mice were found alive: one is fertile and of approximately normal size; the other is dwarfed and infertile. [b]Dead mouse (male) was smaller than littermates.

CHAPTER FOUR

DISCUSSION

LHX3 is a LIM-homeodomain transcription factor necessary for proper development of the pituitary and central nervous system. Patients with mutations in coding regions of the *LHX3* gene have complex syndromes including combined pituitary hormone deficiency and nervous system defects resulting in symptoms such as dwarfism, thyroid insufficiency, infertility, and developmental delay. Previously, we demonstrated that the human *LHX3* gene has a 3′ enhancer region that includes recognition elements for ISL1 and PITX1, and can direct transcription in the developing pituitary and spinal cord tissues in similar patterns to endogenous LHX3 expression (Mullen et al., 2012).

To understand enhancer-directed transgene expression, I used 3′ enhancer-LacZ mouse models to determine which pituitary hormone-expressing cell types also showed enhancer-directed expression and endogenous LHX3 expression. Interestingly, both native LHX3 protein expression and 3′ human *LHX3* gene enhancer-directed transgene were noted in αGSU-expressing cells but not in GH-positive cells in P0 pituitaries (Figure 3.1). This data prompted us to make 3′ human *LHX3* gene enhancer-Cre mice to use the Cre-loxP system to further study the action of the enhancer in pituitary and spinal cord development by lineage tracing. The lineage tracing method allows detection of the early time points during development where transgene expression occurs. Therefore, using $ELHX3^{Cre}$ mice, we can identify anterior pituitary or neuronal cell types in which the 3′ enhancer is expressed.

$ELHX3^{cre/+}/R26R^{LacZ/+}$ mice express the β-galactosidase reporter gene in some, but not all, anterior pituitary hormone-secreting cells. In the pituitary, the enhancer-DTA

activity is mostly active in αGSU-expressing cells: the LHβ-, FSHβ-, and TSHβ-secreting gonadotropes and thyrotrope cell types. This expression pattern matches the endogenous LHX3 protein and that noted with direct expression of enhancer-*LacZ* transgenes. Enhancer-directed activity (as revealed by "direct" expression of enhancer-*LacZ* transgenes, and "indirectly" via the actions of enhancer-*Cre* transgenes in LacZ, YFP, or DTA reporter mice) and the endogenous protein were not detected in GH-expressing pituitary somatotropes, numerically the most abundant hormone-secreting cell type. This result was unexpected considering the deficiencies of GH in pediatric CPHD patients with *LHX3* mutations and *Lhx3* knockout mouse embryos.

There are several possible explanations for these observations. It may be that LHX3 is transiently expressed in developing somatotropes and such expression is sufficient to guide the determination of this and the related lactotrope lineage. However, the 3′ *LHX3* enhancer-*Cre* lineage tracing experiments using LacZ or DTA appear to indicate that the tested human 3′ *LHX3* enhancer does not play a role in establishing the somatotrope lineage. Others have demonstrated that pituitary somatotropes are susceptible to Cre-mediated targeting approaches (Nasonkin et al., 2009). This would suggest that there are other *cis*-acting elements involved in human *LHX3* gene expression. Alternately, LHX3 may not exert its effects on somatotrope development directly; rather, it may act indirectly through its expression in surrounding pituitary cells. Pituitary cell types are extensively networked and these cell-cell interactions may play significant roles in such developmental influences [reviewed in (Lee and Pfaff, 2001; Hashimoto et al., 2005; Kelberman et al., 2009; Davis et al., 2010)]. It is important to also consider some limitations of these observations. It is possible that there are levels of

LHX3 in somatotropes below the level of detection of the immunohistochemistry approaches used here. In addition, this study uses a human *LHX3* enhancer sequence. It is conceivable that the differences in observed pituitary expression represent differences between mouse and human *LHX3* expression patterns or differences in gene control mechanisms between the two species. Several studies have shown that transgene DNA sequences, rather than the host animal environment, typically determine the transcriptional outcome. For example, the rat *gonadotropin releasing hormone receptor* gene displays the native rat gene expression pattern in transgenic mouse models rather than the mouse pattern (Schang et al., 2011; Schang et al., 2013); human aromatase promoters guide a "humanized" expression pattern in transgenic mice (Zhao et al., 2012); and human genes show human-like expression patterns in transgenic mice (Willson et al., 2008). The observations here therefore may represent the actions of the human gene enhancer sequence rather than the mouse nuclear environment.

