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A role of the LIM-homeobox gene *Lhx2* in the regulation of pituitary development

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ABSTRACT

The mammalian pituitary gland originates from two separate germinal tissues during embryonic development. The anterior and intermediate lobes of the pituitary are derived from Rathke's pouch, a pocket formed by an invagination of the oral ectoderm. The posterior lobe is derived from the infundibulum, which is formed by evagination of the neuroectoderm in the ventral diencephalon. Previous studies have shown that development of Rathke's pouch and the generation of distinct populations of hormone-producing endocrine cell lineages in the anterior/intermediate pituitary lobes is regulated by a number of transcription factors expressed in the pouch and by inductive signals from the ventral diencephalon/infundibulum. However, little is known about factors that regulate the development of the posterior pituitary lobe. In this study, we show that the LIM-homeobox gene *Lhx2* is extensively expressed in the developing ventral diencephalon, including the infundibulum and the posterior lobe of the pituitary. Deletion of *Lhx2* gene results in persistent cell proliferation, a complete failure of evagination of the neuroectoderm in the ventral diencephalon, and defects in the formation of the distinct morphological features of the infundibulum and the posterior pituitary lobe. Rathke's pouch is formed and endocrine cell lineages are generated in the anterior/intermediate pituitary lobes of the *Lhx2* mutant. However, the shape and organization of the pouch and the anterior/intermediate pituitary lobes are severely altered due to the defects in development of the infundibulum and the posterior lobe. Our study thus reveals an essential role for *Lhx2* in the regulation of posterior pituitary development and suggests a mechanism whereby development of the posterior lobe may affect the development of the anterior and intermediate lobes of the pituitary gland.

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Introduction

The pituitary gland regulates metabolism, growth, reproduction, and reaction to stress by secreting hormones in response to signals from the hypothalamus. It is composed of three lobes. The anterior and intermediate lobes contain corticotrope, gonadotrope, lactotrope, somatotrope, thyrotrope, and melanotrope lineages that are specialized to produce adrenocorticotropic hormone (ACTH), luteinizing hormone (LH) or follicle stimulating hormone (FSH), prolactin (PRL), growth hormone (GH), thyroid stimulating hormone (TSH), and melanocyte stimulating hormone (MSH), respectively. The posterior lobe of the pituitary is directly connected to the ventral hypothalamus and contains neuroglial cells called pituicytes and axon terminals from the hypothalamus that release oxytocin or vasopressin upon stimulation by brain activities.

During embryonic development, the anterior/intermediate and posterior lobes of the pituitary originate from two separate germinal regions. The anterior/intermediate lobes are derived from an invagination of the oral ectoderm called Rathke's pouch, whereas the posterior lobe is derived from the infundibulum, an evagination of the neuroectoderm lining the floor of the ventral diencephalon. In spite of their separate origins, Rathke's pouch and the infundibulum maintain a close contact during development. Early tissue recombination studies have suggested that the neuroectoderm of the ventral diencephalon provides inductive signals that are required for the growth and differentiation of Rathke's pouch (Daikoku et al., 1982; Watanabe, 1982). Since then we have learned more about the nature of such signals. Secreted molecules including fibroblast growth factor FGF8, bone morphogenetic protein BMP4, and the Wnt protein Wnt5A are expressed in the ventral diencephalon and play important roles in controlling the growth of Rathke's pouch and the specification of various pituitary endocrine cell lineages (Cha et al., 2004; Ericson et al., 1998; Norlin et al., 2000; Takuma et al., 1998; Treier et al., 1998; also see Zhu et al., 2007, for a comprehensive review).

In addition to signaling molecules, intricate cascades of transcriptional networks are involved in the formation of Rathke's pouch and the generation of the various endocrine cell lineages in the anterior

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and intermediate lobes of the pituitary (for reviews, see Scully and Rosenfeld, 2002; Zhu et al., 2007). Notably, the bicoid-related transcription factors Pitx1 and Pitx2 are required for early cell proliferation, survival, and differentiation of the developing anterior/intermediate pituitary lobes (Charles et al., 2005; Kioussi et al., 2002; Suh et al., 2002). Downstream of the Pitx factors, Lhx3 and other closely related LIM-homeodomain factors such as Lhx4 and Islet1 are involved in the control of cell survival and growth of Rathke's pouch (Ellsworth et al., 2008; Li et al., 1996; Mullen et al., 2007; Raetzman et al., 2002; Sheng et al., 1996, 1997; Takuma et al., 1998; Zhao et al., 2006). Other transcription regulators, including the paired-like homeodomain factors Hesx1 (Rpx) and Prop1, the notch signaling mediator Rbp-j, and the notch signaling effectors Hes1and Hes5, also play important roles in the formation, growth or patterning of Rathke's pouch, or in the specification of specific endocrine cell lineages (Dattani et al., 1998; Kita et al., 2007; Martinez-Barbera et al., 2000; Nasonkin et al., 2004; Raetzman et al., 2007; Sornson et al., 1996; Ward et al., 2005; Zhu et al., 2006). Later in pituitary development, the transcription factor Pit1, which is regulated by a notch signaling pathway and Prop1 (Zhu et al., 2006), as well as the canonical Wnt/β-catenin pathway (Olson et al., 2006), is required for terminal differentiation of specific endocrine cell lineages such as lactotropes, somatotropes, and thyrotropes (Camper et al., 1990; Dasen et al., 1999; Li et al., 1990). Under the control of Pit1 the transcription factor Math3 is required specifically for the maturation and proliferation of somatotropes (Zhu et al., 2006). The transcription factor Tbx19, on the other hand, is required for the differentiation of corticotropes and melanotropes (Pulichino et al., 2003a,b). More recently, the histone lysine demethylase LSD1 has been shown to control later pituitary cell lineage determination and differentiation as a key component of complexes for either gene activation or repression (Wang et al., 2007).

While the molecular mechanisms governing the formation of Rathke's pouch and the anterior/intermediate pituitary lobes have thus been studied in considerable detail, less is known regarding the development of the posterior lobe and its influence on the formation of the pouch-derived structures of the pituitary gland. Previous studies have revealed that deletion of the transcription factor Nkx2.1 leads to a loss of the infundibulum and the posterior pituitary lobe and a complete elimination of *Fgf8* expression in the ventral diencephalon, consequently resulting in growth arrest of Rathke's pouch (Kimura et al., 1996; Takuma et al., 1998). On the other hand, deletion of the transcription factor Sox3 causes a ventral extension of the expression domains of *Fgf8* and *Bmp4* and results in a defect in the evagination of the infundibulum, dysmorphology of Rathke's pouch, and hypopituitarism (Rizzoti et al., 2004). In addition, loss of the posterior pituitary lobe has also been observed in double mutant lacking both Hes1 and Hes5 (Kita et al., 2007) and in mutant lacking the paired-like homeodomain factor Rx (Medina-Martinez et al., 2009; Zhang et al., 2000).

In this study, we examine the function of LIM-homeodomain transcription factor Lhx2 in pituitary development. Lhx2 has previously been shown to play essential roles in development of the telencephalon and retina (Monuki et al., 2001; Porter et al., 1997; Saha et al., 2007). Here we find that *Lhx2* mRNA is extensively expressed in the developing mouse ventral diencephalon including the infundibulum and the posterior lobe of the pituitary. Our analysis of loss-of-function mutants reveals an essential role for *Lhx2* in the formation of the posterior lobe and its involvement in regulation of the development of the anterior and intermediate lobes of the pituitary.

