Model of pediatric pituitary hormone deficiency separates the endocrine and neural functions of the LHX3 transcription factor in vivo

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Edited* by Michael G. Rosenfeld, University of California at San Diego, La Jolla, CA, and approved November 22, 2010 (received for review July 1, 2010)

The etiology of most pediatric hormone deficiency diseases is poorly understood. Children with combined pituitary hormone deficiency (CPHD) have insufficient levels of multiple anterior pituitary hormones causing short stature, metabolic disease, pubertal failure, and often have associated nervous system symptoms. Mutations in developmental regulatory genes required for the specification of the hormone-secreting cell types of the pituitary gland underlie severe forms of CPHD. To better understand these diseases, we have created a unique mouse model of CPHD with a targeted knockin mutation (*Lhx3* W227ter), which is a model for the human *LHX3* W224ter disease. The *LHX3* gene encodes a LIM-homeodomain transcription factor, which has essential roles in pituitary and nervous system development in mammals. The introduced premature termination codon results in deletion of the carboxyl terminal region of the LHX3 protein, which is critical for pituitary gene activation. Mice that lack all LHX3 function do not survive beyond birth. By contrast, the homozygous *Lhx3* W227ter mice survive, but display marked dwarfism, thyroid disease, and female infertility. Importantly, the *Lhx3* W227ter mice have no apparent nervous system deficits. The *Lhx3* W227ter mouse model provides a unique array of hormone deficits and facilitates experimental approaches that are not feasible with human patients. These experiments demonstrate that the carboxyl terminus of the LHX3 transcription factor is not required for viability. More broadly, this study reveals that the in vivo actions of a transcription factor in different tissues are molecularly separable.

endocrinology | growth | reproduction | metabolism

The development of the mammalian anterior pituitary gland is an exemplary model system for understanding the pathways regulating organogenesis and the establishment of specialized cell types (1, 2). The pituitary coordinates developmental and physiological processes, including the stress response, metabolism, growth, reproductive fitness, and lactation through the secretion of hormones from specialized cell types. Pituitary corticotropes secrete adrenocorticotropin (ACTH), gonadotropes produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH), somatotropes release growth hormone (GH), thyrotropes produce thyroid-stimulating hormone (TSH), and lactotropes secrete prolactin (PRL).

During pituitary organogenesis, the development of differentiated cell types requires a complex cascade of signaling proteins and transcription factors (1, 2). A critical regulator of pituitary organogenesis, LHX3, is a LIM-homeodomain transcription factor that is required for both pituitary and central nervous system development (3–5). The LHX3 protein has two LIM domains in the amino (N) terminus that mediate protein–protein interactions, a centrally located homeodomain that binds DNA, and *trans*-activation domains within the carboxyl (C) terminus that are involved in pituitary gene regulation (6, 7). *Lhx3* knockout ($Lhx3^{-/-}$) mice are stillborn or die within 24 h of birth, likely because of nervous system deficits, and they lack anterior and intermediate pituitary lobes with four of the five hormone-secreting anterior cell types absent (only a small population of corticotropes remain) (4, 5). Consistent with this phenotype, molecular studies have shown that LHX3 binds 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* (6, 8–11). In addition to the pituitary, LHX3 is expressed in the developing brain, spinal cord, and inner ear, and the specification of interneuron and ventral motor neuron fates is impaired in mice lacking LHX3/4 function (3, 12).

Autosomal recessive mutations in the human *LHX3* gene are associated with pediatric hormone deficiency diseases featuring insufficiencies in GH, TSH, LH, FSH, and PRL hormones, sometimes coupled with syndromic features such as pituitary gland dysmorphogenesis, ACTH deficiency, mental retardation, and sensorineural hearing loss (13–17). In addition to combined pituitary hormone deficiency (CPHD), most patients with *LHX3* mutations also present with a rigid cervical spine resulting in limited head rotation, a phenotype that is presumed to result from deficits in LHX3 function during early spinal cord neuron specification. Treatment for the patients involves hormone replacement therapy, including GH, T_4, and sex steroid therapy. Family members heterozygous for *LHX3* mutation alleles are asymptomatic as are $Lhx3^{+/-}$ mice (4, 5, 13–18). Molecular analyses of the predicted aberrant proteins from *LHX3* gene mutations show that protein function is disabled or reduced (16, 18, 19).

Of the 10 *LHX3* mutations described to date, one type (W224ter) is associated with pituitary hormone deficiency without cervical spine rigidity, defining a unique form of the disease. This mutation, found in four siblings from a consanguineous Lebanese family, introduces a premature stop codon (W224ter) predicted to cause loss of the C terminus of the LHX3 protein that contains activation domains critical for pituitary gene regulation (6, 7, 16). The N-terminal LIM domains and homeodomain are required for multiprotein complexes in spinal cord development (3, 12, 20). Together, these observations suggest the hypothesis that the molecular functions of LHX3 in the nervous system and pituitary are separable in vivo. To test this hypothesis, and to provide a model to examine the molecular and cellular events that accompany CPHD disease progression (an invasive approach that is not possible in patients), we created a mouse model of the human *LHX3* W224ter disease.