Even within the αGSU-expressing cell lineages that demonstrate activity of the 3´ human *LHX3* enhancer, there is spatial specificity of action. Based on the observation of αGSU-expressing cells in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice, LHβ, FSHβ, and TSHβ cell types have different expression sensitivities for the 3′ enhancer. At e14.5, TSHβ and αGSU cells are reduced only in the caudomedial region, but not the rostral tip TSHβ and αGSU cells. This data suggests that enhancer expression in the caudomedial αGSU and TSHβ cells different from enhancer activity in the rostral αGSU and TSHβ cells and therefore contain different gene-regulatory environments. This result indicates that the enhancer is most active in the central caudomedial area of the AP but not in the rostral tip area. Within the caudomedial area, the PIT-1 transcription factor is required for establishment

of the thyrotrope, lactotrope and somatotrope cell types, but an earlier thyrotrope population in the rostral tip region is PIT-1-independent (Dasen et al., 1999). The *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ experiments demonstrate that the enhancer is not active in most of the PIT-1 expressing cells, including the GH, PRL producing cells, since PIT-1 expression is not dramatically affected by DTA activity. However, the TSHβ-staining data suggests that the enhancer is active in central caudomedial area thyrotropes but not in the rostral tip thyrotropes. Corticotrope ACTH hormone and corticotrope-restricted T-box transcription factor TPIT (Lamolet et al., 2001; Pulichino et al., 2003) expression levels were not notably reduced in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice. This indicates that corticotropes are largely unaffected in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice and this data correlates with the lack of *LHX3* in corticotrope cells during mouse anterior pituitary development [reviewed in (Drouin et al., 2007)].

Interestingly, I found that the 3′ human *LHX3* enhancer-DTA activity is notable in LH-expressing gonadotropes. The pituitary gonadotrope is a complex cell type that secretes hormones with multiple actions that are required for many aspects of development, physiology, and behavior [reviewed in (Brown and McNeilly, 1999)]. However the mechanisms that determine the gonadotrope cell lineage are not fully understood. This study is the first to determine the fraction of bi-hormonal FSHβ/LHβ cells in gonadotropes in the developing mouse pituitary. Whereas LH-expressing gonadotrope numbers were notably affected by human *LHX3* enhancer guided DTA activity with ~90% of the cells missing compared to controls, the impact on FSHβ-expressing gonadotropes, although significant, is considerably less and a majority (~80% of total FSHβ) remains. I observed the number of FSHβ positive cells is larger population

than bi-hormonal (FSHβ/LHβ) gonadotrope cells. The possible physiological role of a FSH only cell is that it may be simply a cell that does FSH-related activities [e.g. spermatogenesis or gonad development; reviewed in (Simoni et al., 1997)]. Further experiments are needed to test FSH level at postnatal stages or puberty period to confirm above hypothesis. Previous studies have also examined gonadotrope populations in the developing pituitaries. For example, in accord with our results, a study of human gonadotropes demonstrated that whereas LH-only cells are very rare (0.1-0.3% of total gonadotropes), bi-hormonal and FSH-only cell types are more common (Pope et al., 2006). Our data suggest that gonadotrope population is mostly composed of bi-hormonal (FSHβ/LHβ) and single FSHβ-expressing cells in developing mouse pituitaries and that the human *LHX3* 3′ enhancer-guides gene expression to bi-hormonal gonadotrope cells.

Multiple pituitary transcription factors contribute to the signals that restrict gonadotrope cells to their specific fates [reviewed in (Lee and Pfaff, 2001; Hashimoto et al., 2005; Kelberman et al., 2009; Davis et al., 2010)]. For example, recent studies have shown that GATA2, LHX3 and ISL1 interact *in vivo* to regulate rat *gonadotropin-releasing hormone (GNRH) receptor (Gnrhr)* promoter activity (Charles et al., 2006; Schang et al., 2013). GNRH is expressed in the hypothalamus and is transported to the anterior pituitary and binds to the GNRH receptor (GnRHR) in gonadotrope cells. This stimulates the synthesis and release of LH and FSH. It is likely that LHX3, GATA2 (and perhaps other pituitary-expressed GATA factors such as GATA3 or 4 (Charles et al., 2006; Lo et al., 2011)), SF1, and ISL1 contribute selectively to establishing different gonadotrope subtypes over time. Experiments using Cre targeting under transcriptional control of the *Gnrhr* gene, as similarly observed here for LHX3 enhancer action,