Materials and methods

Generation and genotyping of mouse embryos

Heterozygous mice carrying a null mutant allele of *Lhx2* ($Lhx2^{+/-}$) have been previously generated (Porter et al., 1997) and maintained in a C57BL6/J background. Animals were housed in an NICHD facility following NIH guidelines. To obtain embryos, the $Lhx2^{+/-}$ mice were mated and females were checked daily for the presence of copulation plugs. Noon of the day when a plug was observed was designated as embryonic day (E) 0.5. Tissue samples from embryos of various developmental stages were collected and fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.4) at 4 °C overnight. After fixation, the tissue was washed in phosphate-buffered saline (pH 7.4) and either dehydrated through a series of ethanol solutions with ascending concentrations and embedded in paraffin or incubated in 20% sucrose in phosphate-buffered saline (PBS) (pH 7.4) and frozen in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA).

To determine genotypes, either yolk sac or tail tip biopsy was collected and digested in a DirectPCR lysis solution (Viagen, Los Angeles, CA) following the manufacturer's suggested procedure. The resulting tissue lysate was analyzed by PCR using the following oligonucleotide primers: *Lhx2* wild-type allele, 5′-gagtgcaggattgggctcagc; 5′-ggtacccactgtacaacaagc. *Lhx2* mutant allele, 5′-acggggtcatcgacgaaatgg; 5′-ttaagggccagctcattcctcc.

Histology, in situ hybridization, and immunohistochemistry

Paraffin-embedded or frozen mouse embryonic tissue was sectioned at a thickness of 5 or 16 μm and collected on silanated microscopic slides (KD Medical, Columbia, MD). For histological analysis, the paraffin sections were stained by hematoxylin and eosin (Sigma, St. Louis, MO). *In situ* hybridization analysis was performed on paraffin or frozen sections by previously published procedures (Robinson et al., 1991; Schaeren-Wiemers and Gerfin-Moser, 1993), using ^{33}P-UTP-labeled or digoxigenin-UTP-labeled RNA probes. The probes used include *Lhx2* (Porter et al., 1997), *Six3* (Oliver et al., 1995), *Nkx2.1* (Sussel et al., 1999), *Wnt5a* (Zhao et al., 1999), *Bmp4* (Takuma et al., 1998), and *Fgf8* (Takuma et al., 1998). Immunohistochemical analysis was performed on paraffin sections using antibodies directed against vasopressin (AB1565, Millipore, Billerica, MA, 1:5000), calbindin (AB1778, Millipore, Billerica, MA, 1:1000), CRABP2 (Protein Tech Group, Inc. Chicago, IL, 1:50), Pitx1 (kindly provided by Dr. Jacques Drouin, Institut de Recherches Cliniques de Montréal, Montréal, Quebec, Canada, 1:80), Pitx2 (kindly provided by Dr. Jeffrey Murray, University of Iowa, Iowa City, IA, 1:4), Sox3 (Zhang et al., 2003) (kindly provided by Dr. Michael Klymkowsky, University of Colorado, Boulder, CO, 1:500), Lhx3 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, 1:400), Islet1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, 1:600), TSH [National Hormone and Peptide Program (NHPP), Torrance, CA, 1:1500], PRL (NHPP, Torrance, CA, 1:2000), ACTH (A1927, Sigma, St. Louis, MO, 1:200), GH (NHPP, Torrance, CA, 1:2000), and MSH (AB5087, Millipore, Billerica, MA, 1:10000). As described previously (Zhao et al., 2006, 2008), the paraffin sections were treated for antigen retrieval by boiling in an antigen unmasking solution (Vector Laboratory, Burlingame, CA) and immunostained for vasopressin, Pitx1, Pitx2, Lhx3, Islet1, TSH, PRL, ACTH, GH, and MSH or processed in a PickCell 2100 retriever with R-Buffer A (Electron Microscopy Sciences, Hatfield, PA) and stained for Sox3 and CRABP2. Sections were incubated in 0.1% trypsin (Sigma, St. Louis. MO) in phosphate-buffered saline prior to staining of calbindin. The immunostaining was carried out using an ABC *elite* or an M.O.M kit (Vector Laboratory, Burlingame, CA) following the manufacturer's suggested procedures.

Analysis of cell proliferation and apoptosis

Cells undergoing proliferation were labeled and detected as previously described (Zhao et al., 2006). Briefly, pregnant females were injected intraperitoneally with saline containing 5′-bromo-2′-deoxy-uridine (BrdU) (Sigma, St. Louis, MO; 0.1 mg per gram of body weight). One hour later, embryos were processed and sectioned as described above. BrdU-labeled cells were detected by staining the

sections using a peroxidase conjugated antibody directed against BrdU (Roche, Indianapolis, IN). For quantification of proliferating cells in the infundibulum in E11.5 and E12.5 embryos, we selected two mid-sagittal sections through the pituitary from two control and two mutant embryos for each stage and counted BrdU-positive nuclei in an area of the neuroectoderm spanning 100 μm on both sides of the most caudal-ventral point of the infundibulum (see Figs. 4A–D). Student's *t*-test was performed to determine statistic significance of the difference between the control and the mutant. For detection of cells undergoing apoptosis, the sections were analyzed by TUNEL staining using an APO-BrdU-IHC kit (Millipore, Billerica, MA).

Results

Detection of Lhx2 mRNA in the developing ventral diencephalon and posterior lobe of the pituitary

Lhx2 is widely expressed in various regions of the developing central nervous system, including the retina, forebrain, midbrain, hindbrain, and spinal cord (Bulchand et al., 2003; Monuki et al., 2001; Nakagawa and O'Leary, 2001; Saha et al., 2007; Xu et al., 1993). In an effort to establish a role for this gene in the development of the pituitary gland, we performed a more detailed analysis of *Lhx2* expression in pituitary primordium, i.e., the ventral diencephalon/infundibulum and the oral ectoderm/Rathke's pouch.

Abundant levels of *Lhx2* mRNA were detected in the midline neuroectoderm of the ventral diencephalon, including a region that is in direct contact with the underlying oral ectoderm at E9.5 (Fig. 1A) and E10.5 (Fig. 1B). At E11.5, *Lhx2* mRNA is continuously expressed in the neuroectoderm of the ventral diencephalon that undergoes evagination to form the infundibulum (Fig. 1C). At this stage, a definitive Rathke's pouch develops from an invagination of the oral ectoderm (Fig. 1C). By E12.5, the expression of *Lhx2* mRNA persists in what can be discerned as the posterior lobe of the pituitary (Fig. 1D). Notably, while *Lhx2* mRNA is abundantly detected in the neural tissue from which the posterior lobe is derived, it is absent or barely detectable in the midline oral ectoderm and Rathke's pouch, the primordium of the anterior and intermediate lobes of the pituitary (Figs. 1A–D).

Histological defects of the pituitary in Lhx2 mutant embryos

To investigate the role of *Lhx2* in pituitary development, we performed histological analysis of the developing pituitary in a previously generated *Lhx2*-null mutant strain (Porter et al., 1997) by hematoxylin and eosin staining. At E10.5, a rudimentary pouch is present in both control (wild-type or $Lhx2^{+/-}$) and *Lhx2* homozygous mutant ($Lhx2^{-/-}$) embryos (Figs. 2A, E). However, the neuroectoderm of the ventral diencephalon in $Lhx2^{-/-}$ mutants fails to show any sign of evagination by bending dorsally as seen in control embryos (Figs. 2A, E). By E11.5, it becomes evident that the neuroectoderm of the ventral diencephalon in $Lhx2^{-/-}$ mutant fails to evaginate and to form the infundibulum that is normally observed in the controls (Figs. 2B, F). Microscopic examination under high magnification showed that while the cells in the infundibulum normally contain nuclei with an elongated shape (Fig. S1A), many cells in the comparable region of the *Lhx2* mutant contain nuclei with a round shape and are more densely populated (Fig. S1B). As a consequence of the failed evagination, later during development (E12.5 and E13.5), the $Lhx2^{-/-}$ mutants lack a morphologically distinctive posterior lobe as compared to the controls (Figs. 2C–D and G–H).

