Lhx4 and *Prop1* are required for cell survival and expansion of the pituitary primordia

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SUMMARY

Deficiencies in the homeobox transcription factors LHX4 and PROP1 cause pituitary hormone deficiency in both humans and mice. *Lhx4* and *Prop1* mutants exhibit severe anterior pituitary hypoplasia resulting from limited differentiation and expansion of most specialized cell types. Little is known about the mechanism through which these genes promote pituitary development. In this study we determined that the hypoplasia in *Lhx4* mutants results from increased cell death and that the reduced differentiation is attributable to a temporal shift in *Lhx3* activation. In contrast, *Prop1* mutants exhibit normal cell proliferation and cell survival but show evidence of defective dorsal-ventral patterning. Molecular genetic analyses reveal that *Lhx4* and *Prop1* have overlapping functions in early pituitary development. Double mutants exhibit delayed corticotrope specification and complete failure of all other anterior pituitary cell types to differentiate. Thus, *Lhx4* and *Prop1* have critical, but mechanistically different roles in specification and expansion of specialized anterior pituitary cells.

Key words: Pituitary development, Proliferation, Cell death, Lhx3, CPHD, Mouse

INTRODUCTION

The pituitary gland is known as the master gland because it controls the release of hormones that affect diverse processes including growth, metabolism and fertility. Many of the genes necessary for normal development and function of the specialized hormone producing cells of the anterior pituitary lobe have been identified (Dasen and Rosenfeld, 2001; Watkins-Chow and Camper, 1998) The key genes fall into several groups: transcription factors, secreted signaling molecules and receptors. Each transcription factor shown to be important in mouse pituitary development is also a cause of congenital pituitary hormone deficiency in humans, a common condition occurring with a frequency of 1 in 4000 births (Procter et al., 1998; Vimpani et al., 1977). The excellent correspondence of mouse and human pituitary phenotypes suggests that mouse studies will be ideal to characterize the undefined genetic interactions between these transcription factors.

The pituitary gland develops from an invagination of oral ectoderm known as Rathke's pouch (Burrows et al., 1999; Watkins-Chow and Camper, 1998). The pouch is in contact with the ventral diencephalon, infundibulum or prospective posterior lobe, and surrounding mesenchyme. Each of these adjacent tissues secrete signaling molecules that establish spatial orientation and identity of the pouch. Experimental evidence supports the involvement of BMP4, FGF8, FGF10, SHH, BMP2, WNT4, and chordin (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998). FGF8 and FGF10 produced by the infundibulum, and BMP2 from the ventral mesenchyme, constitute opposing signals that activate region-specific expression of the lim homeodomain transcription factors LHX3 and ISL1 in the dorsal and ventral regions of the pouch respectively (Ericson et al., 1998). The stereotypic pattern of activation of these transcription factors is critical for determination of the corticotropes (cells that synthesize adrenocorticotropic hormone or ACTH) and the cells that produce αGSU, the common subunit of thyrotropin (TSH) and the gonadotropins (luteinizing hormone, LH, and follicle stimulating hormone, FSH). Very few additional links have been made between signaling molecules, transcription factor activation, and the specification of hormone producing cells.

The roles of transcription factors in cell specification have been illuminated by both molecular and genetic studies (Dasen and Rosenfeld, 2001; Watkins-Chow and Camper, 1998). Related transcription factors have overlapping functions in early development. The bicoid type transcription factors *Pitx1* and *Pitx2* are expressed throughout the oral ectoderm at the earliest stages of pituitary specification (Suh et al., 2002). *Pitx2* is required for expansion of the pouch and has a later role in specification of gonadotropes and expansion of the *Pit1* lineage: thyrotropes, somatotropes and lactotropes (Suh et al., 2002). *Pitx1* has a minor role in expansion of the individual specialized cells, affecting only the relative proportions of each cell type (Szeto et al., 1999). Deficiency in both *Pitx1* and *Pitx2* causes an earlier arrest in development than caused by lesions in either gene alone (Suh et al., 2002).

Classical genetic analysis of the LIM gene family reveals similar roles to those of the PITX family (Sheng et al., 1997; Sheng et al., 1996). Both *Lhx3* and *Lhx4* are expressed in Rathke's pouch. The *Lhx3*-deficient phenotype is similar to that of *Pitx2*, consisting of pouch hypoplasia and absence of most of the pituitary cell types (Sheng et al., 1996). The phenotype of *Lhx4* mutants suggests a minor role for this gene relative to *Lhx3*. *Lhx4*-deficient pituitaries exhibit correct specification of all five hormone producing cell types, but the expansion of each specialized cell is reduced dramatically (Sheng et al., 1997). Loss of both *Lhx3* and *Lhx4* causes earlier developmental arrest; only rudimentary pouch formation is detected (Sheng et al., 1997). The mechanism leading to hypoplasia in *Lhx3* and *Lhx4* mutants is not defined.

Another gene important for pouch expansion is *Prop1*, a *paired*-like homeobox transcription factor gene that is only expressed for a short period during mouse embryogenesis. Ames dwarf mice (*Prop1^{df}*) have a missense mutation in the homeodomain of *Prop1* that reduces transcriptional activity (Sornson et al., 1996). The pituitaries exhibit hypoplasia with less than 1% the normal levels of thyrotropes, somatotropes and lactotropes (Gage et al., 1996). The hypoplasia is presumably due to the failure to activate the prototype POU-homeodomain transcription factor PIT1, which is responsible for the specification of those three cell lineages (Camper et al., 1990; Li et al., 1990). Serum gonadotropins are reduced in *Prop1^{df}* mutants, although the role of *Prop1* in gonadotrope differentiation is not clear (Tang et al., 1993).

Mutations in *PROP1* are the most common cause of combined pituitary hormone deficiency (CPHD) in humans (Cogan et al., 1998; Mendonca et al., 1999; Wu et al., 1998). The clinical features of patients vary considerably, and they are not explained completely by genotype-phenotypical correlations, suggesting that genetic interactions with variants in other genes may be one cause of heterogeneity (Flück et al., 1998). To investigate this idea we characterized the phenotypes of mice homozygous for mutations in *Prop1* and *Lhx4*, examining the effects on cell proliferation, cell death, and expression of other critical transcription factor genes. We found that *Lhx4* and *Prop1* have overlapping functions in early pituitary development, although the two genes have different mechanisms of causing hypoplasia. *Lhx4* is required for cell survival and timely activation of *Lhx3*, while *Prop1* is important for patterning dorsal-ventral expansion.