Results

Generation of $Lhx3^{W227ter/W227ter}$ Mice. To generate a knockin mouse model of the human disease caused by the *LHX3* W224ter mutation, a targeting construct was made, which introduced the point mutation at the conserved, equivalent position (W227ter) in the

Author contributions: S.C.C., R.E.M., A.D.S., K.W.S., and S.J.R. designed research; S.C.C., R.E.M., and A.D.S. performed research; S.C.C., R.E.M., A.D.S., K.W.S., and S.J.R. analyzed data; and S.C.C., R.E.M., A.D.S., K.W.S., and S.J.R. wrote the paper.

Conflict of interest statement: S.C.C., R.E.M., and S.J.R. declare no conflicts of interest. A.D.S. and K.W.S. are employees of Eli Lilly and Company and may own stock or stock options in the company.

*This Direct Submission article had a prearranged editor.

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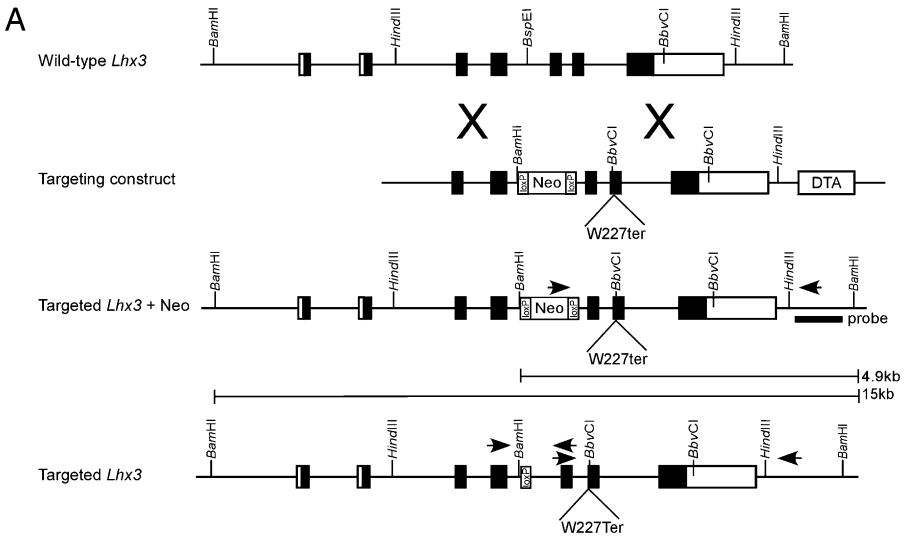
This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1009501108/-/DCSupplemental.

mouse locus (Fig. 1 *A* and *B*). The point mutation created a *Bbv*CI restriction site, and the "Neo" cassette for ES cell selection was flanked by loxP sites. This allowed later removal of the cassette to precisely model the human disease and to return the gene to as close to its native form as possible [Neo (−)]. PCR, restriction digests, and Southern blotting were used to identify correctly targeted ES cells and to determine the genotype of the animals produced (Fig. S1 *A–D*). Matings designed to produce homozygous *Lhx3*$^{W227ter/W227ter}$ Neo (+) animals did not yield any viable *Lhx3*$^{W227ter/W227ter}$ mice, suggesting the presence of the Neo cassette results in functional inactivation of the gene (Fig. 1*C*). Crosses designed to generate *Lhx3*$^{W227ter/W227ter}$ Neo (−) animals generated homozygous pups at the expected frequencies (Fig. 1*C*)

***Lhx3*$^{W227ter/W227ter}$ Mice Are Viable and Dwarfed.** Wild-type, heterozygote, and homozygote *Lhx3*$^{W227ter/W227ter}$ Neo (−) animals were produced at the predicted 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, there was some postnatal loss with several homozygote stillbirths (Fig. 1*C*), and a small fraction of *Lhx3*$^{W227ter/W227ter}$ mice exhibited wasting and died between the ages of P21 and P60.