demonstrated efficient ablation of LH-expressing but not FSH-positive gonadotropes (Wen et al., 2010). These authors also noted that the targeted LHβ positive cells were bi-hormonal cells that also made FSH and GnRHR. Our data suggest that the 3′ human *LHX3* enhancer is notably active in LH-expressing gonadotropes and that FSH-only (but not bi-hormonal) gonadotropes do not possess a transcriptional milieu that mediates the actions of this enhancer. The 3′ human *LHX3* enhancer is directing LHX3 expression to the appropriate cell types including gonadotrope cells. Further experiments are needed to determine the expression of GnRHR in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice and to compare expression levels of GnRHR with LHβ and FSHβ. This experiment will give us better understanding about the 3′ enhancer-DTA action in gonadotrope subtypes whether GnRHR is expressing all gonadotrope cells or only in bi-hormonal cells.

Our immunohistochemistry analysis showed that the 3′ human *LHX3* enhancer-DTA specifically ablates pituitary cells expressing the GATA2 transcription factor that serves as an upstream regulator of gonadotrope lineages. Consistent with the observations for αGSU and TSHβ, GATA2 cells were notably reduced in the caudomedial portion of the AP rather than in the rostral tip area. In the AP, GATA2 is postulated to be involved in the differentiation of gonadotrope and thyrotrope cell lineages (Dasen et al., 1999). GATA2 expression is detectable at e10.5 at the ventral boundary of Rathke's pouch and persists through adulthood in an expression pattern coincident with αGSU cells (Dasen et al., 1999). It has been shown that GATA2 can activate the expression of SF1 (Steger et al., 1994) which in turn stimulates αGSU and LHβ expression, but not FSHβ expression (Brown and McNeilly, 1999). *In vitro* cell transfection assays suggest that GATA2 can regulate the human *αGSU* gene in αT3 gonadotrope cells (Steger et al., 1994) and that

inhibition of endogenous GATA2 and GATA4 expression decreases expression of the *LHβ* gene in LβT2 cells (Lo et al., 2011). These studies indicate a role for the GATA2 transcription factor in regulating LHβ expression. Pituitary-specific *Gata2* knockout mice are smaller than wild type littermates and contain low levels of thyrotrope and gonadotrope cell lineages after birth (Charles et al., 2006). *αGSU* promoter-DTA tox176 mice have complete ablation of transcripts of αGSU, TSHβ, and LHβ using *in situ* hybridization detection (Burrows et al., 1996). However, neither αGSU nor GATA2 positive cells are completely ablated in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice. Taken together, this data suggests the interpretation that GATA2 action, either directly or indirectly, is related to the actions of LHX3 mediated by the 3′ gene enhancer in the caudomedial region of the AP. However GATA2-, αGSU- and TSHβ-expressing cells in the rostral tip area of the AP are independent of LHX3 expression guided by the 3′ *LHX3* enhancer. The results of this study indicate that the enhancer ablation of caudomedial GATA2 cells leads to absent or low populations of thyrotropes and gonadotropes, especially LH-expressing cells at later embryonic time points. The corticotropes, somatotropes, and lactotropes appear to be mostly unchanged in pattern and number in the developing anterior pituitary.

Bioinformatic searches of potential GATA2 binding sites in 3′ enhancer region of the human *LHX3* were performed with programs such as MatInspector (Quandt et al., 1995). There are several putative GATA binding sites in the 3′ enhancer region. Further research will be needed to test the role of GATA factor such as GATA2. Experiments might include *in vivo* luciferase assays using LβT2 mouse gonadotrope cells to test if GATA2 can increase luciferase activity when co-transfected with 3′ enhancer-luciferase constructs. Subsequent mutation of possible GATA2 binding sites would be needed to

test whether these GATA2 binding sites are important for gene transcription and which GATA2 sites are necessary. *In vitro* gel shift assay may also be needed to confirm GATA2 binds to the possible GATA binding sites in the 3′ enhancer. Chromatin immunoprecipitation assays could be performed using embryonic mouse pituitary tissue or culture pituitary cells to determine whether GATA factors are present at the indicated enhancer sequences. Further to test the function of these GATA2 binding sites using *in vivo* analysis, putative GATA2 binding sites will be mutated in the enhancer in transgenic mouse models such as enhancer-LacZ mice.