MATERIALS AND METHODS

Mutant animals

Ames dwarf mice (DF/B-*df/df*) were originally obtained from Dr A. Bartke (Southern Illinois University, Carbondale, IL), and have been maintained in our colony. *Lhx4* mutant mice on a mixed CF1, 129, BL6 background were a gift from Dr S. Potter (University of Cincinnati, Cincinnati, OH). Double heterzygotes were generated by mating the *Prop1^{+/df}* and *Lhx4^{+/−}* mice and intercrossing the doubly heterozygous progeny to generate double mutant embryos (*Prop1^{df/df}*, *Lhx4^{−/−}*). For each experiment, 3-5 mice of the different genotypes were examined.

Histology and Immunohistochemistry

Embryos were generated from matings of double heterozygous mice. The day the copulatory plug was detected was taken as day 0.5.

Embryos were fixed for 2 to 24 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2). All samples were washed in PBS, dehydrated in a graded series of ethanol and embedded in paraffin. 6-10 µm sections were prepared and either stained with Hematoxylin and Eosin or processed as described below.

In order to detect cell proliferation, pregnant mice were injected intraperitoneally with bromodeoxyuridine (BrdU) at 10 mg/g body weight, 2 hours before embryo removal (Nowakowski et al., 1989). After epitope retrieval in 2 N HCl, BrdU incorporation was examined with a rat anti-BrdU antibody (1:200; Harlan) and a TRITC-labeled secondary antibody (1:200; Jackson Immunoresearch). For cell counts, a FITC-labeled secondary antibody was used and the sections were counterstained with the nuclear counterstain 7-Aminoactinomycin D (7-AAD, Molecular Probes). The percentage of proliferating cells was determined by sectioning the entire pituitary region in the sagittal plane and staining every other slide with antibodies specific for BrdU. The total number of cells in Rathke's pouch and the anterior lobe were tallied, and the number of cells with BrdU immunoreactivity was ascertained. Three to four mice were examined for each genotype.

Proliferation was also assessed by immunostaining with an antibody that detects phosphorylated histone 3 (PH3, 1:200; Upstate Biotechnologies) and a FITC-labeled secondary antibody (1:200; Jackson Immunoresearch).

Programmed cell death in the embryos was detected by the TUNEL method using the FragEL kit (Oncogene Research Products) according to the manufacturer's protocol. Sections were counterstained with Methyl Green (Vector Labs).

The transcription factors expressed during pituitary gland development were examined by immunostaining for these factors with specific antibodies, PITX1 (1:1500; J. Drouin, Institut de Recherches Cliniques de Montreal, Montreal, Quebec, Canada), LHX3 (1:1000, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA), ISL1 (1:600, DSHB) SF-1 (1:1500, K. Morohashi, National Institute for Basic Biology, Myodaiji-cho, Okazaki, Japan). Biotinylated secondary antibodies were used in conjunction with avidin and biotinylated peroxidase (Vectastain MOM (mouse), guinea pig, rabbit and human kits; Vector Laboratories). Diaminobenzidine (Sigma), which produces a brown precipitate, was used as the chromogen. Slides were counterstained with either Hemotoxylin or Methyl Green (Vector Labs).

The pituitary cell populations in embryonic day (E) 18.5 mouse embryos were analyzed by immunohistochemistry with antibodies against each of the pituitary hormone markers. Immunostaining was carried out with polyclonal antisera against α-GSU (1:200), rat PRL (1:2000, AFP1050B), rat GH (1:1000, AFP411S), rat LHβ (1:1000, AFP22238790GPOLHB), rat TSHβ (1:1000, AFP1274789) (National Hormone and Pituitary Program, NIDDK, Bethesda, MD), and human ACTH (1:1000, Dako).

RESULTS

Lhx4 is necessary for the correct temporal expression of *Lhx3* and *Isl1*

Two genes known to be critical for the formation of the anterior lobe are *Lhx4* and *Prop1*. To investigate the mechanism of action of these genes in pituitary gland development and to test for genetic interactions, we analyzed single mutant mice and mice deficient in both *Prop1* and *Lhx4*. By E12.5 of mouse development, Rathke's pouch has separated from the oral ectoderm and cartilage has begun to proliferate beneath it (Fig. 1A). The anterior lobe has begun to form at the rostral and ventral aspect of the pouch. At this time *Prop1^{df/df}* pituitaries are morphologically indistinguishable from wild type (Fig.

1B). In contrast, the *Lhx4*$^{-/-}$ pouches are substantially smaller than wild type and there is no evidence of anterior lobe formation (Fig. 1C). The morphology of the double mutant pouches is identical to that of *Lhx4* mutants at this stage (Fig. 1D). To determine the mechanism by which these genes promote anterior lobe formation, we examined the expression pattern of other critical transcription factors. LHX3 immunoreactivity is detected throughout the pouch and in the anterior lobe of both the wild-type and *Prop1* mutant mice at E12.5 (Fig. 1E,F). Thus, LHX3 protein and mRNA expression are coincident (Sornson et al., 1996). In contrast, only a few cells express LHX3 in the *Lhx4* mutants, and these cells are restricted to the dorsal-most aspect of the pouch (Fig. 1G). Mice deficient in both *Prop1* and *Lhx4*, have no LHX3-positive cells at E12.5 (Fig. 1H). Thus, *Lhx4* is necessary for proper *Lhx3* expression, and *Prop1* contributes to this process.