Despite the defect in the development of the posterior lobe, a structure resembling a definitive pouch forms in the $Lhx2^{-/-}$ mutants at E11.5, but the pouch fails to grow dorsally as seen in the controls (Figs. 2B, F). It instead extends in a rostral–caudal direction beneath the floor of the diencephalon (Fig. 2F). At E12.5 and E13.5, the ventral portion of the pouch in control embryos expands rostrally to form the anterior lobe of the pituitary (Figs. 2C–D). By contrast, the dorsal portion of the mutant pouch expands and then grows ventrally into the lumen of the pouch (Figs. 2G–H).

E15.5 is the latest stage when viable $Lhx2^{-/-}$ mutant embryos can be collected. Thereafter, $Lhx2^{-/-}$ mutants die *in utero* due to a severe defect in definitive erythropoiesis (Kioussi et al., 2002; Porter et al., 1997). At this terminal stage, severe morphological defects of the pituitary were observed in the mutants (Figs. 3D–F) as compared to the controls (Figs. 3A–C). The various components connecting the hypothalamus and the anterior/intermediate lobes of the pituitary, including the medial eminence, pituitary stalk, and the posterior pituitary lobe, are all missing in the mutants (Figs. 3D–F). Instead we observed a large mass of cells occupying the area between the third ventricle and the anterior/intermediate lobes of the pituitary (Figs. 3D, E). The anterior and intermediate pituitary lobes in the mutants are highly disorganized. The anterior lobe lacks a rostral tip, and we often observed an increased number of vascular elements carrying immature red blood cells and mononuclear cells (Figs. 3E, F). In summary, our histological analysis revealed that a loss of *Lhx2* function impairs proper evagination of the neuroectoderm in the ventral diencephalon and results in severe morphological defects in the developing pituitary gland.

Molecular analysis of pituitary defects in Lhx2 mutant embryos

To determine whether the presence of the large mass of cells in the infundibular region of the ventral diencephalon of *Lhx2* mutants results from an increase in cell proliferation, we used BrdU to label cells undergoing proliferation at E11.5, E12.5, E13.5, and E14.5. This analysis revealed a dramatic increase in cell proliferation in the

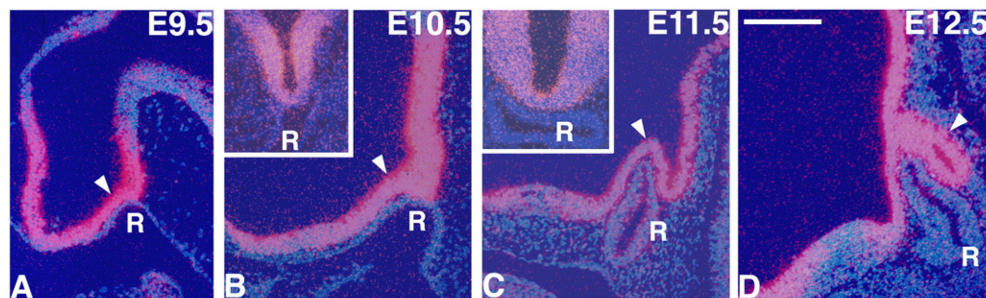

Fig. 1. Expression of *Lhx2* in the developing ventral diencephalon and posterior lobe of the pituitary gland. (A–D) *Lhx2* mRNA was detected by *in situ* hybridization on sagittal sections through the developing pituitary of E9.5 (A), E10.5 (B), E11.5 (C), and E12.5 (D) mouse embryos. Rostral is to the left. Insets in panels B, C show part of a coronal section through the pituitary. Arrowheads in panels A, B point at an area that directly abuts Rathke's pouch (R). Arrowheads in panels C and D point at the evaginating neuroectoderm of the ventral diencephalon and the posterior lobe of the pituitary, respectively. Scale bar in panel D represents 200 μm for all panels.

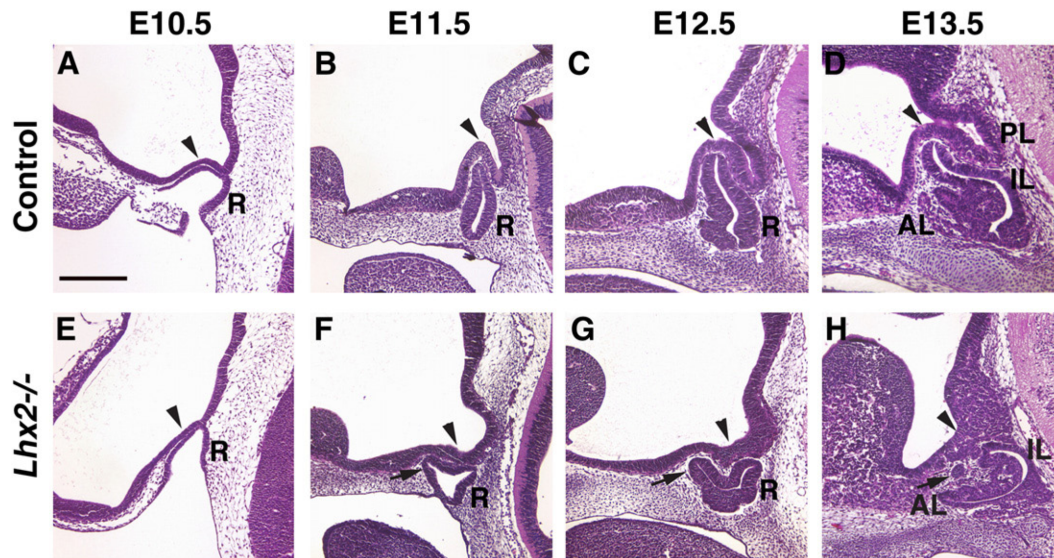

Fig. 2. Defects in morphogenesis of the pituitary gland in *Lhx2*$^{-/-}$ mutants. (A–H) Sagittal sections through the developing pituitary of E10.5 (A, E), E11.5 (B, F), E12.5 (C, G), and E13.5 (D, H) control (A–D) and *Lhx2*$^{-/-}$ mutant (E–H) embryos were stained by hematoxylin and eosin. Rostral is to the left. In contrast to control embryos (A–D), the neuroectoderm of the ventral diencephalon in *Lhx2*$^{-/-}$ mutants (E–H) fails to evaginate to form the posterior lobe (PL) of the pituitary. Arrowheads in panels A–D point at the part of the neuroectoderm bending dorsally directly above the dorsal pole of Rathke's pouch in the control embryos. Arrowheads in panels E–H point at a comparable region in the mutant embryos that fails to evaginate. Rathke's pouch (R) in *Lhx2*$^{-/-}$ mutants (E–H) initially forms but fails to extend dorsally. The dorsal part of the pouch extends instead along the rostral–caudal axis and then grows ventrally into the lumen of the pouch, resulting in misshapen anterior (AL) and intermediate lobes (IL) of the pituitary gland (arrows). Scale bar in panel A represents 200 μm for all panels.

infundibular region of the *Lhx2* mutants as compared to the controls in all these stages (Fig. 4). By counting BrdU-positive nuclei in a region spanning 100 μm on both sides of the most ventral–caudal point of the infundibulum (see Fig. 4), we estimated that there are about 3 and 8-fold increases in number of BrdU-positive cells in the *Lhx2* mutants as compared to those in the controls at E11.5 and E12.5, respectively (E11.5, average ± standard deviation: control 11 ± 4, mutant 34 ± 10, $P = 0.004$, $N = 4$; E12.5, average ± standard deviation: control 3 ± 1, mutant 25 ± 7, $P = 0.006$, $N = 4$).

