Graduate School ETD Form 9
(Revised 12/07)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Stephanie C. Colvin

Entitled An Animal Model of Pediatric Combined Pituitary Hormone Deficiency Disease

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

Stephen F. Konieczny Chair	S.J. Rhodes
Emily Walvoord	Teri Belecky-Adams
B.P. Herring	Randall Roper

To the best of my knowledge and as understood by the student in the *Research Integrity and Copyright Disclaimer (Graduate School Form 20)*, this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Approved by Major Professor(s): S.J. Rhodes

Approved by: N. Lees 6/9/10
Head of the Graduate Program Date

Graduate School Form 20
(Revised 1/10)

PURDUE UNIVERSITY
GRADUATE SCHOOL

Research Integrity and Copyright Disclaimer

Title of Thesis/Dissertation:
An Animal Model of Pediatric Combined Pituitary Hormone Deficiency Disease

For the degree of Doctor of Philosophy

I certify that in the preparation of this thesis, I have observed the provisions of *Purdue University Teaching, Research, and Outreach Policy on Research Misconduct (VIII.3.1)*, October 1, 2008.*

Further, I certify that this work is free of plagiarism and all materials appearing in this thesis/dissertation have been properly quoted and attributed.

I certify that all copyrighted material incorporated into this thesis/dissertation is in compliance with the United States' copyright law and that I have received written permission from the copyright owners for my use of their work, which is beyond the scope of the law. I agree to indemnify and save harmless Purdue University from any and all claims that may be asserted or that may arise from any copyright violation.

Stephanie C. Colvin
Printed Name and Signature of Candidate

06/12/10
Date (month/day/year)

*Located at http://www.purdue.edu/policies/pages/teach_res_outreach/viii_3_1.html

AN ANIMAL MODEL OF COMBINED PITUITARY HORMONE DEFICIENCY DISEASE

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Stephanie C. Colvin

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

August 2010

Purdue University

Indianapolis, Indiana

I dedicate this dissertation to my family, especially…

To my Dad, for instilling his favorite motto “Endeavor to Persevere”

To my Mom, without your love, wisdom, and encouragement, I would not be the person I am today

To my brother, Dustin, who continues to teach me aspects about life in ways no one else can

To my husband, Scott, for your love, encouragement, and understanding

To my daughter, Cate, for being so wonderfully you

ACKNOWLEDGMENTS

It is a privilege to acknowledge the members of my graduate committee: Dr. Stephen Konieczny, Dr. Simon Rhodes, Dr. Paul Herring, Dr. Teri Belecky-Adams, Dr. Randall Roper, and Dr. Emily Walvoord. Your insight and advice was extremely valuable to my success throughout my graduate education. I would also like to extend my gratitude to my friends and colleagues in the Rhodes lab, both past and present: Dr. Jesse Savage, Dr. Chad Hunter, Rachel Mullen, Tafadzwa Mwashita, Marin Garcia, Dr. Zachary Neeb, Christine Hammer, Aaron Showalter, Qi 'Sophia' Liu, Krystal Renner, Brooke West, Dr. Kyle Sloop, Soyoung Park, Raleigh Malik, and Dr. Kelly Prince. I want to thank members of the departments where the Rhodes lab has called home, Dr. N. Douglas Lees, Dr. Dring Crowell, Dr. Kathleen Marrs, Dr. Guoli Dai, Suzanne Merrel, and Erin McDaniel of the IUPUI Department of Biology, and Tracy McWilliams, Marlene Brown, Joyce Lawrence, Dr. David Basile, Dr. Glenn Bohlen, Dr. Richard Day, Dr. Patricia Gallagher, Dr. Steven Kempson, Dr. Frederick Pavalko, Dr. Michael Sturek, Dr. Patrick Fueger, Dr. Christine Quirk, Dr. Suzanne Young, Dr. Min Zhang, Emily Blue, Rebekah Jones, Ketrija Touw, Dr. Ryan Widau, and Dr. Jiliang 'Leo' Zhou of the IU Department of Cellular and Integrative Physiology. Finally, it is a pleasure to thank my friend and mentor, Dr. Simon Rhodes, without whose advice and encouragement the completion of this dissertation would have been impossible. Thank you, Simon, for your

sagacity, your guidance, allowing me the freedom to think independently, and for being such a strong advocate for me.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Adrenocorticotropic hormone	ACTH
Alpha glycoprotein subunit	αGSU
Alpha melanocyte-stimulating hormone	αMSH
Arginine vasopressin	AVP
Base pair	bp
Bone morphogenetic protein	BMP
Calf intestinal alkaline phosphatase	CIAP
Central nervous system	CNS
Combined pituitary hormone deficiency	CPHD
Complementary DNA	cDNA
Corticotropin-releasing hormone	CRH
Days post coitum	dpc
Diaminobenzidine	DAB
Diphtheria toxin-A	DTA
Embryonic stem cells	ES cells
Enzyme-linked immunosorbant assay	ELISA
Fibroblast growth factor	FGF
Follicle-stimulating hormone	FSH

Follicle-stimulating hormone beta	FSHβ
Forkhead box	FOX
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Gonadotropin-releasing hormone	GnRH
Gonadotropin-releasing hormone receptor	GnRH-R
Growth hormone	GH
Growth hormone-releasing hormone	GHRH
Growth hormone-releasing hormone receptor	GHRHR
High mobility group	HMG
Holoprosencephaly	HPE
Homeodomain	HD
Immunohistochemistry	IHC
Insulin growth factor-1	IGF-1
Kilobases	kb
Kilodaltons	kDa
Luria-Bertani broth	LB
LIM homeobox 3	LHX3
LIM homeobox 4	LHX4
LIM homeodomain	LIM-HD
Luteinizing hormone	LH
Luteinizing hormone beta	LHβ
Magnetic resonance imaging	MRI
Nuclear factor-1	NF1

Open reading frame	ORF
Oral ectoderm	OE
Orthodenticle homeobox 2	OTX2
Oxytocin	OT
Paired homeobox	PAX
Phosphate buffered saline	PBS
Phosphoglycerate kinase	PGK
Polymerase chain reaction	PCR
Pro-opiomelanocortin	POMC
Prolactin	PRL
Prophet of Pit-1	PROP1
Reverse transcriptase-PCR	RT-PCR
Sex-determining region Y	SRY
Sine oculis homeobox	SIX
Sonic hedgehog	SHH
SRY-box	SOX
Specificity protein-1	SP-1
Steroidogenic factor 1	SF1
Thyroid-stimulating hormone	TSH
Thyroid-stimulating hormone beta	TSHβ
Thyrotropin-releasing hormone	TRH
Triiodothyronine	T_3

Thyroxine	T_4
Wingless/integrated protein	WNT

ABSTRACT

Colvin, Stephanie C. Ph.D., Purdue University, August 2010. An Animal Model of Pediatric Combined Pituitary Hormone Deficiency Disease. Major Professor: Simon J. Rhodes.

LHX3 is a LIM-homeodomain transcription factor that has essential roles in pituitary and nervous system development in mammals. Children who are homozygous for recessive mutations in the *LHX3* gene present with combined pituitary hormone deficiency disease (CPHD) characterized by deficits of multiple anterior pituitary hormones. Most *LHX3* patients also present with additional defects associated with the nervous system including a characteristic limited head rotation and sometimes deafness. However, of the 10 types of *LHX3* mutation described to date, one mutation type (W224ter) does not result in the limited head rotation, defining a new form of the disease. W224ter patients have CPHD but do not have nervous system symptoms. Whereas other mutations in *LHX3* cause loss of the entire protein or its activity, the W224ter mutation causes specific loss of the carboxyl terminal of the LHX3 protein—a region that we have shown to contain critical regulatory domains for pituitary gene activation. To better understand the molecular and cellular etiology of CPHD associated with *LHX3* gene mutations, I have generated knock-in mice that model the human *LHX3* W224ter disease.

The resulting mice display marked dwarfism, thyroid disease, female infertility, and reduced male fertility. Immunohistochemistry, real-time quantitative polymerase chain reaction (PCR), and enzyme-linked immunosorbant assays (ELISA) were used to measure hormones and regulatory factor protein and RNA levels, an approach which is not feasible with human patients. We have generated a novel mouse model of human pediatric CPHD. Our findings are consistent with the hypothesis that the actions of the LHX3 factor are molecularly separable in the nervous system and pituitary gland.

CHAPTER ONE

INTRODUCTION

1.1 The Pituitary Gland and the Physiological Importance of Its Hormones

The pituitary gland, or hypophysis, is a composite organ located at the base of the brain within a bony pocket termed the sella turcica. This endocrine organ is derived from two different embryonic origins. The neurohypophysis is derived from the ventral diencephalon and is therefore neuroectodermal in origin. It includes the pars nervosa (posterior pituitary), the infundibular stalk, and the median eminence. At the same time during development, an invagination of the oral ectoderm into a rudimentary structure, termed Rathke's pouch, gives rise to the development of the adenohypophysis. The adenohypophysis consists of the pars distalis (anterior pituitary), the pars intermedia (intermediate pituitary), and the pars tuberalis (Figure 1.1).

These two different embryonic structures give rise to the three different lobes of the pituitary gland, and each lobe has its own physiological functions. The posterior lobe of the pituitary is composed of neuronal axons that descend from the hypothalamus. Two different peptide hormones, arginine vasopressin (AVP) and oxytocin (OT), are synthesized within the hypothalamus and exported to the posterior pituitary for secretion. Neural signals from the hypothalamus initiate the release of these hormones from the

posterior pituitary into the bloodstream. Arginine vasopressin, or anti-diuretic hormone, serves to control water retention within the body, while oxytocin has roles in lactation and uterine contraction during childbirth. The intermediate lobe of the pituitary is responsible for the production and secretion of α-melanocyte-stimulating hormone (αMSH), which has roles in skin pigmentation. While a more defined role for the intermediate lobe has been identified in amphibians, the human intermediate lobe appears to be less important as it is sometimes absent in adults, and when present, consists only of a thin layer of cells between the posterior and anterior lobes. The anterior lobe of the pituitary contains five hormone-secreting cell types that are responsible for the secretion of six different hormones. The corticotropes secrete adrenocorticotropin (ACTH), which is a product of the pro-opiomelanocortin (*POMC*) gene and acts on the adrenal glands to assist the body in its response to stress. The gonadotropes discharge both luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both of which function to regulate the development, growth, and maturation of the reproductive system. The somatotropes release growth hormone (GH), which has a role in metabolism in addition to the regulation of the growth of muscle, bone, and other organs. The thyrotropes produce thyroid-stimulating hormone (TSH), which acts on the thyroid to promote the production and secretion of thyroxine (T_4) and triiodothyronine (T_3). The lactotropes secrete prolactin (PRL), which plays a major role in lactation (Figure 1.1). Three of these hormones, TSH, LH, and FSH, are heterodimeric in nature and consist of a common α-glycoprotein subunit (αGSU) and a unique β-subunit (TSHβ, LHβ, and FSHβ). The release of these hormones from the anterior pituitary is tightly regulated through the secretion of both inhibiting and releasing hormones from the hypothalamus. These

inhibiting and releasing hormones are released into the blood stream from the hypothalamus and travel to the anterior pituitary where they bind to the surface membrane receptors of the hormone-secreting cells to promote or inhibit the secretion of those hormones (Figure 1.1). For example, once corticotropin-releasing factor is secreted from the hypothalamus, it travels to the anterior pituitary where it binds to the corticotropin-releasing factor receptor on the corticotrope cells to promote the release of ACTH.

1.2 Signaling Events and Transcription Factors That Regulate the Development of the Pituitary

Numerous studies across several different species have shown that inductive signaling events between the ventral diencephalon and Rathke's pouch must occur for proper pituitary development (Figure 1.2) (Daikoku, Chikamori et al. 1982; Watanabe 1982; Watanabe 1982; Watanabe 1982; Kawamura and Kikuyama 1995; Gleiberman, Fedtsova et al. 1999). Diffusible signaling molecules produced by the ventral diencephalon and the oral ectoderm that have been found to have significant effects on the induction and growth of Rathke's pouch include members of the bone morphogenetic protein (BMP), fibroblast growth factor (FGF), wingless/integrated (WNT), and hedgehog families. Extrinsic signals and transcription factors expressed from the ventral diencephalon and developing infundibulum important for pouch development include BMP4, FGF8, FGF10, FGF18, WNT5a, SOX3 and NKX2.1 (Kimura, Hara et al. 1996; Ericson, Norlin et al. 1998; Takuma, Sheng et al. 1998; Norlin, Nordstrom et al. 2000; Ohuchi, Hori et al. 2000; Alatzoglou, Kelberman et al. 2009). Sonic hedgehog (SHH) is another extrinsic signal

necessary for early pituitary development. SHH expression is absent in the cells of oral ectoderm that invaginate to form Rathke's pouch; however, its expression is maintained in the ventral diencephalon and the oral ectoderm surrounding Rathke's pouch during early pituitary development (Treier, Gleiberman et al. 1998; Treier, O'Connell et al. 2001). Intrinsic signals within the pouch itself are also important for the proper development of the pituitary. WNT4 is expressed in the oral ectoderm and developing Rathke's pouch in addition to BMP2 and BMP7 also being expressed in the pouch during early pituitary development (Ericson, Norlin et al. 1998; Treier, Gleiberman et al. 1998). Rathke's pouch eventually separates from the oral ectoderm with the dorsal cells of the pouch destined to populate the intermediate lobe of the pituitary and the ventral cells of the pouch acquiring an anterior pituitary cell fate. The combination of these extrinsic and intrinsic signaling molecules establishes opposing dorsal-to-ventral and ventral-to-dorsal signaling gradients. These opposing signaling gradients prime transcription factors to be expressed in a spatial manner within the developing pouch and set up the stratification of the developing hormone-secreting cell types with the corticotropes, somatotropes, and lactotropes appearing in the dorsal side of the anterior lobe, the thyrotropes located in the central part of the lobe, and the gonadotropes occupying the ventral part of the anterior lobe (Dasen, O'Connell et al. 1999; Kioussi, O'Connell et al. 1999; Scully and Rosenfeld 2002). While this regionalization of the hormone-secreting cell types of the anterior pituitary persists in birds, teleost fish, amphibians, and reptiles, it is not retained in adult mammalian pituitaries.

The specification of the unique identities of the hormone-secreting cell types within the anterior pituitary is dependent on a transcription factor cascade that is

facilitated by the signaling gradients established between the ventral diencephalon and Rathke's pouch (Figure 1.2). Several transcription factors have been found to be requisite for the appropriate development and maintenance of the mature anterior pituitary gland including GLI2, OTX2, SOX2, PAX6, SIX3, SIX6, PITX1, PITX2, HESX1, LHX3, LHX4, PROP1, PIT-1, and SF1.

GLI2 is a member of the GLI family of transcription factors which are known as mediators of SHH signaling in vertebrates (Ruiz i Altaba, Palma et al. 2002). The GLI transcription factors are composed of a centrally located DNA-binding domain, a transcription activation domain located within the carboxyl terminus of the protein, and 5 C2-H2 zinc fingers. GLI2 has a broad expression pattern throughout most of the mesoderm and ectoderm early in development and is later expressed in the developing somites and limbs (Mo, Freer et al. 1997). It has important roles in the appropriate development of a variety of systems including the developing nervous system, the developing skeletal system, and limb buds and can act as both a transcriptional activator and repressor (Mo, Freer et al. 1997; Aza-Blanc, Lin et al. 2000; Roessler, Du et al. 2003). Mice homozygous for a null allele of the *Gli2* gene die embryonically with defects in the development of the brain and spinal cord, no floor plate, and some mild craniofacial defects (Mo, Freer et al. 1997; Ding, Motoyama et al. 1998; Matise, Epstein et al. 1998; Park, Bai et al. 2000). Mutations within the human *GLI2* gene have been found in a heterozygous state and are inherited in an autosomal dominant manner. The mutations appear to ablate the function of the *GLI2* allele in which they are found. These patients present with varying forms of holoprosencephaly (HPE) with the pituitary and facial structures the most sensitive to the reduction in GLI2 activity as the common

features between these patients were hypoplastic/aplastic anterior pituitaries and variable craniofacial abnormalities (Roessler, Du et al. 2003).

The Orthodenticle homeobox 2 (*OTX2*) gene encodes a paired-class homeodomain transcription factor and is expressed in the neuroepithelium of most of the forebrain and midbrain during development (Simeone, Acampora et al. 1993). Targeted disruptions within the murine *Otx2* gene result in defects in gastrulation and no anterior head or brain structures leading to embryonic lethality (Acampora, Mazan et al. 1995; Ang, Jin et al. 1996). *Otx2* expression has also been found in the developing eye, pituitary, hypothalamus, brain, and thalamus, and human patients with mutations within the *OTX2* gene display variable phenotypes representing abnormalities within these regions (Ragge, Brown et al. 2005; Hever, Williamson et al. 2006; Dateki, Fukami et al. 2008; Diaczok, Romero et al. 2008; Wyatt, Bakrania et al. 2008; Henderson, Williamson et al. 2009; Tajima, Ohtake et al. 2009; Dateki, Kosaka et al. 2010). OTX2 appears to have a role in pituitary development as it is expressed in the developing pituitary, some human patients with mutations within the *OTX2* gene display pituitary insufficiency, and *in vitro* studies have shown that OTX2 is capable of binding and activating transcription from the *HESX1*, *PIT1*, and *GNRH1* promoters (Diaczok, Romero et al. 2008; Henderson, Williamson et al. 2009; Tajima, Ohtake et al. 2009; Dateki, Kosaka et al. 2010).

A member of the SRY-related high mobility group (HMG) box family of transcription factors, SOX2, has been shown to have important roles early in pituitary development. *Sox2* is expressed early in mouse development with roles in the developing CNS, sensory placodes, branchial arches, and gut endoderm including the esophagus, the

trachea, and the inner ear (Collignon, Sockanathan et al. 1996; Wood and Episkopou 1999; Williamson, Hever et al. 2006; Hume, Bratt et al. 2007). *Sox2* expression is found throughout Rathke's pouch as early as e11.5 in the developing mouse embryo; however, by e18.5, its expression is found scattered throughout the pouch and within the proliferating cells of the dorsal zone (Kelberman and Dattani 2006). In the adult anterior pituitary, *Sox2* expression is maintained in a small population of cells lining the pituitary cleft and scattered throughout the parenchyma and represent progenitor cells within the pituitary as they are able to differentiate into all pituitary cell types. Cells that continue to express *Sox2* in the adult pituitary gland are proposed to have a role in the plasticity of the gland in its response to changing physiological demands (Fauquier, Rizzoti et al. 2008). Disruptions within the murine *Sox2* gene result in some embryonic and perinatal lethality (Avilion, Nicolis et al. 2003; Ferri, Cavallaro et al. 2004). The surviving mice demonstrate the importance of *Sox2* in the developing nervous system and pituitary with defects in behavior, various brain structures, and bifurcated Rathke's pouch with decreases in somatotropes and gonadotropes (Kelberman and Dattani 2006; Alatzoglou, Kelberman et al. 2009). Mutations within the human *SOX2* gene are associated with bilateral anophthalmia, or severe microphthalmia, developmental delay, esophageal atresia, sensorineural hearing loss, and genital abnormalities in male patients in addition to hypoplastic anterior pituitaries. Although the patients present with hypoplastic pituitaries, they all have a normal GH response and are diagnosed with isolated gonadotropin deficiency (Alatzoglou, Kelberman et al. 2009).

PAX6 is a member of the conserved paired homeodomain PAX transcription factor family. Transcription of the *Pax6* gene leads to the production of a transcription

factor containing both a paired DNA-binding domain and a homeodomain. PAX6 expression is found in the developing eye, nervous system, pancreas, and pituitary, and studies in both human patients and mouse models with mutations in *PAX6* show that it appears to be necessary for the proper development of those structures (Bentley, Zidehsarai et al. 1999; Terzic and Saraga-Babic 1999; Dohrmann, Gruss et al. 2000). In the developing pituitary, PAX6 plays a role in the dorsal-to-ventral signaling gradient that establishes the proper cell-type development within the anterior pituitary. In mice lacking any functional *Pax6* alleles, this dorsal-to-ventral gradient is disrupted and these mice exhibit significantly reduced numbers of the dorsal somatotropes and lactotropes while there is an increase in ventral thyrotropes and gonadotropes (Kioussi, O'Connell et al. 1999).

Members of the *SIX* family of transcription factors were identified through their homology to the *Drosophila sine oculis* homeobox gene. Two members of this family, *Six3* and *Six6*, appear to have roles in pituitary development (Cheyette, Green et al. 1994). *Six3* expression is found within the anterior neural plate early in development, and later in derivatives of the anterior neural plate, including the ectoderm of the nasal cavity, the olfactory placode, Rathke's pouch, and regions of the optic recess, hypothalamus, optic vesicles, retina, and lens placode (Oliver, Mailhos et al. 1995; Bovolenta, Mallamaci et al. 1998). The exact role of *Six3* in pituitary development has been difficult to elucidate as Rathke's pouch is never induced in $Six3^{-/-}$ mice (Lagutin, Zhu et al. 2003). Recently, however, severe pituitary dysmorphogenesis resulting in hypopituitarism has been observed in $Six3^{+/-}$; $Hesx1^{Cre/-}$ doubly heterozygous mice demonstrating that *Six3* plays an essential role in normal pituitary development (Gaston-Massuet, Andoniadou et

al. 2008). Human patients with mutations within the homeodomain-encoding region of the *SIX3* gene present with varying forms of holoprosencephaly (HPE), which is a severe malformation involving failure of the brain to separate into left and right halves (Wallis, Roessler et al. 1999). *Six6* expression is more restricted than the expression of *Six3*, and is found in the developing hypothalamus, pituitary, neural retina, optic chiasm, and optic stalk (Jean, Bernier et al. 1999). Retinal hypoplasia, optic nerve defects, and pituitary hypoplasia are observed in animals lacking SIX6. A role for SIX6 has also been found during development in the repression of genes that encode cell cycle inhibitors suggesting that SIX6 promotes the proliferation of precursor cells within the developing retina and pituitary (Li, Perissi et al. 2002).

Two bicoid-related homeodomain transcription factors, PITX1 and PITX2, play important roles in several developmental pathways including limb, heart, and pituitary organogenesis (Drouin, Lamolet et al. 1998; Gage, Suh et al. 1999). In pituitary development, PITX1 was first identified as a protein partner capable of synergistically activating pituitary target genes with another homeodomain transcription factor, PIT-1. It was also determined that PITX1 has a role in the activation of the *POMC* gene in corticotrope cells (Szeto, Ryan et al. 1996; Tremblay, Lanctot et al. 1998). Expression of PITX1 occurs early in pituitary development, and studies have shown that it is able to activate transcription from several pituitary hormone promoters including *PRL*, *LHβ*, *FSHβ*, *TSHβ*, *GnRHR*, and *GH* (Lamonerie, Tremblay et al. 1996; Szeto, Ryan et al. 1996; Lanctot, Lamolet et al. 1997; Tremblay, Lanctot et al. 1998; Lanctot, Gauthier et al. 1999; Jeong, Chin et al. 2004). Synergy between PITX1 and other pituitary transcription factors, including SF1 and PIT-1, has to occur for the proper activation of its

target genes (Tremblay, Lanctot et al. 1998). *In vitro* and *in vivo* experiments have shown PITX1 to have a role in the proper expression of LHX3 and αGSU (Tremblay, Lanctot et al. 1998; Charles, Suh et al. 2005). Mice lacking any functional PITX1 protein support these data with defects in gonadotrope, thyrotrope, and corticotrope development as well as defects in hindlimb and craniofacial development (Lanctot, Moreau et al. 1999; Szeto, Rodriguez-Esteban et al. 1999; Charles, Suh et al. 2005).

PITX2 is also important in pituitary development as evidenced by *Pitx2*$^{-/-}$ knockout mice. *Pitx2* null mice display abnormalities in pituitary development in addition to abnormalities in left-right asymmetry, heart, tooth, eye, and craniofacial development (Semina, Reiter et al. 1996; Gage and Camper 1997; Logan, Pagan-Westphal et al. 1998; Ryan, Blumberg et al. 1998; Yoshioka, Meno et al. 1998; Gage, Suh et al. 1999; Lin, Kioussi et al. 1999). A gene-dosage requirement for *Pitx2* has been determined through studies done on mice bearing hypomorphic *Pitx2* alleles. Pitx2 hypomorphs reveal that PITX2 is necessary for proper development and expansion of Rathke's pouch and the proper expression of HESX1 and PROP1 during pouch development. These mice also reveal the importance of PITX2 for the appropriate differentiation of gonadotropes, thyrotropes, somatotropes, and lactotropes (Gage, Suh et al. 1999; Suh, Gage et al. 2002). The mechanism through which pituitary cell proliferation is regulated by PITX2 is mediated by the WNT/beta-catenin pathway. Signaling from the WNT/β-catenin pathway converts PITX2 from repressor to activator allowing it to activate transcription from the target genes *cyclin D2*, *cyclin D1*, and *c-Myc* which promote pituitary cell proliferation (Kioussi, Briata et al. 2002; Baek, Kioussi et al. 2003). Mutations within the human *PITX2* gene cause phenotypes similar to that seen in the *Pitx2*$^{-/-}$ mice with eye defects,

pituitary abnormalities, dental hypoplasia, and craniofacial defects which are associated with Reiger syndrome seen in human patients (Semina, Reiter et al. 1996).

HESX1, or RPX, is a paired homeodomain transcription factor necessary for proper pituitary development. It is expressed early in the developing neural plate before being restricted to Rathke's pouch (Thomas, Johnson et al. 1995; Hermesz, Mackem et al. 1996). HESX1 is the earliest pituitary marker within the invaginating oral ectoderm with expression noted around e9.0 in the developing mouse pituitary. However, its expression is downregulated as differentiation of the hormone-secreting cell types within the anterior pituitary occurs, so that by e15.5, HESX1 is no longer detectable (Gage, Brinkmeier et al. 1996; Hermesz, Mackem et al. 1996). Two other transcription factors in pituitary development have been linked to the strong expression of HESX1, initially followed by its expression being extinguished as the cell types differentiate. LHX3 has been shown to be requisite for initial HESX1 expression during early pituitary development, and PROP1 has been shown to repress HESX1 expression in time for the differentiation of the specific pituitary cell types (Gage, Brinkmeier et al. 1996; Hermesz, Mackem et al. 1996; Sheng, Zhadanov et al. 1996). Homozygous *Hesx1*$^{-/-}$ knockout mice have forebrain defects, defects in olfactory development, bifurcations in Rathke's pouch, and defects in eye development in addition to other various brain abnormalities (Dattani, Martinez-Barbera et al. 1998). A human condition similar to this mouse model is septo-optic dysplasia, which can present itself as any combination of optic nerve hypoplasia, pituitary hypoplasia, and midline abnormalities of the brain, such as the corpus callosum and septum pellucidum. Mutations within the human *HESX1* gene have been associated

with this disorder in several patients (Dattani, Martinez-Barbera et al. 1998; Thomas, Dattani et al. 2001; Sobrier, Maghnie et al. 2006).