Another LIM homeodomain transcription factor, ISL1, is initially expressed throughout Rathke's pouch at e9.5 and becomes restricted to the developing anterior lobe by E12.5. There is no change in *Isl1* expression in *Prop1* mutant mice relative to wild type at E12.5 (Fig. 1I,J). *Isl1* is expressed correctly throughout the pouch at E10.5 in the *Lhx4*$^{-/-}$ mice and the *Lhx4, Prop1* double mutants (data not shown), but it fails to be properly down regulated and restricted to the ventral side of the pouch at E12.5 (Fig. 1K,L). Instead, the expression

is weak and distributed throughout the primordium, with the highest expression in the dorsal aspect of *Lhx4* mutants (Fig. 1K, inset). Despite these differences in expression of *Isl1* and *Lhx3*, the two bicoid homeodomain transcription factors *Pitx1* and *Pitx2* are expressed normally in all cases examined: *Prop1*$^{df/df}$, *Lhx4*$^{-/-}$, and double mutants (Fig. 1M-P and data not shown). These data indicate that *Lhx4* is important for activation of *Lhx3,* which is known to be critical in restricting *Isl1* expression to the ventral aspect of the pituitary gland. *Prop1* is not required for the activation or pattern of *Lhx3* and *Isl1* expression, but it acts together with *Lhx4* in the maintenance of *Lhx3* expression. In addition, *Pitx1* and *Pitx2* are upstream or independent of *Lhx4* and *Prop1*.

Expansion of the anterior lobe is blocked in double mutant Rathke's pouches

The anterior pituitary lobe normally expands substantially between E12 and E14.5 (compare Fig. 1A and Fig. 2A). Although initiation of pituitary development and activation of transcription factor gene expression in *Prop1*$^{df/df}$ mice appears normal at E12.5, the prospective anterior lobe is clearly hypocellular at E14.5, and Rathke's pouch has expanded to produce an abnormal, overgrown, branched morphology (Fig. 2B). The minute pouch evident in *Lhx4*$^{-/-}$ mice at E12.5 has progressed to a larger pouch by E14.5, but the anterior lobe is
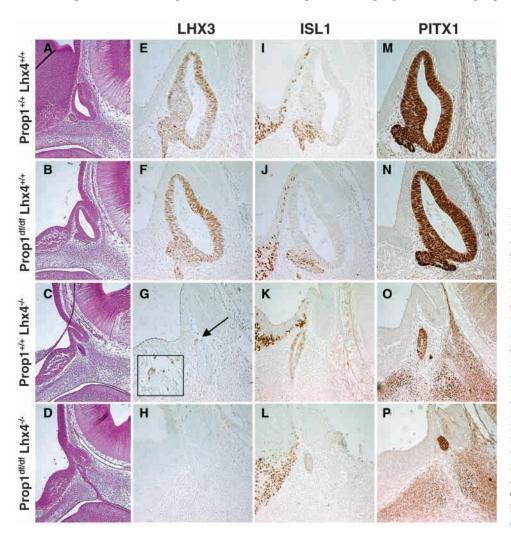

Fig. 1. *Prop1* and *Lhx4* are necessary for normal temporal and spatial activation of *Lhx3* and *Isl1*. (A-D) Embryos collected at E12.5 were genotyped as described in Materials and Methods, sectioned in the sagittal plane, and stained with Hematoxylin and Eosin (H&E). (E-P) Immunohistochemistry was used to detect LHX3 (E-H), ISL1 (I-L), and PITX1 (M-P) in sections from each genotype. LHX3 is present throughout Rathke's pouch in wild-type and *Prop1*$^{df/df}$ mice (E,F), but only a few LHX3-positive cells were present in *Lhx4* mutants (arrow and inset, G), and no positive cells were detected in double mutants (H). (I-L) ISL1 is restricted to the ventral part of the anterior lobe in wild-type and *Prop1*$^{df/df}$ mice (I,J) but it is expressed in the most dorsal area of *Lhx4* mutant pituitaries (K) and in double mutants (L). (M-P) PITX1 is present in the pituitaries of all the genotypes examined.
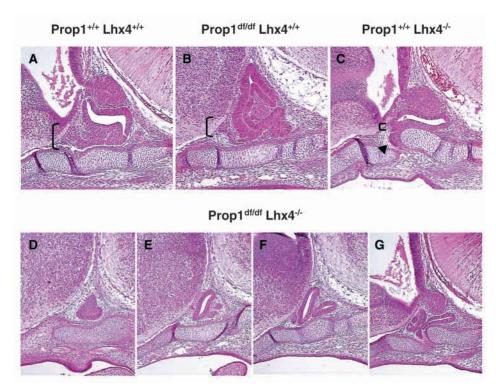
Fig. 2. *Prop1* normally represses dorsal growth and activates ventral expansion. Midsagittal sections of embryos collected at E14.5 were stained with H&E to reveal the dorsal overgrowth in *Prop1$^{df/df}$* (B) relative to wild type (A) and *Lhx4$^{-/-}$* (C). The anterior lobe is marked with a bracket. Limited anterior lobe expansion is evident in *Prop1* and *Lhx4* mutants, but no anterior lobe development is detected in *Prop1$^{df/df}$, Lhx4$^{-/-}$* double mutants (D-G) in sections spanning the lateral through midsagittal regions. The number of lumens is increased in double mutants relative to any of the other genotypes.

still barely discernible as a slight thickening in the ventral region or as a small anterior lobe (Fig. 2C). Sometimes the pouch in *Lhx4$^{-/-}$* mice appears dysmorphic with a second, smaller pouch located ventrally and bisecting the underlying cartilage. The pituitary morphology of *Lhx4* mutants is more variable than that of *Prop1* mutants or double mutants, suggesting that *Lhx4* mutants are more sensitive to genetic background, environment or stochastic events than either the *Prop1* single mutants or the *Prop1, Lhx4* double mutants. Mice lacking both *Prop1* and *Lhx4* never have any evidence of anterior lobe formation at E14.5 (Fig. 2D-G). The dysmorphology of Rathke's pouch characteristic of the *Prop1$^{df/df}$* single mutants is also apparent in double mutants, but it is less dramatic. The ventral dysmorphology typical of *Lhx4$^{-/-}$* mutants is more pronounced in the double mutants. These data indicate that *Prop1* and *Lhx4* have overlapping functions in specifying normal pouch morphology and interact to stimulate anterior pituitary lobe expansion. Since the dorsal dysmorphology is reduced in double mutants relative to *Prop1$^{df/df}$* mice, *Lhx4* must be permissive for the abnormal dorsal pouch expansion. The dysmorphic features typical of *Prop1$^{df/df}$* mice shift more ventrally in the absence of *Lhx4*.