We further characterized the pituitary defects in *Lhx2*$^{-/-}$ mutants by analysis of molecular marker expression. A previous study has shown that the calcium binding protein calbindin is expressed in differentiated immature pituicytes in the posterior lobe of the pituitary and has proposed that this protein may be involved in regulation of calcium-mediated functions in these cells (Abe et al., 1991). Consistent with the previous study, we detected calbindin in many cells in the infundibulum of control embryos at E12.5 (Fig. 5A). However, there were no detectable calbindin-positive cells in the

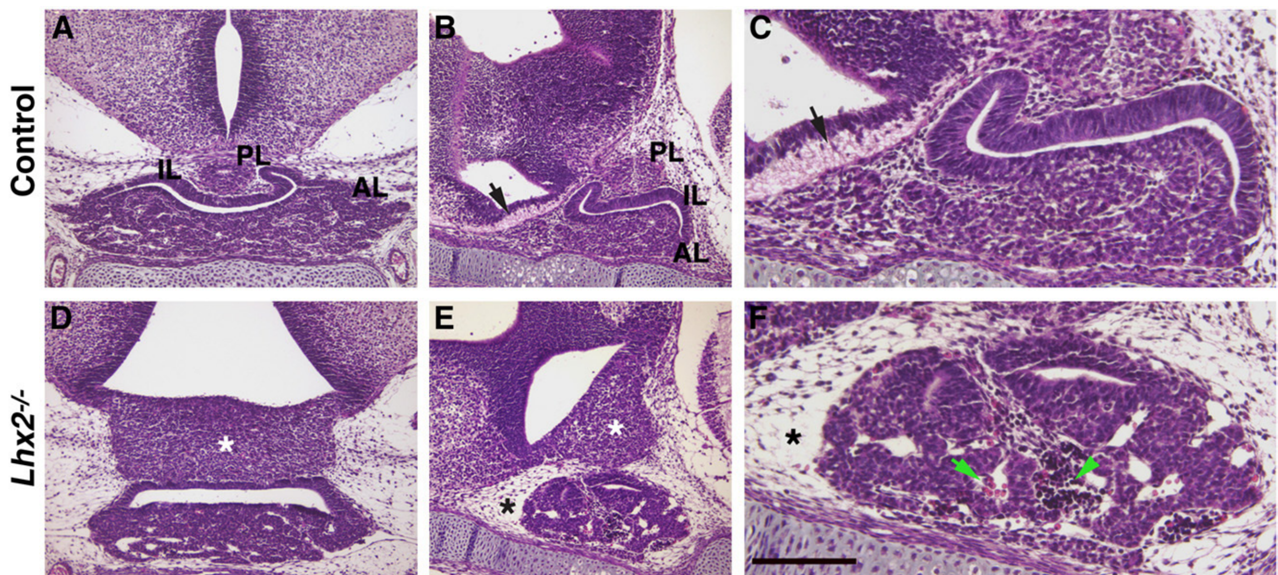

Fig. 3. Histological defects of the pituitary gland in *Lhx2*$^{-/-}$ mutants at E15.5. Coronal (A, D) and sagittal (B, C, E, F) sections through the pituitary of control (A–C) and *Lhx2*$^{-/-}$ mutant (D–F) embryos were stained with hematoxylin and eosin. Panels C and F are enlargements of the anterior/intermediated lobes of the pituitary shown in panels B and E, respectively. In *Lhx2*$^{-/-}$ mutants, a dense group of cells (indicated by white asterisks in panels D, E) occupy the area between the third ventricle and the anterior/intermediate lobes. The median eminence (pointed out by black arrows in panels B, C) and the posterior lobe (PL) of the pituitary are missing. The anterior (AL) and intermediate (IL) lobes of the pituitary in *Lhx2*$^{-/-}$ mutants are severely disorganized. The rostral tip of the anterior lobe is missing (as indicated by black asterisks in E, F), and there is an increased presence of blood vessels containing immature red blood cells (green arrow in F) and mononuclear cells (green arrowhead in F). Scale bar in panel F represents 200 μm for panels A, B, D, E and 70 μm for panels C, F.

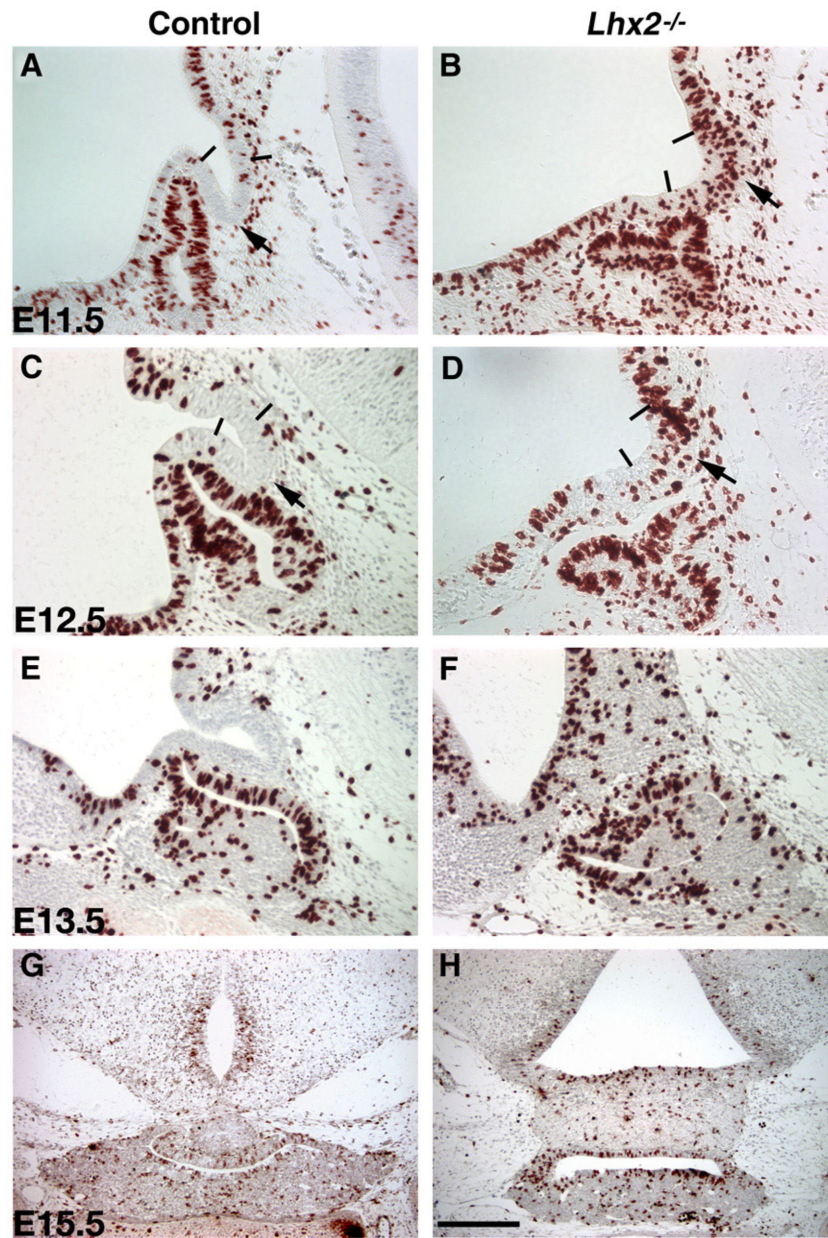

Fig. 4. Increased cell proliferation in the area of the infundibulum of *Lhx2*$^{-/-}$ mutants. Anti-BrdU staining of sagittal (A–F) or coronal (G, H) sections through the pituitary of E11.5 (A, B), E12.5 (C, D), E13.5 (E, F), and E15.5 (G, H) control (A, C, E, G) and *Lhx2*$^{-/-}$ mutant (B, D, F, H) embryos. Black bars in panels A–B and C–D, which are placed 100 μm from the most ventral–caudal point of the infundibulum (indicated by black arrows), mark the areas of the infundibulum where BrdU-positive cells were counted. Scale bar in panel H represents 100 μm for panels A–F and 200 μm for panels G–H.

comparable region of *Lhx2*$^{-/-}$ mutants (Fig. 5B). These results thus suggest that a loss of *Lhx2* function causes prolonged proliferation and failure of proper differentiation of the cells in the infundibulum that results in defects in formation of the posterior lobe of the pituitary.