At birth, *Lhx3*$^{W227ter/W227ter}$ homozygote mice resembled their heterozygote and wild-type counterparts; however, poor growth in homozygotes was noticed as early as P10. This size difference reached significance by P21 and continued into adulthood with the dwarfism more marked in the males (Fig. 2). To characterize the growth deficiency of the *Lhx3*$^{W227ter/W227ter}$ mice, immunohistochemical staining for hormone markers was performed using pituitaries from mice at P1 and at 12 wk of age. Whereas the anterior pituitary lobes from the adult *Lhx3*$^{W227ter/W227ter}$ mice are severely hypoplastic compared with those of adult wild-type animals, their posterior lobes are similar in size (Fig. 3*A*). GH and the β-subunit of TSH (TSHβ) are notably reduced at P1 in the *Lhx3*$^{W227ter/W227ter}$ pituitaries (Fig. 3 *B* and *D*), with some of the mutant mouse pituitaries exhibiting almost no GH staining (Fig. 3*B*, *Inset*). GH-producing somatotrope cell densities for the *Lhx3*$^{W227ter/W227ter}$ pituitaries at 12 wk are similar to those of wild-type controls, but the mutant pituitaries are hypoplastic (Fig. 3*C*). TSHβ is reduced at 12 wk in *Lhx3*$^{W227ter/W227ter}$ pituitaries (Fig. 3*E*). Consistent with these observations, real-time quantitative PCR measurements demonstrated a reduction of *Gh* transcript levels in *Lhx3*$^{W227ter/W227ter}$ mice at 6 and 12 wk of age (representing pubertal and adult animals, respectively; Fig. 3 *F* and *G*). Because pituitary GH acts on the liver to produce insulin-like growth factor 1 (IGF1) and TSH promotes the production of thyroxine (T_4) from the thyroid, serum hormone levels of total IGF1 and total T_4 were measured in the mice. Serum IGF1 levels are significantly reduced at 6 wk and lower at 12 wk (Fig. 3 *H* and *I*). Total T_4 is significantly reduced at both time points in *Lhx3*$^{W227ter/W227ter}$ mice (Fig. 3 *J* and *K*). Further, the thyroid glands of *Lhx3*$^{W227ter/W227ter}$ mice have reduced follicle size indicative of hypothyroidism (e.g., Fig. 3 *L* and *M*).



C

	+/+ 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%)

Fig. 1. *Lhx3*$^{W227ter/W227ter}$ mice are viable. (*A*) Strategy for knocking in the W227ter mutation. The wild-type *Lhx3* allele, targeting construct, *Lhx3* allele with the point mutation, and LoxP-PGK-Neo ("Neo") cassette knocked in (targeted *Lhx3* + Neo), and *Lhx3* allele with the point mutation after removal of the Neo cassette (targeted *Lhx3*) are shown. Coding regions are denoted by black boxes. The W227ter mutation introduces a *Bbv*CI enzyme site. Predicted DNA fragments that hybridize with the indicated probe during Southern analysis (Fig. S1) are indicated. Primers for PCR analysis (Fig. S1) of ES cells and animals are represented as black arrowheads. DTA, diphtheria toxin A coding sequence. (*B*) The W227ter mutation results in a truncated LHX3 (LHX3a W227ter) protein lacking the carboxyl terminal *trans*-activation domain. (*C*) *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 wk of age when genotyping occurred.

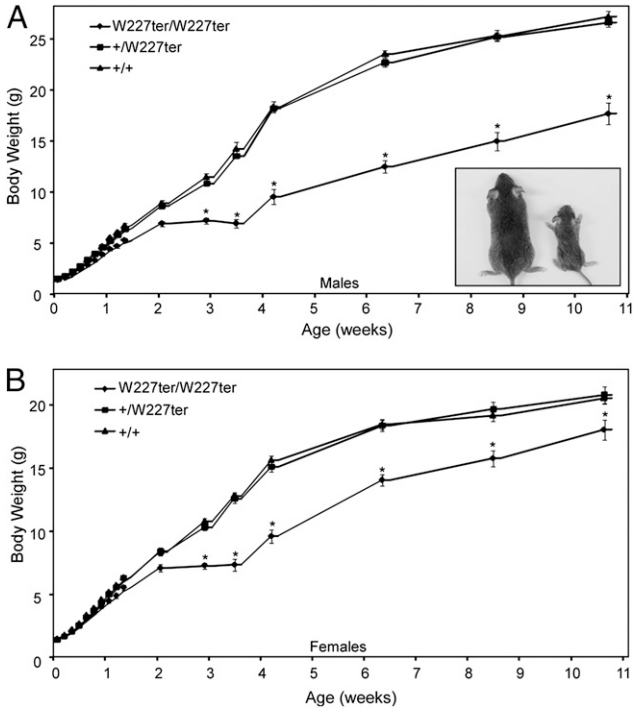


Fig. 2. *Lhx3*$^{W227ter/W227ter}$ mice are dwarfed. (*A* and *B*) In comparison with wild-type and heterozygote controls, growth deficiency of both male (*A*) and female (*B*) *Lhx3*$^{W227ter/W227ter}$ mice is evident around postnatal day 10 (P10) and becomes significant at P21. Asterisks (*) indicate a significant difference between homozygote *Lhx3*$^{W227ter/W227ter}$ compared with wild-type (+/+) and heterozygote controls with $P < 0.05$ (ANOVA). $N \geq 11$ for each time point and each genotype. Error bars are ±SEM. *Inset* shows wild-type *Lhx3*$^{+/+}$ (*Left*) and *Lhx3*$^{W227ter/W227ter}$ (*Right*) male litter mates at 8 wk old.