Lhx3 expression is eventually activated in *Lhx4$^{-/-}$* and in *Prop1, Lhx4* double mutants

By E14.5 *Lhx3* expression has formed a dorsal-ventral gradient with strong expression in every cell of the intermediate lobes of wild-type and *Prop1*-deficient mice (Fig. 3A,B). Weaker LHX3 immunostaining is detected in discrete cells scattered through the anterior lobes of these mice. Despite the presence of little or no LHX3-positive cells at E12.5 in *Lhx4* mutants or double mutants, the normal pattern of *Lhx3* expression is established in these mice by E14.5, including the dorsal-ventral gradient (Fig. 3C,D).

Mice mutant in both *Lhx3* and *Lhx4* form only a pouch rudiment and fail to initiate development of the anterior pituitary lobe (Sheng et al., 1997). The arrest in development is more profound than in *Prop1* or *Lhx4* single or double mutants. Thus, the eventual activation of *Lhx3* probably is a major contributor to the initiation of anterior lobe formation in *Lhx4$^{-/-}$* mice and probably accounts for the milder phenotype of *Lhx4* mutants. *Lhx3* expression, however, is not sufficient to rescue anterior lobe formation in mice that are deficient in both *Lhx4* and *Prop1* (Fig. 3D,H,L). This is consistent with each transcription factor activating a set of genes rather than a linear pathway.

To assess pituitary cell specification in mice lacking both *Prop1* and *Lhx4*, we analyzed the expression of two early markers of cell differentiation: ACTH and αGSU (standard nomenclature, chorionic gonadotropin alpha, *Cga*). ACTH is produced by processing of pro-opiomelanocortin, or POMC, in the anterior lobe via proteolytic cleavage. In the intermediate lobe POMC is processed differently to produce melanocyte stimulating hormone and endorphins. The ACTH antibody reacts with the POMC protein precursor. POMC normally appears first in the anterior lobe at E14.5 (Fig. 3E) and later is detectable in intermediate lobe melanotropes (E15.5-E16.5) (Elkabes et al., 1989; Liu et al., 1992). Immunopositive cells are readily detected in the prospective anterior lobes of *Prop1*- and *Lhx4*-deficient mice at E14.5 (Fig. 3F,G), indicating that neither gene is essential for initiating corticotrope specification. No POMC expression is detected in double mutants at E14.5, however, suggesting that *Prop1* and *Lhx4* have overlapping roles in beginning the process of corticotrope differentiation (Fig. 3H).

In wild-type mice αGSU transcripts are detected throughout Rathke's pouch at e9, are later confined to the rostral tip of the anterior lobe by E12.5, and are restricted to thyrotropes and

gonadotropes from late gestation through adulthood (Japon et al., 1994). As expected, αGSU is detected in the rostral tip of wild-type pituitaries at E14.5 with some expansion caudally along the ventral aspect (Fig. 3I). This pattern of expression is unaltered in *Prop1*-deficient mice (Fig. 3J). *Lhx4* mutants also have αGSU-positive cells, although there are far fewer than in wild-type pituitaries. (Fig. 3K). In contrast, no αGSU protein is detectable in double mutants (Fig. 3L). This confirms the morphological observation that no anterior lobe specification is occurring in double mutant pituitaries.

only scattered positive cells in the intermediate lobe and absolutely no positive cells in the presumptive anterior lobe (Fig. 4H). This suggests that *Prop1* and *Lhx4* are important for specification of corticotropes, and they interact to promote or maintain this cell type. The reduction in corticotropes is consistent with the idea that proper expression of *Lhx3* and *Isl1* is essential for activation of *Pomc* expression, and with the occasional deficiency of ACTH in humans with *PROP1* mutations (Pernasetti et al., 2000).

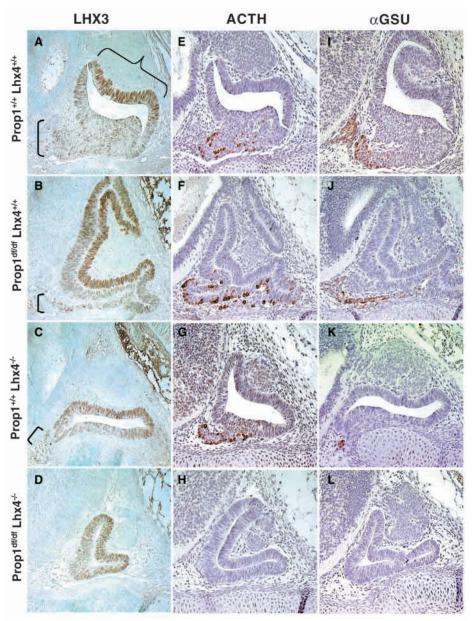At birth αGSU is detected in the caudomedial region where

Corticotrope and gonadotrope differentiation fails in double mutants

The well-defined lobular structure of the pituitary gland is evident at birth (Fig. 4A). The infundibulum has developed into the posterior lobe, and although little expansion of the intermediate lobe has taken place, the anterior lobe has expanded all along the rostral to caudal aspect of the lumen of Rathke's pouch. The abnormal growth of the dorsal aspect of the pouch has persisted and expanded in *Prop1df/df* mutants (Fig. 4B). The development of the pouch in *Lhx4* mutants fails to progress much beyond E14.5 (compare Fig. 2C and Fig. 4C). Surprisingly, the double mutant pouch appears larger than that of the *Lhx4* single mutants, but the expansion extends laterally rather than dorsally or ventrally (Fig. 4D).