From our data, LHβ cells are typically bi-hormonal cells and this bi-hormonal population is ~45% of all gonadotrope cells in the developing pituitaries normal mice. In addition, I tested SF1 expression in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice. SF1 is known to act downstream of *Gata2* and SF1 expression is reduced following ablation of GATA2 (Charles et al., 2006). In normal pituitaries, virtually all LHβ-positive cells are SF1-positive. By contrast, FSHβ-expressing cells are not always SF1-expressing and a few pituitary cells (less than 5% of total SF1-positive cells) are SF1-positive but do not express either LHβ or FSHβ (data not shown). We assume that these SF1-only expressing cells are gonadotrope precursor cells which may develop after birth or during puberty. Out data suggest that the SF1 transcription factor expression is reduced in the remaining bi-hormonal gonadotropes in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice.

Previous studies have shown that LHX3 is expressed in the developing spinal cord and is involved in MN and V2 IN specification (Tsuchida et al., 1994; Sharma et al., 1998; Thaler et al., 2002; Al-Mosawie et al., 2007; Peng et al., 2007). For example, in *Lhx3::Cre*; *ROSA26::YFP* mice: a $Lhx3^{Cre}$ mice has been generated by inserting Cre

cassette into the 3′ UTR of the *Lhx3* gene (Sharma et al., 1998), YFP is expressed in a subset of V2 INs as well as in MNs at P0 (Sharma et al., 1998; Al-Mosawie et al., 2007; Lundfald et al., 2007; Peng et al., 2007). Mice lacking the *Lhx3* gene fail to generate proper MNs and V2 INs (Sharma et al., 1998). LHX3 appears to be a primary transcription factor required to direct specification of V2 INs by forming V2-tetramer complex; however MN specification requires both ISL1 and LHX3 in the MN hexamer complex (Thaler et al., 2002). Our previous study demonstrated that the 3′ *LHX3* enhancer region contains multiple nervous system-specific enhancer modules and transcriptional activity guided by the 3′ enhancer is similar to the time frame of endogenous LHX3 expression in the spinal cord at e9.5 (Mullen et al., 2012).

In this study, we showed by 3′ enhancer-Cre-mediated DTA ablation that endogenous LHX3 expression is reduced in mediolateral portion of the mid cervical region of sagittal spinal cord sections. Also only few LHX3 positive cells are detected in the mouse V2a (CHX10-positive; GATA2-negative) glutamatergic excitatory INs (Kimura et al., 2006; Lundfald et al., 2007; Peng et al., 2007) area suggesting that the 3′ human *LHX3* enhancer guides LHX3 expression in this spinal cord cell type. Similar V2a IN expression data have been described for transgenic animals expressing a *Chx10* promoter-DTA transgene (Crone et al., 2008; Crone et al., 2009). The number of MNs, as determined by HB9 and ISL1 co-expression, were not significantly reduced but $ISL1^{+}$ INs were notably reduced in $ELHX3^{Cre/+}/R26^{DTA/+}$ mice. These data suggest that 3′ *LHX3* enhancer mediated expression of LHX3 is necessary for V2a IN expression but not MN expression. In *Lhx3* null mice, ISL1 is still activated at e9.5 (Sheng et al., 1997) and suggests that LHX3 is not crucial element for ISL1 expression. It is possible the ISL1

alone expression is sufficient to activate MNs in the developing spinal cord or other *trans*-acting factors to *LHX3* gene maybe more critical than LHX3 expression guided by 3′ enhancer. We conclude that other gene regulatory elements are required for LHX3 expression in MNs.

In this study, we use $R26^{DTA}$ mice containing a gene encoding an attenuated diphtheria toxin A subunit, which has a point mutation that results in a glycine to aspartic amino acid substitution at residue 128 (Maxwell et al., 1987). This single amino acid substitution makes DTA ~30-fold less active than native DTA (Breitman et al., 1990). Attenuated DTA has been used to study cell lineage in the pancreas, pituitary, lens, etc., [reviewed in (Saito et al., 2001)]. Studies have shown that transgenic mouse models which express attenuated DTA guided by the pituitary αGSU promoter did not affect viability and αGSU cells were ablated; LHβ and TSHβ cells were absent, but GH cells were not changed (Burrows et al., 1996). Few studies have compared the native diphtheria toxin and the attenuated version (Maxwell et al., 1987; Lang and Bishop, 1993; Lowell et al., 1993). These studies have suggested that the attenuated toxin is able to ablate specific cells without killing many of the transgenic founders. This led to the interpretation that our data using DTA mouse model have less bystander cell killing.