The paired-class homeodomain transcription factor, Prophet of Pit-1 (PROP1), is specifically expressed in the developing anterior pituitary (Sornson, Wu et al. 1996; Sloop, McCutchan Schiller et al. 2000). Molecular studies analyzing the PROP1 protein show that it is capable of acting as both transcriptional activator and repressor with a conserved carboxyl-terminus containing the trans-activation domain and the homeodomain demonstrating repressive properties (Showalter, Smith et al. 2002; Guy, Hunter et al. 2004). The importance of proper *Prop1* gene expression can be seen in both naturally occurring and genetically engineered mouse models. The Ames dwarf mouse is the result of a spontaneous mutation within the homeodomain of the *Prop1* gene. These mice display deficits in somatotrope, lactotrope, and thyrotrope cells in addition to reduced gonadotropin hormone levels (Tang, Bartke et al. 1993; Andersen, Pearse et al. 1995; Gage, Lossie et al. 1995; Gage, Brinkmeier et al. 1996; Sornson, Wu et al. 1996). The transcription factor PIT-1 is also absent in these mice suggesting that PROP1 is epistatic to PIT-1 in the transcriptional cascade involved in anterior pituitary development (Tang, Bartke et al. 1993; Andersen, Pearse et al. 1995; Gage, Lossie et al. 1995; Gage, Brinkmeier et al. 1996; Sornson, Wu et al. 1996). The engineered *Prop1*$^{-/-}$ knockout mice have a similar phenotype to the Ames dwarf mice. However, in addition to the hormone deficiencies, deletion of *Prop1* can be lethal as approximately 50% of *Prop1* null mice die from respiratory distress syndrome (RDS) at birth (Nasonkin, Ward et al. 2004). More roles for PROP1 throughout pituitary development have been elucidated through further evaluation of *Prop1*-deficient mice and gain of function

experiments. These studies have shown that PROP1 plays a part in pituitary vascularization, the onset of puberty, and tumorigenesis within the pituitary (Cushman, Watkins-Chow et al. 2001; Vesper, Raetzman et al. 2006; Ward, Stone et al. 2006). Analyses of mice homozygous for mutations in both *Prop1* and *Lhx4*, two transcription factors important in pituitary development, demonstrate an overlapping role early in pituitary development to promote the expansion of Rathke's pouch and later for the appropriate differentiation of both corticotrope and gonadotrope cell types (Raetzman, Ward et al. 2002). Mutations within the human *PROP1* gene are the most common known cause of combined pituitary hormone deficiency (CPHD), which is diagnosed when the production of growth hormone and at least one other hormone produced from the anterior pituitary is defective (Wu, Cogan et al. 1998).

The first pituitary transcription factor to be identified was PIT-1 (POU1F1), which is a homeodomain transcription factor (Bodner, Castrillo et al. 1988; Ingraham, Chen et al. 1988). The proper development of the somatotrope, lactotrope, and thyrotrope cell lineages rely on PIT-1 expression within the anterior pituitary. PIT-1 acts within these cells to turn on the appropriate genes while repressing expression of inappropriate hormone genes (Dasen, O'Connell et al. 1999; Scully, Jacobson et al. 2000). Within the somatotrope, lactotrope, and thyrotrope cells, target genes of PIT-1 include *GH*, *GHRHR*, *PRL*, *TSHβ*, and *thyroid hormone receptor beta type 2* (Steinfelder, Hauser et al. 1991; Rhodes, Krones et al. 1996; Iguchi, Okimura et al. 1999; Miller, Godfrey et al. 1999). PIT-1 is also able to bind to and activate transcription from its own promoter and a distal enhancer, which reinforces the commitment of the pituitary cell lineages dependent on PIT-1 expression (Rhodes, Chen et al. 1993; Rhodes, Krones et al. 1996). Two naturally

occurring mouse models exist that demonstrate the importance of PIT-1 in the development and specification of the somatotrope, lactotrope, and thyrotrope cell types. The Snell (*dw*) and Jackson (dw^J) dwarf mice carry recessive point or null mutations in *Pit-1*, respectively. These mice exhibit hypoplastic pituitaries with a dearth of somatotropes, lactotropes, and thyrotropes (Li, Crenshaw et al. 1990). Human patients with mutations within their *PIT-1* gene are diagnosed with CPHD and exhibit a phenotype similar to the dwarf mice lacking GH, TSH, and PRL (Tatsumi, Miyai et al. 1992).

Steroidogenic factor 1 (SF1) is an orphan nuclear receptor transcription factor involved in the development of several endocrine tissues. SF1 has been shown to be an important regulator involved in both the hypothalamus-pituitary-adrenal axis and the hypothalamus-pituitary-gonad axis, with a role in the gene regulation of the enzymes that produce the sex steroids (Morohashi and Omura 1996). Mice lacking any functional SF1 protein die postnatally with defects in ventromedial hypothalamic nuclei, absent gonadal and adrenal tissues, a lack of pituitary gonadotrope cells, and therefore undetectable levels of LHβ, FSHβ, and GnRHR (Ingraham, Lala et al. 1994; Luo, Ikeda et al. 1994; Sadovsky, Crawford et al. 1995; Shinoda, Lei et al. 1995). Mice with targeted disruption of SF1 just within the pituitary gondadotropes display hypogonadotropic hypogonadism, which suggests that proper expression of SF1 within the pituitary is necessary for normal development of reproductive organs (Zhao, Bakke et al. 2001).

1.3 The LIM-Homeodomain Transcription Factor LHX4 in Pituitary Development

LHX4 is a member of the LIM-homeodomain transcription factor family that contains two LIM domains that permit protein-protein interactions, and a centrally located homeodomain that serves as the DNA-binding motif of the protein (Hunter and Rhodes 2005). The human *LHX4* gene spans over 45 kilobases on chromosome 1 and its transcription is controlled by a TATA-less promoter(s), which contains recognition elements for specificity protein 1 (Sp1) (Machinis, Pantel et al. 2001; Liu, Fan et al. 2002; Yaden, Garcia et al. 2006; Liu, Luo et al. 2008). *In vitro* experiments have determined that LHX4 is capable of binding to promoters and activating transcription of several genes involved in pituitary development including *αGSU*, *GH*, *PRL*, *PIT-1,* and *FSHβ* (Sloop, Dwyer et al. 2001; Kawamata, Sakajiri et al. 2002; West, Parker et al. 2004; Machinis and Amselem 2005; Castinetti, Saveanu et al. 2008).

While LHX3 and LHX4 share several similarities in protein structure, their expression patterns are different during development, and double- and single-gene targeting in mice reveals the individual importance of each protein during development. Expression of *Lhx4* (or *Gsh4*) during mouse development is found in the hindbrain, the cerebral cortex, the pituitary gland, and the spinal cord (Li, Witte et al. 1994; Liu, Fan et al. 2002). It is detected early in the development of Rathke's pouch at e9.5, and becomes more concentrated in the area of the pouch that will become the anterior pituitary by e12.5 while Lhx3 expression continues throughout the entire pouch. *Lhx4* gene expression is considerably reduced throughout development with little detected in adult pituitaries whereas *Lhx3* expression continues throughout development into adulthood (Sheng, Moriyama et al. 1997). Mice homozygous for a targeted gene disruption of *Lhx4*

die shortly after birth with lungs that do not inflate (Li, Witte et al. 1994). *Lhx4*$^{+/-}$ (heterozygous) mice appear to be unaffected. Similar to the *Lhx3*$^{-/-}$ mice, a definitive Rathke's pouch forms in the *Lhx4*$^{-/-}$ mice, and growth of the pouch is arrested at this stage. While proliferation of the pouch is impaired and the anterior pituitary is severely hypoplastic, the pouch of *Lhx4*$^{-/-}$ mice contains cells of all the differentiated cell types, which is not the case with the *Lhx3*$^{-/-}$ mice where only a few corticotropes remain within the anterior pituitary (Li, Witte et al. 1994; Sheng, Moriyama et al. 1997). Apoptosis of pituitary precursor cells appears to be responsible for the hypoplastic anterior pituitary in the *Lhx4* null mice (Raetzman, Ward et al. 2002). Expression of *Lhx3* is impaired in *Lhx4*$^{-/-}$ and *Lhx4*/*Prop1* double knockout mice, which indicates a role for *Lhx4* in the proper expression of *Lhx3*, which is aided by *Prop1* (Raetzman, Ward et al. 2002). The combination of the importance of LHX4 for cell survival and proper expression of LHX3 indicates that LHX4 is necessary for appropriate expansion of the pouch during development. *Lhx3* and *Lhx4* appear to have more overlapping roles earlier in pouch development as Rathke's pouch in double knockouts does not progress beyond an early rudimentary stage, whereas it develops into a more definitive pouch structure in the single knockouts (Sheng, Moriyama et al. 1997). *Lhx4* also has an important role in the development of ventral motor neurons, as does *Lhx3* (Sharma, Sheng et al. 1998).

Lhx3 and *Lhx4* appear to play separate roles in the function of pituitary stem/progenitor cells and gland maintenance. Although LHX4 expression is downregulated after anterior pituitary development, anterior pituitary stem/progenitor cells contained within a side population of the adult gland have been shown to express

LHX4. However, LHX3 expression was restricted to the main population of pituitary cells (Chen, Hersmus et al. 2005).

Human mutations within the *LHX4* gene have been found to exist in a heterozygous state (Table 1). Patients with mutations within *LHX4* are diagnosed with CPHD with variable hormone deficiencies. All patients heterozygous for a *LHX4* mutation present with some degree of GH and TSH deficiency, but deficiency in the other anterior pituitary hormones (LH, FSH, ACTH, and PRL) is more variable. Hypoplasia of the anterior lobe, ectopic posterior pituitary, structural abnormalities of the sella turcica, chiari malformations in the brain, and respiratory distress syndrome are other variable features associated with *LHX4* mutations (Machinis, Pantel et al. 2001; Tajima, Hattori et al. 2007; Castinetti, Saveanu et al. 2008; Pfaeffle, Hunter et al. 2008; Tajima, Yorifuji et al. 2009).

The fact that *LHX4* mutations exist in human patients in a heterozygous state suggests that the aberrant proteins being produced are acting in a dominant negative fashion, or haploinsufficiency is taking place, meaning the mutations within the *LHX4* gene result in reduced gene function and the effectiveness of the LHX4 protein produced from the functional allele is below the threshold necessary for developmental steps requiring LHX4. To date, studies suggest the latter is taking place with some evidence pointing to the instability of aberrant LHX4 proteins produced due to the mutation present in the gene (Machinis and Amselem 2005; Castinetti, Saveanu et al. 2008; Pfaeffle, Hunter et al. 2008; Tajima, Yorifuji et al. 2009). The phenotype of human patients with *LHX4* mutations does not correspond to the observation of apparently normal pituitary physiology of $Lhx4^{+/-}$ mice (Li, Witte et al. 1994; Sheng, Moriyama et al.

1997; Raetzman, Ward et al. 2002). However, factors that could be contributing to this difference include the difference in biology between humans and rodents, genetic background effects, gene inactivation, and epigenetic effects. Compound heterozygosity within human patients is something else to consider; meaning, human patients could have another mutation within another gene important for *LHX4* gene function throughout development, although none have been described to date.

1.4 The LIM-Homeodomain Transcription Factor LHX3 in Pituitary and Nervous System Development

LHX3 is another member of the LIM-homeodomain transcription factor family. The human LHX4 and LHX3 proteins are very similar, sharing 63% amino acid identity with 75%-95% homology observed in the LIM domains and the HD (Hunter and Rhodes 2005; Mullen, Colvin et al. 2007). In addition to the LIM domains and the homeodomain, LHX3 also contains a major *trans*-activation domain for pituitary gene activation in its carboxyl terminus (Sloop, Dwyer et al. 2001). In both rodents and humans, transcription of the gene encoding *LHX3* is regulated by two TATA-less GC-rich promoters that lie upstream of exons Ia and Ib, and involves the actions of specificity protein-1 (Sp1) and nuclear factor I (NFI) (Yaden, Garcia et al. 2006). Studies involving *in vitro* and *in vivo* methods have determined that *Lhx4*, *Pitx1*, *Pitx2*, *Sox2*, and FGFs, such as FGF8, are involved in the direct and indirect activation of *Lhx3* (Takuma, Sheng et al. 1998; Tremblay, Lanctot et al. 1998; Raetzman, Ward et al. 2002; Charles, Suh et al. 2005; Rajab, Kelberman et al. 2008) (Figure 1.2). Transcription of the *LHX3* gene produces two mRNA transcripts from which three protein isoforms are translated. The LHX3a and

LHX3b isoforms result from alternate use of exons Ia and Ib and therefore differ in their amino termini (Zhadanov, Bertuzzi et al. 1995; Sloop, Meier et al. 1999). The M2-LHX3 isoform is a truncated isoform resulting from an internal methionine codon within the LHX3a transcript (Zhadanov, Copeland et al. 1995; Sloop, Meier et al. 1999; Sloop, Dwyer et al. 2001). These protein isoforms have different DNA binding and gene activation abilities and are therefore proposed to have individual roles during development (Sloop, Meier et al. 1999; Bridwell, Price et al. 2001; Sloop, Dwyer et al. 2001; Savage, Yaden et al. 2003).

In mice, *Lhx3* maps to the proximal region of chromosome 2 and is an early marker for pituitary development. It is expressed as early as e8.5, and found in Rathke's pouch and the closing neural tube around e9.5. Expression of *Lhx3* is also found in the developing hindbrain, spinal cord, pineal gland, and the vestibular epithelium of the inner ear (Seidah, Barale et al. 1994; Bach, Rhodes et al. 1995; Zhadanov, Bertuzzi et al. 1995; Sheng, Zhadanov et al. 1996; Sharma, Sheng et al. 1998; Hume, Bratt et al. 2007). *Lhx3* is required for proper pituitary development and motor neuron specification as evidenced by the phenotype of the $Lhx3^{-/-}$ knockout mice (Sheng, Zhadanov et al. 1996; Sheng, Moriyama et al. 1997; Sharma, Sheng et al. 1998). These mice are stillborn, or die within 24 hours of birth, and lack anterior and intermediate pituitary structures with four of the five hormone-secreting cell types absent—only a small population of corticotropes remain (Sheng, Zhadanov et al. 1996; Sheng, Moriyama et al. 1997). Consistent with this phenotype, molecular studies have shown that LHX3a is able to bind directly to promoter/enhancer regions of several genes involved in pituitary development and function including *αGSU*, *TSHβ*, *FSHβ*, *PRL*, gonadotropin releasing hormone receptor,

and *PIT-1* (Bach, Rhodes et al. 1995; Sloop, Meier et al. 1999; West, Parker et al. 2004; McGillivray, Bailey et al. 2005; Granger, Bleux et al. 2006). It is also required for proper expression of FOXL2, which is a transcription factor with implicated roles in the differentiation of pituitary cells that express *αGSU* (Ellsworth, Egashira et al. 2006) (Figure 1.2). Spinal cord motor neuron development is also impaired in $Lhx3^{-/-}/Lhx4^{-/-}$ mice, and further evidence indicates a role for LHX3 in the specification of interneuron and ventral motor neuron fates during development (Sharma, Sheng et al. 1998).

1.5 Diseases Associated With Mutations Within the *LHX3* Gene

The human *LHX3* gene contains seven coding exons with six introns, and maps to chromosome 9 at 9q34.3 (Sloop, Showalter et al. 2000; Sloop, Walvoord et al. 2000). Ten autosomal recessive mutations within the human *LHX3* gene have been reported thus far. These include missense mutations, intragenic deletions, nonsense mutations, and a complete gene deletion (Table 1.2). In accordance with observations of the *Lhx3* knockout mice, human patients with mutations in *LHX3* present with deficiencies in GH, TSH, LH, FSH, and PRL hormones, and are diagnosed with CPHD (Netchine, Sobrier et al. 2000; Bhangoo, Hunter et al. 2006; Pfaeffle, Savage et al. 2007; Rajab, Kelberman et al. 2008; Kristrom, Zdunek et al. 2009). MRI analyses demonstrate variable pituitary morphology in patients with *LHX3* mutations with some patients having hypoplastic pituitaries, others having enlarged pituitaries, and still others having normal pituitary morphology. In addition to CPHD, almost all mutations in *LHX3* cause patients to present with a rigid cervical spine resulting in limited head rotation. This phenotype is presumably due to the role of LHX3 in spinal cord motor neuron specification. In mice,

Lhx3 expression has been shown to play a role in directing spinal motor neuron axon projections to the ventral side of the neural tube (Sharma, Sheng et al. 1998; Thaler, Lee et al. 2002). A subset of patients with *LHX3* mutations have also been diagnosed with some form of mental deficiency (reviewed by Colvin, Mullen et al. 2009; Kristrom, Zdunek et al. 2009). More recently, Rajab et al. (2008) have published reports on two new mutations (p.K50X and an intragenic deletion) that extend the phenotype associated with mutations within the *LHX3* gene (Rajab, Kelberman et al. 2008). These patients present with ACTH deficiency in addition to loss of the other anterior pituitary hormones, and they were found to exhibit sensorineural hearing loss ranging from mild/moderate to complete deafness. The same group also reported this sensorineural hearing loss in the patients with the p.Y111C and 23 bp deletion mutations originally described by Netchine et al (2000). The authors also found LHX3 to be expressed in the developing human inner ear supporting a role for LHX3 in ear development (Rajab, Kelberman et al. 2008). Future studies will help determine whether ACTH deficiency and hearing loss are common or more variable features associated with mutations within the *LHX3* gene. Treatment for the endocrine symptoms of these patients involves hormone replacement therapy, including recombinant GH, T_4, and gonadotropin therapy. Family members heterozygous for the *LHX3* mutations are asymptomatic, which agrees with $Lhx3^{+/-}$ mice (reviewed by Colvin, Mullen et al. 2009).

The position of most of the described mutations lies within the region of the gene that encodes the LIM domains or the homeodomain of the protein, thereby causing structural abnormalities within the protein (Table 1.2). Several described mutations are expected to result in no production of any LHX3 proteins. Other mutations may result in

the production of severely truncated proteins or messenger RNA transcripts that could be targets of nonsense-mediated decay resulting in the loss of LHX3 protein. Molecular analyses done with the predicted aberrant proteins of some of the more subtle *LHX3* gene mutations show that LHX3 protein function is disabled or reduced if the protein is produced at all (Sloop, Parker et al. 2001; Savage, Hunter et al. 2007; Colvin, Mullen et al. 2009).

Of the 10 types of *LHX3* mutation described to date, one mutation type (W224ter) does not result in the limited head rotation, defining a new form of the disease. This mutation, found in four siblings from a consanguineous Lebanese couple, is a guanine to adenine substitution at position 672 of the *LHX3*a ORF within exon 5 of the *LHX3* gene. This substitution introduces a premature stop codon predicted to cause loss of the carboxyl terminus of the LHX3 protein (W224ter) (Pfaeffle, Savage et al. 2007). Functional tests with this protein showed that while it did not retain its ability to activate the *αGSU* promoter, there was a synergistic effect with PIT1 on the *PRL* promoter in 293T cells, although it was reduced. This protein also displayed reduced DNA binding ability, indicating that it may retain some continuing function in the pituitary (Pfaeffle, Savage et al. 2007). The endocrine phenotype of these patients seemed consistent with these results because although they were diagnosed with CPHD, they were not diagnosed until the ages of 14 and 15 years, indicating that there may have been some lingering pituitary function that decreased with time. At the ages of 9 and 8 years, they were diagnosed with secondary hypothyroidism and thyroxine replacement was initiated. However, the thyroxine replacement apparently had no significant impact on their height velocity because at 14 and 15 years of age, they presented with growth failure, and were

finally tested and diagnosed with CPHD (Pfaeffle, Savage et al. 2007). However, unlike the other patients with *LHX3* mutations, these patients were unique in that they did not present with limited head rotation, indicating that not all *LHX3* mutations are associated with a rigid cervical spine, and MRI analyses showed these patients had pituitaries of normal size and location.

This study and these experiments provide us with a lot of information as to how this *LHX3* mutation could be causing these patients' CPHD. However, there are limitations to this type of study; mainly, that we are unable to directly examine these patients' LHX3 proteins, thereby limiting our understanding of the mechanism of LHX3 in development. It is possible that the premature termination codon of this mRNA *in vivo* could result in its degradation via nonsense-mediated decay resulting in no LHX3 protein, although the delayed phenotype of these patients suggests that some LHX3 function is retained. LHX3 plays an important role in the development of ventral motor neurons in the spinal cord. Previous work indicates that the LIM domains and homeodomain of LHX3 are required for the formation of a multiprotein complex necessary for spinal cord motor neuron development (Sharma, Sheng et al. 1998; Thor, Andersson et al. 1999; Thaler, Lee et al. 2002). Although the carboxyl terminus of LHX3 contains the major *trans*-activation domain of the protein that could allow regulation of LHX3 function and location through signaling pathways, the LIM domains also appear to contain some activation function that may serve a role in the nervous system (Parker, Sandoval et al. 2000; Sloop, Showalter et al. 2000; Rhodes, Kator et al. 2005). For the patients with the W224ter mutation, the intact LIM domains and homeodomain may be sufficient for nervous system and some pituitary development, which would explain the

absence of limited neck rotation and abnormal pituitary morphology. However, although it is less severe and its development somewhat retarded, these patients with W224ter mutations still present with pituitary insufficiency demonstrating the importance of the carboxyl terminus in overall pituitary function. Our laboratory has been investigating the molecular functions of the carboxyl terminus of LHX3 and found this region to contain critical activation/repression domains, targets for post-translational modification, and intracellular targeting signals (Parker, Sandoval et al. 2000; Sloop, Showalter et al. 2000; Sloop, Dwyer et al. 2001; Parker, West et al. 2005; Savage, Hunter et al. 2007). The milder phenotype of these patients with later onset of hormone deficiency, normal pituitary morphology, and the absence of the limited neck rotation suggests that the actions of LHX3 in the pituitary and nervous system are separable, perhaps mediated by the different functional domains/motifs of the protein, and that the carboxyl terminus of LHX3 is essential for pituitary development. A knock-in mouse model of this human disease was generated to investigate the effects of this particular mutation throughout development at the molecular and cellular level, which is not feasible with human patients.

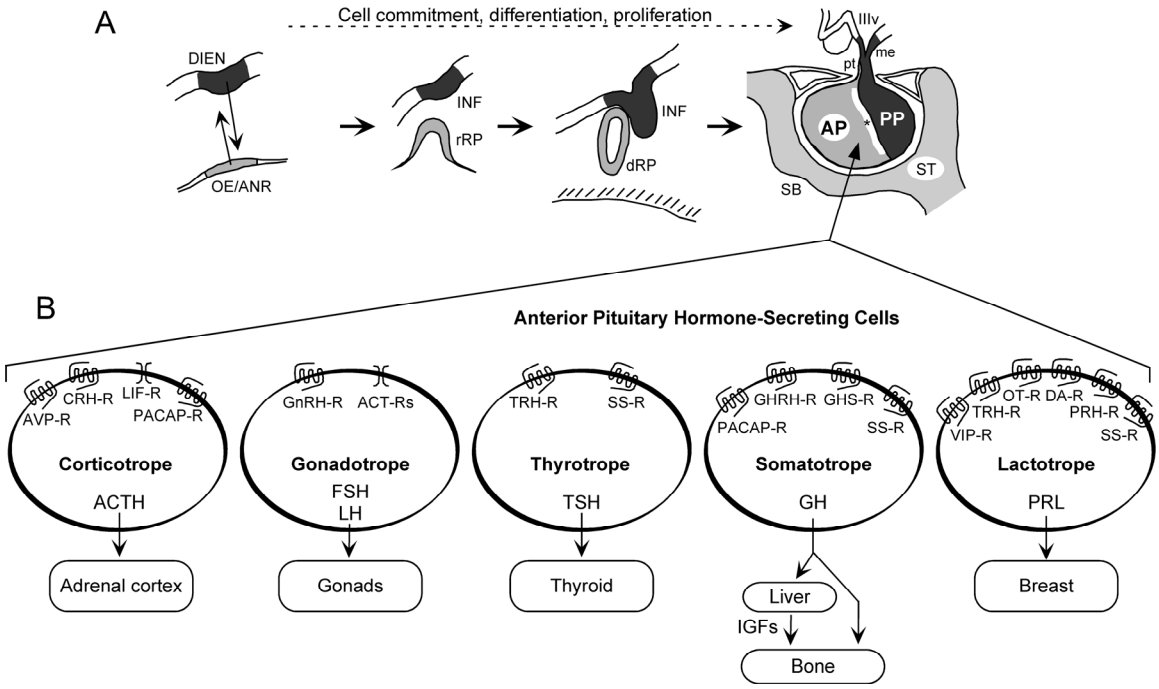


Figure 1.1. Development of the anterior pituitary gland within mammals. **A.** Inductive signaling between and within the ventral diencephalon (DIEN) of the brain and oral ectoderm/anterior neural ridge (OE/ANR) promotes the formation of a rudimentary Rathke's pouch (rRP), and the infundibulum (INF), from which the adenohypophysis and neurohypophysis will later develop, respectively. Later, a definitive Rathke's pouch (dRP) forms which then separates from the oral cavity. The mature pituitary gland is encased by the sella turcica (ST) of the sphenoid bone (SB). AP = anterior pituitary lobe, asterisk = intermediate pituitary lobe, IIIv = third ventricle of the brain, me = median eminence, PP = posterior pituitary, pt = pars tuberalis. **B.** The five anterior pituitary hormone-secreting cell types, their hormone products, and the target organs of the hormones are shown. The receptors that allow regulation of pituitary hormone secretion by hypothalamic and other regulatory hormones are also depicted. ACT = activin, AVP = arginine vasopressin, CRH = corticotropin-releasing hormone, DA = dopamine, GHRH = growth hormone releasing hormone, GHS = growth hormone secretagogue/ghrelin, GnRH = gonadotropin-releasing hormone, LIF = leukemia inhibitory factor, OT = oxytocin, PACAP = pituitary adenylate cyclase-activating polypeptide, PRH = prolactin releasing hormone, SS = somatostatin, TRH = thyrotropin-releasing hormone, VIP = vasoactive intestinal peptide. Adapted from Savage, Yaden et al. 2003.