At birth POMC is expressed in the majority of cells in the intermediate lobe and in scattered cells of the anterior lobe in normal mice (Fig. 4E). The pattern of POMC expression in *Prop1df/df* mice suggests that some of the dorsal overgrowth and dysmorphology comes from expansion of prospective intermediate lobe cells (Fig. 4F). There are more POMC-negative cells in the intermediate lobes of *Prop1df/df* mice than wild-type mice, however, suggesting that *Prop1* deficiency promotes expansion but not differentiation of cells in the intermediate lobe (Fig. 4F). The population of corticotropes is apparently normal in the anterior lobes of *Prop1*-deficient mice (Fig. 4F). POMC-positive cells are detectable in both the prospective intermediate and anterior lobes of *Lhx4* mutant pituitaries, but there are substantially fewer differentiated cells in both regions (Fig. 4G). Surprisingly, POMC is detectable at birth in the double mutants, but its expression is compromised even further than it is in the *Lhx4−/−* mutants, with

Fig. 3. Corticotropes and αGSU expressing cells fail to develop in double mutants. Immunohistochemical detection of LHX3 (A-D), ACTH (E-H), and αGSU (I-L) in sagittal sections of embryos of each genotype at E14.5 reveal the lack of cell specification in double mutants. LHX3 immunoreactivity is present throughout the entire Rathke's pouch of wild type (A) *Prop1df/df* (B), *Lhx4−/−* (C) and double mutants (D). However, ACTH is not detectable in the double mutants (H) compared to the other genotypes (E-G). Similarly αGSU is absent in double mutants (L) but is present in the other genotypes (I-K). In these sections, anterior lobe is marked with a bracket and intermediate lobe with a curved bracket.

TSH producing thyrotropes are located and in the ventral region where gonadotropes arise (Fig. 4I). In *Prop1df/df* mice the αGSU immunoreactivity is confined to the most ventral region (Fig. 4J). This is not unexpected because *Prop1* deficiency leads to a lack of caudo-medial thyrotropes (Gage et al., 1996; Sornson et al., 1996). In *Lhx4* mutants, the αGSU-positive cell population does not expand after E14.5, with only a few positive cells apparent at birth (Fig. 4K). These rare cells are located towards the caudal tip of the anterior lobe. Their identity is not clear, but they may represent thyrotropes that are also detectable in very low numbers (data not shown). These data are in agreement with the observation that, αGSU and TSH are present at greatly reduced numbers in *Lhx4−/−* pituitaries (Sheng et al., 1997). No αGSU-expressing cells are detectable in the pituitaries of double mutant mice (Fig. 4L). In addition, double mutants lack detectable TSH and GH (data not shown). This suggests that final differentiation of thyrotropes and somatotropes is dependent upon *Lhx4* and *Prop1*.

Gonadotrope development can be followed by a series of molecular markers. The earliest marker is αGSU, followed by gonadotropin releasing hormone receptor (*Gnrhr*) transcripts, which precede expression of the beta subunits *Fshb* and *Lhb* (Alarid et al., 1996). Several transcription factors are important, beginning with GATA2, which is critical for expression of *Fshb* and *Lhb,* but also plays a role in the *Pit1* lineage (Dasen et al., 1999). The first definitive marker is the orphan nuclear receptor SF1 (officially known as *Nr5a1*) which is necessary for basal transcription of *Fshb* and *Lhb* (Dorn et al., 1999; Halvorson et al., 1998; Keri and Nilson, 1996). We examined expression of SF1 to better understand gonadotrope development in the single and double mutant mice.

SF1 is detected only in the ventral-most aspect of the pituitaries of normal mice (Fig. 4M). Surprisingly, SF1 is detected in many regions of the *Prop1df/df* pituitaries (Fig. 4N). There are positive cells in the ventral region as expected, but immunoreactive cells extend all along the caudal aspect to the dorsal tip of the organ. Additional positive cells are present on the opposite side of the pouch lumen, in the caudal aspects of the prospective intermediate lobe. Thus, the expression
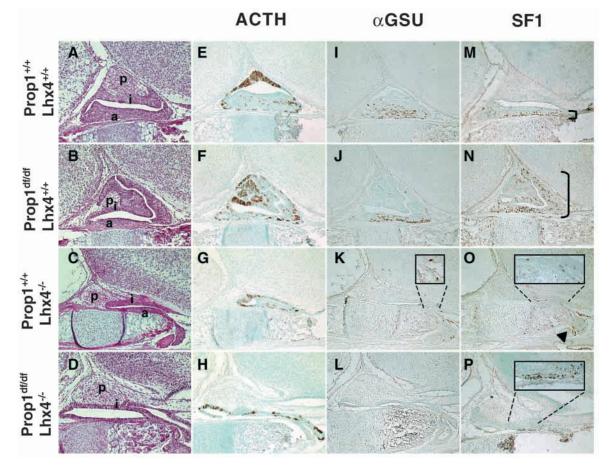

Fig. 4. Double mutants express an intermediate lobe marker and a pregonadotrope marker. H&E-stained mid-sagittal sections of pituitaries at P0 reveals the stunted anterior lobe in *Prop1df/df, Lhx4−/−* double mutants relative to normal mice and single mutants (A-D). p, posterior lobe; I, intermediate lobe; a, anterior lobe. (E-H) ACTH immunoreactivity is normally detected in intermediate lobe melanotropes and anterior lobe corticotropes (E). By P0, ACTH is present in double mutant pouches (H), but it is confined to the dorsal aspect of the primordia in the prospective melanotropes. (I-L) αGSU protein is easily detected in thyrotropes and gonadotropes of wild-type (I) and *Prop1df/df* pituitaries (J). Only isolated cells in *Lhx4−/−* pituitaries express αGSU (inset, K), and αGSU is not evident in any sections of double mutants (L). (M-P) SF1-positive pre-gonadotropes lie along the ventral surface of wild-type anterior lobes (bracket, M). The expression domain of SF1 is greatly expanded dorsally in *Prop1df/df* mice, including areas within the dysmorphic pouch (bracket, N). There are a few isolated SF1-positive cells in *Lhx4−/−* pituitaries (inset and arrowhead, O) and readily detectable SF1 expressing cells in double mutants (inset, P).
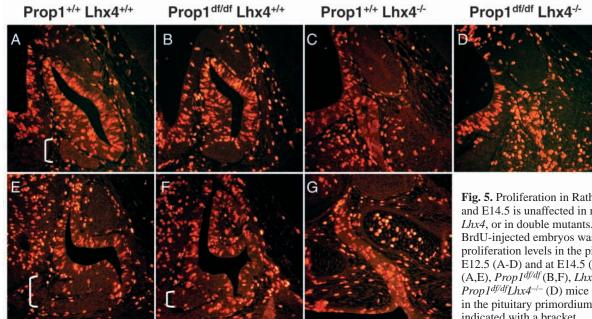
Fig. 5. Proliferation in Rathke's pouch at E12.5 and E14.5 is unaffected in mice lacking *Prop1*, *Lhx4*, or in double mutants. Immunostaining of BrdU-injected embryos was performed to assess proliferation levels in the pituitary mutants at E12.5 (A-D) and at E14.5 (E-G). Wild-type (A,E), *Prop1$^{df/df}$* (B,F), *Lhx4$^{-/-}$* (C,G) and *Prop1$^{df/df}$Lhx4$^{-/-}$* (D) mice all had dividing cells in the pituitary primordium. The anterior lobe is indicated with a bracket.