The *LHX3* 3′ enhancer-mediated DTA cell ablation mouse ($ELHX3^{Cre/+}/R26^{DTA/+}$) model displays extremely low viability (Table 3.1). $ELHX3^{Cre/+}/R26^{DTA/+}$ mice have prenatal and postnatal loss. The low viability of $ELHX3^{Cre/+}/R26^{DTA/+}$ mice may have several causes include loss of essential LHX3 functions in the pituitary and especially the nervous system. Similar pre- and post-natal loss data have been found in *Chx10* promoter-DTA transgenic mice (Crone et al., 2008): mice died shortly after birth or

rarely surviving mice were small and exhibited defects in left-right coordination. These authors also found that survival of Chx10::DTA mice is strain dependent (Crone et al., 2009). Furthermore, loss of viability and phenotypic differences associated with genetic background have been shown in mouse models of pituitary disease (Nasonkin et al., 2004; Prince et al., 2013), kidney disease (Han and Strohl, 2000), and V2a interneuron ablation (Bradley et al., 2006; Crone et al., 2009), for example. The *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice described here are on a C3HeB/FeJ + C3H genetic background. It is possible that different genetic backgrounds may result in higher viability or different experimental results. 3′ enhancer-directed DTA target cells are present not only in the pituitary but also in the nervous system, and these ablated cells both in the pituitary and spinal cord may induce multiple developmental defects during organogenesis. However the exact cause of *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice phenotype is unknown. One of the possible explanations for low viability in *ELHX3*$^{Cre/+}$*/R26*$^{DTA/+}$ mice is that loss of proper interneuron cells or loss of the facial/vestibuloacoustic ganglion complex (or loss of cells in ectopically-expressed locations) may cause nervous system defects.

This study describes cell-specific actions of a downstream enhancer of the human *LHX3* gene during development of the anterior pituitary and spinal cord. This data strongly suggests that the 3′ enhancer region is required for the proper expression of LHX3 in specific hormonal cells and neural cells. The 3′ *LHX3* enhancer region is important for LHX3 expression in GATA2-positive pre-TSHβ, LHβ, and FSHβ hormone cells, and V2a INs. This implies it functions in their differentiation by precisely controlling LHX3 expression. Specifically LHX3 expression guided by the 3′ enhancer is in αGSU-positive/GATA2-positive developing gonadotropes and caudomedial-

thyrotropes, which are located in the caudomedial region of the anterior pituitary. We assume these cells co-express with LHX3. However GH, PRL, ACTH, and rostral tip αGSU- and TSHβ-expressing cells are not directly activated by the 3′ enhancer-guided LHX3 expression. Additionally, excitatory V2a INs exhibit 3′ enhancer-guided LHX3 expression in the developing spinal cord. My studies suggest that action of the 3′ enhancer plays critical roles in guiding LHX3 expression and subsequent specification of specific hormone and neuronal cells during embryogenesis.

It is possible that mutations in non-coding regulatory regions of the human *LHX3* gene might underlie uncharacterized cases of *LHX3*-associated CPHD. To date, in collaborations with pediatric endocrinologists, we have not discovered any genetic changes in *LHX3* gene transcriptional regulatory regions that might cause such disease but our efforts to characterize the molecular genetic basis of *LHX3*-associated CPHD continue. The *ELHX3*^{Cre} mouse model will serve as an important tool in these efforts. Uncovering cell-specific regulation of *LHX3* enhancer activity in the pituitary and nervous system will provide a clear understanding of the biology of the human *LHX3* gene on a cellular level.

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CURRICULUM VITAE

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Dissertation: In vivo Analysis of Human *LHX3* Gene Enhancer Regulation

Advisor: Simon J. Rhodes Ph.D.

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Master of Science in Engineering, Biotechnology

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Thesis: Studies on Antibody Interaction-mediated Protein Folding *in vitro*

Bachelor of Science, Molecular Biology

Dankook University, Seoul, South Korea

Research Experiences

01/2007-09/2007 **Institut Pasteur, Seoul, Korea**

Research Associate in Nano-Biotechnology group

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Primary research project was purifying antifreeze proteins from Arctic and Antarctic psychrophilic bacteria and algae.

Isolated antifreeze protein by 2-DE electrophoresis and purified by chromatography.

03/2003-02/2005 **Graduate School of Biotechnology at Yonsei University**

Main research project was measuring tagged or fusion protein folding rate with its specific antibody.