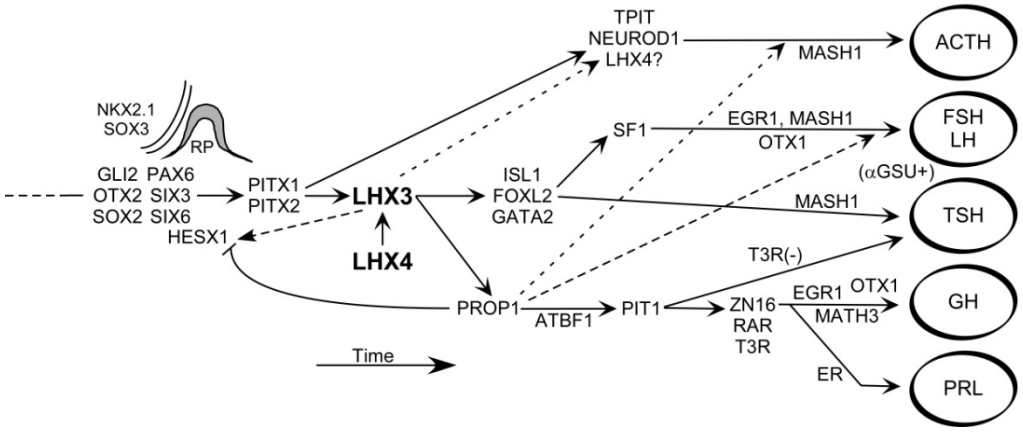
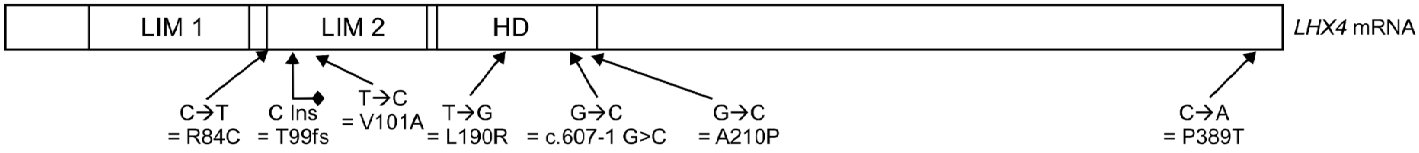


Figure 1.2. Transcriptional regulation of anterior pituitary development. Inductive signaling events between the ventral diencephalon and the oral ectoderm promote the formation of a rudimentary Rathke's pouch (RP). At the same time, these inductive signaling events initiate the expression of a cascade of transcription factors that guide the development of the specialized hormone-secreting cell types of the anterior pituitary. Adapted from Colvin, Mullen et al. 2009.

Table 1.1 Mutations within the *LHX4* gene associated with combined pituitary hormone deficiency diseases. The diagram depicts the locations of the changes encoded by the mutations relative to the *LHX4* mRNA. The positions of the sequences that encode the LIM domains and the homeodomain (HD) are indicated by the boxes. The numbering scheme for the LHX4 protein represented here begins with the first in-frame methionine in the human sequence: the p.P389T mutation in the table is therefore the same as the p.P366T mutation (Tajima, Hattori et al. 2007). Adapted from Colvin, Mullen et al. 2009.

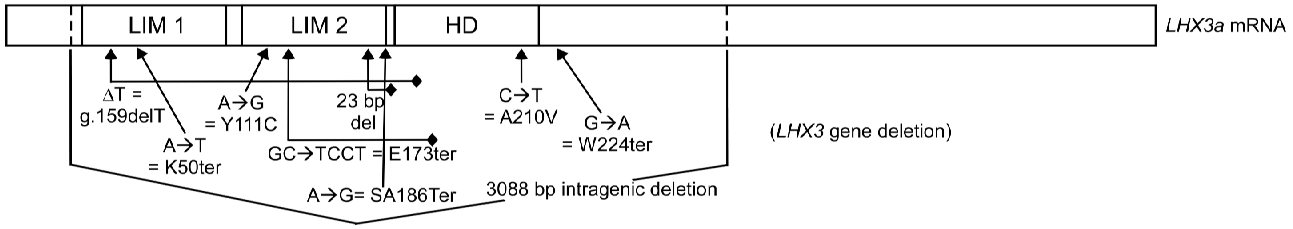
Table 1.1



	c. 607 -1G>C (Machinis et al., 2001; Castinetti et al., 2008)	**p.R84C** (Pfäffle et al., 2008)	**p.L190R** (Pfäffle et al., 2008)	**p.A210P** (Pfäffle et al., 2008)	**p.P389T** (Tajima et al., 2007)	**p.T99fs** (Castinetti et al., 2008)	**p.V101A** (Tajima et al., 2009)
Hormone deficiencies	GH, TSH, LH, FSH, ACTH	GH, TSH, PRL, LH, FSH	GH, TSH, ACTH	GH, TSH, LH, FSH, ACTH	GH, TSH, PRL, LH, FSH, ACTH	GH, TSH, PRL, LH, FSH	GH, TSH, LH, FSH, ACTH
Anterior pituitary morphology	Hypoplastic	Hypoplastic	Hypoplastic	Hypoplastic/Normal	Hypoplastic	Hypoplastic/ Enlarged	Hypoplastic
Location of posterior lobe	Ectopic	Ectopic	Ectopic	Normal	Ectopic	Normal	Ectopic
Sella turcica development	Poor	Normal	Normal	Normal	Poor	Poor/Normal	Normal
Intracranial defects	Chiari malformation	None	None	None	Chiari malformation	Corpus callosum hypoplasia	Chiari malformation
Genetic status	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Location of mutation	Splice acceptor site mutation upstream of exon 5	Missense mutation within exon 3	Missense mutation within exon 4	Missense mutation within exon 5	Missense mutation within exon 6	Frame shift mutation within exon 3	Missense mutation within exon 3

Table 1.2. Mutations within the *LHX3* gene associated with pediatric combined pituitary hormone deficiency diseases. The diagram depicts the locations of the molecular changes encoded by the mutations relative to the *LHX3a* mRNA. The positions of the sequences that encode the LIM domains and the homeodomain (HD) are indicated by the boxes. The extended horizontal lines ending in diamond symbols denote predicted alterations in protein reading frames ending in premature termination of translation. Adapted from Colvin, Mullen et al. 2009.

Table 1.2



	p.Y111C (Netchine et al., 2000; Rajab et al., 2008)	**23bp deletion** (Netchine et al., 2000; Rajab et al., 2008)	**g.159delT** (Bhangoo et al., 2006)	**p.A210V** (Pfäffle et al., 2007)	**p.E173X** (Pfäffle et al., 2007)	**p.W224X** (Pfäffle et al., 2007)	***LHX3* gene deletion** (Pfäffle et al., 2007)	**p.K50X** (Rajab et al., 2008)	**Intragenic deletion of 3088bp** (Rajab et al., 2008)	**p.SA186X** (Kristrom et al., 2009)
Hormone deficiencies	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH	GH, TSH, PRL, LH, FSH	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
Anterior pituitary morphology	Hypoplastic	Enlarged	Hypointense pituitary lesion	Enlarged	Hypoplastic	Normal	Severely hypoplastic/ aplastic	Hypoplastic	Hypoplastic	Severely hypoplastic/ aplastic
Neck rotation	Limited	Limited	Limited	Limited	Limited	Normal	Limited	Limited	Limited	Limited
Hearing deficiencies	Moderate to mild sensorineural hearing loss	Completely deaf	Not reported	Not reported	Not reported	Not reported	Not reported	Sensorineural deafness	Sensorineural hearing loss	Sensorineural hearing loss
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	Some learning difficulties
Genetic status	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous	Homozygous
Location of mutation	Missense mutation within exon 3	Deletion affecting exon 3 and splice-donor site	Deletion within exon 2	Missense mutation within exon 5	Complex mutation within exon 3	Nonsense mutation within exon 5	Complete gene deletion	Nonsense mutation within exon 2	Deletion of exons 2-5	Missense mutation within intron 3 affecting splice- acceptor site

CHAPTER TWO

MATERIALS AND METHODS

2.1 DNA Cloning and Gene Targeting Vector Construction

Mouse embryonic stem cell targeting

Mouse genomic DNA of the 129/Sv strain was obtained as a gift from Emily Blue, P.A. Gallagher Laboratory, Cellular and Integrative Physiology, Indiana School of Medicine (IUSM), Indianapolis, IN. Approximately 5 kb of murine genomic *Lhx3* was used for the homology arms and was generated using *Pfu UltraII* DNA polymerase (Stratagene, La Jolla, CA), the forward primer 5′-atggtttatgggatgggttacccg-3′, and the reverse primer 5′-aaccaacaggtagccaagatcctg-3′. Touchdown PCR parameters were used as follows: 95°C 5 min; 95°C 20 sec, 67°C 20 sec, 72°C 5 min 30 sec for 2 cycles; 95°C 20 sec, 65°C 20 sec, 72°C 5 min 30 sec for 2 cycles; 95°C 20 sec, 63°C 20 sec, 72°C 5 min 30 sec for 2 cycles; 95°C 20 sec, 61.5°C 20 sec, 72°C 5 min 30 sec for 2 cycles; and 95°C 20 sec, 60°C 20 sec, 72°C 5 min 30 sec for 25 cycles. This reaction product was then cloned into the StrataClone PCR Cloning Vector using the StrataClone Blunt PCR Cloning Kit (Stratagene), and the W227ter mutation was introduced using site-directed mutagenesis (QuickChange II kit, Stratagene) and the following primers:

5′-cgctggccggcagcgctgaggacagtatttccgc-3′ and 5′-gcggaaatactgtcctcagcgctgccggccagcg-3′ (Figure 2.1A). Further manipulation required cloning the 5′ and 3′ homology arms into the PGK-neolox-2DTA vector, which was obtained as a gift from Dr. Stephen Konieczny, Ph.D., Purdue University, West Lafayette, IN. To clone the 2.9 kb 5′ homology arm, the StrataClone vector containing the 5 kb of murine genomic *Lhx3* was first digested with *Hind*III and *Bsp*EI restriction enzymes (Figure 2.1A). The resulting sticky ends were then blunted using the Klenow enzyme (New England Biolabs, Ipswich, MA), and the 5′ arm was isolated by agarose gel electrophoresis and purified using Qiaquick gel extraction columns (Qiagen, Valencia, CA). The PGK-neolox-2DTA vector was first digested with *Not*I (Figure 2.1B), the sticky ends were blunted with Klenow enzyme (New England Biolabs), and the vector was then treated with calf intestinal alkaline phosphatase (CIAP). The 5′ arm was then ligated into the PGK-neolox-2DTA vector, and its presence was confirmed through a series of test digests and DNA sequencing. To clone the 2.2 kb 3′ arm, the StrataClone vector containing the 5 kb of murine genomic *Lhx3* was digested with the *Bsp*EI restriction enzyme (Figure 2.1A). The resulting sticky ends were then blunted using the Klenow enzyme (New England Biolabs). This product was then *Hind*III digested, and the 3′ arm was isolated by agarose gel electrophoresis and purified using the Qiaquick gel extraction columns (Qiagen). The 3′ arm was ligated into a *Hind*III site within the PGK-neolox-2DTA vector containing the 5′ arm that had been previously digested (Figure 2.1B). The integrity of this construct was confirmed through a series of test digests and DNA sequencing.

The gene targeting vector plasmid was purified via CsCl preparation, linearized by *Kpn*I digestion, and two 50-µg aliquots at 1 µg/µl were submitted to the Transgenic

and Knock-Out Mouse Core of the IUSM for transfection into CCE 916 embryonic stem (ES) cells originally derived from a 129SvEv mouse. Twenty-five micrograms of linearized construct was electroporated into 10 million cells. The cells were then plated at one million cells per 10 cm plate, and G418 selection was started 48 hours post electroporation at 600 μg/ml. G418, or Geneticin, is an aminoglycoside antibiotic produced by *Micromonospora rhodorangea* which functions to block protein synthesis through interference with the 80S subunit of the ribosome. The *Neomycin resistance* (NeoR) gene provides resistance to G418 by encoding an aminoglycoside 3′-phosphotransferase, APH 3′ II, which inhibits the function of G418. Four days after electroporation, the G418 selection was lowered to 300 μg/ml and maintained for 10 to 12 days. Individual ES cells were picked 10 days after electroporation and expanded on 24-well plates for a few days. After expansion, a portion of the cells were frozen for later use and the remaining cells were expanded on 24 well plates for screening. To screen the cells, the cell culture medium was removed and 600 μl of cell lysis solution (Qiagen) was added per well followed by a 2-3 hour incubation at 37°C. The ES cell lysates were then transferred to labeled microcentrifuge tubes for future screening. Of 191 129/Sv G418-resistant ES cell clones received from the core, five were found to be positive via PCR and Southern blotting for both the Neo cassette and the point mutation and negative for the DTA cassette. After screening, the cells were expanded into injection stocks and 15 to 18 cells were injected into 3.5 dpc C57BL/6J blastocysts. The blastocysts were then transferred to 2.5 dpc pseudopregnant Swiss Webster female mice, and resulting high percentage male chimeras were bred to C57BL/6J females to generate heterozygous mice.

DNA sequencing

DNA sequencing was performed at the Biochemistry Biotechnology Facility at the Indiana School of Medicine using a PerkinElmer DNA sequencer. The DNA templates were submitted using the recommended guidelines from the sequencing facility, and the sequence analyses and assembly were performed using the DNASIS (Hitachi Software Engineering, San Francisco, CA) software.

2.2 Identification of Homologous Recombinants

PCR

Genomic DNA was obtained from ES cell lysates by adding 4 µl of RNase A solution (4 mg/ml) to 600 µl ES cell lysate. The RNase A was mixed with the ES cell lysates by inverting the tubes 25 times and the tubes were then incubated at 37°C for 45 minutes. Following this incubation, the samples were allowed to cool to room temperature for 15 minutes before 265 µl of protein precipitation solution (Qiagen) was added to the lysates. The lysates were then vortexed vigorously for 20 seconds followed by centrifugation at 13,000 rpm for 10 minutes. Supernatant containing the DNA was then poured into a clean 2.0 ml microcentrifuge tube and 800 µl isopropanol was added. The samples were mixed by inverting 50 times before centrifuging at 13,000 rpm for 5 minutes to pellet the DNA. After centrifugation, the ethanol was poured off the pellet and 800 µl 70% ethanol was added. The tubes were then inverted several times to wash the pellet before centrifuged at 13,000 rpm for 1 minute. The ethanol was poured off and the

pellets were allowed to air dry for 10-15 minutes before being resuspended in 100 µl TE buffer (20 mM Tris, 1 mM EDTA). DNA was stored at –20°C until ready for use.

Genomic DNA from ES cell lysates received from the core was initially analyzed via PCR for the presence of the point mutation and the "Neo" cassette using *FastStart HiFi* Taq polymerase (Roche, Indianapolis, IN) and the following oligonucleotides: 5′-tggacatgcgagtggtgcaggtca-3′, 5′-aaccaacaggtagccaagatcctg-3′. The PCR parameters were 95°C 5 min.; 95°C 30 sec, 60°C 30 sec, and 72°C 2:20 min for 30 cycles. The resulting product was then digested with *Bbv*CI enzyme to verify the presence of the point mutation in a heterozygous state with expected band sizes of 1,430 bp, 1,280 bp, 620 bp, and 150 bp if the point mutation was present, and 1,430 bp and 620 bp if the point mutation was not present. The absence of the DTA cassette was also confirmed through PCR using *GoTaq* polymerase (Promega, Madison, WI), the primers 5′-aaggcgtagcctctaaatgcgaga-3′ and 5′-caacatcatcaggatccatggcga-3′, and the following PCR parameters: 95°C 5 min; 95°C 30 sec, 60°C 30 sec, and 72°C 45 sec for 30 cycles; the expected product size if the DTA cassette was present was 750 bp.

Five ES cell lysates were positive for the point mutation and the Neo cassette and negative for the DTA cassette. Southern blotting was then used to screen genomic DNA from these five ES cell lysates and toe biopsies from the first set of heterozygous mice produced at the core facility to confirm the presence of the endogenous and targeted alleles. The probe used was located within the *Lhx3* gene just downstream of the 3′ homology arm and was amplified using the primers 5′-caggatcttggctacctgttggtt-3′ and 5′-acttgctcatccctcataccagca-3′, and the following PCR parameters: 95°C 5 min; 95°C 30 sec, 60°C 30 sec, and 72°C 45 sec for 30 cycles; the expected product size was 720 bp. A vial

of frozen ES cells from each of the five positive ES cell lysates was sent to the Purdue University Cancer Center Transgenic Mouse Core Facility for chromosome counting. Of the number of cells counted within each lysate submitted, 70% of those cells needed to have a normal chromosome complement (40 chromosomes) to be considered acceptable for chimera production. Four of the five ES cell vials submitted fit these guidelines and were considered appropriate for blastocyst injection and chimera production.

Southern blot

Ten micrograms of DNA prepared from ES cell lysates as mentioned above was precipitated with 1 ml 100% EtOH and centrifuged at 13,000 rpm for 2 min. The pellet was then washed with 1 ml of 70% EtOH. After a quick spin down, the EtOH was removed and the pellet was allowed to air dry before being resuspended in 20 µl sterile water. After the DNA was digested with *Bam*HI overnight, the DNA fragments were separated on a 0.8% agarose/1× Tris-Acetate gel in 1×TAE (40 mM Tris, 0.1142% glacial acetic acid, 2 mM EDTA) running buffer at 30 volts overnight. The gel was then stained with 0.5 µg/ml ethidium bromide for 20 min, and a picture of the gel was taken with a rule placed next to it for future orientation. Once excess gel had been trimmed off, the gel was depurinated in a 1:50 dilution of 32-38% HCl for 15 min, neutralized in 0.4 N NaOH for 15 min, and transferred overnight onto a Hybond-XL charged nylon membrane (GE Healthcare, Piscataway, NJ) that had been pre-soaked in 0.4 N NaOH. The next morning, the membrane was neutralized in 100 ml neutralization buffer (SSPE [0.15M NaCl, 10 mM NaH_2PO_4, 1.25 mM EDTA], 1M Tris pH 7.5) for 15 min, then pre-hybridized with FBI buffer (SSPE [0.225M NaCl, 0.1M NaH_2PO_4, 1.875 mM EDTA],

10% PEG 8000, 7% SDS) at 65°C for 4 hours. During this time, fresh probe was made using the DECAprime II Random Primed DNA Labeling Kit (Ambion, Austin, TX) according to manufacturer's instructions. Unincorporated ^{32}P-labeled nucleotides were removed using illustra ProbeQuant G-50 Micro Columns (GE Healthcare). Radiolabeled probe was then counted and 1.5×10^6 cpm/ml denatured probe in 20 ml FBI buffer was allowed to hybridize with the membrane at 65°C overnight in a roller bottle at 4 rpm. The next day, the membrane was washed twice in 20 ml 1 × SSPE, 1% SDS for 15 min each wash at 65°C. The third wash was done at room temperature with 100 ml 1 × SSPE, 1% SDS for 15 min. The membrane was then exposed to Kodak BioMax MR film (Carestream Health, Inc., Rochester, NY) with an intensifying screen and stored at –80°C overnight before analysis.

2.3 Genotyping and Breeding of Knock-In Mice

Wild-type C57BL/6J female mice (The Jackson Laboratory, Bar Harbor, ME) were bred to male chimeras at the core facility to generate F1 heterozygote mice. Genomic DNA from tail biopsies from these heterozygotes was screened via PCR and Southern blot for the presence of the point mutation (see above). Of these heterozygotes, the males were transferred from the core to the LARC animal facility for further analyses. The female heterozygotes at the core were bred to male B6.FVB-Tg(EIIa-Cre)C5379Lmgd/J mice (The Jackson Laboratory). This mouse line contains a *Cre* transgene paired with the adenovirus EIIa promoter to target the expression of *Cre* recombinase in the early mouse embryo and was used to promote the excision of the Neo cassette from the *Lhx3* gene locus. Progeny from this cross were transferred to the LARC

animal facility and screened via PCR with *GoTaq* polymerase (Promega) and primers flanking the Neo cassette: 5′-aggttgcccgctttgagagaatta-3′ and 5′-tgacctgcaccactcgcatgtcca-3′. The PCR parameters used were 95°C 5 min; 95°C 30 sec, 60°C 30 sec, and 72°C 2:20 min for 30 cycles. The products generated from this PCR were used to genotype the animals: if the Neo cassette was still present, the expected band size was 2,270 bp, if the Neo cassette had been excised, the expected band size was 630 bp, and the expected band size for wild-type *Lhx3* was 470 bp. Genomic DNA obtained from tail biopsies of weanlings was also screened for the presence of *Cre* recombinase using *GoTaq* polymerase (Promega), the forward primer 5′-ccaatttactgaccgtacacc-3′, the reverse primer 5′-gtacgtgagatatctttaaccctgat-3′, and the following PCR parameters: 95°C 5 min; 95°C 30 sec, 60°C 30 sec, and 72°C 30 sec for 30 cycles. If *Cre* recombinase was present, the expected band size was approximately 500 bp.

Mice were housed in a specific pathogen-free environment under controlled conditions of temperature and light (12 hours lights on/12 hours lights off), and were provided free access to tap water and commercial mouse chow. Some homozygote dwarf mice were provided with daily wet feed on the bottom of the cage due to their small size. The Indiana University Committee on Use and Care of Animals approved all procedures done using the 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. Mouse age was designated such that the morning after conception was embryonic day 0.5 (e0.5) and the day of birth was postnatal day 1 (P1).

Breeding into specific mouse strains

All animal analyses were done on progeny of an N2 intercross with a mixed background of approximately 75% C57BL/6J and 25% 129/Sv. Fertility studies were carried out so that wild-type, heterozygote, and homozygote mice were housed as single breeding pairs for at least 4 months while fecundity was measured. The frequency of birth, number of offspring, and genotype were recorded. Mice also are being bred with wild-type C57BL/6J and 129/Sv (The Jackson Laboratory) for six generations to analyze the dwarf phenotype on each of these backgrounds.

2.4 Histology and Immunohistochemistry

Harvested tissues were fixed in 4% paraformaldehyde in 1 × phosphate buffered saline (PBS [137 mM NaCl, 5.4 mM KCl, 16.2 mM Na_2HPO_4, 2.9 mM KH_2PO_4]) for 1 to 24 hours at room temperature. Fixed P1 neonates were washed in 1× PBS, dehydrated, and embedded in paraffin. Whole pituitaries from adult 12 week old animals were washed in 1× PBS, set in 20% sucrose in PBS overnight, embedded in O.C.T. compound (Sakura Finetek, Torrence, CA), and stored at –80°C for future cryosectioning. Paraffin-embedded and frozen tissues were all cut at 10 μm and all sections were rehydrated, and antigen unmasked (boiled in 10 mM citric acid at pH 6.0 for 10 min) before immunostaining. Paraffin sections were deparaffinized through several xylene washes prior to rehydration and antigen unmasking.

Both frozen and paraffin sections were immunostained using polyclonal antibodies for human ACTH (1:1,000 diaminobenzidine [DAB]; Sigma, St. Louis, MO) or (1:500 Fluor.) (AFP-39032082), rat αGSU (1:500 DAB) or (1:100 Fluor.) (AFP-

66P9986), rat GH (1:1000 DAB) or (1:500 Fluor.) (AFP-5672099), rat LHβ (1:800 DAB) or (1:400 Fluor.) (AFP-571292393), rat TSHβ (1:1000 DAB) or (1:500 Fluor.) (AFP-1274789), mouse PRL (1:100 Fluor.) (AFP-131078) (National Hormone and Pituitary Program, Torrance, CA), and mouse LIM-3 (1:1000 DAB) (Chemicon, Temecula, CA). Biotinylated secondary antibody was used with avidin and biotinylated peroxidase or fluorescent avidin alone (Vectastain rabbit kit; Vector Laboratories, Burlingame, CA). The chromogen DAB was used for some immunostaining reactions.

2.5 RNA Analyses

RNA extraction/cDNA synthesis

Mouse pituitaries from juvenile 6 week old animals and adult 12 week old animals were harvested and immediately placed in RNA*later* solution (Ambion). RNA was isolated according to manufacturer's instructions using an RNeasy Mini Kit (Qiagen), and pituitaries were homogenized using 1-ml 25G$^{5/8}$ syringes (Becton Dickinson, Franklin Lakes, NJ). An on-column DNase I digestion was performed as suggested in the protocol (Qiagen). cDNA was synthesized using Multiscribe reverse transcriptase (RT) and random primers according to the protocol within the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Quantitative PCR analyses

cDNA reverse transcription (RT) reactions were carried out in triplicate as described (see above). Negative control reactions that contained no RT enzyme were also performed. The relative abundance of the genes of interest was determined using real-time quantitative PCR with the 5′ fluorogenic nuclease assay and an ABI 7900 PRISM (Applied Biosystems). The 5′ terminus of the fluorogenic probes was labeled with 6-carboxy-fluorescein. The 3′ terminus of the *36B4* probe contained a non-fluorescent quencher—black-hole quencher-1 (Biosearch Technologies, Inc., Novato, CA). Primer and probe sequences were as follows: *36B4* forward primer, 5′-ggcccgagaagacctcctt-3′, *36B4* reverse primer, 5′-tcaatggtgcctctggagatt-3′, and *36B4* TaqMan probe, 5′-ccaggctttgggcatcaccacg-3′. The *Lhx3a* (Mm00521922_m1), *Lhx3b* (Mm01333633_m1), *Lhx3a & b* (Mm01330618_g1), *Lhx4* (Mm00521928_m1), *Gh* (Mm00433590_g1), *Fshβ* (Mm00433361_m1), *Pou1f1* (Mm00476852_m1), *Tshβ* (Mm00437190_m1), *Pomc* (Mm00435874_m1), *Prl* (Mm00599949_m1), *αGsu* (Mm00438189_m1), *Pitx1* (Mm00440824_m1), *Lhβ* (Mm00656868_g1), and *Gnrhr* (Mm00439143_m1) primers and probes were synthesized by Applied Biosystems, and the 3′ terminus of these probes contained the quenching molecule minor-groove binder (MGB). PCRs were run in triplicate 20 µl reactions that contained Universal Master Mix (Applied Biosystems), 10 pmol of each forward and reverse primer, 3.5 pmol probe, and 4 µl diluted cDNA from the RT reactions. The two-step PCR cycling parameters were as follows: 50°C 2 min for one cycle, 95°C 10 min for one cycle, and 95°C 15 sec and 60°C 1 min for 40 cycles. Data were normalized by determining the relative abundance of *36B4* mRNA. The *36B4* gene encodes an acidic ribosomal phosphoprotein P0 (RPLP0). This phosphoprotein

associates tightly with the smaller 40S subunit of the ribosome. The 5′ open reading frame of *36B4* cDNA is highly conserved across tissues and species, and therefore, it is used frequently as a reference gene for normalizing data in real-time quantitative PCR assays. To allow for better comparison between groups at the different time points examined, wild-type data were normalized to 1 and heterozygote and homozygote data were subsequently adjusted.

2.6 Microscopy

Fluorescent images were obtained 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 (Nikon Instruments, Inc.) were used to process the images. Light images of whole pituitaries were obtained using a Leica MZ 6 microscope and a CCD camera (PL A662, PixeLINK, Ottawa, Ontario, Canada) with PixeLINK Capture software.

2.7 Hormone Analyses

Sera collection

Trunk blood was taken after decapitation and placed in 1.5 ml snap-cap polypropylene microcentrifuge tubes containing 100 µl heparin (Baxter Healthcare Corporation, Deerfield, IL). The samples were then spun down at 5,000 rpm for 10 min at 4°C after which sera was collected and placed in a new 1.5 ml microcentrifuge tube. The sera samples were then stored at –20°C in a non-frost freezer until hormone analyses

could be performed. Enzyme-linked immunosorbent assays (ELISA), or enzyme immunoassays (EIA), were used to analyze levels of mouse insulin-like growth factor 1 (IGF-1) (Immunodiagnostic Systems, Scottsdale, AZ, AC-42F1), human T4 (thyroxine) (ALPCO, Salem, NH, 25-TT4HU-EO1), human testosterone (DRG International, Inc., Mountainside, NJ, EIA-1559), and mouse/rat prolactin (Calbiotech, Spring Valley, CA, PR063F-100) hormone levels within the mouse serum according to the manufacturer's instructions for each kit.