In addition to dwarfism, *Lhx3*$^{W227ter/W227ter}$ mice have a characteristic dysmorphic craniofacial shape similar to that found in human patients with *LHX3* mutations and growth hormone deficiencies (15–17, 21). Although they are small in size and tend to cluster with litter mates (likely for body warmth because of hypothyroidism), *Lhx3*$^{W227ter/W227ter}$ mice appear to interact and eat normally. A modified Preyer hearing test was performed and, consistent with the *LHX3* W224ter patients (16), *Lhx3*$^{W227ter/W227ter}$ mice do not display hearing deficits (*SI Materials and Methods*).

Female *Lhx3*$^{W227ter/W227ter}$ Mice Are Infertile. Human patients with the W224ter mutation have reduced FSH, LH, and PRL hormone deficiencies but because of their age and hormone therapies, their inherent reproductive potential is unknown (16). 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 S1). Over a 4-mo period, the percentage of breeding pairs producing offspring (productive matings), the average number of litters produced, and the mean litter sizes were recorded so that relative fecundity (RF) could be calculated. The *Lhx3*$^{+/W227ter}$ heterozygote animals had no reduction in fertility, producing litters on average every 24 d (RF = 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 these pairs produced litters. Whereas the *Lhx3*$^{W227ter/W227ter}$ male mice could produce litters of comparable size to those mice of the heterozygote crossings, they produced litters at a reduced rate (RF = 18.5). Female *Lhx3*$^{W227ter/W227ter}$ mice are infertile and have never produced litters (Table S1).

Examination of the pituitary glands of P1 and 12-wk males and females revealed deficiencies in LHβ- and FSHβ-producing gonadotrope cells (Fig. S2 *A–C*). As with GH, some *Lhx3*$^{W227ter/W227ter}$ animals displayed very reduced LHβ or FSHβ staining (Fig. S2*A*, *Center*) and in some LHβ or FSHβ was barely detectable (*Right*). The size and weights of the seminal vesicles of male *Lhx3*$^{W227ter/W227ter}$ mice are significantly proportionally smaller than wild types at 6 and 12 wk of age, indicative of reduced testosterone production (Fig. S2*D*). 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 with the wild-type controls. Histological examination in 6-wk-old male *Lhx3*$^{W227ter/W227ter}$ mice showed reduced secretions in the seminal vesicles and prostate, and spermatogenesis was decreased or absent with no mature sperm apparent within the testes or epididymis (Fig. S2). Together, these data demonstrate a delay in sexual maturation of male *Lhx3*$^{W227ter/W227ter}$ mice compared with wild-type controls.

Prolactin Deficiency in *Lhx3*$^{W227ter/W227ter}$ Animals. The ovaries of infertile *Lhx3*$^{W227ter/W227ter}$ female mice appear to function normally with corpora lutea and mature and immature eggs similar to that observed in the wild-type females at 6 wk of age. This suggests that ovarian failure is unlikely to be the primary cause of their infertility. The uteri of female *Lhx3*$^{W227ter/W227ter}$ mice are proportionally smaller and weigh significantly less than those of wild-type females (Fig. S2*E*). Histological examination revealed that the uterus of *Lhx3*$^{W227ter/W227ter}$ female mice is immature compared with wild-type controls at 6 wk of age (Fig. S2*F*). In the wild-type females, the thickness and keratinization of the uterine wall changed with estrus; however, the uteri of the mutant female mice did not reflect the varying stages of estrus. The uterine wall of the mutant female mice was thinner with no keratinization (Fig. S2*F*). Further, as observed for PRL null and PRL receptor null female mice (22, 23), after mating with normal males, *Lhx3*$^{W227ter/W227ter}$ knockin mice have vaginal plugs but do not enter a pseudopregnant state.

There is a striking loss of lactotrope cells in both male and female *Lhx3*$^{W227ter/W227ter}$ mice. Immunostaining demonstrates a deficiency in PRL-producing cells of the pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice at 1 d after birth (P1) (Fig. 4*A*) and essentially a lack of detectable PRL by 12 wk of age (Fig. 4*B*). Western blot experiments analyzing whole pituitaries confirmed very low or undetectable PRL protein in the pituitaries of adult *Lhx3*$^{W227ter/W227ter}$ mice compared with wild-type controls (Fig. 4*C*). Consistent with these observations, the level of *Prl* gene transcript is markedly reduced in the pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice at both 6 wk (Fig. 4*D*) and 12 wk of age (Fig. 4*E*). Further, circulating PRL hormone concentrations measured by ELISA in serum are significantly reduced at 6 and 12 wk in the *Lhx3*$^{W227ter/W227ter}$ mice (Fig. 4 *F* and *G*). Together, these data demonstrate profound PRL deficiency in the *Lhx3*$^{W227ter/W227ter}$ mice consistent with the observed female infertility.