domain of SF1 is extended substantially in *Prop1$^{df/df}$* pituitaries, but expression of SF1 alone is not sufficient to activate the α-subunit. Few positive SF1 cells are detected in the *Lhx4* mutants (Fig. 4O). These are located caudally, as expected on the basis of the location of the αGSU-positive cells in these animals (Fig. 4K). Surprisingly, the double mutants express substantially more SF1 than the *Lhx4* single mutants (Fig. 4P). SF1-positive cells are as numerous in double mutant pituitaries as they are in wild-type mice, and they are appropriately confined to the ventral region. These data suggest that *Lhx4* is an activator of *Sf1* expression, and *Prop1* is a repressor. In double mutants the failure of *Prop1* to repress *Sf1* transcription dominates over the lack of activation by *Lhx4*. Thus, additional activators of *Sf1* are probably present in double mutant mice.

LH and FSH immunoreactivity are normally detectable late in gestation (Japon et al., 1994). *Prop1$^{df/df}$* pituitaries possess near wild-type numbers of gonadotropes at birth**.** Gonadotropes are only rarely identified in *Lhx4* mutants, consistent with the low level of SF1 expression (Sheng et al., 1997) (data not shown). Neither of the gonadotropins are detectable in the double mutant pituitaries (data not shown). The failure to activate transcription of *Fshb* and *Lhb*, despite the expression of *Sf1*, suggests that gonadotrope differentiation is initiated but arrests before it is complete.

Proliferation in Rathke's pouch is unaffected by loss of *Prop1* and *Lhx4*

To test whether the hypocellular anterior lobes of *Prop1$^{df/df}$* and *Lhx4$^{-/-}$* mice result from a failure of cell proliferation, we labeled dividing cells with the nucleoside analog, bromodeoxyuridine (BrdU). BrdU is incorporated into the DNA of dividing cells and can readily be detected with an epitope-specific antibody. Fetuses were collected at E12.5 and E14.5, 2 hours after injecting the mothers with BrdU. The mitotic index, calculated as the number of BrdU-labeled cells divided by the total number of nuclei, is essentially the same

in wild-type (40.3±4%) and *Prop1*-deficient mice (38.0±7%) at E12.5 (Fig. 5A,B). A greater number of dividing cells are present in the dorsal pouch, in close juxtaposition to the infundibulum. Also, most of the proliferation is occurring near the lumen of the pouch. The mitotic index is slightly lower than normal in *Lhx4* (30.1±4%) and double mutant pituitaries (Fig. 5C,D). Independent experiments using an antibody to phosphorylated histone H3, a marker of mitosis (Hendzel et al., 1997), produced similar results (data not shown). At E14.5 the number of mitotic cells appeared similar to that at E12.5 (Fig. 5E,G). The amount and location of dividing cells detected in the mouse embryonic pituitary is in agreement with values reported for the rat (Ikeda and Yoshimoto, 1991). Our data suggest that cell proliferation does not completely explain the hypocellular anterior pituitaries of *Prop1* or *Lhx4* single or double mutants.

Lhx4 is essential for the survival of cells in Rathke's pouch

We examined mutant pituitaries for evidence of apoptosis using the TUNEL method. Very few apoptotic cells are evident in normal or *Prop1* mutant pituitaries at E12.5 (Fig. 6A,B) or 14.5 (Fig. 6E,F). Thus, there is no evidence of either decreased cell proliferation or enhanced cell death in developing *Prop1*-deficient mice. The number of cells in the mutant gland is approximately the same as in wild type, but the distribution of cells along the dorsal-ventral axis is different, suggesting a role for *Prop1* in dorsal-ventral patterning. In contrast, dying cells are detectable throughout the pituitary primordia of *Lhx4$^{-/-}$* and double mutant mice at E12.5 (Fig. 6C,D). At E14.5 TUNEL-labelled cells are only present in the small portion of primordia that lies ventral to the cartilage and is still connected to the oral ectoderm (Fig. 6G,H). Little or no cell death is found in the dorsal aspect of the *Lhx4* mutant pitutiaries at this stage. Thus, the hypocellularity of the anterior lobe in *Lhx4$^{-/-}$* mice is primarily attributable to failure of pituitary precursor cells to survive.

DISCUSSION

A concert of signaling molecules and transcription factors work together to specify the 5 cell types of the anterior pituitary gland. Although many genes regulating pituitary development have been identified, the ways they fit together in pathways that lead to anterior lobe development remain to be defined. In order to determine the genetic hierarchy of genes controlling pituitary development, we created mice doubly mutant in two unrelated genes that are individually necessary for expansion of committed pituitary cell types. Our studies demonstrate the importance of *Prop1* and *Lhx4* in patterning Rathke's pouch and cell survival, and reveal the relationship of these genes to expression of another key homeobox gene, *Lhx3*.

Importance of Lim homeodomain genes in pituitary development

The LIM homeobox genes *Lhx3* and *Lhx4* are important in controlling the formation of Rathke's pouch. *Lhx3* is essential for formation of an anterior and intermediate lobe (Sheng et al., 1996). *Lhx4* mutants have a less severe phenotype than *Lhx3* mutants; all five anterior cells types are present, albeit severely reduced in number (Sheng et al., 1997). We have found that mice doubly mutant in these genes arrest in pouch formation before E12.5, indicating an early cooperative function of these 2 related genes in pituitary organogenesis. The role of the LIM homeodomain genes in pituitary development appears to be in specifying the oral ectoderm to form a definitive pituitary primordium. In neuronal development, *Lhx3* and *Lhx4* have a distinctly different role than in the pituitary. Motor neurons are generated in appropriate numbers in mice lacking both *Lhx3* and *Lhx4*, but the identity of ventrally projecting motorneurons is not specified and the fate of the existing motorneurons is shifted to dorsally projecting (Sharma et al., 1998). Although the studies of both pituitary and motorneurons described the phenotype in the absence of *Lhx3* and *Lhx4*, little is known about the pathways through which these genes act during development.