Western blotting

Pituitaries from adult 12 week old animals were lysed in ice-cold RIPA Buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 0.01 M N_3PO_4, pH 7.2, 2 mM EDTA and 50 mM NaFl) supplemented with protease inhibitors (Sigma). The samples were sonicated at level 6 for 10 seconds and incubated on ice for 10 minutes. After the incubation, samples were centrifuged full speed for 3 minutes and the supernatant was stored at -80°C. The protein concentration was determined by Bradford assay.

Harvested pituitary tissue (30 μg) was run at 100 V on a 12% acrylamide gel and transferred to a charged PVDF membrane in western transfer buffer (192 mM glycine, 2 mM Tris, 20% methanol). The membrane was blocked in 5% nonfat dry milk (NFDM, Carnation) in TBST (Tris-Buffered Saline tween (154 mM NaCl, 52 mM Tris, 1% Tween 20) with shaking. After blocking, the membrane was washed with TBST and incubated with primary antibodies LHβ (NHPP, 1:600) and PRL (Santa Cruz, 1:200) in TBST overnight at 4°C. The membrane was washed with TBST and then incubated with

peroxidase conjugated secondary antibody (1:25,000) in TBST for 1 hour. The membrane was then incubated in SuperSignal West Dura Extended Duration Substrate (Pierce) for 1 minute with gentle mixing prior to exposure. (Western blot information written and obtained by Raleigh E. Malik).

2.8 General Molecular Techniques

Ligations and transformations

DNA fragments to be ligated were examined for size and abundance by agarose gel electrophoresis. DNA fragments were joined using 1 µl T4 DNA ligase (Roche) in a total volume of 15 µl buffered solution at 16°C overnight. Five µl of the ligation reaction or 2-5 ng of plasmid preparation were transformed into bacterial cells to generate clones. Plasmid DNA was then added to 50 µl of chemically competent *E. coli* DH5α cells (Invitrogen) on ice. This mixture was heat shocked at 42°C for 45 sec and placed on ice again for 2 min. Eight hundred microliters of LB broth supplemented with 0.2% glucose and 10 mM $MgCl_2$ was added and this mixture was incubated at 37°C for 45-60 min with shaking at 150 rpm. After incubation, cultures were centrifuged at 2,400 × g for 2 min to pellet the cells. The supernatants were aspirated, and the remaining cells were resuspended in 200 µl fresh LB broth. Cell suspensions 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°C overnight with shaking at 250 rpm in LB broth with appropriate antibiotics. To pellet cells, 1.5 ml of liquid culture was centrifuged at 16,000 × g for 1 minute. After the supernatant was aspirated, 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 minutes. The addition of 200 µl solution 2 (0.2 M NaOH, 0.5% SDS) lysed the cells, and this was followed by gentle mixing and incubation on ice for 5 min. One hundred seventy-five microliters of solution 3 (3 M KOAc, 11.5% (v/v) glacial acetic acid) was added followed by an additional incubation on ice for 5 min before the solution was centrifuged at 16,000 × g for 10 min. After the addition of 300 µl of a 1:1 phenol/chloroform solution, the sample was then centrifuged at 16,000 × g for 10 min. The upper aqueous phase was carefully removed and placed in a new 1.5 ml microcentrifuge tube before the addition of 1 volume of room temperature isopropanol to precipitate nucleic acids. The sample was centrifuged at 16,000 × g for 10 min and the pellet washed with 75% ethanol. The pellet was dried at 37°C for 5 min and resuspended in 30 µl TER (100 µg/ml RNaseA in 1 × TE buffer [10 mM Tris-Cl, pH 7.4, 1 mM EDTA]).

Between 2-5 µl of the plasmid preparations were digested with restriction endonucleases using the recommended buffer conditions (New England Biolabs). Restriction digests were separated on agarose/1× Tris-borate gels to select properly constructed plasmids.

Gel purification of DNA fragments

DNA fragments to be gel purified were separated on 0.7% or 1.0% agarose/Tris-borate gels. Gel slices including the fragments of interest were excised using razor blades and long wavelength ultraviolet light. Gel purification was performed with regular Qiaquick or MinElute gel extraction columns (Qiagen) 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.

2.9 Statistical Analyses

Quantitative PCR data and hormone data were analyzed using a two-tailed Student's *t*-test for unpaired samples using Excel (Microsoft Corp., Redmond, VA). Data points from the homozygous *Lhx3*$^{W227ter/W227ter}$ knock-in animals were compared to data points obtained for the *Lhx3*$^{+/+}$ wild-type control animals. The data were considered significantly different when $P<0.05$. Data used in the weight charts were analyzed using a one-way ANOVA to compare data points obtained for animals wild-type (+/+), heterozygous (+/W227ter), and homozygous (W227ter/W227ter) for the *Lhx3*$^{W227ter/W227ter}$ mutation. Tthe data were considered significantly different when $P<0.05$.

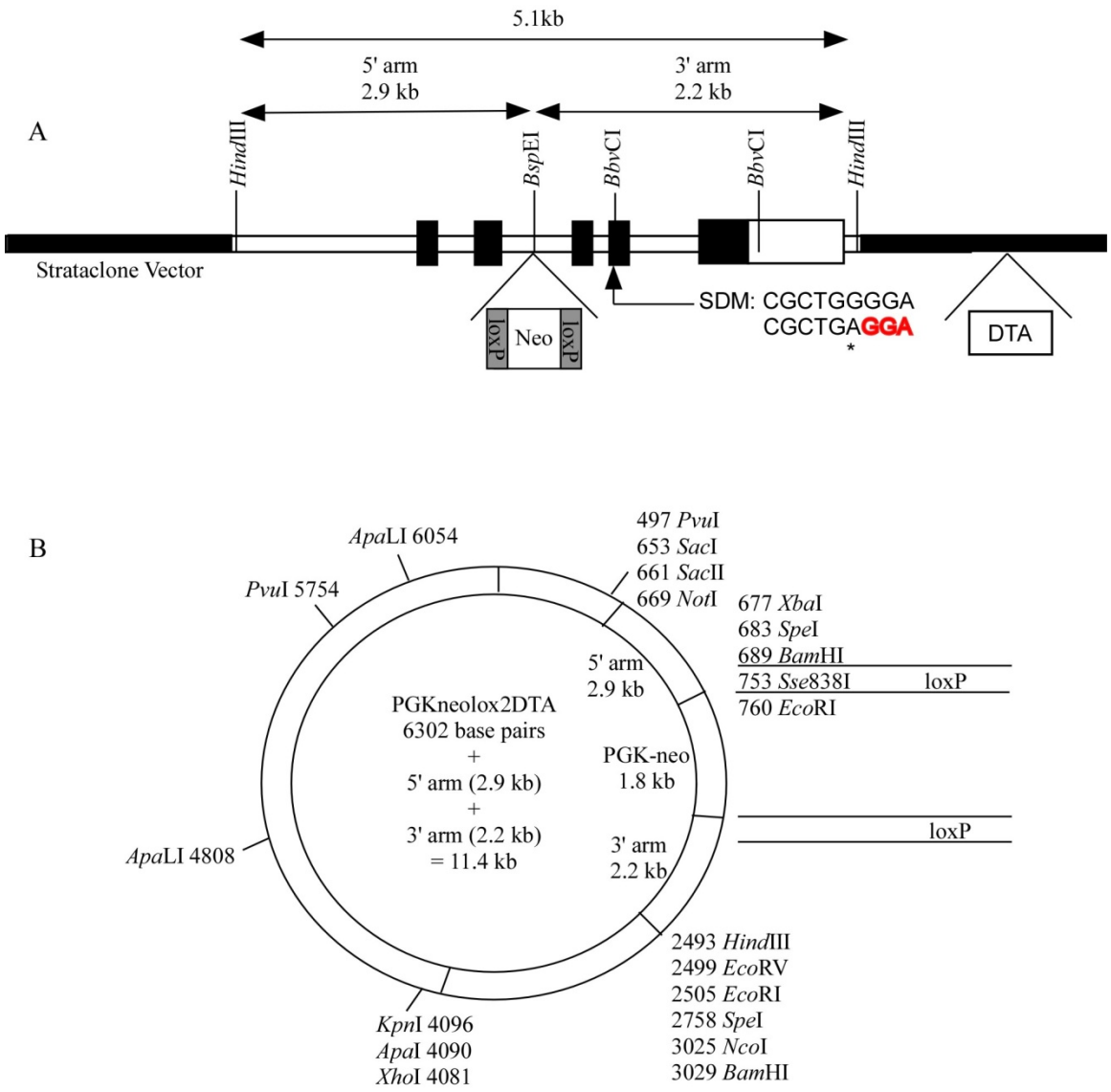


Figure 2.1. Generating the Targeting Construct. **A.** Five kilobases of mouse genomic *Lhx3* was isolated via PCR and cloned into a StrataClone vector. The point mutation was introduced at the appropriate location within the gene using site-directed mutagenesis. **B.** Through a series of digests, the 5 kb of genomic *Lhx3* within the StrataClone vector in panel A was split into two homology arms. The homology arms were then ligated into the PGKneolox2DTA vector as shown.

CHAPTER THREE

A MOUSE MODEL OF HUMAN PEDIATRIC COMBINED PITUITARY HORMONE DEFICIENCY DISEASE

3.1 Introduction

Patients with mutations within the *LHX3* gene are diagnosed with CPHD and typically present with other syndromic features, including limited head rotation, in addition to pituitary hormone deficiencies. Patients with the *LHX3*W224ter mutation present with the expected hormone deficiencies, but they do not have the limited head rotation thereby defining a new form of the disease (Pfaeffle, Savage et al. 2007). The location of this particular mutation within the LHX3 protein is interesting in that it occurs just beyond the homeodomain and results in a truncated LHX3 protein that lacks the carboxyl terminus which has been shown to contain the critical activation domain necessary to activate pituitary target genes. Patient observation and molecular work done with this truncated protein suggest our hypothesis, which is that the actions of LHX3 within the nervous system and pituitary appear to be separable *in vivo* with the carboxyl terminus of LHX3 being essential for the proper development of the anterior pituitary gland.

3.2 Results

Generation of *Lhx3*$^{W227ter/W227ter}$ mice

A knock-in mouse model of the human disease caused by the *LHX3*W224ter mutation was generated to investigate the effects of this particular mutation throughout development at the molecular and cellular level, an analysis which is not feasible with human patients. A targeting construct was generated and site-directed mutagenesis was used to introduce the point mutation at the appropriate place within the 129/Sv mouse *Lhx3* gene (Figure 3.1A). While the conserved tryptophan is located at position 224 within the human LHX3 protein sequence, the equivalent tryptophan within the mouse LHX3 protein sequence is at position 227. The introduction of this point mutation created a *Bbv*CI restriction site and PCR, restriction digest, and Southern blotting were used to identify correctly targeted ES cells, and later, the genotype of the animals produced (Figure 3.1 A-D). Correctly targeted clones were injected into C57BL/6J blastocysts and the resulting male chimeras were bred to female C57BL/6J mice to generate F1 heterozygotes. At this point, two different lines of mice were made: those that would retain the "Neo" cassette, and those that would have the "Neo" cassette removed from their *Lhx3* genomic locus using Cre-lox technology. The F1 male heterozygotes were paired with female C57BL/6J mice to generate N2 heterozygotes that were intercrossed to produce *Lhx3*$^{W227ter/W227ter}$ mice that still retained the "Neo" cassette (+Neo animals). The F1 female heterozygotes were bred with male B6.FVB-Tg(EIIa-Cre)C5379Lmgd/J mice (The Jackson Laboratory) to generate N2 heterozygotes in which the "Neo" cassette had been excised. The EIIa-Cre gene is expressed ubiquitously within the mouse embryo

prior to its implantation within the uterine wall. These N2 heterozygotes were intercrossed to produce *Lhx3*$^{W227ter/W227ter}$ mice which lacked the "Neo" cassette (-Neo animals). Several litters produced by the (+Neo) animals did not yield any viable *Lhx3*$^{W227ter/W227ter}$ mice, thus this line was not maintained (Figure 3.1F). However, the (-Neo) line of animals produced *Lhx3*$^{W227ter/W227ter}$ pups available for analyses. Since the *Lhx3* locus of the (-Neo) animals was closest to its native form and to that of the human patients, this line of animals was continued and all experiments were performed on the offspring of the N2 intercross of mixed genetic background within the (-Neo) line of animals.

Lhx3$^{W227ter/W227ter}$ mice survive and exhibit a dwarf phenotype

Wild-type, heterozygote, and homozygote animals were produced at the appropriate 1:2:1 Mendelian ratio with *Lhx3*$^{+/W227ter}$ heterozygotes appearing no different from their wild-type litter mates. Very little prenatal loss was observed, however, some postnatal loss was noted with several homozygote stillbirths (Figure 3.1F), and a few *Lhx3*$^{W227ter/W227ter}$ mice exhibited wasting and eventually died between the ages of P21 and P60. Initially, *Lhx3*$^{W227ter/W227ter}$ homozygote mice resembled their heterozygote and wild-type counterparts, however, growth insufficiency was noticed as early as P10 with a significant difference in the size of the *Lhx3*$^{W227ter/W227ter}$ homozygote mice noted around P21 and continuing into adulthood (Figure 3.2). The anterior pituitary of adult *Lhx3*$^{W227ter/W227ter}$ mice is severely hypoplastic when compared to that of adult wild-type animals, while their posterior lobes appear to be similar in size (Figure 3.3A). To further examine the growth deficiency of the *Lhx3*$^{W227ter/W227ter}$ mice, immunohistochemical

staining (IHC) with hormone markers was done on pituitaries of mice at P1 and at 12 weeks old. IHC staining shows that GH- and TSHβ-producing cells are reduced at P1 (Figure 3.3B and D). The GH staining in the *Lhx3*$^{W227ter/W227ter}$ pituitaries at P1 varied with some mutant pituitaries exhibiting very little GH staining (inset picture of Figure 3.3B). While there appears to be similar densities of GH-producing cells staining at 12 weeks between the wild-type and the *Lhx3*$^{W227ter/W227ter}$ pituitaries, the mutant pituitaries are still hypoplastic (Figure 3.3C). TSHβ-positive cells are reduced at 12 weeks in the *Lhx3*$^{W227ter/W227ter}$ pituitaries (Figure 3.3E). Real-time quantitative PCR analyses revealed a deficiency in *Gh* transcript in *Lhx3*$^{W227ter/W227ter}$ mice at both 6 weeks of age (Figure 3.3F) and at 12 weeks of age (Figure 3.3G), and a decrease in transcript levels for the alpha subunit of TSH (*αGsu*) was also seen at these time points (Figure 3.4A-B). However, *Tshb* transcript is increased at both 6 weeks of age (Figure 3.4C) and at 12 weeks of age (Figure 3.4D) in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice.

Since pituitary GH acts on the liver to produce insulin-like growth factor 1 (IGF-1), and pituitary TSH promotes the production of thyroxine (T_4) from the thyroid, serum hormone levels of total IGF-1 and total T_4 were measured in the mice at a 6 week time point (representing juvenile mice at a pubertal time point) and at a 12 week time point (representing adult mice finished with puberty). Serum IGF-1 levels are relatively similar to the *Gh* transcript levels. At the 6 week time point, both *Gh* transcript and IGF-1 hormone levels are reduced significantly in *Lhx3*$^{W227ter/W227ter}$ mice (Figure 3.3F and 3.3H). At the 12 week time point, *Gh* transcript and IGF-1 hormone levels are still lower in *Lhx3*$^{W227ter/W227ter}$ mice, but it is no longer significant (Figure 3.3G and 3.3I). Total T_4 was significantly lower at both time points in the *Lhx3*$^{W227ter/W227ter}$ mice (Figure 3.3J-K).

Histological examination of the thyroid glands of the mice showed reduced follicle size which is indicative of hypothyroidism (Figure 3.3L-M).

Fertility is compromised in *Lhx3*$^{W227ter/W227ter}$ mice

Given the hormone deficiencies of the human patients, we expected similar hormone deficiencies in the *Lhx3*$^{W227ter/W227ter}$ mice, with LH, FSH, and PRL deficits possibly leading to reduced fertility. Test matings were set up with wild-type males paired with *Lhx3*$^{W227ter/W227ter}$ female mice, wild-type females paired with *Lhx3*$^{W227ter/W227ter}$ male mice, and male and female *Lhx3*$^{+/W227ter}$ heterozygote pairs were used as controls (Table 1). Over a four month period, the percentage of breeding pairs producing offspring (productive matings), the average number of litters produced, and the mean litter size were recorded so that relative fecundity (R.F.) could be calculated as we have previously described (Savage, Mullen et al. 2007)(Silver 1995). The *Lhx3*$^{+/W227ter}$ heterozygote animals did not have any apparent reduction in fertility producing litters on average every 24 days (R.F. = 24.4). Of the control heterozygote crossings (n = 18) and the *Lhx3*$^{W227ter/W227ter}$ male mice paired with wild-type females (n = 10), 100% of those pairs produced litters. While the *Lhx3*$^{W227ter/W227ter}$ male mice were able to produce litters of comparable size to those mice of the heterozygote crossings, they produced litters at a reduced rate (R.F. = 18.5). The female *Lhx3*$^{W227ter/W227ter}$ mice paired with wild-type males (n = 9) never produced any litters (Table 1).

To further examine the reduced fertility of the male *Lhx3*$^{W227ter/W227ter}$ mice and the infertility of the female *Lhx3*$^{W227ter/W227ter}$ mice, IHC staining at P1 and at 12 weeks of age of male and female *Lhx3*$^{W227ter/W227ter}$ pituitaries revealed deficiencies in LHβ-

(Figure 3.5A-B), and FSHβ-producing cells (data not shown). As with GH, variation was seen with the LHβ staining within the *Lhx3*$^{W227ter/W227ter}$ pituitaries at P1 with some mutant pituitaries displaying very little staining. Real time quantitative PCR analyses at 6 weeks of age determined that the transcript levels of both *Lhb* (Figure 3.4E) and *Fshb* (Figure 3.4G) were not significantly different in the knock-in mice compared to the wild-type and heterozygote controls. However, at 12 weeks of age, *Lhb* (Figure 3.4F) and *Fshb* (Figure 3.4H) transcript levels were both increased in the *Lhx3*$^{W227ter/W227ter}$ mice. And, as previously mentioned, transcript levels of the alpha subunit of both LH and FSH (*αGsu*) was down at both 6 weeks and 12 weeks of age (Figure 3.4A-B). *Gnrhr* transcript was also measured in the mice at 12 weeks of age, but no significant difference was observed in *Gnrhr* transcript levels between the *Lhx3*$^{W227ter/W227ter}$ mice and the wild-type and heterozygote controls (Figure 3.4I).

The seminal vesicles of male *Lhx3*$^{W227ter/W227ter}$ mice are proportionally smaller than wild-type male mice at both 6 weeks of age (Figure 3.5C) and at 12 weeks of age (Figure 3.5D). Further analyses of juvenile males (6 weeks old) show a difference between the weights of the seminal vesicles of the male *Lhx3*$^{W227ter/W227ter}$ mice and those of wild-type controls, with the seminal vesicles of the mutant mice weighing significantly less. This is indicative of reduced testosterone production in the male *Lhx3*$^{W227ter/W227ter}$ mice. A reduction in the size of the prostate and testes of the male *Lhx3*$^{W227ter/W227ter}$ mice was also consistently noted, but was not significant compared to wild-type controls. Histological examination of the seminal vesicles (Figure 3.5G) and prostate (Figure 3.5I) showed reduced secretions in these structures of the male *Lhx3*$^{W227ter/W227ter}$ mice, while in the testes, spermatogenesis was either decreased or absent with no mature sperm apparent

within the testes (Figure 3.5H) or epididymis, implying sexual immaturity of the male *Lhx3*$^{W227ter/W227ter}$ mice at 6 weeks of age compared to wild-type controls. Ovarian failure does not appear to be the primary cause of the infertility noted in the female *Lhx3*$^{W227ter/W227ter}$ mice as the ovaries of the mutant mice appear to be functioning normally with corpora lutea present along with both mature and immature eggs similar to that observed in the wild-type females at 6 weeks of age (Figure 3.5G). However, a severe lack of PRL is evident in both male and female *Lhx3*$^{W227ter/W227ter}$ mice. IHC staining at both P1 and 12 weeks of age demonstrate a deficiency in PRL-producing lactotrope cells of the pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice (Figure 3.6A-B). The *Prl* transcript is strikingly down in the pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice at both 6 weeks (Figure 3.6C) and 12 weeks (Figure 3.6D) of age. Corresponding with the decrease in transcript level, PRL hormone levels are also down in the *Lhx3*$^{W227ter/W227ter}$ mice at these time points (Figure 3.6E-F). Western blot data confirm a near absence of PRL protein in the pituitaries of adult *Lhx3*$^{W227ter/W227ter}$ mice compared to wild-type controls (Figure 3.6G) (western blot data obtained by Raleigh Malik). Histological examination of the uteri of female *Lhx3*$^{W227ter/W227ter}$ mice revealed a proportional significant difference in the weight and size, with the uteri of the *Lhx3*$^{W227ter/W227ter}$ mice weighing significantly less than those of wild-type females (Figure 3.5E). Closer examination revealed a more immature uterus of *Lhx3*$^{W227ter/W227ter}$ female mice compared to that of wild-type controls at 6 weeks of age (Figure 3.5F). In the wild-type females, the thickness and keratinization of the uterine wall changed with estrous; however, the uteri of the mutant female mice did not seem to reflect the varying stages of estrous. The uterine wall of the mutant female mice was thinner with no keratinization.

Other pituitary hormone analyses

In addition to the expected deficiencies in hormones in the knock-in mice, IHC staining in pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice indicated that ACTH-positive cells were reduced in the knock-in mice at both P1 (Figure 3.7A) and at 12 weeks (Figure 3.7B). *Pomc* transcript levels were also decreased at 12 weeks although not significantly (Figure 3.7C).

Altered gene expression in *Lhx3*$^{W227ter/W227ter}$ mice

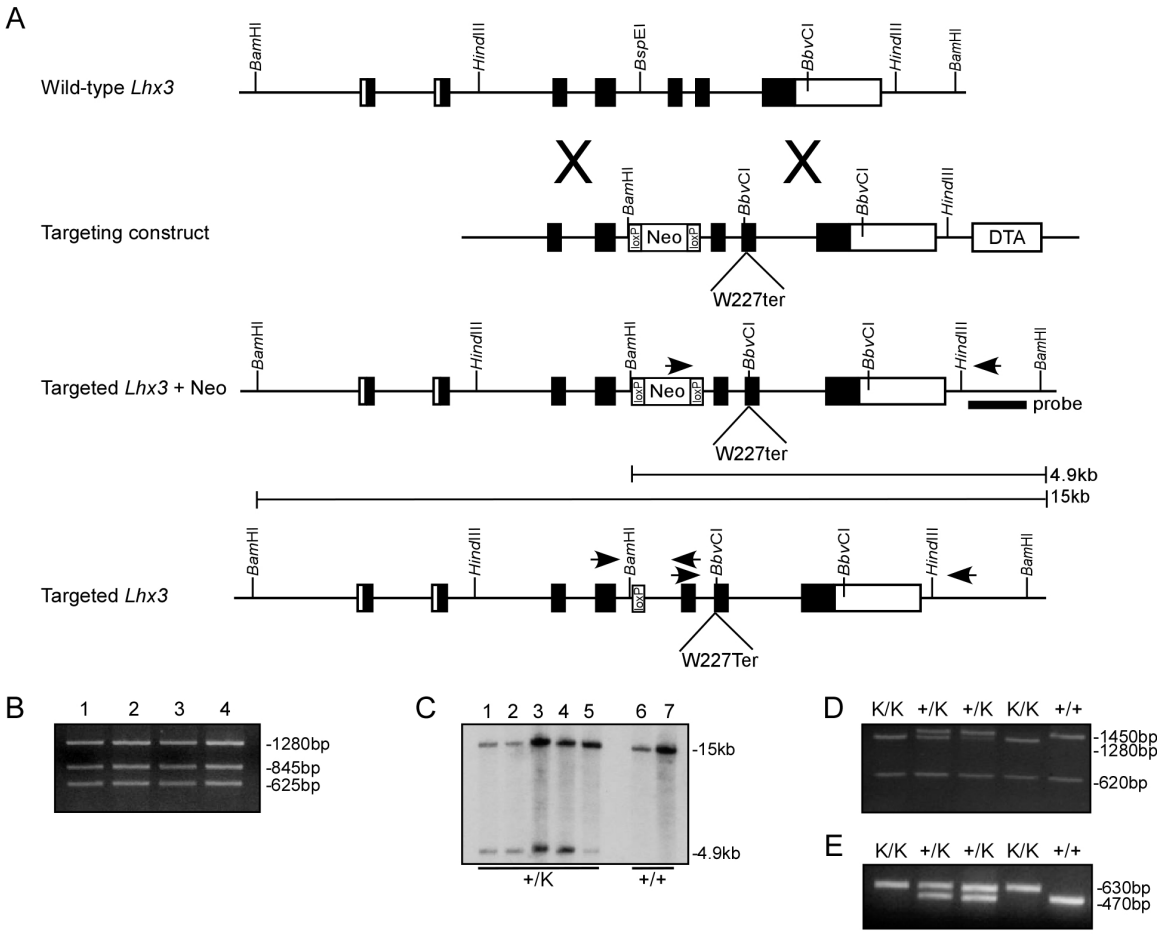
Real time qPCR analyses done at 12 weeks of age revealed a difference in gene expression of several different genes involved in pituitary development within the pituitaries of *Lhx3*$^{W227ter/W227ter}$ knock-in mice. Using a Taqman probe placed within the *Lhx3* transcript so that both the *Lhx3a* and the *Lhx3b* transcripts would be measured, real-time qPCR analyses demonstrated a significant decrease in *Lhx3a&b* transcript levels in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice at both 6 weeks (Figure 3.8A) and 12 weeks (Figure 3.8B) of age. Also, mice heterozygous for the knock-in mutation have reduced levels of *Lhx3a&b* transcript with this reduction significant at 6 weeks (Figure 3.8A). *Lhx4* transcript levels were also examined, but no significant difference was found between the knock-in mice and the wild-type and heterozygote controls (Figure 3.8E). *Pit-1* transcript levels were significantly decreased in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice (Figure 3.8C); however, *Pitx1* transcript levels were significantly increased in pituitaries of *Lhx3*$^{W227ter/W227ter}$ knock-in mice (Figure 3.8D).

Table 3.1. Reproductive performance of *Lhx3* W227ter mice.

	Number of pairs[a]	Average number of litters[b]	Average litter size	Relative fecundity[c]
+/W227ter males x +/W227ter females	18	4.2±1	5.8±2.4	24.4
W227ter/W227ter males x +/+ females	10	2.8±1.1	6.6±2.0	18.5
+/+ males x W227ter/W227ter females	9	0	0	0

[a]All mice were maintained as breeding pairs for a minimum of four months.
[b]Average number of litters produced from breeding pairs paired together over a four month mating period.
[c]Relative fecundity is calculated as (productive matings) x (litter size) x (number of litters) (Silver 1995).