Discussion

We have generated mice that recapitulate the human pediatric compound hormone deficiency disease caused by the W224ter mutation in the *LHX3* gene. Whereas the *Lhx3* knockout mice die within 24 h of birth (4), *Lhx3*$^{W227ter/W227ter}$ knockin mice are born at the expected frequencies and are viable. As with human patients and mice possessing one functioning *Lhx3* allele (4, 16, 19), *Lhx3*$^{W227ter/+}$ heterozygotes appear to be phenotypically normal. However, homozygous *Lhx3*$^{W227ter/W227ter}$ mice are deficient in GH, TSH, IGF1, T_4, LH, FSH, and PRL, resulting in dwarfism, reduced fertility in males, and infertility in the females. This is a similar array of pituitary hormone deficiencies to the *LHX3* W224ter human patients (16, 19) (with the caveat that more invasive analyses are possible with the animal model) and a unique array of hormone deficiencies for a mouse model of CPHD. Immunostaining of pituitaries of *Lhx3*$^{W227ter/W227ter}$ mice indicated that the numbers of ACTH-producing corticotrope cells are reduced in the knockin mice (Fig. S3). ACTH deficiency is not reported in human patients with this mutation (16). However, other mutations in the *LHX3* gene have been linked to ACTH deficiency (15, 17) and *Lhx3*$^{-/-}$ mice have a reduced population of corticotropes (4). The *Lhx3*$^{W227ter/W227ter}$ mice do not exhibit overt effects of this reduction in ACTH and adrenal morphology appears normal (Fig. S3). However, this result, coupled with the observations that ACTH deficiency can manifest during adoles-