We have also examined the expression of transcription factors Nkx2.1, Sox3, Six3, and the cellular retinoic acid binding protein CRABP2 in *Lhx2* mutants. These factors have previously been shown to be expressed in the ventral diencephalon including the infundibulum (Oliver et al., 1995; Rizzoti et al., 2004; Ruberte et al., 1992; Takuma et al., 1998). Our analysis revealed that the expression of these factors appears not affected in *Lhx2* mutant embryos (Figs. S2 and 8A, B).

Calbindin is also detected at E12.5 in the rostral–ventral tip of Rathke's pouch both in control and in *Lhx2*$^{-/-}$ mutant embryos (Figs. 5A–B). By E15.5, most of the calbindin-positive cells in control embryos are distributed at the rostral tip of the anterior lobe of the pituitary (Fig. 5C). In *Lhx2*$^{-/-}$ mutants, many calbindin-positive cells are still present, but they are abnormally located along the ventral floor of the anterior lobe of the pituitary (Fig. 5D). Thus, the loss of *Lhx2* leads to an abnormal cell distribution in the anterior lobe of the pituitary, which may result in the loss of the rostral tip.

Vasopressin immunostaining confirmed the loss of the medial eminence and posterior lobe of the pituitary in *Lhx2*$^{-/-}$ mutants. At E15.5, this marker is specifically detected in axons in the medial eminence and the posterior lobe of the pituitary in control embryos (Fig. 5E) but is completely absent in *Lhx2*$^{-/-}$ mutants (Fig. 5F).

Increased cell apoptosis in the ventral diencephalon of Lhx2 mutant embryos

We performed TUNEL staining to assess whether the cells that fail to differentiate properly in the ventral diencephalon of the *Lhx2*$^{-/-}$ mutants undergo apoptosis. There are very few, if any, TUNEL-positive

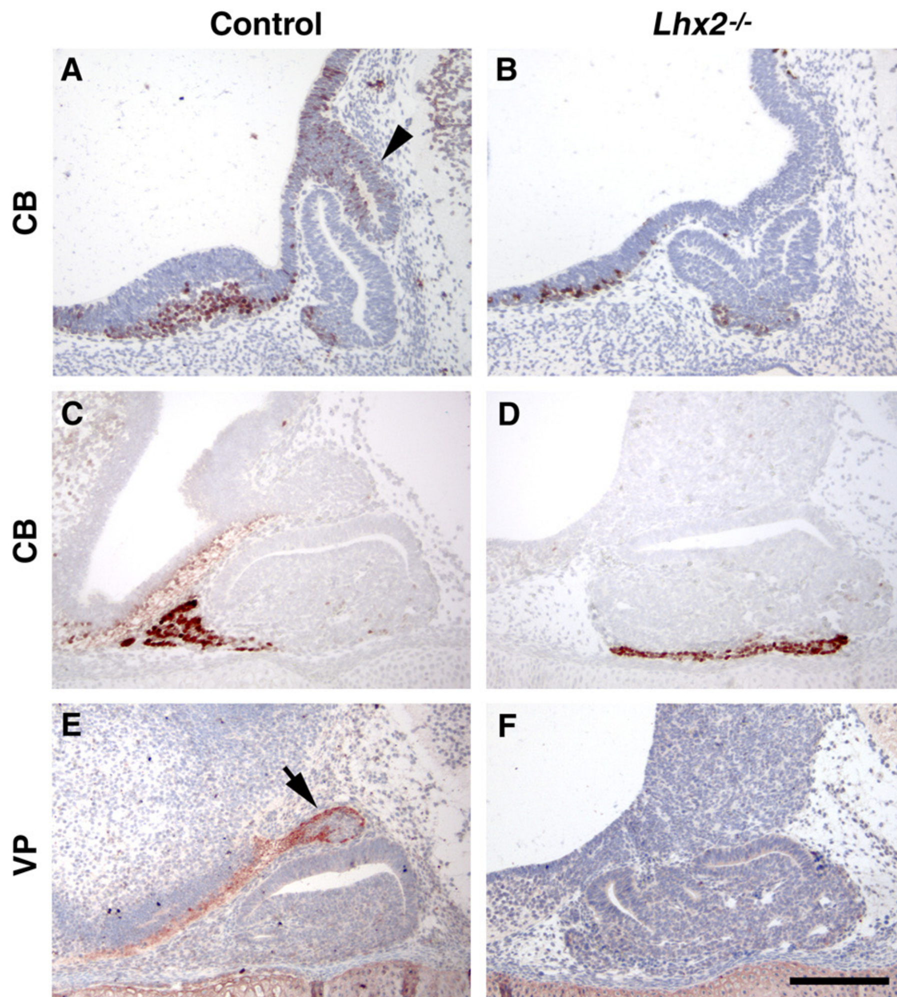

Fig. 5. Molecular marker analysis of pituitary defects in *Lhx2*$^{-/-}$ mutants. (A–B) Sagittal sections through the developing pituitary of E12.5 control (A) and *Lhx2*$^{-/-}$ mutant (B) embryos stained with an antibody to calbindin (CB). (C–F) Sagittal sections through the pituitary of E15.5 control (C, E) and *Lhx2*$^{-/-}$ mutant (D, F) embryos stained with antibodies to calbindin (CB) (C, D) and vasopressin (VP) (E, F), respectively. Arrowhead in panel A points at CB-positive cells in the posterior lobe of the pituitary. Arrow in panel E points at the VP-positive axons in the posterior lobe of the pituitary. Scale bar in panel F represents 200 μm for panels A and B and 140 μm for panels C–F.

cells in the posterior lobe or ventral diencephalon in control embryos at E13.5 (Fig. 6A) and E15.5 (Fig. 6C). By contrast, at both stages, many TUNEL-positive apoptotic cells were present in the large abnormal mass occupying the ventral diencephalon of *Lhx2*$^{-/-}$ mutants (Figs. 6B, D).

Analysis of pituitary endocrine cell lineages in Lhx2 mutant embryos

The anterior/intermediate lobes of the pituitary are severely disorganized in *Lhx2*$^{-/-}$ mutants. This raises the question whether the various pituitary endocrine cell lineages are properly generated in the mutants. To address this question, we first examined the expression of several transcription factors that are important for the generation of these lineages. These include Pitx1, Pitx2 (Charles et al., 2005; Kioussi et al., 2002; Suh et al., 2002), and Lhx3 (Ellsworth et al., 2008; Sheng et al., 1996, 1997; Zhao et al., 2006). Immunostaining using specific antibodies showed that all these factors are expressed in the anterior/intermediate pituitary lobes of both control and *Lhx2*$^{-/-}$ mutant embryos at E13.5 (Fig. S3). Consistent with this result, immunostaining for TSH, PRL, ACTH, GH, and MSH revealed that the lineages of endocrine cells that produce these hormones are present in the anterior/intermediate lobes of the pituitary in both control (Figs. 7A–E) and *Lhx2*$^{-/-}$ mutant (Figs. 7F–J) embryos at E15.5. There is, however, a notable difference. The distribution of endocrine cell lineages is drastically different in the anterior/intermediate pituitary lobes of the *Lhx2*$^{-/-}$ mutants as compared to the controls (Fig. 7). Many of these hormone-producing cells are ectopically distributed in the middle of the lumen. These cells are most likely derived from the dorsal portion of the Rathke's pouch, which, as shown further above, abnormally invades the lumen earlier during development (Fig. 2H).