Figure 3.1. *Lhx3*$^{W227ter/W227ter}$ mice are viable. **A.** Strategy for knocking in the W227ter point mutation using homologous recombination. The wild-type *Lhx3* allele, the targeting construct, the *Lhx3* allele with the point mutation and LoxP-"Neo" cassette knocked in (Targeted *Lhx3* + Neo), and the *Lhx3* allele with the point mutation and after removal of the Neo cassette (Targeted *Lhx3*) are shown. Coding regions of *Lhx3* exons are denoted by black boxes. The W227ter point mutation introduces a novel *Bbv*CI restriction enzyme site used for genotyping. Predicted DNA fragments that hybridize with the indicated probe during Southern blot analysis are shown. Primers used for PCR analysis of genomic DNA obtained from ES cells and animals are represented as black arrowheads. **B.** Diagnostic PCR analysis demonstrates correct targeting of four representative ES cell lines. A 2750 kb fragment was amplified using the primers shown above and was then digested with the *Bbv*CI to demonstrate the presence of both the Neo cassette and the point mutation. **C.** Southern blot analysis using *Bam*HI confirms the presence of both the wild-type *Lhx3* allele (15 kb) and the targeted *Lhx3* allele (4.9 kb). **D.** PCR to confirm the generation of the expected genotypes. A 2050 kb fragment digested with *Bbv*CI enzyme to confirm the presence of the point mutation. Littermates were either wild-type (+/+), heterozygous (+/K), or homozygous for the W227ter knock-in mutation (K/K). **E.** Genotyping to confirm removal of the Neo cassette following crossing to Cre-expressing mice. Expected band sizes are 470 bp for wild-type and 630 bp for the knock-in. **F.** *Lhx3*$^{W227ter/W227ter}$ mice are viable with almost no prenatal loss and little postnatal loss. By contrast, *Lhx3*$^{W227ter/W227ter}$ mice retaining Neo sequences have high rates of prenatal and postnatal death. Asterisk (*) indicates the number of mice viable at 2 weeks of age when genotyping occurred.



F

	+/+ Number (%)	+/W227ter Number (%)	W227ter/W227ter Number (%)
Total Neo (-) animals	208 (23.6%)	474 (53.7%)	200 (22.7%)
Total Neo (+) animals	53 (39%)	81 (59.6%)	2 (1.5%)
Viable* Neo (-) animals (2 weeks)	204 (24.3%)	464 (55.4%)	170 (20.3%)
Viable* Neo (+) animals (2 weeks)	53 (39.6%)	81 (60.4%)	0 (0%)

Figure 3.1

Figure 3.2. *Lhx3*$^{W227ter/W227ter}$ mice are dwarfed. **A-B.** In comparison to wild-type and heterozygote controls, growth deficiency of both male (**A**) and female (**B**) *Lhx3*$^{W227ter/W227ter}$ mice is evident around postnatal day 10 (P10) and becomes significant at P21. Asterisks (*) indicate a significant difference between homozygote *Lhx3*$^{W227ter/W227ter}$ compared to wild-type and heterozygote controls with $p<0.05$. As it proved to be difficult to weigh the same population of animals at every time point examined, the depicted n values represent the minimum number of animals weighed at a given time point. **C.** The picture represents *Lhx3*$^{+/+}$ (left) and *Lhx3*$^{W227ter/W227ter}$ male littermates at 8 weeks old.

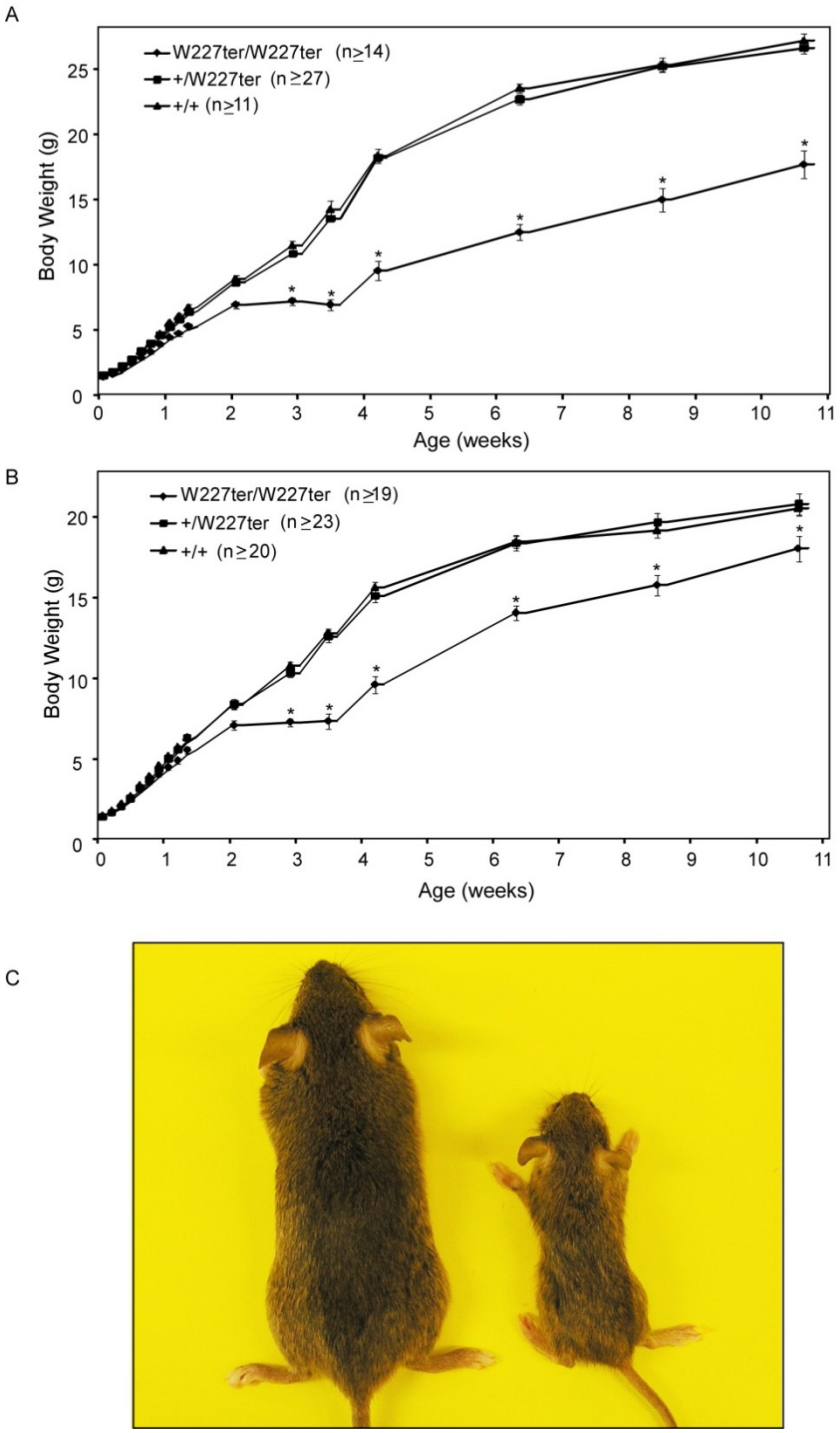

A
W227ter/W227ter (n≥14)
+/W227ter (n≥27)
+/+ (n≥11)
Body Weight (g)
Age (weeks)
B
W227ter/W227ter (n≥19)
+/W227ter (n≥23)
+/+ (n≥20)
Body Weight (g)
Age (weeks)
C

Figure 3.2

Figure 3.3. Deficiencies in the growth hormone and thyroid hormone pituitary signaling axes underlie dwarfism in *Lhx3*$^{W227ter/W227ter}$ mice. **A.** The anterior lobe (AL) of 12 week old adult *Lhx3*$^{W227ter/W227ter}$ pituitary gland is hypoplastic compared that of wild- type (+/+) animals while the posterior lobes (PL, dotted oval) are of similar size. Scale bar = 1 mm. **B-E.** Immunostaining of P1 (**B** and **D**) and 12 week old pituitaries (**C** and **E**) reveals deficiencies in GH- and TSHβ-producing cells of the *Lhx3*$^{W227ter/W227ter}$ pituitaries. The inset in panel B shows an additional example of an *Lhx3*$^{W227ter/W227ter}$ animal with negligible GH-producing cells. Scale bars = 200 μm. **F-G.** Real-time quantitative PCR analyses show that the *Gh* transcript is decreased in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) at both 6 weeks (**F**) and 12 weeks (**G**) of age. Data are mRNA levels normalized to a control transcript (*36b4*). **H-K.** Hormone analyses of trunk blood serum indicate a decrease in both IGF-1 and T4 levels in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) compared to their wild type (+/+) and heterozygote (+/KI) counterparts at both 6 weeks (**H** and **J**) and 12 weeks (**I** and **K**) of age. **L-M.** Thyroid follicles of *Lhx3*$^{W227ter/W227ter}$ mutant mice (**M**) are smaller compared to those of wild type mice (**L**) indicating impaired thyroid function. Scale bar = 50 μm. Asterisks (*) indicate significance when compared to wild type controls with $p<0.05$.

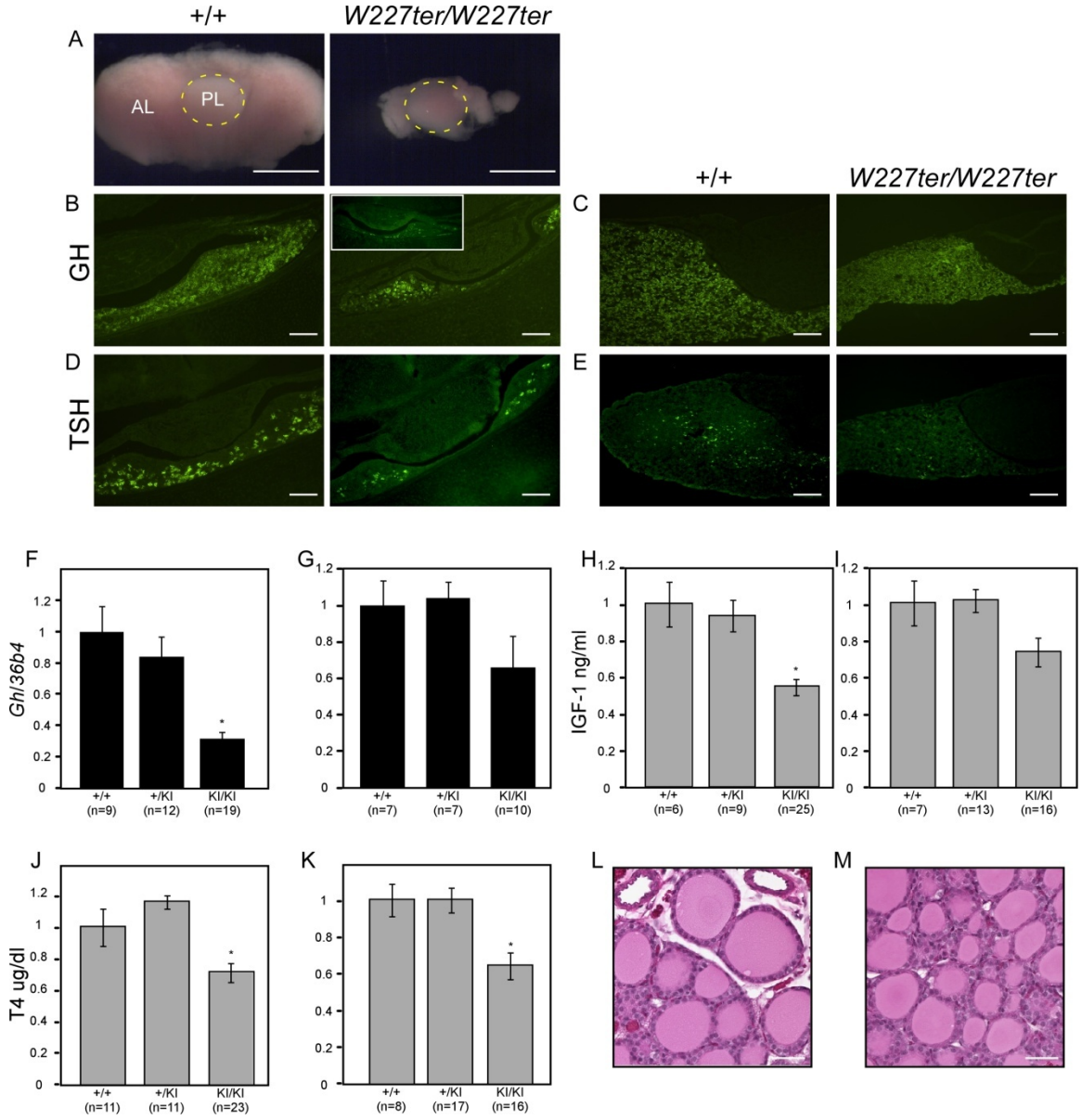

+/+
W227ter/W227ter
A
AL
PL
B
GH
C
+/+
W227ter/W227ter
D
TSH
E
F
Gh/36b4
G
H
IGF-1 ng/ml
I
+/+ (n=9)
+/KI (n=12)
KI/KI (n=19)
+/+ (n=7)
+/KI (n=7)
KI/KI (n=10)
+/+ (n=6)
+/KI (n=9)
KI/KI (n=25)
+/+ (n=7)
+/KI (n=13)
KI/KI (n=16)
J
T4 ug/dl
K
L
M
+/+ (n=11)
+/KI (n=11)
KI/KI (n=23)
+/+ (n=8)
+/KI (n=17)
KI/KI (n=16)

Figure 3.3

Figure 3.4. Altered expression of dimeric hormone transcripts. **A-B.** Real-time quantitative PCR analyses show that the *αGsu* (*Cga*) transcript is decreased in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) at 6 weeks (**A**) and at 12 weeks (**B**) of age. However, the *Tshb* transcript is increased in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) at both 6 weeks (**C**) and 12 weeks (**D**) of age. At 6 weeks of age, *Lhb* (**E**) and *Fshb* (**G**) transcript in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) are not significantly different from wild-type (+/+) and heterozygote (+/KI) controls, but at the 12 week time point, both *Lhb* (**F**) and *Fshb* (**H**) transcript levels are significantly increased in the knock-in mice compared to wild-type controls. **I.** No significant difference in *Gnrhr* transcript levels was observed in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) compared to wild-type (+/+) animals at 12 weeks of age. Asterisks (*) indicate significance when compared to wild type controls with $p<0.05$.

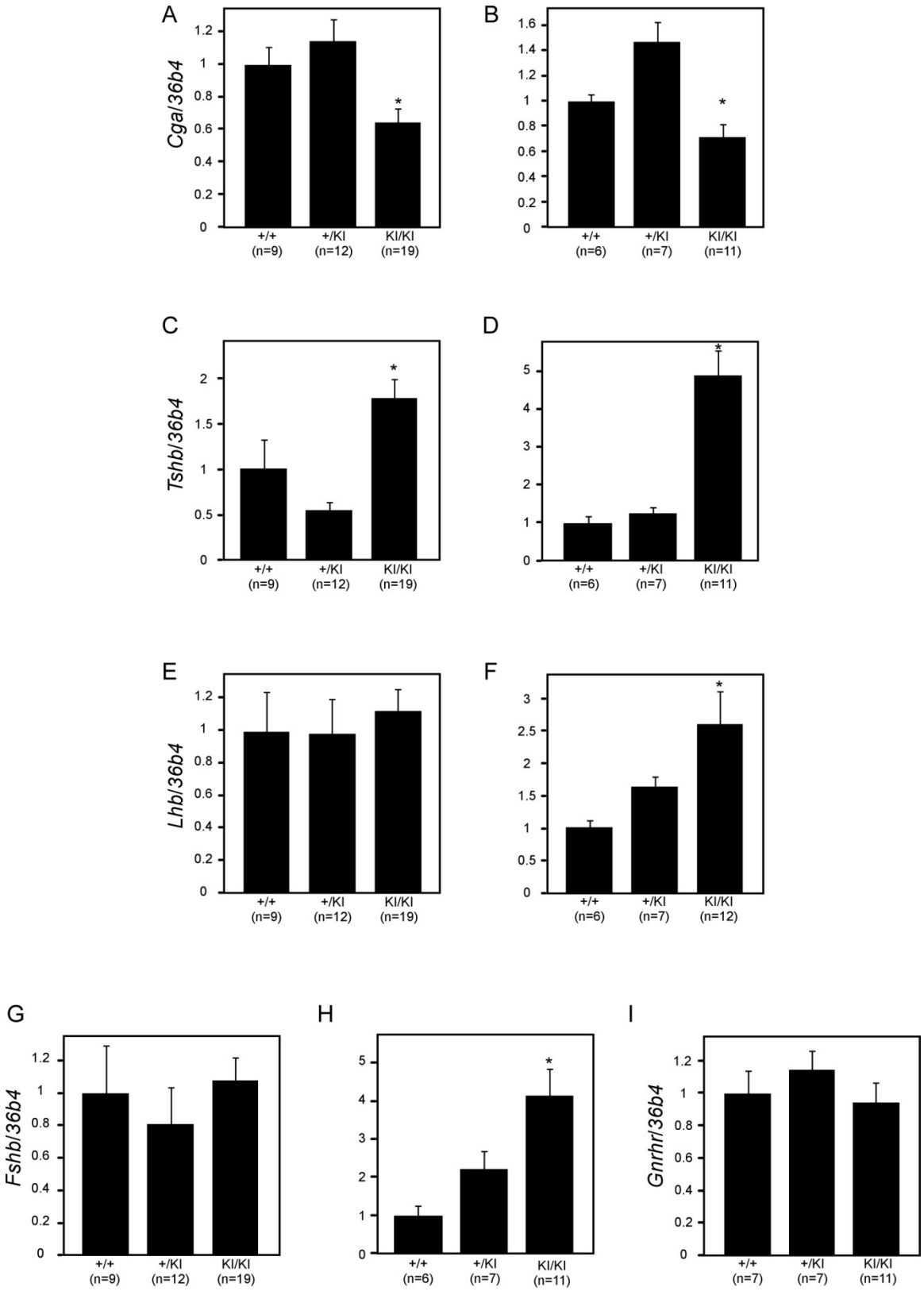

A
Cga/36b4
1.2
1
0.8
0.6
0.4
0.2
0
*
+/+ (n=9)
+/KI (n=12)
KI/KI (n=19)
B
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+/+ (n=6)
+/KI (n=7)
KI/KI (n=11)
C
Tshb/36b4
2
1.5
1
0.5
0
*
+/+ (n=9)
+/KI (n=12)
KI/KI (n=19)
D
5
4
3
2
1
0
*
+/+ (n=6)
+/KI (n=7)
KI/KI (n=11)
E
Lhb/36b4
1.2
1
0.8
0.6
0.4
0.2
0
+/+ (n=9)
+/KI (n=12)
KI/KI (n=19)
F
3
2.5
2
1.5
1
0.5
0
*
+/+ (n=6)
+/KI (n=7)
KI/KI (n=12)
G
Fshb/36b4
1.2
1
0.8
0.6
0.4
0.2
0
+/+ (n=9)
+/KI (n=12)
KI/KI (n=19)
H
5
4
3
2
1
0
*
+/+ (n=6)
+/KI (n=7)
KI/KI (n=11)
I
Gnrhr/36b4
1.2
1
0.8
0.6
0.4
0.2
0
+/+ (n=7)
+/KI (n=7)
KI/KI (n=11)

Figure 3.4

Figure 3.5. Sexual maturation and fertility are impaired in *Lhx3*$^{W227ter/W227ter}$ mice. **A-B.** Immunostaining reveals reduced numbers of LH-producing gonadotrope cells in pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice at both P1 (**A**) and at 12 weeks (**B**). Scale bars = 200 μm. **C-D.** Seminal vesicles in male *Lhx3*$^{W227Ter/W227Ter}$ mice are smaller than those of wild type male mice at both 6 weeks (**C**) and 12 weeks (**D**) of age. Scale bars = 0.5 cm. **E.** The uteri of female *Lhx3*$^{W227ter/W227ter}$ mutant mice are significantly smaller than wild-type controls at 6 weeks of age. Scale bar = 1 cm. **F-G.** Histological examination indicates the uteri of female *Lhx3*$^{W227ter/W227ter}$ mutant mice are immature compared to wild-type controls (+/+) (**F**) while the ovaries of female *Lhx3*$^{W227ter/W227ter}$ mutant mice appear similar to wild-type controls (**G**). Scale bars = 100 μm. **H-J.** Histological analyses of seminal vesicles (**H**), testes (**I**), and prostates (**J**) of *Lhx3*$^{+/+}$ and *Lhx3*$^{W227ter/W227ter}$ male mice at 6 weeks of age indicate delayed sexual maturity of the *Lhx3*$^{W227ter/W227ter}$ mice compared to wild type (+/+) controls. Scale bars = 100 μm.

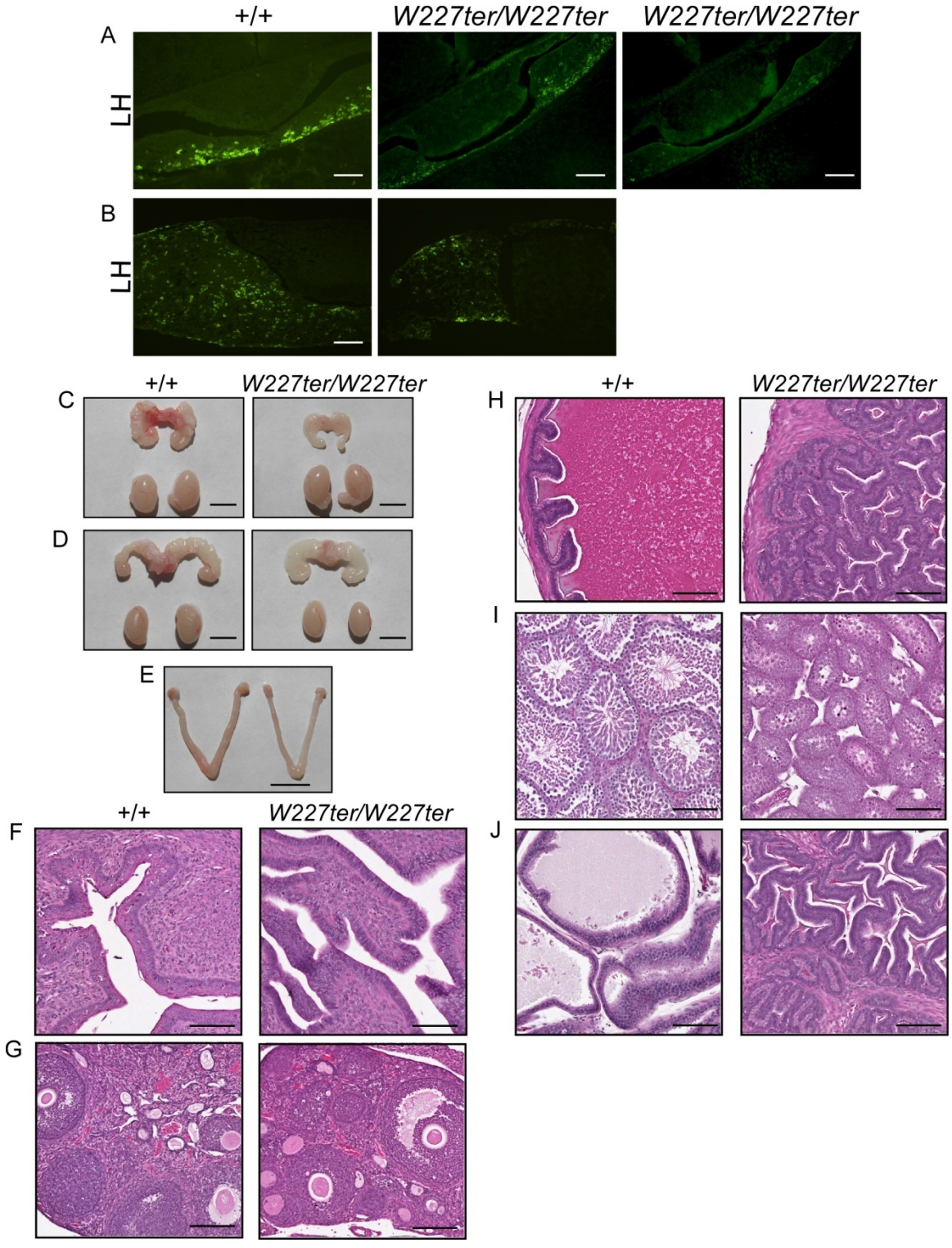

+/+
W227ter/W227ter
W227ter/W227ter
A
LH
B
LH
C
+/+
W227ter/W227ter
D
E
H
+/+
W227ter/W227ter
I
J
F
+/+
W227ter/W227ter
G

Figure 3.5

Figure 3.6. PRL deficiency and infertility in *Lhx3*$^{W227ter/W227ter}$ female mice. **A-B.** PRL immunostaining of pituitaries of P1 (**A**) and 12 week old (**B**) mice show a paucity of PRL-producing lactotrope cells in *Lhx3*$^{W227ter/W227ter}$ mice. Scale bars = 200 μm. **C-D.** Real-time quantitative PCR analyses demonstrate a striking reduction in *Prl* transcript in *Lhx3*$^{W227ter/W227ter}$ pituitaries (KI/KI) relative to their wild type (+/+) and heterozygote (+/KI) counterparts at both 6 weeks (**C**) and at 12 weeks (**D**) of age. Data are mRNA levels normalized to a control transcript (*36b4*). **E-F.** Hormone analyses also show a dramatic decrease in serum PRL in *Lhx3*$^{W227ter/W227ter}$ mutant mice at both 6 weeks (**E**) and 12 weeks (**F**). Asterisk (*) indicates significance compared to wild type controls with $p<0.05$ or less. **G.** Western blot detection of PRL protein in pituitaries confirms the PRL deficiency in the *Lhx3*$^{W227ter/W227ter}$ mutant mice compared to wild type controls. Blots were reprobed with an anti-GAPDH antibody as a loading control.