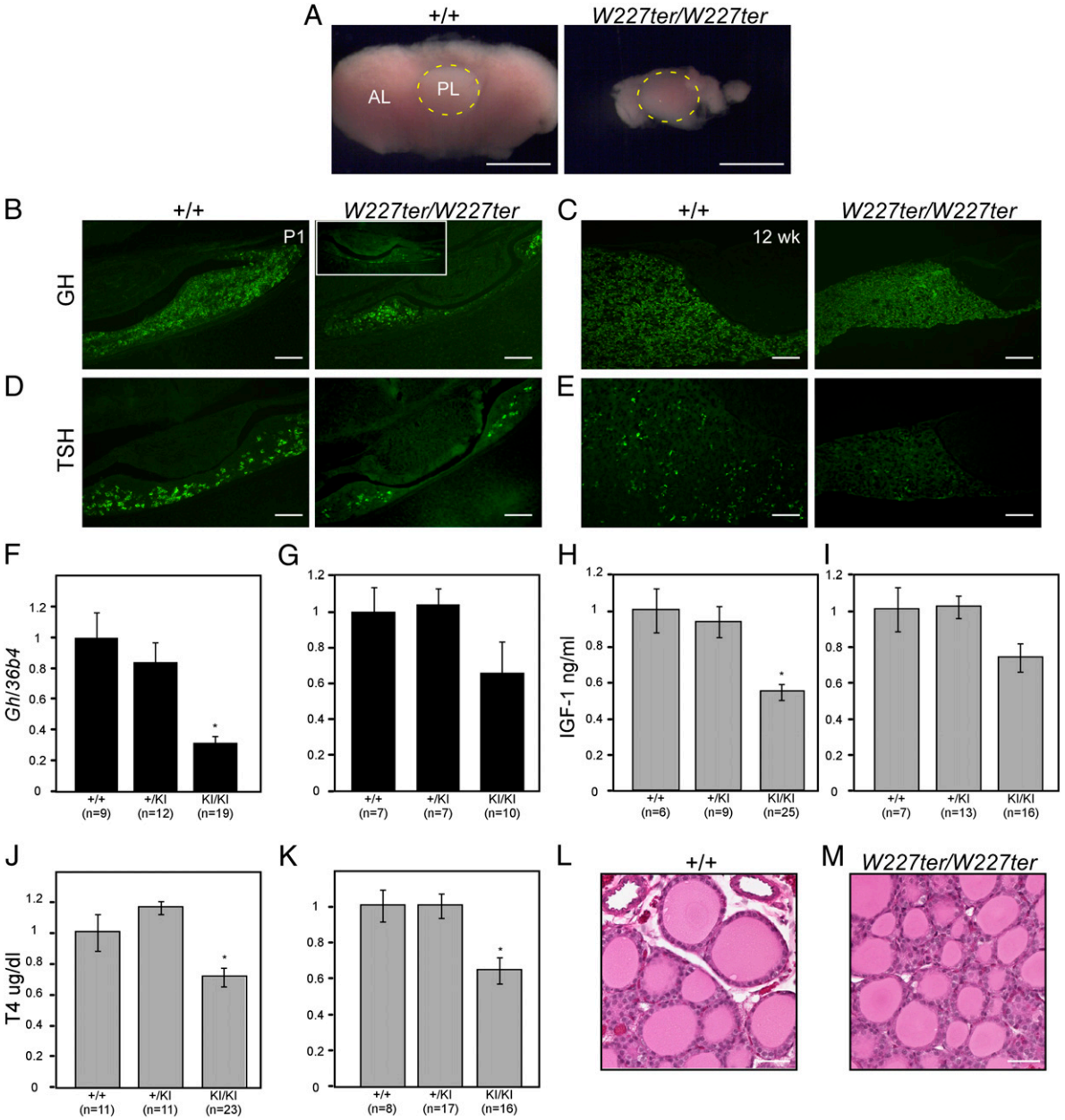


Fig. 3. Deficiencies in the GH and thyroid hormone pituitary axes underlie dwarfism in *Lhx3*$^{W227ter/W227ter}$ mice. (*A*) The anterior lobe (AL) of 12-wk-old *Lhx3*$^{W227ter/W227ter}$ pituitary gland is hypoplastic compared with that of wild-type (+/+) animals, whereas the posterior lobes (PL, dotted oval) are of similar size. (Scale bar, 1 mm.) (*B–E*) Immunostaining of P1 (*B* and *D*) and 12-wk-old pituitaries (*C* and *E*) reveals deficiencies in GH- and TSHβ-producing cells in the *Lhx3*$^{W227ter/W227ter}$ pituitaries. *Inset* in *B* shows an example of an *Lhx3*$^{W227ter/W227ter}$ animal with negligible GH-producing cells. (Scale bars, 200 μm.) (*F* and *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 wk (*F*) and 12 wk (*G*) of age. Data are mRNA levels normalized to a control transcript (*36b4*). Error bars are ±SEM. (*H–K*) Hormone analyses of trunk blood serum indicate a decrease in both IGF1 and T_4 levels in the *Lhx3*$^{W227ter/W227ter}$ mutant mice (KI/KI) compared with their wild-type (+/+) and heterozygote (+/KI) counterparts at both 6 wk (*H* and *J*) and 12 wk (*I* and *K*) of age. (*L* and *M*) Thyroid follicles of *Lhx3*$^{W227ter/W227ter}$ mutant mice (M) are smaller compared with those of wild-type mice (L), indicating impaired thyroid function. (Scale bar, 50 μm.) Asterisks (*) indicate significance compared with wild-type controls with $P < 0.05$.

cence or adulthood as a late complication of CPHD associated with mutations in other pituitary transcription factor genes such as *PROP1* (24–26), suggests that ongoing monitoring of the HPA axis in patients with *LHX3* mutations is warranted. The growth insufficiency of the *Lhx3*$^{W227ter/W227ter}$ knockin mice is not as severe as that of some dwarf models such as the Snell and Jackson mice that carry a mutation in the *Pit-1* gene, the Ames dwarf (*Prop1*^{df}), and the *Prop1* knockout mice (*Pit-1*$^{dw-J}$) (27–31).

The *Lhx3*$^{W227ter/W227ter}$ knockin mice also have compromised fertility. The male knockin mice have delayed reproductive development and reduced fertility, whereas female mice are infertile. Immunostaining indicates reduced levels of LH and FSH in both sexes of *Lhx3*$^{W227ter/W227ter}$ mice. Male *Lhx3*$^{W227ter/W227ter}$ mice have poor seminal vesicle, prostate, and testicular development, consistent with a delay in puberty and low testosterone production due to LH deficiency (32, 33). Although puberty is delayed in the male knockin mice, they are eventually able to reproduce at a reduced rate compared with controls. Overall, the impaired fecundity in the male *Lhx3*$^{W227ter/W227ter}$ mice is likely due to reduced gonadotropin levels and not the loss of PRL, as