Dorsal–ventral polarity of Rathke's pouch is maintained in Lhx2 mutant embryos

Rathke's pouch shows a dorsal–ventral polarity by expression of various marker genes in development (Kioussi et al., 1999). The general disorganization and ectopic distribution of endocrine cells observed in the anterior and intermediate lobes of the pituitary in *Lhx2*$^{-/-}$ mutants prompted us to examine whether the dorsal–ventral polarity is disrupted due to the failure of proper dorsal extension of Rathke's pouch earlier during development. In normal development, the transcription factor gene *Six3* is expressed in the ventral diencephalon including the infundibulum and the dorsal region of the pouch (Fig. 8A). Based on our BrdU labeling experiments, cell proliferation is mostly confined to this dorsal region (Zhao et al., 2006; Figs. 4A, C). The transcription factor Islet1 is expressed in the ventral region of the pouch (Zhao et al., 2006; Fig. 8C). Our analysis of E12.5 embryos showed that despite the change in shape of Rathke's pouch in *Lhx2*$^{-/-}$ mutants, the *Six3*- and BrdU-positive cells are

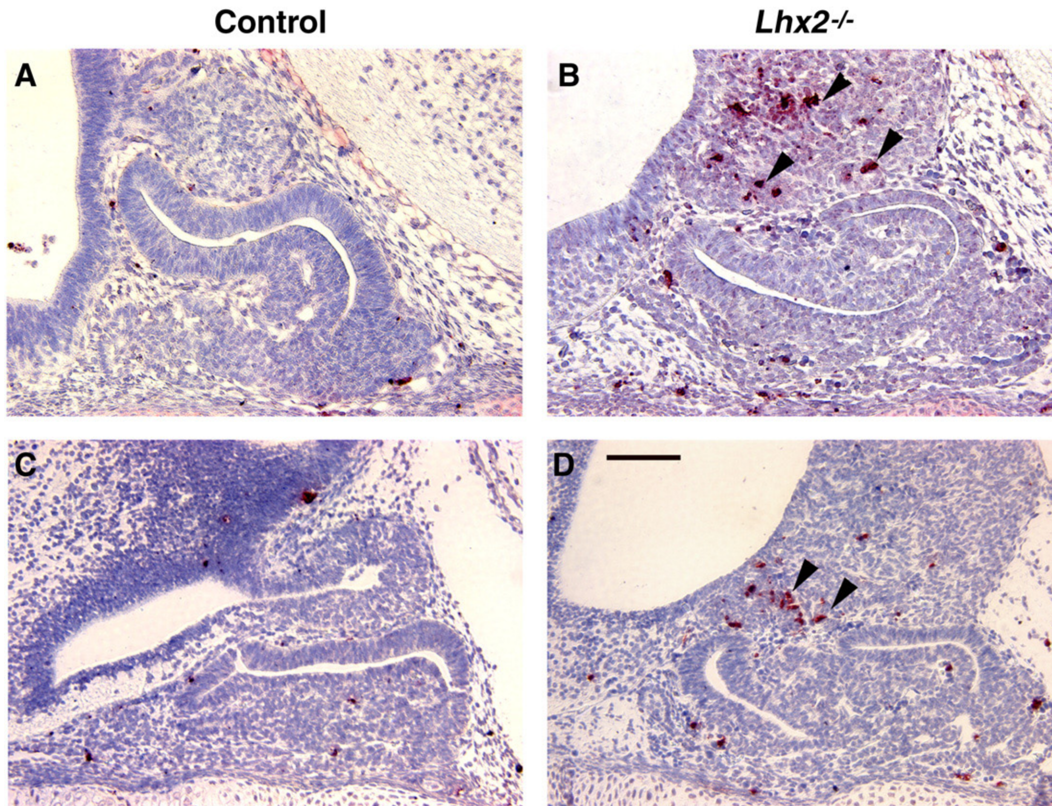

Fig. 6. Increased cell apoptosis in the ventral diencephalon of *Lhx2*$^{-/-}$ mutants. TUNEL staining of sections from E13.5 (A, B) and E15.5 (C, D) embryos shows an increase of cell apoptosis in the ventral diencephalic region adjacent to the anterior/intermediate pituitary lobes in *Lhx2*$^{-/-}$ mutants (B, D, indicated by arrowheads) as compared to the controls (A, C). Scale bar in panel D represents 100 μm for all panels.

mostly localized in the dorsal region (Figs. 4D and 8B) whereas the Islet1-positive cells are mostly localized in the ventral region (Fig. 8D) of the pouch. The dorsal–ventral polarity of the pouch is thus maintained in *Lhx2*$^{-/-}$ mutants.

Expression of genes encoding signaling molecules in the pituitary of Lhx2 mutant embryos

Early in pituitary development, the ventral diencephalon/infundibulum regulates the formation or generation of endocrine cell lineages of Rathke's pouch through inductive signaling mechanisms mediated by several classes of molecules including BMPs, Wnts, and FGFs. It stands to reason that the defective formation of the infundibulum and the posterior lobe in *Lhx2*$^{-/-}$ mutants may perturb these essential early signaling mechanisms. We therefore analyzed the expression of genes encoding these molecules in the ventral diencephalon of the mutants.

At E11.5, *Wnt5a* mRNA is broadly expressed in the neuroectoderm in the ventral diencephalon, including the domain that is undergoing evagination to form the infundibulum (Fig. 9A). *Bmp4* mRNA is detected in the neuroectoderm of the ventral diencephalon as well as in the dorsal portion of Rathke's pouch and the mesenchyme of the cephalic flexure (Fig. 9B). Despite the failure of evagination, *Wnt5a* and *Bmp4* mRNAs are both detected in the

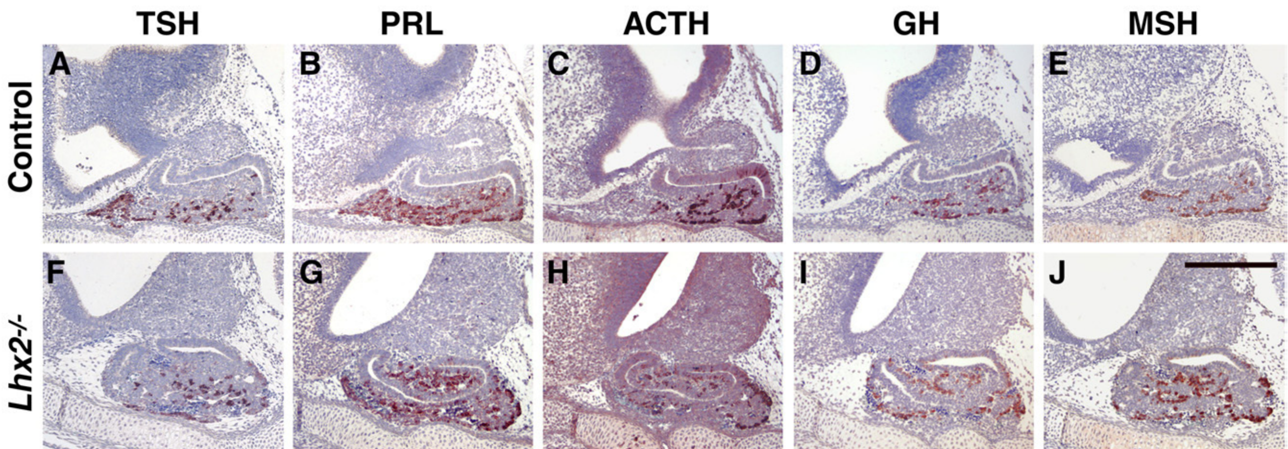

Fig. 7. Analysis of endocrine cell lineages of the pituitary gland in *Lhx2*$^{-/-}$ mutants. Immunostaining of sagittal sections through the developing pituitary of E15.5 embryos shows the presence but abnormal distribution of the various endocrine cell lineages, including thyrotropes (A, F), lactotropes (B, G), corticotropes (C, H), somatotropes (D, I), and melanotropes (E, J) in *Lhx2*$^{-/-}$ mutants (F–J) as compared to the controls (A–E). Scale bar in panel J represents 300 μm for all panels.