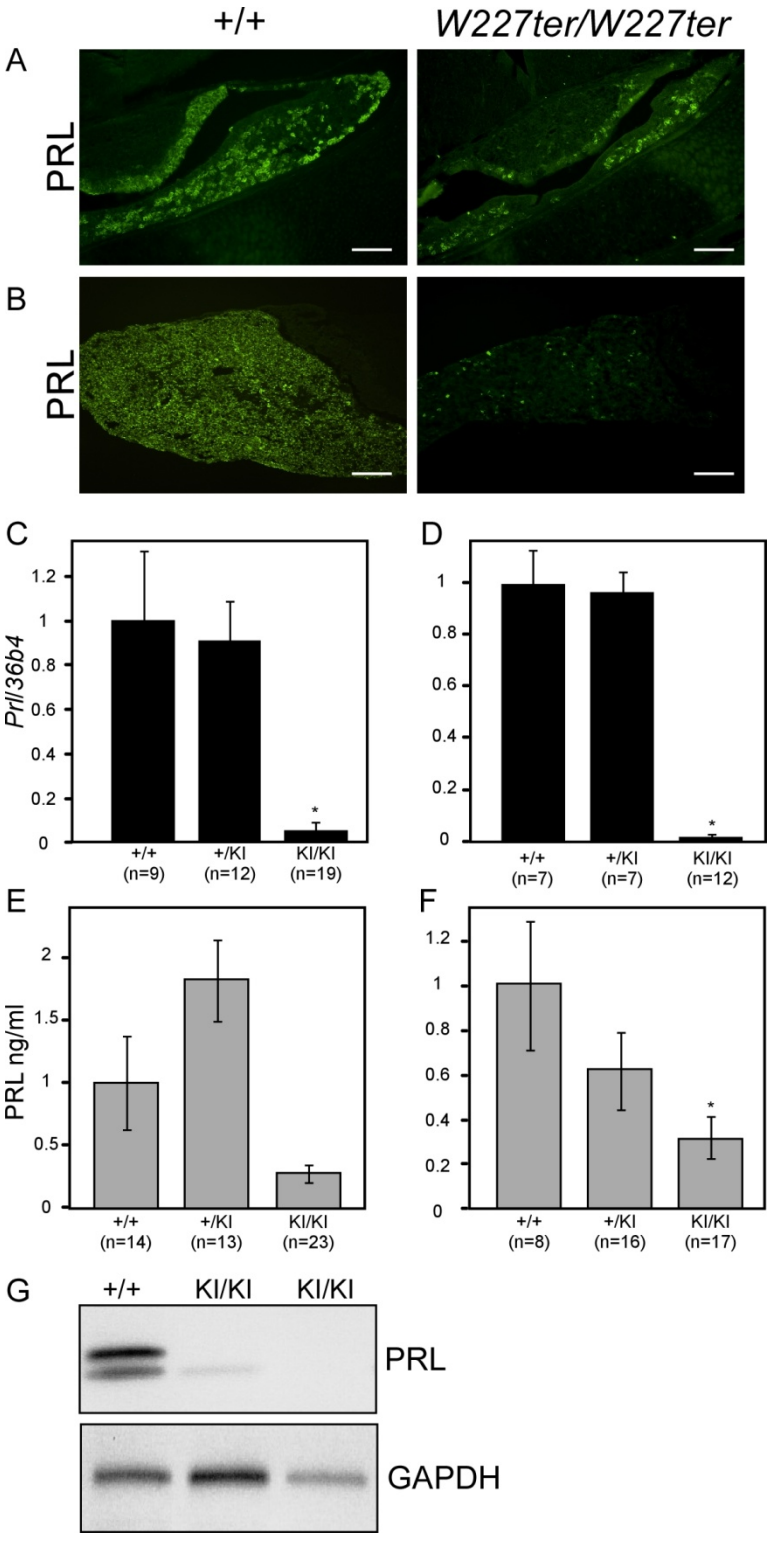

+/+
W227ter/W227ter
A
PRL
B
PRL
C
Prl/36b4
1.2
1
0.8
0.6
0.4
0.2
0
*
+/+ (n=9)
+/KI (n=12)
KI/KI (n=19)
D
1
0.8
0.6
0.4
0.2
0
*
+/+ (n=7)
+/KI (n=7)
KI/KI (n=12)
E
PRL ng/ml
2
1.5
1
0.5
0
+/+ (n=14)
+/KI (n=13)
KI/KI (n=23)
F
1.2
1
0.8
0.6
0.4
0.2
0
*
+/+ (n=8)
+/KI (n=16)
KI/KI (n=17)
G
+/+
KI/KI
KI/KI
PRL
GAPDH

Figure 3.6

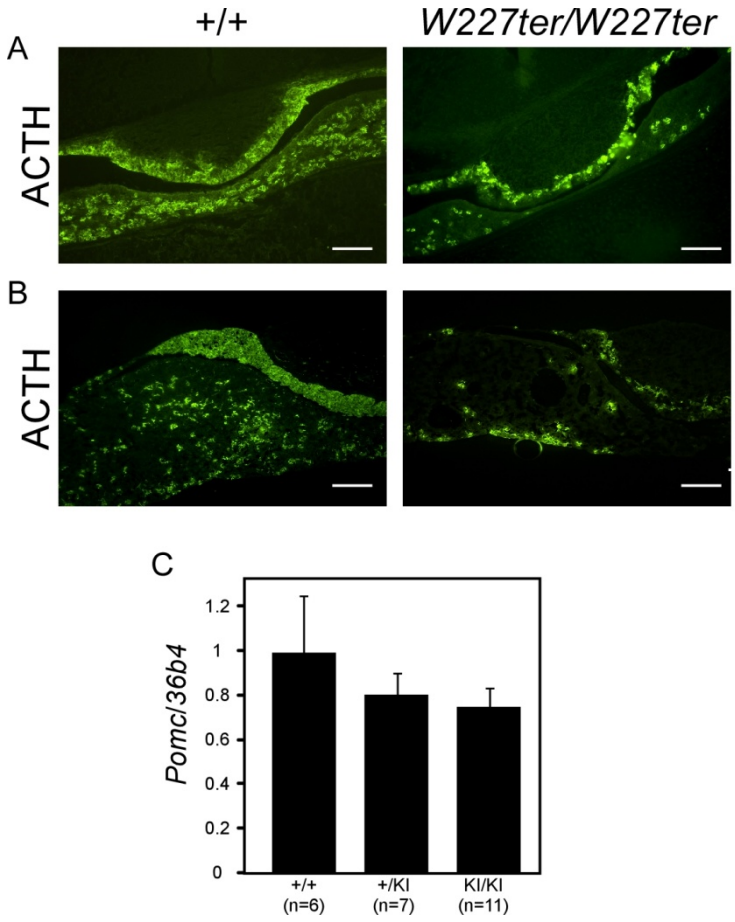


Figure 3.7. The corticotrope population is decreased in pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice. **A-B.** Immunostaining of P1 (**A**) and 12 week old pituitaries (**B**) reveals a deficiency in ACTH-producing cells of the *Lhx3*$^{W227ter/W227ter}$ pituitaries. Scale bars = 200 µm. **C.** Real-time quantitative PCR analyses show that the *Pomc* transcript is slightly decreased in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) at 12 weeks of age, although this decrease is not significant.

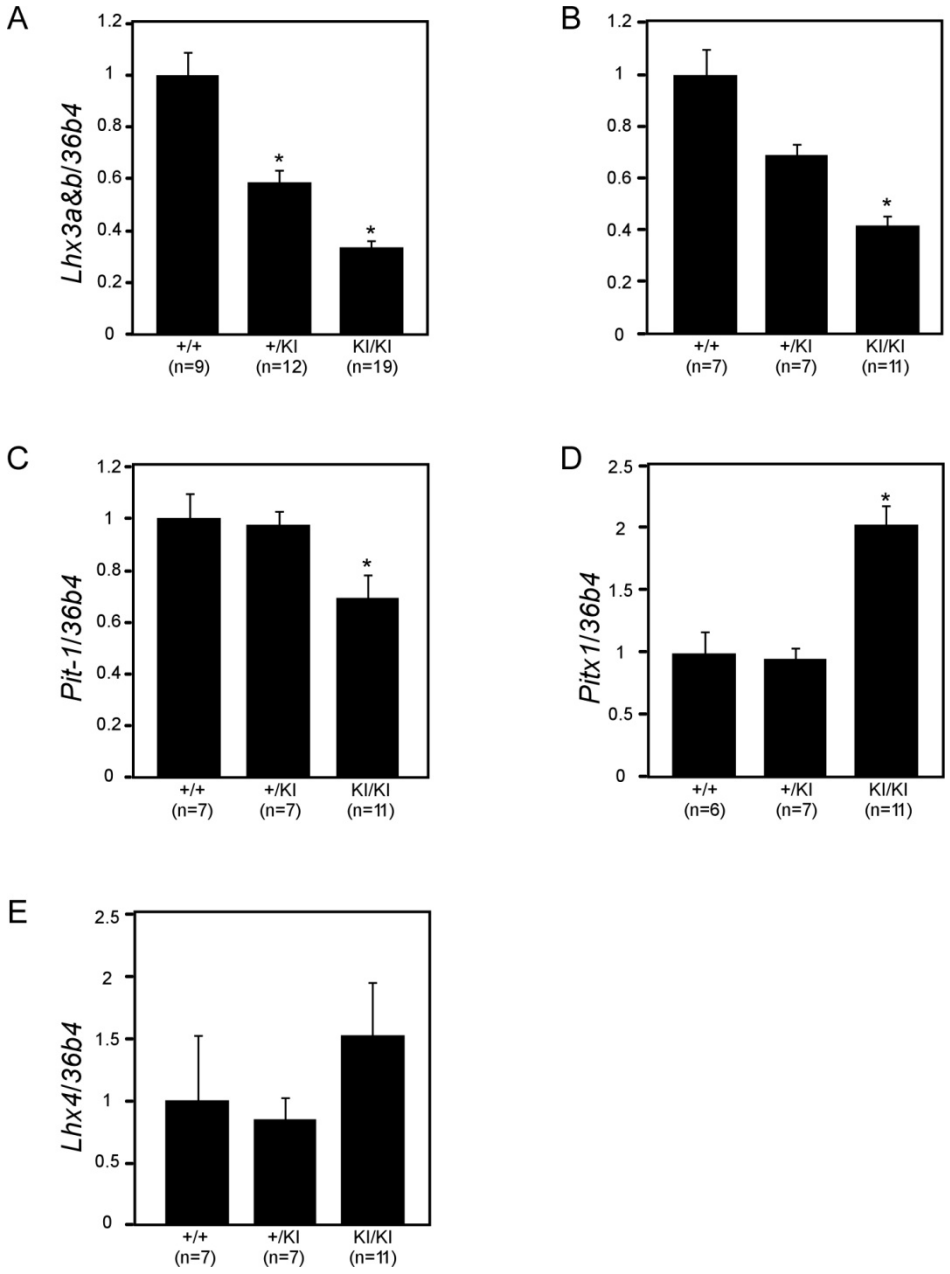


Figure 3.8. Altered gene expression of transcription factors involved in pituitary development. **A-B.** Examination of transcript levels of *Lhx3a&b* via real-time qPCR revealed a significant decrease in both *Lhx3*$^{+/W227ter}$ heterozygote (+/KI) and *Lhx3*$^{W227ter/W227ter}$ homozygote (KI/KI) mice at both 6 weeks (**A**) and 12 weeks of age (**B**). **C.** *Pit-1* transcript was found to be decreased in the knock-in mice at 12 weeks of age. **D.** Transcript levels of *Pitx1* were increased in *Lhx3*$^{W227ter/W227ter}$ mutant mice in comparison to wild-type controls (+/+). **E.** *Lhx4* transcript levels were not significantly different in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice compared to wild-type controls. Asterisks (*) indicate significance when compared to wild type controls with $p<0.05$.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

We have made a new dwarf mouse model that models the human disease caused by the W224ter mutation within the *LHX3* gene. As for the *Lhx3* knockout mice that die within 24 hours of birth (Sheng, Zhadanov et al. 1996), the *Lhx3*$^{W227ter/W227ter}$ knock-in mice survive and are represented in the expected numbers in the offspring from heterozygous crosses. Mice heterozygous for the *Lhx3*W227ter mutation appear to be phenotypically normal in comparison to wild-type animals, in accordance with observations of patient and animals with only one functioning *Lhx3* allele (Sheng, Zhadanov et al. 1996; Pfaeffle, Savage et al. 2007; Savage, Hunter et al. 2007). However, the homozygous knock-in mice have reduced levels of GH, TSH, IGF-1, T_4, LH, FSH, and PRL, which manifests phenotypically in the mice with dwarfism, reduced fertility in the male knock-in mice, and infertility in the female knock-in mice. The truncated LHX3 protein resulting from the *Lhx3*W227ter mutation appears to have similar actions in both mice and humans as the hormone deficiencies and phenotype of the *Lhx3*$^{W227ter/W227ter}$ knock-in mice mimics that of the human patients (Pfaeffle, Savage et al. 2007; Savage, Hunter et al. 2007). In addition to the expected deficiencies in hormones in the knock-in mice, IHC staining in pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice indicated that ACTH-positive cells were reduced in the knock-in mice at both P1 and at 12 weeks of age. *Pomc*

transcript levels were also decreased at 12 weeks of age in the knock-in mice. While ACTH deficiency is not associated with this particular mutation in the human patients, other mutations within the *LHX3* gene have been linked with ACTH deficiency and the population of corticotropes that is found in the *Lhx3*$^{-/-}$ mice is diminished (Sheng, Zhadanov et al. 1996; Colvin, Mullen et al. 2009). More experiments are necessary to determine if the reduction in the ACTH-producing cells within the pituitaries of the *Lhx3*$^{W227ter/W227ter}$ knock-in mice results in lower serum ACTH levels and potentially reduced serum corticosterone levels.

The growth insufficiency seen in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice is likely due to the deficiencies in GH, IGF-1, TSH, and T_4 as all of these proteins appeared to be significantly reduced in the knock-in mice when compared to wild-type controls. However, real-time qPCR analyses revealed an increase in *Tshb* transcript levels at both 6 weeks and 12 weeks of age. This is presumably due to a feedback mechanism where the low circulating T_3 and T_4 hormone levels are feeding back to the pituitary in an attempt to increase pituitary output of TSH as has been shown by Shupnik et al. (1985). However, IHC staining demonstrated a decrease in thyrotrope cell numbers within the anterior pituitaries of the knock-in mice at both P1 and 12 weeks of age, and real-time qPCR revealed a decrease in transcript levels of the alpha subunit (*αGsu*) of TSH at both 6 weeks and 12 weeks of age. Overall, something is impairing the ability of the pituitary to release appropriate levels of TSH, whether it is the paucity of thyrotrope cells or the potential rate-limiting decrease in the production of αGSU, although this would be a different mechanism to the model mentioned by Shupnik et al. (1985) in which it is the beta subunit of TSH that is considered the rate-limiting step in the production of TSH

hormone. This fits with known roles of LHX3 in both cell proliferation (Sheng, Moriyama et al. 1997; Raetzman, Ward et al. 2002; Ellsworth, Butts et al. 2008) and gene activation of both αGSU and TSHβ (Bach, Rhodes et al. 1995; Sloop, Meier et al. 1999).

The growth insufficiency of the *Lhx3*$^{W227ter/W227ter}$ knock-in mice, while significant when compared to wild-type animals, does not appear to be as severe as the growth insufficiency seen in other dwarf mouse models. At 8 weeks of age, the *Lhx3*$^{W227ter/W227ter}$ knock-in mice appear to be approximately twice the size of Snell dwarf mice (*Pit-1*^{dw}), the Ames dwarf mice (*Prop1*^{df}), the *Prop1* knockout mice, and the Jackson dwarf mice (*Pit-1*$^{dw-J}$) (Eicher and Beamer 1980; Li, Crenshaw et al. 1990; Gage, Lossie et al. 1995; Gage, Roller et al. 1996; Nasonkin, Ward et al. 2004). This could be due to varying hormone levels across the different dwarf mouse models. The pituitary hypoplasia and hormone deficiency in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice does not appear to be as severe as that seen in the aforementioned dwarf mouse models (Eicher and Beamer 1980; Li, Crenshaw et al. 1990; Gage, Lossie et al. 1995; Gage, Roller et al. 1996; Nasonkin, Ward et al. 2004). This indicates that the truncated LHX3W227ter protein retains some residual function *in vivo*, which is supported by several *in vitro* assays demonstrating some function of this truncated protein (Savage, Hunter et al. 2007). The hypocellularity of the pituitaries of the *Lhx3*$^{W227ter/W227ter}$ knock-in mice needs to be investigated further and more experiments need to be done to determine if the hypocellularity is due to an increase in apoptosis or a decrease in proliferation during pituitary development.

The *Lhx3*$^{W227ter/W227ter}$ knock-in mice also have compromised fertility. The male knock-in mice have reduced fertility while the female knock-in mice are completely infertile. IHC indicates reduced levels of LHβ- and FSHβ-producing cells in the

Lhx3$^{W227ter/W227ter}$ mice at 12 weeks of age, but real-time qPCR analyses showed an increase in *Lhb* and *Fshb* transcript at this time point. As for TSH, this is presumably due to a feedback mechanism where the low circulating sex steroid hormone levels are feeding back to the pituitary in an attempt to increase pituitary output of LH and FSH. However, IHC staining demonstrated a decrease in gonadotrope cells within the anterior pituitaries of the knock-in mice at both P1 and 12 weeks of age, and real-time qPCR revealed a decrease in transcript levels of the alpha subunit (*αGsu* = *Cga*) of both of LH and FSH hormones at both 6 weeks and 12 weeks of age. Again, it is not clear whether it is the decrease in gonadotrope cells within the anterior pituitaries or the potential rate-limiting decrease in the production of αGSU that is causing the presumed decreased pituitary output of LH and FSH in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice. However, while IHC indicates LH and FSH are reduced in the knock-in mice, histological evidence suggests these hormones are not completely absent. The male knock-in mice have seminal vesicles that are proportionally smaller and weigh significantly less than their wild-type counterparts at 6 weeks of age. At the same time point, histological examination of the seminal vesicles and prostate glands of male mutant mice show a decrease in secretous material and the testes show no evidence of spermatogenesis. These data indicate a delay in puberty and testosterone production in the male *Lhx3*$^{W227ter/W227ter}$ knock-in mice presumably due to LH deficiency as observed in LHβ$^{-/-}$ mice (Kumar, Wang et al. 1997; Ma, Dong et al. 2004). The size of the seminal vesicles of the knock-in male mice at 12 weeks is still smaller than those of their wild-type counterparts, indicating lower testosterone levels. At 6 weeks of age, the sizes of the testes and prostate glands of the male knock-in mice are consistently smaller compared to those of the wild-

type controls presumably due to decreases in LH and FSH as male mice with a complete knockout of either LHβ or FSHβ also have smaller testes and prostate glands (Kumar, Wang et al. 1997; Ma, Dong et al. 2004). However, although puberty appears to be delayed in the male knock-in mice, they are eventually able to reproduce, albeit at a reduced rate compared to heterozygote controls. This pubertal delay is presumably due to the decrease in LH hormone levels which then leads to reduced testosterone levels. While LH appears to be reduced, it is not completely absent in the male *Lhx3*$^{W227ter/W227ter}$ knock-in mice as indicated by their reduced fertility; by contrast, LHβ-null male mice are sterile (Ma, Dong et al. 2004). This suggests that enough LH is present for the male *Lhx3*$^{W227ter/W227ter}$ knock-in mice to eventually become fertile. Although PRL becomes almost completely absent in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice, the lack of PRL does not appear to be the cause of reduced fertility in the male knock-in mice because PRL knockouts are fully fertile and able to reproduce at the same rate as wild-type controls (Horseman, Zhao et al. 1997). More experiments are necessary to further investigate the cause of the reduced fertility of the male *Lhx3*$^{W227ter/W227ter}$ knock-in mice. These might include measuring serum LH levels, and measuring serum and testicular testosterone levels at various timepoints with the male *Lhx3*$^{W227ter/W227ter}$ knock-in mice in an attempt to determine the point in time at which these hormone levels reach the necessary threshold for reproduction to occur.

In contrast to the male *Lhx3*$^{W227ter/W227ter}$ knock-in mice having only reduced fertility, the female knock-in mice are infertile. As mentioned previously, the *Lhx3*$^{W227ter/W227ter}$ mice have reduced levels of LH- and FSH-producing cells, however, histological analyses indicate there are enough circulating gonadotropins for normally-

functioning ovaries in the female *Lhx3*$^{W227ter/W227ter}$ knock-in mice whereas the ovaries of both the LHβ knockout mice and the FSHβ knockout mice have abnormal folliculogenesis and an absence of corpora lutea indicating no ovulation is taking place (Kumar, Wang et al. 1997; Ma, Dong et al. 2004). PRL levels are strikingly low in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice. Within the ovary, PRL is required for the proper maintenance of the corpus luteum. The PRL knockout mice appear to ovulate normally, as do the female *Lhx3*$^{W227ter/W227ter}$ knock-in mice. However, PRL knockout female mice are also infertile (Horseman, Zhao et al. 1997). One of the important roles of PRL in reproduction is that it stimulates the production of progesterone by the corpus luteum within the ovaries, which is then responsible for implantation of embryos, maintenance of pregnancy, and the inhibition of ovulation (Bole-Feysot, Goffin et al. 1998). We believe the infertility of the female *Lhx3*$^{W227ter/W227ter}$ mice is due to the PRL deficiency seen in these mice. If the pituitary glands of the female *Lhx3*$^{W227ter/W227ter}$ knock-in mice are not producing adequate amounts of PRL, then the corpus luteum is probably not receiving enough PRL support and the appropriate signals for implantation that are dependent on progesterone and estrogen signaling cannot occur (Ormandy, Camus et al. 1997). More experiments need to be done with the female knock-in mice to verify that implantation is impaired, for example, implanting wild-type embryos into the uteri of female *Lhx3*$^{W227ter/W227ter}$ knock-in mice to determine if implantation can occur. If possible, embryos flushed from the oviducts of female *Lhx3*$^{W227ter/W227ter}$ knock-in mice should also be implanted into the uteri of wild-type female mice to determine that the infertility of the female *Lhx3*$^{W227ter/W227ter}$ knock-in mice is due to an implantation problem versus a problem with the eggs being viable. Serum progesterone and estrogen levels should also

be measured in the mice to verify a disruption in hormone signaling. It would be interesting to attempt to rescue this reproductive phenotype seen in the female *Lhx3*$^{W227ter/W227ter}$ knock-in mice with exogenous PRL hormone treatments.

Histological examination of the uteri of female *Lhx3*$^{W227ter/W227ter}$ mice suggests that estrous is impaired in these animals. The uterine wall of 6 week old wild-type mice displayed varying thicknesses and levels of keratinization representing the various stages of estrous; however, the uterine wall of knock-in mice displayed only one kind of phenotype: a thin uterine wall with no keratinization suggestive of an immature uterus. The uteri of female knock-in mice were proportionally smaller and weighed significantly less than those of wild-type female mice at 6 weeks of age, and overall appeared thinner compared to uteri of wild-type female mice. However, the reduced size of the uteri of *Lhx3*$^{W227ter/W227ter}$ mice does not appear to be as drastic as the uteri of LHβ- and FSHβ-knockout mice (Kumar, Wang et al. 1997; Ma, Dong et al. 2004). It is not obvious why the estrous cycles of the knock-in mice are impaired as there appears to be enough LH and FSH present for normally functioning ovaries. It is possible that there is a threshold amount of LH and FSH required for appropriate estrous cycling; however, PRL knockout mice and PRLR knockout mice with normal LH and FSH levels also have aberrant estrous cycles (Horseman, Zhao et al. 1997; Ormandy, Camus et al. 1997). Typically, after a vaginal plug is observed, the female mice will go into a pseudopregnant state and refrain from mating for about 12 days. However, in both the PRL$^{-/-}$ and PRLR$^{-/-}$ female mice, a pseudopregnant state was never observed after vaginal plugs were noted as the animals mated again 3-4 days later. This indicates PRL may have a role for the establishment and maintenance of pseudopregnancy (Gunnet and Freeman 1983;

Horseman, Zhao et al. 1997; Ormandy, Camus et al. 1997). Additional experiments are needed to determine how often the female knock-in mice go into estrous using vaginal smear cytology and how frequently they mate. Serum LH and FSH levels should also be measured.

Several genes within the transcription factor cascade essential for proper anterior pituitary development were examined in the mice at 12 weeks of age via real-time qPCR, and aberrant expression of every gene examined was noted in the *Lhx3*$^{W227ter/W227ter}$ mice. A decrease in the *Lhx3a* and *Lhx3b* transcripts was noted in both heterozygous and knock-in mice, with the decrease in the knock-in mice significantly different from wild-type controls. It appears as though the *Lhx3* transcript containing the introduced point mutation may be subjected to some nonsense-mediated decay and/or instability, although more experiments need to be done to confirm this. Also, assuming the reduction in *Lhx3*W227ter transcript levels results in reduced LHX3W227ter protein levels, the overall reduction in LHX3W227ter protein levels or the effect of the mutation itself could be affecting the ability of the LHX3 protein to autoregulate its own transcription. Although it is not known if LHX3 is capable of regulating transcription of its own gene, other transcription factors are known to do this, for example, the PIT-1 transcription factor is capable of activating transcription of its own gene (Rhodes, Krones et al. 1996). However, enough truncated protein must be produced for the *Lhx3*$^{W227ter/W227ter}$ mice to be viable as mice homozygous for a null *Lhx3* allele die within 24 hours of birth (Sheng, Zhadanov et al. 1996). The truncated LHX3W227ter protein does not appear to be exerting any dominant negative effects as *Lhx3*$^{+/W227ter}$ heterozygous mice appear to be unaffected. *Lhx4* transcript levels were also examined in the *Lhx3*$^{W227ter/W227ter}$ mice to test

the hypothesis that LHX4 expression levels may be increased in an effort to compensate for any loss of LHX3 expression and/or function in the knock-in mice. However, no significant difference in *Lhx4* transcript levels was noted in the *Lhx3*$^{W227ter/W227ter}$ mice. The PIT-1 transcription factor acts downstream of LHX3 within the transcription factor cascade involved in anterior pituitary development (Colvin, Mullen et al. 2009). It has been shown to be essential for the proper development of the thyrotrope cells, somatotrope cells, and lactotrope cells within the anterior pituitary (Li, Crenshaw et al. 1990; Dasen, O'Connell et al. 1999; Scully and Rosenfeld 2002). *Pit-1* transcript levels were found to be significantly reduced in the *Lhx3*$^{W227ter/W227ter}$ mice in comparison to wild-type controls. As LHX3 has been shown to be able to bind and activate transcription from the *Pit-1* promoter (Bach, Rhodes et al. 1995), this reduction in *Pit-1* transcript seen in the knock-in mice could be due to the reduction in overall production of LHX3 protein in the knock-in mice or due to the fact that there are less cells of the Pit-1 lineage in the knock-in mouse pituitaries. Another possibility is that the truncated LHX3 protein itself has a reduced ability to bind and activate the *Pit-1* promoter in the knock-in mice, as some *in vitro* work suggests this truncated protein may not retain full function (Bach, Rhodes et al. 1995; Pfaeffle, Savage et al. 2007; Savage, Hunter et al. 2007). PITX1 is a transcription factor thought to act upstream of LHX3 within the cascade of genes involved in pituitary development. *Pitx1* transcript levels were significantly increased in *Lhx3*$^{W227ter/W227ter}$ mice compared to wild-type controls. Several possible scenarios may explain this increase in *Pitx1* transcript. As mentioned previously, PITX1 has been shown to have a role in the activation of several genes involved in pituitary development and/or function. It has a role in the activation of the *POMC* gene, and it is able to bind and

activate transcription of *PRL*, *LHβ*, *FSHβ*, *TSHβ*, *GnRHR*, and *GH* (Lamonerie, Tremblay et al. 1996; Szeto, Ryan et al. 1996; Lanctot, Lamolet et al. 1997; Tremblay, Lanctot et al. 1998; Lanctot, Moreau et al. 1999; Jeong, Chin et al. 2004). PITX1 has also been shown to have a role in the proper expression of LHX3 and αGSU (Tremblay, Lanctot et al. 1998; Charles, Suh et al. 2005). All of the pituitary targets of PITX1 have altered expression in the *Lhx3*$^{W227ter/W227ter}$ mice, with most of them being decreased (*Lhb*, *Fshb*, and *Tshb* transcript levels being exceptions). Perhaps *Pitx1* transcript levels are increased in *Lhx3*$^{W227ter/W227ter}$ mice in an effort to increase production of its pituitary target genes, for example, the increases seen in *Lhb*, *Fshb*, and *Tshb* transcript levels. However, the mechanism causing this increase in *Pitx1* transcript is not known.