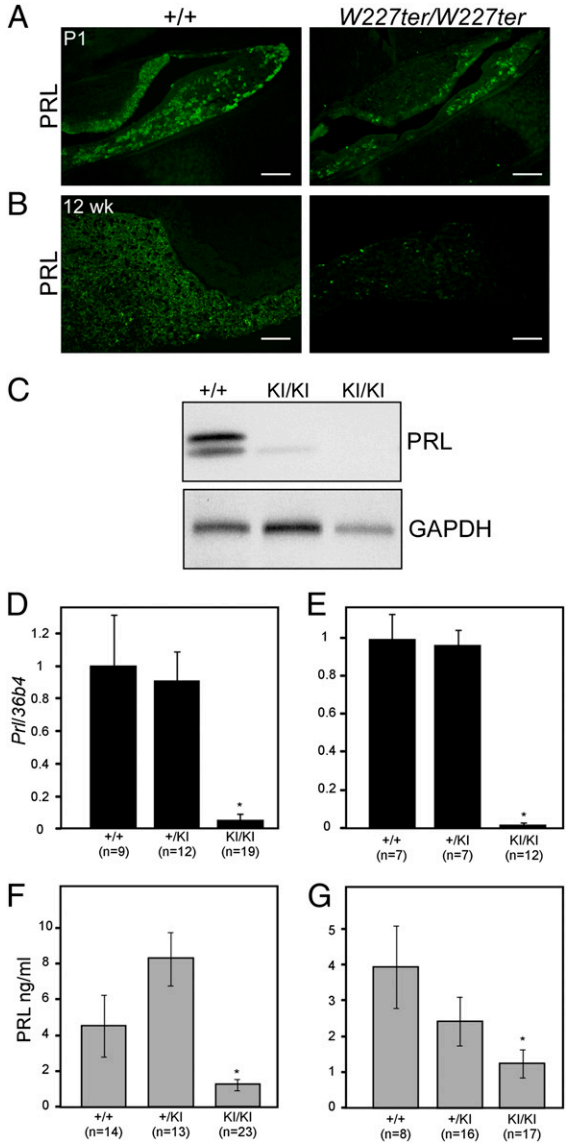


Fig. 4. PRL deficiency and infertility in *Lhx3*$^{W227ter/W227ter}$ female mice. (*A* and *B*) Anti-PRL immunostaining of pituitaries of P1 (*A*) and 12 wk (*B*) mice show a paucity of PRL-producing lactotrope cells in *Lhx3*$^{W227ter/W227ter}$ mice especially at 12 wk. (Scale bars, 200 μm.) (*C*) Western blot detection of PRL protein in 12-wk pituitaries confirms the PRL deficiency in the *Lhx3*$^{W227ter/W227ter}$ mutant mice compared with wild-type controls. Blots were reprobed with an anti-GAPDH antibody as a loading control. (*D* and *E*) Real-time quantitative PCR analyses demonstrate a striking reduction in *Prl* transcript in *Lhx3*$^{W227ter/W227ter}$ pituitaries (KI/KI) relative to wild-type (+/+) and heterozygote (+/KI) counterparts at 6 wk (*D*) and at 12 wk (*E*) of age. Data are mRNA levels normalized to a control transcript (*36b4*). (*F* and *G*) Hormone analyses by ELISA also show a decrease in serum PRL in *Lhx3*$^{W227ter/W227ter}$ mutant mice at both 6 wk (*F*) and 12 wk (*G*). Error bars are ±SEM. Asterisk (*) indicates significance compared with wild-type controls with $P < 0.05$ or less (Student's two-tailed *T*-test).

LHβ knockout male mice are sterile (33), but male PRL knockouts are fertile (22).

Female *Lhx3*$^{W227ter/W227ter}$ knockin mice are infertile with reduced gonadotropins and loss of PRL with apparent normal ovarian morphology. By contrast, the ovaries of LHβ and FSHβ knockout mice have abnormal folliculogenesis and an absence of corpora lutea, indicating a lack of ovulation (32, 33). The uterine walls of knockin mice do not display the varying thicknesses and levels of keratinization representative of the various stages of estrus, and the structure is thin and poorly developed with no keratinization, suggestive of an immature uterus. We hypothesize that PRL deficiency is the critical parameter for female *Lhx3*$^{W227ter/W227ter}$ infertility and that the reduced levels of LH and FSH may not be below critical thresholds for some reproductive functions but the loss of PRL, as in *Prl* null and *prolactin receptor* null mice (22, 23), results in profound female infertility. PRL is required for the maintenance of the corpus luteum in the ovary and stimulation of the production of progesterone by the ovaries to generate a receptive environment for implantation of embryos, in addition to other roles such as the inhibition of ovulation. Like *Lhx3*$^{W227ter/W227ter}$ mice, PRL knockout females appear to ovulate normally but also are infertile (22, 23, 34).

Studies of *Lhx3* knockout mice and animals with reduced *Lhx3* expression demonstrate that LHX3 is involved in the formation of the definitive Rathke's pouch (the primordial structure in pituitary development), pituitary organ fate commitment, lineage specification, cell expansion, and differentiation. Moreover, an absence of *Lhx3* function results in reduced proliferation and increased apoptosis (4, 5, 35, 36). The *Lhx3*$^{W227ter/W227ter}$ knockin mice demonstrate that the amino terminal and homeodomain of the LHX3 protein are sufficient for its early roles in pituitary and nervous system development but reveal that the carboxyl terminus is required for establishment of normal populations of the differentiated hormone-secreting cell types and maintenance of the lactotrope lineage.