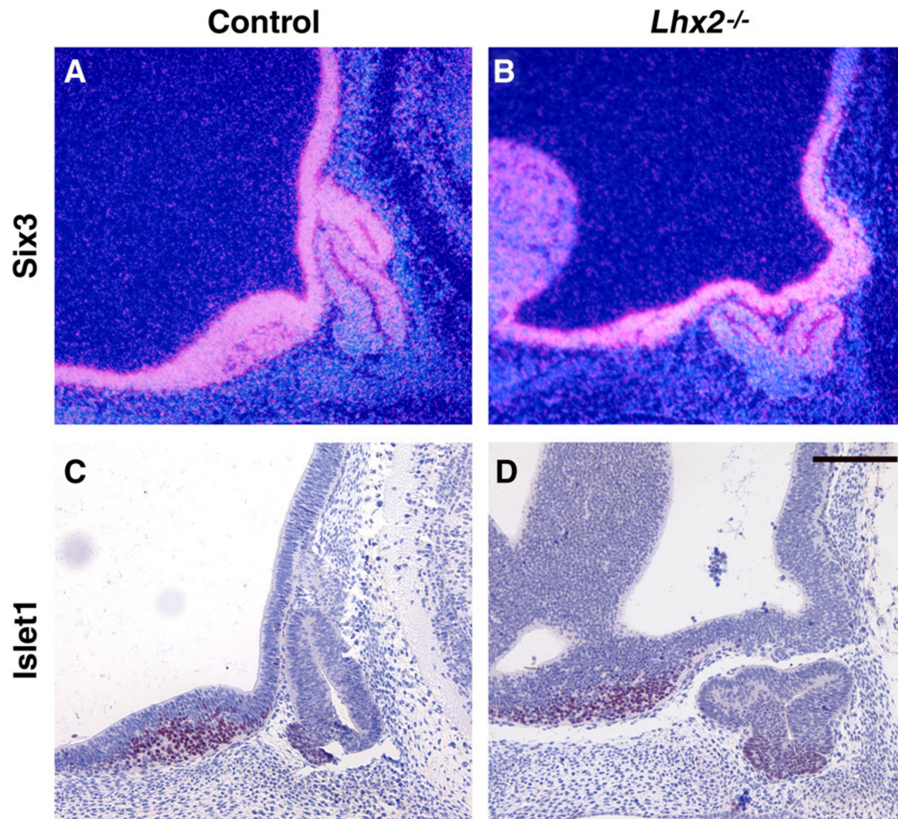

Fig. 8. Dorsal–ventral polarity of Rathke's pouch is maintained in *Lhx2*$^{-/-}$ mutants. *In situ* hybridization analysis of *Six3* (A, B) and immunostaining of Islet1 (C, D) of sagittal sections through the developing pituitary of E12.5 embryos shows that the dorsal–ventral polarity of Rathke's pouch is preserved in *Lhx2*$^{-/-}$ mutants (B, D) and comparable to the controls (A, C). Scale bar in panel D represents 170 μm for all panels.

ventral diencephalon of *Lhx2*$^{-/-}$ mutants (Figs. 9D, E) in patterns similar to those in the controls (Figs. 9A, B). The expression of *Fgf8* in the ventral diencephalon is more restricted than that of *Wnt5a*. The *Fgf8*-expressing domain ends rostrally right at the border where the evagination of the neuroectoderm occurs (Figs. 9C, C′). Interestingly, *Fgf8* mRNA is not only detected in the ventral diencephalon of the *Lhx2*$^{-/-}$ mutants but its expression domain extends rostrally as well, overlying the pouch that fails to grow dorsally (Figs. 9F, F′). This extension of *Fgf8* expression domain in the ventral diencephalon of the *Lhx2* mutants was also observed earlier at E10.5, before or

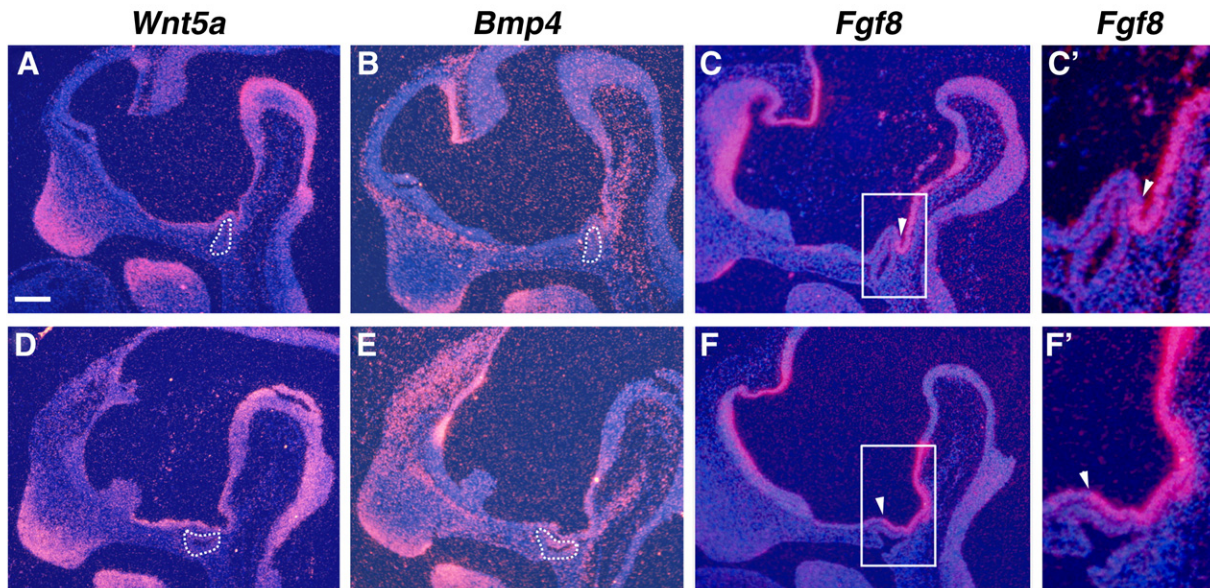

Fig. 9. Analysis of expression of signaling molecules in *Lhx2*$^{-/-}$ mutants. *In situ* hybridization analysis of sagittal sections through the developing ventral diencephalon and Rathke's pouch of E11.5 embryos shows that *Wnt 5a* (A, D) and *Bmp4* (B, E) mRNAs are expressed normally in *Lhx2*$^{-/-}$ mutants (D, E) as compared to the controls (A, B). *Fgf8* (C, C′, F, F′) mRNA is detected in the ventral diencephalon of both mutant (F, F′) and control (C, C′) embryos, but the expression domain extends more rostrally in the mutants as compared to the controls (arrowheads). Rathke's pouch shown in panels A, B, D, E is outlined with a white dotted line. Panels C′ and F′ are enlargements of the outlined areas shown in panels C and F, respectively. Scale bar in panel A represents 200 μm for panels A–F and 100 μm for panels C′ and F′.

around the time when evagination of the neuroectoderm about to start (Fig. S4).