No nervous system defects have been found in the *Lhx3*$^{W227ter/W227ter}$ mice. While the *LHX3*W224ter mutation does not cause a nervous system phenotype in the human patients, other mutations within the human *LHX3* gene are associated with a rigid cervical spine resulting in limited head rotation of the patients. The nervous system phenotype seen in human patients with LHX3 mutations is presumably due to the role of *LHX3* within the developing nervous system. Previous work indicates that the LIM domains and homeodomain of LHX3 are required for the formation of a multiprotein complex necessary for spinal cord motor neuron development, and in animals lacking both LHX3 and LHX4, V2 interneurons and ventral motor neurons do not develop properly (Sharma, Sheng et al. 1998; Thor, Andersson et al. 1999; Thaler, Lee et al. 2002). Since the *Lhx3*$^{W227ter/W227ter}$ mice supposedly have normal expression of LHX4, perhaps the lack of nervous system phenotype in the knock-in mice is due to compensation of LHX4 within the V2 interneuron and ventral motor neuron cells of the developing spinal cord. It would

be beneficial to examine the expression of the LHX4 transcription factor within the nervous system of the *Lhx3*$^{W227ter/W227ter}$ mice to first determine if its expression pattern is normal compared to wild type controls. Second, in order to determine more accurately whether the mutant LHX3W227ter protein is capable of functioning in the developing nervous system on its own without compensation by LHX4, the *Lhx3*$^{W227ter/W227ter}$ mice should be crossed with *Lhx4*$^{+/-}$ heterozygous animals and progeny should be bred to produce *Lhx3*$^{W227ter/W227ter}$/*Lhx4*$^{-/-}$ mice. Analyses of the nervous systems of these animals should give further insight as to whether the LHX3W227ter protein is able to retain function within the developing nervous system. Another problem with analyzing the nervous system in the *Lhx3*$^{W227ter/W227ter}$ mice is that it is not certain how this rigid cervical spine resulting in limited head rotation seen in the human patients would present itself phenotypically within mice. One way to examine this would be to generate mice containing a knock-in mutation of the LHX3A210V mutation that is known to cause this neck phenotype seen in the human patients. However, as these mice would presumably have normal LHX4 in the nervous system, the nervous system phenotype may not present itself phenotypically until the animals were bred to obtain an *Lhx3*$^{A210V/A210V}$/*Lhx4*$^{-/-}$ genotype. If it were possible to generate mice with similar nervous system defects as those seen in the human patients, it would prove difficult to examine the extent of the phenotype. One benefit to investigating the molecular effects these mutations exert within mice is that the cell populations within the developing spinal cord can be examined using IHC at various time points. However, there are presumed limitations when it comes to measuring the level of neck rigidity and impaired head rotation in mice versus humans. Human patients are asked to sit in a chair and look at various points within the room. This

kind of task cannot be performed as easily with mice. However, some attempt must be made to measure various behavioral tasks to determine whether this limited head rotation seen in the human patients can also be seen in the mice. For example, are the mutant knock-in animals able to reach around and groom themselves properly, do they exhibit normal cage behavior (can they rear up to reach food and/or water), would they respond differently than their wild type counterparts during a startle response task. Further exmaination into the nervous system of the *Lhx3*$^{W227ter/W227ter}$ mice is warranted.

Given the limited examination into the nervous systems of the *Lhx3*$^{W227ter/W227ter}$ mice, again, no nervous system defects have been observed in these mice to date. Work done by other groups indicates that the LIM domains and homeodomain of LHX3 are required for the formation of a multiprotein complex necessary for spinal cord motor neuron development (Sharma, Sheng et al. 1998; Thor, Andersson et al. 1999; Thaler, Lee et al. 2002). And although the carboxyl terminus of LHX3 contains the major *trans*-activation domain of the protein that could allow regulation of LHX3 function and location through signaling pathways, the LIM domains also appear to contain some activation function that may serve a role in the nervous system (Parker, Sandoval et al. 2000; Sloop, Showalter et al. 2000; Parker, West et al. 2005). The fact that the mice survive into adulthood indicates that the truncated LHX3 protein is not only being made, but that it is retaining enough function to produce viable mice. Mice without any functional LHX3 protein are stillborn or die within 24 hours after birth, and *Lhx3*$^{Cre/Cre}$ mice with reduced *Lhx3* mRNA and protein levels recapitulate the phenotype seen in *Lhx3* knockout mice in that they do not survive within 24 hours of birth and display similar pituitary problems (Sheng, Zhadanov et al. 1996; Zhao, Morales et al. 2006). This

indicates that there may be some threshold level of LHX3 necessary for survival that the *Lhx3*$^{W227ter/W227ter}$ mice are attaining whereas the *Lhx3*$^{Cre/Cre}$ mice fall below this threshold level. While the *Lhx3*$^{-/-}$ mice and the *Lhx3*$^{Cre/Cre}$ mice do not express any or enough LHX3 to survive, the ex act cause of death in the *Lhx3*$^{Cre/Cre}$ and the *Lhx3*$^{-/-}$ mice has not been determined. It has been speculated that these animals die from impaired brain stem function due to a lack of LHX3 expression in precursor cells of the Raphe nuclei and reticular formation (Zhadanov, Bertuzzi et al. 1995; Sheng, Zhadanov et al. 1996). Although the exact role of LHX3 in the developing brain stem has yet to be determined, this emphasizes the importance of LHX3 expression in the developing nervous system. Previous data has demonstrated that the LIM domains and the homeodomain of LHX3 are vital for protein-protein interaction in the LIM code that specifies V2 interneurons from motor neurons in the developing spinal cord (Sharma, Sheng et al. 1998; Thaler, Lee et al. 2002), and data from our laboratory indicates that the LHX3W224Ter protein is able to act in synergy with its partner proteins to activate transcription from the *Hb9* motor neuron enhancer (Savage, Hunter et al. 2007). IHC staining with both motor neuron (Hb9) and V2 interneuron markers (CHX10) in the developing spinal cords of *Lhx3*$^{W227ter/W227ter}$ knock-in mice revealed no abnormalities (data not shown). While an attempt was made to detect the LHX3W227ter mutant protein within the pituitaries and nervous systems of the knock-in animals, most antibodies made for the LHX3 protein are polyclonal for the carboxyl terminus of the protein. Since the LHX3W227ter mutant protein does not retain the carboxyl terminus, these antibodies could not be used for detection. One antibody that was said to react with the amino terminus of the LHX3 protein did not give reliable staining results; however, if a good antibody for the amino-

terminus of LHX3 were available, or if the mice had been made with a Myc or HA tag attached to Lhx3, it would be beneficial to use chromatin immunoprecipitation (ChIP) assays to determine if the LHX3W227ter protein is able to bind to its pituitary targets with the same efficiency as its nervous system targets. Even without a method for detection of the LHX3W227ter protein, ChIP assays could be used to determine whether target genes of LHX3 were in a transcriptionally active state using an acetylated histone H3 antibody, and whether more target genes of LHX3 in the nervous system would be active versus those in the pituitary gland. However, the lack of a nervous system phenotype in the *Lhx3*$^{W227ter/W227ter}$ knock-in mice support the hypothesis that the LIM domains and the homeodomain of LHX3 are all that are required for it to function properly during nervous system development. However, the carboxyl terminus of LHX3 is necessary for the proper development of the anterior pituitary as the hormone deficiencies seen in both the human patients and the knock-in mice suggests.

BIBLIOGRAPHY

BIBLIOGRAPHY

Acampora, D., S. Mazan, Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A., Brûlet, P.: Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation. Development 121(1995) 3279-3290.

Alatzoglou, K. S., Kelberman, D., Dattani, M.T.: The role of SOX proteins in normal pituitary development. J Endocrinol 200 (2009) 245-258.

Andersen, B., Pearse, R.V. 2nd, Jenne, K., Sornson, M., Lin, S.C., Bartke, A., Rosenfeld, M.G.: The Ames dwarf gene is required for Pit-1 gene activation. Dev Biol 172 (1995) 495-503.

Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L., Rossant, J.: A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. Development 122 (1996) 243-252.

Avilion, A. A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., Lovell-Badge, R.: Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17 (2003) 126-140.

Aza-Blanc, P., Lin, H.Y., Ruiz i Altaba, A., Kornberg, T.B.: Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. Development 127 (2000) 4293-4301.

Bach, I., Rhodes, S.J., Pearse, R.V., 2nd, Heinzel, T., Gloss, B., Scully, K.M., Sawchenko, P.E. and Rosenfeld, M.G.: P-Lim, a LIM homeodomain factor, is expressed during pituitary organ and cell commitment and synergizes with Pit-1. Proc Natl Acad Sci U S A 92 (1995) 2720-4.

Baek, S.H., Kioussi, C., Briata, P., Wang, D., Nguyen, H.D., Ohgi, K.A., Glass, C.K., Wynshaw-Boris, A., Rose, D.W. and Rosenfeld, M.G.: Regulated subset of G1 growth-control genes in response to derepression by the Wnt pathway. Proc Natl Acad Sci U S A 100 (2003) 3245-50.

Bentley, C.A., Zidehsarai, M.P., Grindley, J.C., Parlow, A.F., Barth-Hall, S. and Roberts, V.J.: Pax6 is implicated in murine pituitary endocrine function. Endocrine 10 (1999) 171-7.

Bhangoo, A.P., Hunter, C.S., Savage, J.J., Anhalt, H., Pavlakis, S., Walvoord, E.C., Ten, S. and Rhodes, S.J.: Clinical case seminar: a novel LHX3 mutation presenting as combined pituitary hormonal deficiency. J Clin Endocrinol Metab 91 (2006) 747-53.

Bodner, M., Castrillo, J.L., Theill, L.E., Deerinck, T., Ellisman, M. and Karin, M.: The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55 (1988) 505-18.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., Kelly, P.A.: Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19 (1998) 225-268.

Bovolenta, P., Mallamaci, A., Puelles, L. and Boncinelli, E.: Expression pattern of cSix3, a member of the Six/sine oculis family of transcription factors. Mech Dev 70 (1998) 201-3.

Bridwell, J.A., Price, J.R., Parker, G.E., McCutchan Schiller, A., Sloop, K.W. and Rhodes, S.J.: Role of the LIM domains in DNA recognition by the Lhx3 neuroendocrine transcription factor. Gene 277 (2001) 239-50.

Castinetti, F., Saveanu, A., Reynaud, R., Quentien, M.H., Buffin, A., Brauner, R., Kaffel, N., Albarel, N., Guedj, A.M., El Kholy, M., Amin, M., Enjalbert, A., Barlier, A., Brue, T.: A novel dysfunctional LHX4 mutation with high phenotypical variability in patients with hypopituitarism. J Clin Endocrinol Metab 93 (2008) 2790-2799.

Charles, M.A., Suh, H., Hjalt, T.A., Drouin, J., Camper, S.A. and Gage, P.J.: PITX genes are required for cell survival and Lhx3 activation. Mol Endocrinol 19 (2005) 1893-903.

Chen, J., Hersmus, N., Van Duppen, V., Caesens, P., Denef, C. and Vankelecom, H.: The adult pituitary contains a cell population displaying stem/progenitor cell and early embryonic characteristics. Endocrinology 146 (2005) 3985-98.

Cheyette, B.N., Green, P.J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S.L.: The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. Neuron 12 (1994) 977-96.

Collignon, J., Sockanathan, S., Hacker, A., Cohen-Tannoudji, M., Norris, D., Rastan, S., Stevanovic, M., Goodfellow, P.N., Lovell-Badge, R.: A comparison of the properties of Sox-3 with Sry and two related genes, Sox-1 and Sox-2. Development 122 (1996) 509-520.

Colvin, S. C., Mullen, R.D., Pfaeffle, R.W., Rhodes, S.J.: LHX3 and LHX4 transcription factors in pituitary development and disease. Pediatr Endocrinol Rev (2009) 283-290.

Cushman, L.J., Watkins-Chow, D.E., Brinkmeier, M.L., Raetzman, L.T., Radak, A.L., Lloyd, R.V. and Camper, S.A.: Persistent Prop1 expression delays gonadotrope differentiation and enhances pituitary tumor susceptibility. Hum Mol Genet 10 (2001) 1141-53.

Daikoku, S., Chikamori, M., Adachi, T. and Maki, Y.: Effect of the basal diencephalon on the development of Rathke's pouch in rats: a study in combined organ cultures. Dev Biol 90 (1982) 198-202.

Dasen, J.S., O'Connell, S.M., Flynn, S.E., Treier, M., Gleiberman, A.S., Szeto, D.P., Hooshmand, F., Aggarwal, A.K. and Rosenfeld, M.G.: Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. Cell 97 (1999) 587-98.

Dateki, S., Fukami, M., Sato, N., Muroya, K., Adachi, M., Ogata, T.: OTX2 mutation in a patient with anophthalmia, short stature, and partial growth hormone deficiency: functional studies using the IRBP, HESX1, and POU1F1 promoters. J Clin Endocrinol Metab 93 (2008) 3697-3702.

Dateki, S., Kosaka, K., Hasegawa, K., Tanaka, H., Azuma, N., Yokoya, S., Muroya, K., Adachi, M., Tajima, T., Motomura, K., Kinoshita, E., Moriuchi, H., Sato, N., Fukami, M., Ogata, T.: Heterozygous orthodenticle homeobox 2 mutations are associated with variable pituitary phenotype. J Clin Endocrinol Metab 95 (2010) 756-764.

Dattani, M.T., Martinez-Barbera, J.P., Thomas, P.Q., Brickman, J.M., Gupta, R., Martensson, I.L., Toresson, H., Fox, M., Wales, J.K., Hindmarsh, P.C., Krauss, S., Beddington, R.S. and Robinson, I.C.: Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. Nat Genet 19 (1998) 125-33.

Diaczok, D., Romero, C., Zunich, J., Marshall, I., Radovick, S.: A novel dominant negative mutation of OTX2 associated with combined pituitary hormone deficiency. J Clin Endocrinol Metab 93 (2008) 4351-4359.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., Hui, C.C.: Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development 125 (1998) 2533-2543.

Dohrmann, C., Gruss, P. and Lemaire, L.: Pax genes and the differentiation of hormone-producing endocrine cells in the pancreas. Mech Dev 92 (2000) 47-54.

Drouin, J., Lamolet, B., Lamonerie, T., Lanctôt, C., Tremblay, J.J.: The PTX family of homeodomain transcription factors during pituitary developments. Mol Cell Endocrinol 140 (1998) 31-36.

Eicher, E. M., Beamer, W.G.: New mouse dw allele: genetic location and effects on lifespan and growth hormone levels. J Hered 71 (1980) 187-190.

Ellsworth, B.S., Egashira, N., Haller, J.L., Butts, D.L., Cocquet, J., Clay, C.M., Osamura, R.Y. and Camper, S.A.: FOXL2 in the pituitary: molecular, genetic, and developmental analysis. Mol Endocrinol 20 (2006) 2796-805.

Ericson, J., Norlin, S., Jessell, T.M. and Edlund, T.: Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Development 125 (1998) 1005-15.

Fauquier, T., Rizzoti, K., Dattani, M., Lovell-Badge, R., Robinson, R.C.: SOX2-expressing progenitor cells generate all of the major cell types in the adult mouse pituitary gland. Proc Natl Acad Sci U S A 105 (2008) 2907-2912.

Ferri, A. L., Cavallaro, M., Braida, D., Di Cristofano, A., Canta, A., Vezzani, A., Ottolenghi, S., Pandolfi, P.P., Sala, M., DeBiasi, S., Nicolis, S.K.: Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. Development 131 (2004) 3805-3819.

Gage, P.J., Brinkmeier, M.L., Scarlett, L.M., Knapp, L.T., Camper, S.A. and Mahon, K.A.: The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. Mol Endocrinol 10 (1996) 1570-81.

Gage, P.J. and Camper, S.A.: Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. Hum Mol Genet 6 (1997) 457-64.

Gage, P.J., Lossie, A.C., Scarlett, L.M., Lloyd, R.V. and Camper, S.A.: Ames dwarf mice exhibit somatotrope commitment but lack growth hormone-releasing factor response. Endocrinology 136 (1995) 1161-7.

Gage, P. J., Roller, M.L., Saunders, T.L., Scarlett, L.M., Camper, S.A.: Anterior pituitary cells defective in the cell-autonomous factor, df, undergo cell lineage specification but not expansion. Development 122 (1996) 151-160.

Gage, P. J., Suh, H., Camper, S.A.: The bicoid-related Pitx gene family in development. Mamm Genome 10 (1999) 197-200.

Gage, P.J., Suh, H. and Camper, S.A.: Dosage requirement of Pitx2 for development of multiple organs. Development 126 (1999) 4643-51.

Gaston-Massuet, C., Andoniadou, C.L., Signore, M., Sajedi, E., Bird, S., Turner, J.M., Martinez-Barbera, J.P.: Genetic interaction between the homeobox transcription factors HESX1 and SIX3 is required for normal pituitary development. Dev Biol 324 (2008) 322-333.

Gleiberman, A.S., Fedtsova, N.G. and Rosenfeld, M.G.: Tissue interactions in the induction of anterior pituitary: role of the ventral diencephalon, mesenchyme, and notochord. Dev Biol 213 (1999) 340-53.

Granger, A., Bleux, C., Kottler, M.L., Rhodes, S.J., Counis, R. and Laverriere, J.N.: The LIM-homeodomain proteins Isl-1 and Lhx3 act with steroidogenic factor 1 to enhance gonadotrope-specific activity of the gonadotropin-releasing hormone receptor gene promoter. Mol Endocrinol 20 (2006) 2093-108.

Guy, J.C., Hunter, C.S., Showalter, A.D., Smith, T.P., Charoonpatrapong, K., Sloop, K.W., Bidwell, J.P. and Rhodes, S.J.: Conserved amino acid sequences confer nuclear localization upon the Prophet of Pit-1 pituitary transcription factor protein. Gene 336 (2004) 263-73.

Henderson, R. H., Williamson, K.A., Kennedy, J.S., Webster, A.R., Holder, G.E., Robson, A.G., FitzPatrick, D.R., van Heyningen, V., Moore, A.T.: A rare de novo nonsense mutation in OTX2 causes early onset retinal dystrophy and pituitary dysfunction. Mol Vis 15 (2009) 2442-2447.

Hermesz, E., Mackem, S. and Mahon, K.A.: Rpx: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. Development 122 (1996) 41-52.

Hever, A. M., Williamson, K.A., van Heyningen, V.: Developmental malformations of the eye: the role of PAX6, SOX2 and OTX2. Clin Genet 69 (2006) 459-470.

Horseman, N. D., Zhao, W., Montecino-Rodriguez, E., Tanaka, M., Nakashima, K., Engle, S.J., Smith, F., Markoff, E., Dorshkind, K.: Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J 16 (1997) 6926-6935.

Hume, C. R., Bratt, D.L., Oesterle, E.C.: Expression of LHX3 and SOX2 during mouse inner ear development. Gene Expr Patterns 7 (2007) 798-807.

Hunter, C.S. and Rhodes, S.J.: LIM-homeodomain genes in mammalian development and human disease. Mol Biol Rep 32 (2005) 67-77.

Iguchi, G., Okimura, Y., Takahashi, T., Mizuno, I., Fumoto, M., Takahashi, Y., Kaji, H., Abe, H. and Chihara, K.: Cloning and characterization of the 5'-flanking region of the human growth hormone-releasing hormone receptor gene. J Biol Chem 274 (1999) 12108-14.

Ingraham, H.A., Chen, R.P., Mangalam, H.J., Elsholtz, H.P., Flynn, S.E., Lin, C.R., Simmons, D.M., Swanson, L. and Rosenfeld, M.G.: A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell 55 (1988) 519-29.

Ingraham, H.A., Lala, D.S., Ikeda, Y., Luo, X., Shen, W.H., Nachtigal, M.W., Abbud, R., Nilson, J.H. and Parker, K.L.: The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. Genes Dev 8 (1994) 2302-12.

Jean, D., Bernier, G. and Gruss, P.: Six6 (Optx2) is a novel murine Six3-related homeobox gene that demarcates the presumptive pituitary/hypothalamic axis and the ventral optic stalk. Mech Dev 84 (1999) 31-40.

Jeong, K.H., Chin, W.W. and Kaiser, U.B.: Essential role of the homeodomain for pituitary homeobox 1 activation of mouse gonadotropin-releasing hormone receptor gene expression through interactions with c-Jun and DNA. Mol Cell Biol 24 (2004) 6127-39.

Kawamata, N., Sakajiri, S., Sugimoto, K.J., Isobe, Y., Kobayashi, H. and Oshimi, K.: A novel chromosomal translocation t(1;14)(q25;q32) in pre-B acute lymphoblastic leukemia involves the LIM homeodomain protein gene, Lhx4. Oncogene 21 (2002) 4983-91.

Kawamura, K. and Kikuyama, S.: Induction from posterior hypothalamus is essential for the development of the pituitary proopiomelacortin (POMC) cells of the toad (Bufo japonicus). Cell Tissue Res 279 (1995) 233-9.

Kelberman, D., Dattani, M. T.: The role of transcription factors implicated in anterior pituitary development in the aetiology of congenital hypopituitarism. Ann Med 38 (2006) 560-577.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C.H., Ward, J.M., Gonzalez, F.J.: The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev 10 (1996) 60-69.

Kioussi, C., Briata, P., Baek, S.H., Rose, D.W., Hamblet, N.S., Herman, T., Ohgi, K.A., Lin, C., Gleiberman, A., Wang, J., Brault, V., Ruiz-Lozano, P., Nguyen, H.D., Kemler, R., Glass, C.K., Wynshaw-Boris, A. and Rosenfeld, M.G.: Identification of a Wnt/Dvl/beta-Catenin --> Pitx2 pathway mediating cell-type-specific proliferation during development. Cell 111 (2002) 673-85.

Kioussi, C., O'Connell, S., St-Onge, L., Treier, M., Gleiberman, A.S., Gruss, P. and Rosenfeld, M.G.: Pax6 is essential for establishing ventral-dorsal cell boundaries in pituitary gland development. Proc Natl Acad Sci U S A 96 (1999) 14378-82.

Kriström, B., Zdunek, A.M., Rydh, A., Jonsson, H., Sehlin, P., Escher, S.A.: A novel mutation in the LIM homeobox 3 gene is responsible for combined pituitary hormone deficiency, hearing impairment, and vertebral malformations. J Clin Endocrinol Metab 94 (2009) 1154-1161.

Kumar, T. R., Wang, Y., Lu, N., Matzuk, M.M.: Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15 (1997) 201-204.

Lagutin, O. V., Zhu, C.C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., Russell, H.R., McKinnon, P.J., Solnica-Krezel, L., Oliver, G.: Six3 repression of Wnt signaling in the anterior neuroectoderm is essential for vertebrate forebrain development. Genes Dev 17 (2003) 368-379.

Lamonerie, T., Tremblay, J.J., Lanctot, C., Therrien, M., Gauthier, Y. and Drouin, J.: Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. Genes Dev 10 (1996) 1284-95.

Lanctot, C., Gauthier, Y. and Drouin, J.: Pituitary homeobox 1 (Ptx1) is differentially expressed during pituitary development. Endocrinology 140 (1999a) 1416-22.

Lanctot, C., Lamolet, B. and Drouin, J.: The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. Development 124 (1997) 2807-17.

Lanctot, C., Moreau, A., Chamberland, M., Tremblay, M.L. and Drouin, J.: Hindlimb patterning and mandible development require the Ptx1 gene. Development 126 (1999b) 1805-10.

Li, H., Witte, D.P., Branford, W.W., Aronow, B.J., Weinstein, M., Kaur, S., Wert, S., Singh, G., Schreiner, C.M., Whitsett, J.A. and et al.: Gsh-4 encodes a LIM-type homeodomain, is expressed in the developing central nervous system and is required for early postnatal survival. Embo J 13 (1994) 2876-85.

Li, S., Crenshaw, E.B., 3rd, Rawson, E.J., Simmons, D.M., Swanson, L.W. and Rosenfeld, M.G.: Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. Nature 347 (1990) 528-33.

Li, X., Perissi, V., Liu, F., Rose, D.W. and Rosenfeld, M.G.: Tissue-specific regulation of retinal and pituitary precursor cell proliferation. Science 297 (2002b) 1180-3.

Lin, C.R., Kioussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., Izpisua-Belmonte, J.C. and Rosenfeld, M.G.: Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. Nature 401 (1999) 279-82.

Liu, S., Luo, H., Liu, J., McNeilly, A.S., Cui, S.: Specificity protein 1 (Sp1) plays role in regulating LIM homeodomain transcription factor Lhx4 gene expression. Biochem Biophys Res Commun 366 (2008) 36-41.

Liu, Y., Fan, M., Yu, S., Zhou, Y., Wang, J., Yuan, J. and Qiang, B.: cDNA cloning, chromosomal localization and expression pattern analysis of human LIM-homeobox gene LHX4. Brain Res 928 (2002) 147-55.

Logan, M., Pagan-Westphal, S.M., Smith, D.M., Paganessi, L. and Tabin, C.J.: The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. Cell 94 (1998) 307-17.

Luo, X., Ikeda, Y., Parker, K.L.: A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77 (1994) 481-490.

Ma, X., Dong, Y., Matzuk, M.M., Kumar, T.R.: Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. Proc Natl Acad Sci U S A 101 (2004) 17294-17299.

Machinis, K. and Amselem, S.: Functional relationship between LHX4 and POU1F1 in light of the LHX4 mutation identified in patients with pituitary defects. J Clin Endocrinol Metab 90 (2005) 5456-62.

Machinis, K., Pantel, J., Netchine, I., Leger, J., Camand, O.J., Sobrier, M.L., Dastot-Le Moal, F., Duquesnoy, P., Abitbol, M., Czernichow, P. and Amselem, S.: Syndromic short stature in patients with a germline mutation in the LIM homeobox LHX4. Am J Hum Genet 69 (2001) 961-8.

Matise, M. P., Epstein, D.J., Park, H.L., Platt, K.A., Joyner, A.L.: Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 125 (1998) 2759-2770.

McGillivray, S.M., Bailey, J.S., Ramezani, R., Kirkwood, B.J. and Mellon, P.L.: Mouse GnRH receptor gene expression is mediated by the LHX3 homeodomain protein. Endocrinology 146 (2005) 2180-5.

Miller, T.L., Godfrey, P.A., Dealmeida, V.I. and Mayo, K.E.: The rat growth hormone-releasing hormone receptor gene: structure, regulation, and generation of receptor isoforms with different signaling properties. Endocrinology 140 (1999) 4152-65.

Mo, R., Freer, A.M., Zinyk, D.L., Crackower, M.A., Michaud, J., Heng, H.H., Chik, K.W., Shi, X.M., Tsui, L.C., Cheng, S.H., Joyner, A.L., Hui, C.: Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Development 124 (1997) 113-123.

Morohashi, K.I. and Omura, T.: Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. Faseb J 10 (1996) 1569-77.

Mullen, R.D., Colvin, S.C., Hunter, C.S., Savage, J.J., Walvoord, E.C., Bhangoo, A.P., Ten, S., Weigel, J., Pfaffle, R.W. and Rhodes, S.J.: Roles of the LHX3 and LHX4 LIM-homeodomain factors in pituitary development. Mol Cell Endocrinol 265-266 (2007) 190-5.