Involved in a research project supported by Microbial Genomics & Applications Center, Development of the Cisperone Platform Technology for High Throughput Protein Expression

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Publications and Patent

PUBLICATIONS

Cell-specific actions of a downstream enhancer of the human *LHX3* gene during development of the anterior pituitary and spinal cord
Park, S., Mullen, R.D., Rhodes, S.J.
In preparation

Developmental analysis of influence of genetic background on the *LHX3*W227Ter mouse model of combined pituitary hormone deficiency disease
Prince, K.L., Colvin, S.C., **Park, S.**, Lai, X., Witzmann, F.A., Rhodes, S.J.
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PATENT

Antifreeze activity from Polar Microalgae *Porosira* sp. Patent No.10-2006-0001585

Abstracts

Park, S., Mullen, R.D., and Rhodes, S.J. (2012)
Multiple Enhancers Regulate Expression of the Human *LHX3* gene in the Developing Pituitary, 15th International Congress of Endocrinology, Florence, Italy

Malik, R.E., **Park, S.**, Bechtold-Dalla Pozza, S., Hiedl, S., Roeb, J., Lohse, P., Durán-Prado, M., and Rhodes, S.J. (2012)
A Recessive Mutation Resulting in a Disabling Amino Acid Substitution (T194R) in the LHX3 Homeodomain Causes Combined Pituitary Hormone Deficiency. 15th International Congress of Endocrinology, Florence, Italy

Park, S., Mullen, R.D., and Rhodes, S.J. (2012)
Multiple Enhancers Regulate Expression of the Human *LHX3* gene in the Developing Pituitary. Indiana University School of Medicine Department of Biochemistry and Molecular Biology Annual Research Day, Indianapolis

Park, S., Mullen, R.D., and Rhodes, S.J. (2011)
Multiple Enhancer Regulate Temporal and Spatial Expression of the Human *LHX3* Gene in the Developing Endocrine and Nervous System. Society for Developmental Biology, Chicago

Mullen, R.D., **Park, S.**, and Rhodes, S.J. (2010)
Multiple Cis-Acting Enhancers Regulate Temporal and Spatial Expression of the Human *LHX3* Gene in the Developing Endocrine and Nervous System. Endocrine Society Meeting, San Diego

Mullen, R.D., **Park, S.**, and Rhodes, S.J. (2009)
Distal Enhancer Regulate Temporal and Spatial Expression of the Human *LHX3* Gene in the Developing Endocrine and Nervous System. Indiana University School of Medicine Department of Cellular and Integrative Physiology Department Retreat, Indianapolis

Park, S., Lee, J.M., and Kang, S.H. (2006)
Optimal Growth Conditions and Protein Expression of Psychrophilic Bacteria KOPRI ArB0140 *Moritella* sp. International Cryobiology Meeting, Hamburg, Germany

Park, S., Lee, J.M., and Kang, S.H. (2006)
Protein Expression of Psychrophilic Bacteria, KOPRI-ArB0144 *Moritella* sp. International Polar Science Symposium, Seoul, Korea

Park, S., Choi, S., and Seong, B.L. (2004)
Aminoacyl tRNA synthetase as RNA-mediated Intra-cellular Chaperone. International Conference on Amino-acyl tRNA Synthetases: Ancient Molecules for Future Biology and Medicine, Seoul, Korea

Park, S., Choi, S., and Seong, B.L. (2004)
Studies on Antibodies as Potential Mediator of Protein Folding *in vitro*. New Challenges in Functional Microbiology and Biotechnology, Daegu, Korea

Fellowships and Awards

Biochemistry and Molecular Biology Department Research Day
Excellent Poster Award
Indiana University School of Medicine, 2012

Cellular and Integrative Physiology Research Day
Abstract Award
Indiana University School of Medicine, 2011

Teaching Assistant Fellowship
Yonsei University, 2004

Scholarship for Excellent Student
Yonsei University, 2003 to 2004

Full Scholarship
Dankook University, 1998 to 2002

Research Techniques

Software	Image Master by Unicon V5.10, Vector NTI
Protein Analyses	EMSA assay, western blotting, SDS-PAGE, protein purification and isolation (2DE), protein overexpression (tagged or fusion protein) Equipments: ÄKTA FPLC system (gel filtration, ion exchange, affinity, desalting) HPLC, Ettan IPGphor II IEF system

Murine Transgenesis	Transgene construction, Cre/lox technology
Histology	Mouse embryo and tissue harvesting, cryosection, immunohistochemistry, immunofluorescence microscopy
Molecular work	DNA cloning, site-directed mutagenesis, ChIP analysis, cDNA synthesis, quantitative PCR analysis of gene expression
Cell culture	Cell line maintenance, transient transfection, reporter gene analysis (luciferase)