Discussion

Our analysis has revealed an essential role for *Lhx2* in development of the posterior lobe of the pituitary. *Lhx2* is extensively expressed in the neuroectoderm of the developing ventral diencephalon, including a region that is in close physical contact with Rathke's pouch derived from the oral ectoderm. This region later undergoes evagination and becomes the infundibulum and posterior lobe of the pituitary. In *Lhx2*-null mutant embryos, the neuroectoderm of the ventral diencephalon fails to evaginate and to form the morphologically distinctive infundibulum and posterior lobe of the pituitary. Instead of leaving the cell cycle, the cells in these regions continue to proliferate. Furthermore, these cells fail to express the molecular marker calbindin that is normally detected in the infundibulum. These results suggest that *Lhx2* plays a role in the regulation of proper cell cycle exit and differentiation in the developing ventral diencephalon. The defect in formation of the infundibulum and posterior lobe of the pituitary in *Lhx2* mutant may result from an impairment of this regulation.

While the ventral diencephalon fails to evaginate in *Lhx2* mutants, Rathke's pouch fails to expand dorsally. Instead, the pouch expands along the rostral–caudal axis and subsequently ventrally into the lumen, ultimately resulting in severe morphological defects in the anterior/intermediate lobes of the pituitary. Since *Lhx2* mRNA is absent or barely detectable in the developing pouch and the anterior/intermediate pituitary lobes, these defects are most likely secondary in nature, caused by impaired development of the neuroectoderm of the ventral diencephalon.

The ventral diencephalon/infundibulum is known to influence development of Rathke's pouch and the anterior/intermediate lobes of the pituitary, primarily through expression of inductive signaling molecules (Zhu et al., 2007). Among these are BMP4 and FGF8 signals that are required for the induction or growth of Rathke's pouch (Takuma et al., 1998). Like Lhx2, the Nkx2.1 (T/ebp, Ttf1, Titf1) transcription factor is extensively expressed in the developing ventral diencephalon, but not in Rathke's pouch (Takuma et al., 1998). Deletion of *Nkx2.1* results in an early loss of *Fgf8* expression in the ventral diencephalon that eventually leads to a complete loss of all three lobes of the pituitary gland (Takuma et al., 1998). By contrast, both *Bmp4* and *Fgf8* are expressed in the ventral diencephalon of the *Lhx2* mutant. This is consistent with the fact that Rathke's pouch is present in this mutant and develops to form, albeit abnormally, the anterior/intermediate lobes of the pituitary. Wnt5a is another signaling molecule emanating from the ventral diencephalon to regulate the shape of Rathke's pouch (Cha et al., 2004). However, it seems unlikely that the morphological defects of Rathke's pouch observed in *Lhx2* mutants is caused by a defect in Wnt5a signaling, because *Wnt5a* appears to be normally expressed in the ventral diencephalon of the *Lhx2* mutants. Moreover, the change in morphology of Rathke's pouch in *Lhx2* mutants is distinct from that observed in the *Wnt5a* mutants (Cha et al., 2004). In that mutant, the developing pouch forms branches that are caused by dorsal extension at both the rostral and caudal sides of the evaginating infundibulum. By contrast, the dorsal extension of the pouch is completely blocked in *Lhx2* mutants. Thus, the developmental defects in Rathke's pouch of the *Lhx2* mutants appear to be based on mechanisms other than a direct impairment in cell signaling. It is possible that the failure of evagination of the neuroectoderm of the ventral diencephalon may physically prevent the pouch from extending dorsally.

Our analysis of molecular markers in the developing Rathke's pouch and the anterior/intermediate pituitary lobes has shown that in the absence of *Lhx2* function the various endocrine cell lineages are properly generated, but these cells are abnormally distributed. Based on our observations, we propose a scheme for understanding the morphogenesis of the anterior and intermediate pituitary lobes in normal development and its derailment in the *Lhx2* mutants (Fig. 10). At E12.5, the actively proliferating BrdU- and *Six3*-positive cells are located in the dorsal portion of the pouch in both control and *Lhx2* mutants (Figs. 4C, D and 8A, B), whereas the cells positive for calbindin and Islet1 are located in the ventral portion of the pouch (Figs. 5A, B and 8C, D). During normal development, cells gradually leave the cell cycle and move ventrally along the side of the pouch adjacent to the lumen, thus forming the large cell mass in the anterior pituitary lobe later in development (Figs. 2D, 3B, C, and 7A–E). This continuous flow of incoming cells pushes the early population of calbindin-positive cells away from the lumen and toward the rostral tip of the anterior pituitary lobe (Fig. 5C). In *Lhx2* mutants, the neuroectoderm of the ventral diencephalon fails to evaginate, and the pouch cannot extend dorsally and instead bulges beneath the floor of the diencephalon. As a result, many cells that leave the cell cycle are unable to move ventrally along the side of the pouch. They instead push ventrally through the middle of the lumen of the pouch and form the ectopic cell mass observed in the mutants (Figs. 2H, 3E–F, and 7F–J). As fewer cells move along the side and reach the ventral region of the pouch, the calbindin-positive cells fail to be pushed rostrally to form the rostral tip of the anterior pituitary lobe. These cells are instead trapped at the ventral surface of the anterior lobe of the pituitary (Fig. 5D).

Little has been known about how evagination of the neuroectoderm in the ventral diencephalon is regulated during development. As observed in both this and a previous study (Rizzoti et al., 2004), the rostral end of the *Fgf8* expression domain in the ventral diencephalon demarcates the border of the neuroectoderm that undergoes evagination at E11.5 (Figs. 9C, C′). In *Lhx2* mutants, the *Fgf8* expression extends into a more rostral region of the neuroectoderm that fails to evaginate normally (Figs. 9F, F′). A similar rostral extension of *Fgf8* expression in the ventral diencephalon has also been observed in *Sox3* mutants, which show a less pronounced evagination of the infundibulum (Rizzoti et al., 2004). This rostral extension of *Fgf8* expression may well contribute to the failure of proper evagination of the neuroectoderm in the ventral diencephalon. However, considering that the defect observed in *Lhx2* mutants

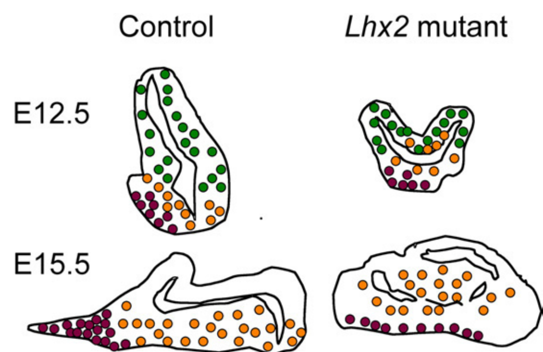

Fig. 10. A schematic drawing showing the morphogenesis of the anterior/intermediate lobes of the pituitary in normal and *Lhx2*$^{-/-}$ mutant embryos. Early in development (E12.5), proliferating cells (green dots) are located in the dorsal region whereas early differentiating cells positive for calbindin (purple dots) are located in the ventral region of Rathke's pouch in both control and the *Lhx2* mutant embryos. In control embryos, the cells leaving the cell cycle (yellow dots) move ventrally along the lumen of the pouch and eventually (E15.5) form the large mass of the anterior lobe, pushing the early population of calbindin-positive cells toward the rostral tip of the anterior lobe. In *Lhx2* mutant embryos, as the pouch fails to extend dorsally, many of the cells leaving the cell cycle are unable to move ventrally along the side of the pouch. These cells instead push ventrally into the lumen of the pouch to form the ectopic mass. As fewer cells reach the ventral region of the pouch, the calbindin-positive cells failed to be pushed rostrally to form the rostral tip of the anterior lobe in *Lhx2* mutants.

is more severe than that in *Sox3* mutants, additional mechanisms controlled by *Lhx2* may be involved in the evagination of the neuroectoderm and the formation of the infundibulum/posterior lobe of the pituitary.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.002.

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