Nasonkin, I.O., Ward, R.D., Raetzman, L.T., Seasholtz, A.F., Saunders, T.L., Gillespie, P.J. and Camper, S.A.: Pituitary hypoplasia and respiratory distress syndrome in Prop1 knockout mice. Hum Mol Genet 13 (2004) 2727-35.

Netchine, I., Sobrier, M.L., Krude, H., Schnabel, D., Maghnie, M., Marcos, E., Duriez, B., Cacheux, V., Moers, A., Goossens, M., Gruters, A. and Amselem, S.: Mutations in LHX3 result in a new syndrome revealed by combined pituitary hormone deficiency. Nat Genet 25 (2000) 182-6.

Norlin, S., Nordstrom, U. and Edlund, T.: Fibroblast growth factor signaling is required for the proliferation and patterning of progenitor cells in the developing anterior pituitary. Mech Dev 96 (2000) 175-82.

Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S. and Itoh, N.: FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. Biochem Biophys Res Commun 277 (2000) 643-9.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N.G., Jenkins, N.A. and Gruss, P.: Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121 (1995) 4045-55.

Ormandy, C. J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., Kelly, P.A.: Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev 11 (1997) 167-178.

Park, H. L., Bai, C., Platt, K.A., Matise, M.P., Beeghly, A., Hui, C.C., Nakashima, N., Joyner, A.L.: Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 127 (2000) 1593-1605.

Parker, G.E., Sandoval, R.M., Feister, H.A., Bidwell, J.P. and Rhodes, S.J.: The homeodomain coordinates nuclear entry of the Lhx3 neuroendocrine transcription factor and association with the nuclear matrix. J Biol Chem 275 (2000) 23891-8.

Parker, G.E., West, B.E., Witzmann, F.A. and Rhodes, S.J.: Serine/threonine/tyrosine phosphorylation of the LHX3 LIM-homeodomain transcription factor. J Cell Biochem 94 (2005) 67-80.

Pfaeffle, R. W., Hunter, C.S., Savage, J.J., Duran-Prado, M., Mullen, R.D., Neeb, Z.P., Eiholzer, U., Hesse, V., Haddad, N.G., Stobbe, H.M., Blum, W.F., Weigel, J.F., Rhodes, S.J.: Three novel missense mutations within the LHX4 gene are associated with variable pituitary hormone deficiencies. J Clin Endocrinol Metab 93 (2008) 1062-1071.

Pfaeffle, R.W., Savage, J.J., Hunter, C.S., Palme, C., Ahlmann, M., Kumar, P., Bellone, J., Schoenau, E., Korsch, E., Bramswig, J.H., Stobbe, H.M., Blum, W.F. and Rhodes, S.J.: Four Novel Mutations of the LHX3 Gene Cause Combined Pituitary Hormone Deficiencies With or Without Limited Neck Rotation. J Clin Endocrinol Metab 92 (2007) 1909-1919.

Raetzman, L.T., Ward, R. and Camper, S.A.: Lhx4 and Prop1 are required for cell survival and expansion of the pituitary primordia. Development 129 (2002) 4229-39.

Ragge, N. K., Brown, A.G., Poloschek, C.M., Lorenz, B., Henderson, R.A., Clarke, M.P., Russell-Eggitt, I., Fielder, A., Gerrelli, D., Martinez-Barbera, J.P., Ruddle, P., Hurst, J., Collin, J.R., Salt, A., Cooper, S.T., Thompson, P.J., Sisodiya, S.M., Williamson, K.A., Fitzpatrick, D.R., van Heyningen, V., Hanson, I.M.: Heterozygous mutations of OTX2 cause severe ocular malformations. Am J Hum Genet 76 (2005) 1008-1022.

Rajab, A., Kelberman, D., de Castro, S.C., Biebermann, H., Shaikh, H., Pearce, K., Hall, C.M., Shaikh, G., Gerrelli, D., Grueters, A., Krude, H., Dattani, M.T. Novel mutations in LHX3 are associated with hypopituitarism and sensorineural hearing loss. Hum Mol Genet 17 (2008) 2150-2159.

Rhodes, S.J., Chen, R., DiMattia, G.E., Scully, K.M., Kalla, K.A., Lin, S.C., Yu, V.C. and Rosenfeld, M.G.: A tissue-specific enhancer confers Pit-1-dependent morphogen inducibility and autoregulation on the pit-1 gene. Genes Dev 7 (1993) 913-32.

Rhodes, S. J., Krones, A., Nelson, C., Rosenfeld, M.G.: Function of the conserved Pit-1 gene distal enhancer in progenitor and differentiated pituitary cells. Mol Cell Endocrinol 124 (1996) 163-172.

Roessler, E., Du, Y.Z., Mullor, J.L., Casas, E., Allen, W.P., Gillessen-Kaesbach, G., Roeder, E.R., Ming, J.E., Ruiz I Altaba, A., Muenke, M. Loss-of-function mutations in the human GLI2 gene are associated with pituitary anomalies and holoprosencephaly-like features. Proc Natl Acad Sci U S A 100 (2003) 13424-13429.

Ruiz i Altaba, A., Palma, V., Dahmane, N.: Hedgehog-Gli signalling and the growth of the brain. Nat Rev Neurosci 3 (2002) 24-33.

Ryan, A.K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Pena, J., Sabbagh, W., Greenwald, J., Choe, S., Norris, D.P., Robertson, E.J., Evans, R.M., Rosenfeld, M.G. and Izpisua Belmonte, J.C.: Pitx2 determines left-right asymmetry of internal organs in vertebrates. Nature 394 (1998) 545-51.

Sadovsky, Y., Crawford, P.A., Woodson, K.G., Polish, J.A., Clements, M.A., Tourtellotte, L.M., Simburger, K. and Milbrandt, J.: Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proc Natl Acad Sci U S A 92 (1995) 10939-43.

Savage, J.J., Mullen, R.D., Sloop, K.W., Colvin, S.C., Camper, S.A., Franklin, C.L. and Rhodes, S.J.: Transgenic mice expressing LHX3 transcription factor isoforms in the pituitary: Effects on the gonadotrope axis and sex-specific reproductive disease. J Cell Physiol (2007).

Savage, J.J., Yaden, B.C., Kiratipranon, P. and Rhodes, S.J.: Transcriptional control during mammalian anterior pituitary development. Gene 319 (2003) 1-19.

Scully, K. M., Jacobson, E.M., Jepsen, K., Lunyak, V., Viadiu, H., Carreire, C., Rose, D.W., Hooshmand, F., Aggarwal, A.K., Rosenfeld, M.G. Allosteric effects of Pit-1 DNA sites on long-term repression in cell type specification. Science 290 (2000) 1127-1131.

Scully, K.M. and Rosenfeld, M.G.: Pituitary development: regulatory codes in mammalian organogenesis. Science 295 (2002) 2231-5.

Seidah, N.G., Barale, J.C., Marcinkiewicz, M., Mattei, M.G., Day, R. and Chretien, M.: The mouse homeoprotein mLIM-3 is expressed early in cells derived from the neuroepithelium and persists in adult pituitary. DNA Cell Biol 13 (1994) 1163-80.

Semina, E.V., Reiter, R., Leysens, N.J., Alward, W.L., Small, K.W., Datson, N.A., Siegel-Bartelt, J., Bierke-Nelson, D., Bitoun, P., Zabel, B.U., Carey, J.C. and Murray, J.C.: Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. Nat Genet 14 (1996) 392-9.

Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H. and Pfaff, S.L.: LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell 95 (1998) 817-28.

Sheng, H.Z., Moriyama, K., Yamashita, T., Li, H., Potter, S.S., Mahon, K.A. and Westphal, H.: Multistep control of pituitary organogenesis. Science 278 (1997b) 1809-12.

Sheng, H.Z., Zhadanov, A.B., Mosinger, B., Jr., Fujii, T., Bertuzzi, S., Grinberg, A., Lee, E.J., Huang, S.P., Mahon, K.A. and Westphal, H.: Specification of pituitary cell lineages by the LIM homeobox gene Lhx3. Science 272 (1996) 1004-7.

Shinoda, K., Lei, H., Yoshii, H., Nomura, M., Nagano, M., Shiba, H., Sasaki, H., Osawa, Y., Ninomiya, Y., Niwa, O. and et al.: Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the Ftz-F1 disrupted mice. Dev Dyn 204 (1995) 22-9.

Showalter, A.D., Smith, T.P., Bennett, G.L., Sloop, K.W., Whitsett, J.A. and Rhodes, S.J.: Differential conservation of transcriptional domains of mammalian Prophet of Pit-1 proteins revealed by structural studies of the bovine gene and comparative functional analysis of the protein. Gene 291 (2002) 211-21.

Silver, Lee M. Mouse Genetics: Concepts and Applications. New York: Oxford University Press, Inc. 1995.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M.R., Nigro, V., Boncinelli, E.: A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. EMBO J 12 (1993) 2735-2747.

Sloop, K.W., Dwyer, C.J. and Rhodes, S.J.: An isoform-specific inhibitory domain regulates the LHX3 LIM homeodomain factor holoprotein and the production of a functional alternate translation form. J Biol Chem 276 (2001a) 36311-9.

Sloop, K.W., McCutchan Schiller, A., Smith, T.P., Blanton, J.R., Jr., Rohrer, G.A., Meier, B.C. and Rhodes, S.J.: Biochemical and genetic characterization of the porcine Prophet of Pit-1 pituitary transcription factor. Mol Cell Endocrinol 168 (2000a) 77-87.

Sloop, K.W., Meier, B.C., Bridwell, J.L., Parker, G.E., Schiller, A.M. and Rhodes, S.J.: Differential activation of pituitary hormone genes by human Lhx3 isoforms with distinct DNA binding properties. Mol Endocrinol 13 (1999) 2212-25.

Sloop, K.W., Showalter, A.D., Von Kap-Herr, C., Pettenati, M.J. and Rhodes, S.J.: Analysis of the human LHX3 neuroendocrine transcription factor gene and mapping to the subtelomeric region of chromosome 9. Gene 245 (2000b) 237-43.

Sloop, K. W., Walvoord, E.C., Showalter, A.D., Pescovitz, O.H., Rhodes, S.J.: Molecular analysis of LHX3 and PROP-1 in pituitary hormone deficiency patients with posterior pituitary ectopia. J Clin Endocrinol Metab 85 (2000) 2701-2708.

Sobrier, M.L., Maghnie, M., Vie-Luton, M.P., Secco, A., di Iorgi, N., Lorini, R. and Amselem, S.: Novel HESX1 mutations associated with a life-threatening neonatal phenotype, pituitary aplasia, but normally located posterior pituitary and no optic nerve abnormalities. J Clin Endocrinol Metab 91 (2006) 4528-36.

Sornson, M.W., Wu, W., Dasen, J.S., Flynn, S.E., Norman, D.J., O'Connell, S.M., Gukovsky, I., Carriere, C., Ryan, A.K., Miller, A.P., Zuo, L., Gleiberman, A.S., Andersen, B., Beamer, W.G. and Rosenfeld, M.G.: Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. Nature 384 (1996) 327-33.

Steinfelder, H.J., Hauser, P., Nakayama, Y., Radovick, S., McClaskey, J.H., Taylor, T., Weintraub, B.D. and Wondisford, F.E.: Thyrotropin-releasing hormone regulation of human TSHB expression: role of a pituitary-specific transcription factor (Pit-1/GHF-1) and potential interaction with a thyroid hormone-inhibitory element. Proc Natl Acad Sci U S A 88 (1991) 3130-4.

Suh, H., Gage, P.J., Drouin, J. and Camper, S.A.: Pitx2 is required at multiple stages of pituitary organogenesis: pituitary primordium formation and cell specification. Development 129 (2002) 329-37.

Szeto, D.P., Rodriguez-Esteban, C., Ryan, A.K., O'Connell, S.M., Liu, F., Kioussi, C., Gleiberman, A.S., Izpisua-Belmonte, J.C. and Rosenfeld, M.G.: Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. Genes Dev 13 (1999) 484-94.

Szeto, D.P., Ryan, A.K., O'Connell, S.M. and Rosenfeld, M.G.: P-OTX: a PIT-1-interacting homeodomain factor expressed during anterior pituitary gland development. Proc Natl Acad Sci U S A 93 (1996) 7706-10.

Tajima, T., Hattori, T., Nakajima, T., Okuhara, K., Tsubaki, J. and Fujieda, K.: A Novel Missense Mutation (P366T) of the LHX4 Gene Causes Severe Combined Pituitary Hormone Deficiency with Pituitary Hypoplasia, Ectopic Posterior Lobe and a Poorly Developed Sella Turcica. Endocr J 54 (2007) 637-41.

Tajima, T., Ohtake, A., Hoshino, M., Amemiya, S., Sasaki, N., Ishizu, K., Fujieda, K.: OTX2 loss of function mutation causes anophthalmia and combined pituitary hormone deficiency with a small anterior and ectopic posterior pituitary. J Clin Endocrinol Metab 94 (2009) 314-319.

Tajima, T., Yorifuji, T., Ishizu, K., Fujieda, K.: A Novel Mutation (V101A) of the LHX4 Gene in a Japanese Patient with Combined Pituitary Hormone Deficiency. Exp Clin Endocrinol Diabetes (2009).

Takuma, N., Sheng, H.Z., Furuta, Y., Ward, J.M., Sharma, K., Hogan, B.L., Pfaff, S.L., Westphal, H., Kimura, S. and Mahon, K.A.: Formation of Rathke's pouch requires dual induction from the diencephalon. Development 125 (1998) 4835-40.

Tang, K., Bartke, A., Gardiner, C.S., Wagner, T.E. and Yun, J.S.: Gonadotropin secretion, synthesis, and gene expression in human growth hormone transgenic mice and in Ames dwarf mice. Endocrinology 132 (1993) 2518-24.

Tatsumi, K., Miyai, K., Notomi, T., Kaibe, K., Amino, N., Mizuno, Y. and Kohno, H.: Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene. Nat Genet 1 (1992) 56-8.

Terzic, J. and Saraga-Babic, M.: Expression pattern of PAX3 and PAX6 genes during human embryogenesis. Int J Dev Biol 43 (1999) 501-8.

Thaler, J.P., Lee, S.K., Jurata, L.W., Gill, G.N. and Pfaff, S.L.: LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. Cell 110 (2002) 237-49.

Thomas, P.Q., Dattani, M.T., Brickman, J.M., McNay, D., Warne, G., Zacharin, M., Cameron, F., Hurst, J., Woods, K., Dunger, D., Stanhope, R., Forrest, S., Robinson, I.C. and Beddington, R.S.: Heterozygous HESX1 mutations associated with isolated congenital pituitary hypoplasia and septo-optic dysplasia. Hum Mol Genet 10 (2001) 39-45.

Thomas, P.Q., Johnson, B.V., Rathjen, J. and Rathjen, P.D.: Sequence, genomic organization, and expression of the novel homeobox gene Hesx1. J Biol Chem 270 (1995) 3869-75.

Thor, S., Andersson, S.G., Tomlinson, A., Thomas, J.B.: A LIM-homeodomain combinatorial code for motor-neuron pathway selection. Nature 397 (1999) 76-80.

Treier, M., Gleiberman, A.S., O'Connell, S.M., Szeto, D.P., McMahon, J.A., McMahon, A.P. and Rosenfeld, M.G.: Multistep signaling requirements for pituitary organogenesis in vivo. Genes Dev 12 (1998) 1691-704.

Treier, M., O'Connell, S., Gleiberman, A., Price, J., Szeto, D.P., Burgess, R., Chuang, P.T., McMahon, A.P. and Rosenfeld, M.G.: Hedgehog signaling is required for pituitary gland development. Development 128 (2001) 377-86.

Tremblay, J.J., Lanctot, C. and Drouin, J.: The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. Mol Endocrinol 12 (1998) 428-41.

Vesper, A.H., Raetzman, L.T. and Camper, S.A.: Role of prophet of Pit1 (PROP1) in gonadotrope differentiation and puberty. Endocrinology 147 (2006) 1654-63.

Wallis, D.E., Roessler, E., Hehr, U., Nanni, L., Wiltshire, T., Richieri-Costa, A., Gillessen-Kaesbach, G., Zackai, E.H., Rommens, J. and Muenke, M.: Mutations in the homeodomain of the human SIX3 gene cause holoprosencephaly. Nat Genet 22 (1999) 196-8.

Ward, R.D., Stone, B.M., Raetzman, L.T. and Camper, S.A.: Cell proliferation and vascularization in mouse models of pituitary hormone deficiency. Mol Endocrinol 20 (2006) 1378-90.

Watanabe, Y.G.: Effects of brain and mesenchyme upon the cytogenesis of rat adenohypophysis in vitro. I. Differentiation of adrenocorticotropes. Cell Tissue Res 227 (1982a) 257-66.

Watanabe, Y.G.: An organ culture study on the site of determination of ACTH and LH cells in the rat adenohypophysis. Cell Tissue Res 227 (1982b) 267-75.

West, B.E., Parker, G.E., Savage, J.J., Kiratipranon, P., Toomey, K.S., Beach, L.R., Colvin, S.C., Sloop, K.W. and Rhodes, S.J.: Regulation of the follicle-stimulating hormone beta gene by the LHX3 LIM-homeodomain transcription factor. Endocrinology 145 (2004) 4866-79.

Williamson, K. A., Hever, A.M., Rainger, J., Rogers, R.C., Magee, A., Fiedler, A., Keng, W.T., Sharkley, F.H., McGill, N., Hill, C.J., Schneider, A., Messina, M., Turnpenny, P.D., Fantes, J.A., van Heyningen, V., FitzPatrick, D.R.: Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. Hum Mol Genet 15 (2006) 1413-1422.

Wood, H. B., Episkopou, V.: Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech Dev 86 (1999) 197-201.

Wu, W., Cogan, J.D., Pfaffle, R.W., Dasen, J.S., Frisch, H., O'Connell, S.M., Flynn, S.E., Brown, M.R., Mullis, P.E., Parks, J.S., Phillips, J.A., 3rd and Rosenfeld, M.G.: Mutations in PROP1 cause familial combined pituitary hormone deficiency. Nat Genet 18 (1998) 147-9.

Wyatt, A., Bakrania, P., Bunyan, D.J., Osborne, R.J., Crolla, J.A., Salt, A., Avuso, C., Newbury-Ecob, R., Abou-Rayyah, Y., Collin, J.R., Robinson, D., Ragge, N.: Novel heterozygous OTX2 mutations and whole gene deletions in anophthalmia, microphthalmia and coloboma. Hum Mutat 29 (2008) 278-283.

Yaden, B.C., Garcia, M., 3rd, Smith, T.P. and Rhodes, S.J.: Two promoters mediate transcription from the human LHX3 gene: involvement of nuclear factor I and specificity protein 1. Endocrinology 147 (2006) 324-37.

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., Inoue, T., Ohuchi, H., Semina, E.V., Murray, J.C., Hamada, H. and Noji, S.: Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. Cell 94 (1998) 299-305.

Zhadanov, A.B., Bertuzzi, S., Taira, M., Dawid, I.B. and Westphal, H.: Expression pattern of the murine LIM class homeobox gene Lhx3 in subsets of neural and neuroendocrine tissues. Dev Dyn 202 (1995) 354-64.

Zhadanov, A. B., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Taira, M., Dawid, I.B., Westphal, H.: Genomic structure and chromosomal localization of the mouse LIM/homeobox gene Lhx3. Genomics 27 (1995) 27-32.

Zhao, L., Bakke, M., Krimkevich, Y., Cushman, L.J., Parlow, A.F., Camper, S.A. and Parker, K.L.: Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope function. Development 128 (2001) 147-54.

Zhao, Y., Morales, D.C., Hermesz, E., Lee, W.K., Pfaff, S.L. and Westphal, H.: Reduced expression of the LIM-homeobox gene Lhx3 impairs growth and differentiation of Rathke's pouch and increases cell apoptosis during mouse pituitary development. Mech Dev 123 (2006) 605-13.

VITA

VITA

Stephanie C. Colvin

Education and Research Experience

- Ph.D., Biology, Purdue University, Indianapolis, IN 46202, 2005-2010.
  Dissertation: "*An Animal Model of Pediatric Combined Pituitary Hormone Deficiency Disease*"

  Advisor: Simon J. Rhodes
  Thesis Committee: Teri Belecky-Adams, Paul Herring, Stephen Konieczny, Randall Roper, Emily Walvoord

- Teaching Assistant, Concepts of Biology I, Indiana University-Purdue University, Indianapolis, IN, 2004-2005.

- B.S., Psychology, Indiana University, Bloomington, IN 47401, 1998-2002.

Awards and Honors

- Outstanding Graduate Student Research Award, Department of Biology, Indiana University-Purdue University Indianapolis, 2010.

- Indiana Clinical and Translational Sciences Institute (CTSI) predoctoral translational research fellowship, 2008-2010.

- First Place, Clinical Category, Annual IUSM and CTSI Scientific Poster Session, 2009.

- Elizabeth Steele Creveling Memorial Scholarship for the Outstanding Doctoral Student in Biology, Department of Biology, Indiana University-Purdue University Indianapolis, 2007.

- IU School of Medicine Cellular and Integrative Physiology - Poster Award Winner, Departmental Retreat, 2007.

- IUPUI Research Investment Fund fellowship, 2005-2006.

- The Central Indiana Chapter of the IU Alumni Association Scholarship, 1999.

- Lions Club Scholarship, 1998.

Scientific Publications

- West, B., Parker, G., Savage, J., Kiratipranon, P., Toomey, K., Beach, L., **Colvin, S.**, Sloop, K., Rhodes, S. Regulation of the *follicle-stimulating hormone beta* gene by the LHX3 LIM-homeodomain transcription factor. *Endocrinology*. 2004 Nov;145(11):4866-79.

- Savage, J., Mullen, R., Sloop, K., **Colvin S.**, Camper, S., Franklin, C., Rhodes, S. Transgenic mice expressing LHX3 transcription factor isoforms in the pituitary: effects on the gonadotrope axis and sex-specific reproductive disease. *J Cell Physiol*. 2007 Jul:212(1):105-17.

- **Colvin, S.**, Mullen, R., Hunter, C., Savage J., Walvoord, E., Bhangoo, A., Ten, S., Weigel, J., Pfäffle, R., and Rhodes, S. Roles of the LHX3 and LHX4 LIM-homeodomain factors in pituitary development. *Mol. Cell. Endocrinol.* 2007 Feb;265-266:190-5.

- **Colvin, S.**, Mullen, R., Pfaeffle, R., Rhodes, S. 2009. LHX3 and LHX4 transcription factors in pituitary development and disease. *Pediatric Endocrinology Reviews*. 2009 Jan; 6 Suppl 2:283-90.

- **Colvin, S.**, Malik, R., Showalter, A., Sloop, K., Rhodes, S. (2010) An animal model of pediatric combined pituitary hormone deficiency disease separates the endocrine and nervous system roles of the LHX3 transcription factor *in vivo*. *In preparation*.

Abstracts

- **Colvin, S.**, Malik, R., Showalter, A., Sloop, K., Rhodes, S. *An Animal Model of Pediatric Combined Pituitary Hormone Deficiency Disease*. The Endocrine Society's 92th Annual Meeting, San Diego, 2010.

- **Colvin, S.**, Malik, R., Rhodes, S. *An Animal Model of Pediatric Combined Pituitary Hormone Deficiency Disease*. The Indiana CTSI Annual Meeting. April 2010.

- **Colvin, S.**, Malik, R., Basden, R., Rhodes, S. *Animal Models of Combined Pituitary Hormone Deficiency Diseases*. The 2009 annual IUSM and CTSI Scientific Poster Session. September 2009. First Place Clinical Category.

- **Colvin, S.**, Mullen, R., Pfaeffle, R., Rhodes, S. *Hypopituitarism*. 11th International Pituitary Congress, Washington, D.C., June 2009.

- **Colvin, S.**, Rhodes, S. *Animal Models of Combined Pituitary Hormone Deficiency Diseases*. Indiana CTSI Pre-doctoral Fellows Meeting. January 2009.

- **Colvin, S.**, Rhodes, S. *Animal Models of Combined Pituitary Hormone Deficiency Diseases*. Indiana CTSI Retreat. January 2009.

- **Colvin, S.**, Rhodes, S. *Animal Models of Combined Pituitary Hormone Deficiency Diseases*. IU Medical School Department of Cellular and Integrative Physiology. Department Retreat. September 2008.

- **Colvin, S.**, Hunter, C., Mullen, R., Savage, J., Bhangoo, A., Pfäffle, R., Rhodes, S. *Animal models of Combined Pituitary Hormone Deficiency Diseases*. IU Medical School Department of Cellular and Integrative Physiology. August 2007 Department Retreat. Poster Award Winner.

- Mullen, R., Savage, J., **Colvin, S.**, Sloop, K., Camper, S., Franklin, C., and Rhodes, S. *Sex-specific reproductive disease and loss of viability in transgenic mice overexpressing LHX3 protein isoforms*. The Endocrine Society's 88th Annual Meeting, Boston, 2006.

- **Colvin, S.**, Hunter, C., Mullen, R., Savage, J., Rhodes, S. *Transcriptional regulation by LIM-homeodomain transcription factors in endocrine organ development and disease*. Indiana University School of Medicine's Biennial Scientific Session. September 13, 2006.

- **Colvin, S.**, Hunter, C., Mullen, R., Savage, J., Bhangoo, A., Pfäffle, R., Rhodes, S. *Analyses of mutations in neuroendocrine genes associated with pituitary hormone deficiency diseases*. IU Medical School Department of Cellular and Integrative Physiology. September 2006 Department Retreat.

- **Colvin, S.**, Hunter, C., Mullen, R., Savage, J., Witzmann, F., Rhodes, S. *Transcriptional regulation by LIM-homeodomain transcription factors in endocrine organ development and disease*. IU Medical School Department of Cellular and Integrative Physiology. September 2006 Department Retreat.

- El-Mounayri, O., **Colvin, S.**, Mullen, R., Rhodes, S., Herring, B. *Dedifferentiation of bladder smooth muscle in LHX3a/b transgenic male mice.* Indiana Center for Vascular Biology and Medicine September 2006 Retreat.

- Hunter, C., Savage, J., Mullen, R., **Colvin, S.**, Walvoord, E., Bhangoo, A., Ten, S., Pfäffle, R., Weigel, J., and Rhodes, S. *Gene regulation by LIM homeodomain transcription factors: association with hormone deficiency diseases*. Adrenal 2006/Molecular Steroidogenesis 5 (AMS) Meeting, Boston MA, June, 2006.

- Savage, J., Hunter, C., Garcia, M., Mullen, R., **Colvin, S.**, Clark, S., Jacob, T., Sloop, K., Smith, T., Franklin, C., Pfäffle, R., Rhodes, S. *LIM homeodomain transcription factors in HPG axis function*. 2005 Endocrine Society Meeting. San Diego, CA.

- Garcia, M., **Colvin, S.**, Hunter, C., Jacob, T., Mullen, R., Mwashita, T., Savage, J., Neeb, Z., Rhodes, S. *Transcriptional regulation in endocrine organ development and disease*. 2nd Annual Cancer Center Research Day, Indiana University Cancer Center and Department of Biology (2004).