Because antibodies to LHX3 primarily recognize the carboxyl terminus that is absent in the truncated protein, it has not been possible to characterize the protein produced in the knockin mice. A recent study indicated that there can sometimes be read through past translation stop codons (37); however, neither the human nor mouse W224/227ter mutations match the required consensus sequences. The W224ter mutation also has some of the characteristics that might result in targeting for nonsense-mediated decay. However, there are exceptions to these rules and the distinct phenotype and viability of the W224ter patients and the W227ter mice indicate that critical LHX3 function is retained.

Many proteins have been demonstrated to interact with LHX3 and related factors (reviewed in refs. 38, 39). These include proteins that interact with the LIM domains and the homeodomain such as NLI, RLIM, SLB, ISL1, and MRG1. Less is known about proteins that interact with the carboxyl terminus, a region that contains activation/repression functions and modification/targeting signals (7, 40, 41). It will be important to determine how specific protein complexes are involved in the roles of LHX3 in the developing pituitary and nervous systems. The *LHX3* W224ter mutation is unique among the known *LHX3* mutations in that it causes specific loss of the carboxyl terminus and does not cause nonpituitary symptoms (16, 19); the other mutations involve changes that impact the LIM domains, the homeodomain, or the entire protein, thereby likely affecting all aspects of LHX3 function (13–17).

No nervous system defects have been found to date in the *Lhx3*$^{W227ter/W227ter}$ mice. Whereas the *LHX3* W224ter mutation does not cause a nervous system phenotype in the human patients, other mutations in the human *LHX3* gene are often associated with a rigid cervical spine, resulting in limited head rotation of the patients and other defects including mental retardation and deafness (13–17). Previous work indicates that the LIM domains and homeodomain of LHX3 are required for the formation of multiprotein complexes necessary for spinal cord neuron development (3, 12, 20). Although the carboxyl terminus of LHX3 contains the major *trans*-activation domain for pituitary gene regulation, the LIM domains also appear to contain some activation function that may serve roles in the developing nervous system (40–42) with the W224ter protein acting with partner proteins to activate transcription from a motor neuron enhancer (19). Assays of *Lhx3* transcripts show that transcript levels are readily detected in wild-type and knockin mice, with levels somewhat reduced in both the heterozygous (normal phenotype) and homozygous animals (affected phenotype) compared with wild type (Fig. S1). It is possible that the observed phenotype of the knockin is due to reduced expression levels but

the survival of the $Lhx3^{W227ter/W227ter}$ mice, in comparison with the inability of $Lhx3^{-/-}$ animals to thrive (4, 35), is consistent with the hypothesis that the truncated LHX3 protein retains sufficient function to produce viable mice.

The exact cause of death in *Lhx3*-deficient mice has not been determined. It has been speculated that these animals die from the impaired brainstem function resulting from a lack of LHX3 expression in precursor cells of the Raphe nuclei and reticular formation (4, 43). Although the exact role of LHX3 in the developing brainstem has yet to be determined, this emphasizes the importance of LHX3 expression in the developing nervous system. The lack of a nervous system phenotype in the $Lhx3^{W227ter/W227ter}$ mice supports the hypothesis that the LIM domains and the homeodomain of LHX3 are sufficient for nervous system development, but are not adequate for effective pituitary organogenesis.

Materials and Methods

Generation of *Lhx3* W227ter Mice. Vector generation, ES cell targeting, and mouse breeding are described in the *SI Materials and Methods*.

Histology and Immunohistochemistry. These were performed as previously described (44). Details are in *SI Materials and Methods*.

RNA Analyses. Quantitative PCR was performed as described (44). Details are in *SI Materals and Methods*.

Hormone Analyses. Hormone assay was performed using standard assays as described in *SI Materials and Methods*.

Western Analyses. Western assays were performed as described (45). Details are in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We are grateful to Drs. S. Camper, G. Dai (Purdue University, Indianapolis), R. Day, P. Fueger, P. Herring (Indiana University, Indianapolis), R. Kineman, S. Konieczny (Purdue University, West Lafayette, IN), and R. Pfäffle for reagents and advice. We thank the Indiana Transgenic and Knockout Mouse Core for expert technical assistance. This work was supported by a grant (to S.J.R.) from the National Institutes of Health (NIH) (HD42024), a fellowship (to S.C.C.) from the Indiana Clinical and Translational Sciences Institute (NIH-National Center for Research Resources TL1RR025759), and a National Science Foundation fellowship (to R.E.M.).

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