Our studies initiated a mechanistic exploration of the cooperation between these genes during pituitary development. In the absence of *Lhx4*, LHX3 protein is only present in a small number of cells in the dorsal most aspect of Rathke's pouch at E12.5. Furthermore, in *Prop1^{df/df}Lhx4^{-/-}* pituitaries, LHX3 is not detectable at E12.5. However, by E14.5 LHX3 is expressed in Rathke's pouch in the appropriate gradient in single and double mutants. This indicates that *Lhx4* is only essential for LHX3 expression early in pituitary specification, and that by E14.5 LHX3 has a phase of expression that is independent of both *Lhx4* and *Prop1*. The initial failure to activate *Lhx3* transcription results in dorsal spread of ISL1 expression and a delay in ACTH expression.

The second phase of LHX3 expression must be important for pituitary development because the *Prop1* and *Lhx4* double mutant mice, which lack both LHX4 and LHX3 until E14.5, have a distinctly different phenotype than the *Lhx3* and *Lhx4* double knockouts. A pouch rudiment forms but remains connected to the oral ectoderm through E15.5 in the *Lhx3, Lhx4* double mutants (Sheng et al., 1997). In contrast, the pouch of *Prop1* and *Lhx4* double mutants is morphologically larger and in the correct position by E15.5. These data suggest that *Prop1* has a repressor function in pouch elaboration and that mice lacking the LIM genes can form a more definitive pouch without *Prop1*.

Pouch expansion relies on *Lhx4*

The mechanism for the hypocellularity observed in both *Lhx4^{-/-}* and *Prop1^{df/df}* pituitaries was an enigma. Specification of all five cell lineages of the anterior pituitary is detectable in the single mutants, but the lineages fail to expand. This was thought to be highly suggestive of a defect in proliferation of the precursor cells. Surprisingly, the mitotic indices are nearly identical in wild type and *Prop1* mutants, and only slightly reduced in *Lhx4* mutants. Although there is no increase in cell death in *Prop1^{dfdf}* mice, the hypoplasia in *Lhx4* mutants is clearly due to a wave of precursor cell death that is mostly completed by E14.5. The
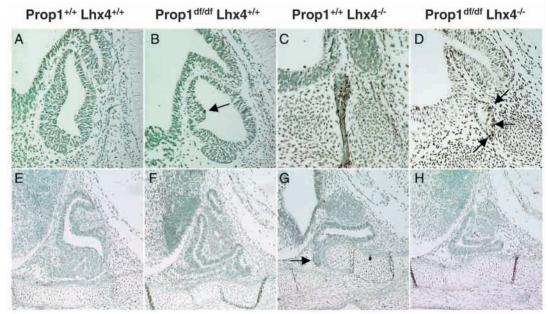

| Prop1^{+/+} Lhx4^{+/+} | Prop1^{df/df} Lhx4^{+/+} | Prop1^{+/+} Lhx4^{-/-} | Prop1^{df/df} Lhx4^{-/-} |

Fig. 6. Rathke's pouch is smaller in *Lhx4^{-/-}* mice owing to cell death at E12.5. Tunnel assay at E12.5 (A-D) and at E14.5 (E-H) demonstrated that only a few cells are dying (arrows) in the pituitary of wild-type (A) and *Prop1^{df/df}* (B) mice. However, numerous dying cells were detected in Rathke's pouch at E12.5 in *Lhx4^{-/-}* (C) and *Prop1^{df/df}Lhx4^{-/-}* (D) mice. At E14.5 no cell death was detected in any of the genotypes: wild type (E), *Prop1^{df/df}* (F), *Lhx4^{-/-}* (G) and *Prop1^{df/df}Lhx4^{-/-}* (H).

anterior lobe begins to undergo limited differentiation after the brief interval of apoptosis, around the time *Lhx3* is finally activated.

Signals sent by the diencephalon are necessary for the oral ectoderm to undergo pouch formation and survival. For example, diencephalon development fails in mice deficient in the transcription factor *T/ebp*, and this results in apoptosis of Rathke's pouch (Takuma et al., 1998). The absence of *T/ebp* causes secondary deficiencies of FGF8 and FGF10, suggesting FGFs may be required for cell survival. *Fgf10* and its receptor *Fgfr2* are also required for survival of oral ectoderm cells (De Moerlooze et al., 2000; Ohuchi et al., 2000). The similar phenotypes of mice deficient in FGF signaling and the *Lhx4−/−* mutants suggest that *Lhx4* is required for response to FGF signaling.

Lhx4 expression is necessary for the early patterning of Rathke's pouch, a process that is also dependent on FGF signaling. Elegant explant culture experiments have shown that FGFs are sufficient for extinguishing ISL1 expression throughout the pouch and for initiation of LHX3 expression (Ericson et al., 1998). The pituitaries of *Lhx4−/−* mice share this failure to regulate ISL1 and LHX3 expression properly, supporting the hypothesis that some *Lhx4* target genes are necessary for the response to FGF signaling. In the absence of *Lhx4*, cell death and inappropriate patterning of Rathke's pouch results. No differences in *Fgf10* mRNA expression are evident in *Prop1df/df*, *Lhx4−/−* or double mutant mice (data not shown). Thus, the cell death and patterning defects characteristic of *Lhx4* mutant mice probably result from a failure to respond to FGF at the level of the receptors, intracellular signaling responses, or transcription factor target genes. Given the complexity of genes involved in FGF signaling (Kato and Sekine, 1999), approaches that screen broadly for differentially expressed genes will be useful for exploring the pathway by which *Lhx4* controls pituitary cell survival and differentiation (Blackshaw et al., 2001; Douglas and Camper, 2000; Douglas et al., 2001).

Cell specification in Rathke's pouch: dorsal versus ventral

The loss of *Prop1* does not significantly affect either cell proliferation or cell survival. We cannot rule out the possibility that differences in these processes exist but were undetected by the techniques we used. However, our methods were sensitive enough to detect a short period of cell death in *Lhx4* mutants and the region-specific differences in proliferation characteristic of normal rodent pituitaries (Ikeda and Yoshimoto, 1991). Moreover, at least one other paired-type gene, *Pax6*, causes hypoplastic organ development without altering the mitotic index or cell death (van Raamsdonk and Tilghman, 2000). We favor the idea that the abnormal dorsal expansion of the pituitary primordia in *Propdf* mutants, which generates the characteristic dorsal dysmorphology and hypercellularity, occurs at the expense of the expansion of the ventral area and prospective anterior lobe.

Very few proliferating cells are detected in the anterior lobe of normal mice. This suggests that the anterior lobe grows through ventral and lateral movement of cells from the highly proliferating zone at the dorsal tip. Target genes of *Prop1* may be required for this ventral movement. Little is known about pituitary cell migration, but failure to leave the dorsal proliferative zone would reduce exposure of precursor cells to critical ventral signals such as BMP2 (Ericson et al., 1998).

In addition to repressing expansion of the dorsal pouch, *Prop1* also restricts anterior lobe differentiation to the more ventral aspect of the gland. The dorsal spreading of SF1 expression in *Prop1df/df* mice is consistent with this idea. *Pax6* is expressed in the dorsal pituitary primordia, in the same
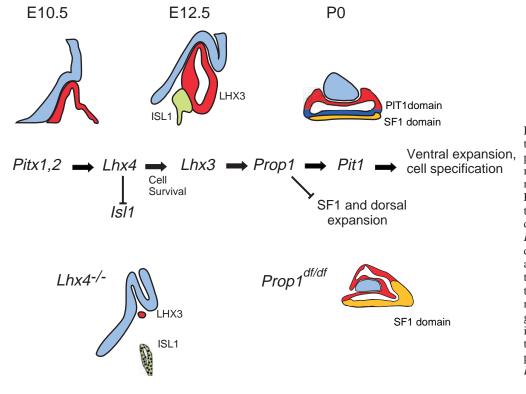

Fig. 7. *Lhx4* and *Prop1* function together in promoting anterior pituitary formation. Analysis of *Lhx4* mutants revealed that this gene is necessary for initial activation of LHX3 expression at the appropriate time and for cell survival. Ames dwarf mutant analysis showed that *Prop1* is essential for repressing dorsal expansion of the primordium and SF1 expression, as well as for the expansion of the anterior lobe. In the absence of both of these genes, no specification of corticotropes, gonadotropes or thyrotropes occurs in the anterior lobe. The schematic of the defects in the *Lhx4* mutant pituitary is depicted at E12.5 and the *Prop1* mutant pituitary at P0.

region as *Prop1*, and it is also involved in restricting SF1 expression to the ventral aspect, although its influence is not as strong as that of *Prop1* (Kioussi et al., 1999). *Prop1* may repress SF1 directly or be required for the response of cells in the primordia to repressive signals such as chordin, which represses BMP signaling along the caudal aspect of the gland. In any case, it is clear that *Prop1* is important in dorsal-ventral patterning at both the morphological and molecular level.

Lhx4 and *Prop1* function together in promoting anterior pituitary formation

We determined that *Lhx4* and *Prop1* interact to control the specification and expansion of the anterior lobe. The observed genetic interaction is not necessarily physical, and is defined by the worsening of the pituitary phenotype in double mutants. By analyzing these mutants we demonstrate that *Lhx4* is essential for cell survival and for LHX3 expression. In addition, we demonstrate that *Prop1* and *Lhx4* act independently or upstream of *Pitx1* and *Pitx2* in activating *Lhx3* expression (Tremblay et al., 1998) (P. J. Gage, H. Suh and S. A. C., unpublished data). These findings add to our understanding of the genetic hierarchy that controls pituitary development (Fig. 7).

Our studies of *Prop1* and *Lhx4* single and double mutant mice may be relevant to understanding the phenotypic heterogeneity in CPHD. The endocrine profile of CPHD patients with mutations in *PROP1* includes undetectable gonadotropin secretion, low GH, PRL and TSH, and occasionally progressive ACTH deficiency, which is very similar to mouse *Prop1* mutants (Cogan et al., 1998; Cushman and Camper, 2001; Deladoey et al., 1999; Parks et al., 1997; Wu et al., 1998). Some juvenile patients have initial enlargement of the pituitary that resolves into hypoplasia (Riepe et al., 2001). It is possible that the dorsal overgrowth observed in *Prop1df/df* pituitaries also occurs in humans with *PROP1* mutations, causing the pituitary area to appear enlarged on magnetic resonance imaging scans. The variability in phenotype extends to patients in the same family harboring identical *PROP1* mutations (Flück et al., 1998). This suggests that there are genetic modifiers of PROP1 function.

Mutations in *LHX4* also cause CPHD in humans (Machinis et al., 2001). Some patients present with GH, TSH and ACTH deficiencies, which is consistent with the hormonal profile of *Lhx4−/−* mice. Patients also have a poorly developed sella turcica, suggesting that the *Lhx4* signaling pathway is necessary for shaping the sphenoid bone. The bone abnormality could be secondary to the incomplete separation of the pituitary primordium from the oral ectoderm, as we observe in *Lhx4−/−* mice. We observed variability in the *Lhx4* mutant phenotype, and one human pedigree includes individuals with CPHD and isolated growth hormone deficiency, consistent with an influence of modifying genes. Given the interaction we observe between *Lhx4* and *Prop1*, and the interactions that have been demonstrated for the related gene pairs *Pitx1* and *Pitx2*, and *Lhx3* and *Lhx4*, it seems likely that variant alleles at any of these loci could contribute to the phenotypic variability observed in patients with deficiencies in *LHX4* or *PROP1*.

Our studies, defining the ways in which the transcription factors PROP1 and LHX4 work together to influence pituitary development, have revealed an interaction between the two genes, and also identified the mechanism behind LHX4 action in pituitary gland development.

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