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WNT signaling affects gene expression in the ventral diencephalon and pituitary gland growth

Mary Anne Potok[1], **Kelly B. Cha**[1], **Andrea Hunt**[1], **Michelle L. Brinkmeier**[1], **Michael Leitges**[2], **Andreas Kispert**[3], and **Sally A. Camper**[1]

[1]Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109

[2]Laboratories for Chronobiology and Signal Transduction, Max Planck Institute of Experimental Endocrinology, 30625 Hannover, Germany

[3]Institut für Molekularbiologie, Medizinische Hochschule Hannover, 30625 Hannover, Germany

Abstract

We examined the role of WNT signaling in pituitary development by characterizing the pituitary phenotype of three WNT knockout mice and assessing the expression of WNT pathway components. *Wnt5a* mutants have expanded domains of *Fgf10* and BMP expression in the ventral diencephalon and a reduced domain of LHX3 expression in Rathke's pouch. *Wnt4* mutants have mildly reduced cell differentiation, reduced POU1F1 expression, and mild anterior lobe hypoplasia. *Wnt4, Wnt5a* double mutants exhibit an additive pituitary phenotype of dysmorphology and mild hypoplasia. *Wnt6* mutants have no obvious pituitary phenotype. We surveyed WNT expression and identified transcripts for numerous *Wnts*, *Frizzleds* and downstream pathway members in the pituitary and ventral diencephalon. These findings support the emerging model that WNT signaling affects the pituitary gland via effects on ventral diencephalon signaling, and suggest additional *Wnt* genes that are worthy of functional studies.

Keywords

Wnt5a; *Wnt4*; *Wnt6*; *Wnt11*; *Wnt16*; mouse knockout; development

Introduction

The pituitary gland is a central organ in the endocrine system of all vertebrates and is responsible for the production and regulation of peptide hormones necessary for growth and development, regulation of thyroid function, lactation, sexual maturation and fertility, and the ability to respond to physiological stresses (Cushman and Camper, 2001). The mature gland is composed of three lobes in rodents, the posterior lobe, the intermediate lobe, and the anterior lobe which is comprised of five major pituitary hormone-producing cell types (Japon et al., 1994).

The posterior lobe is derived from neural ectoderm, whereas the anterior and intermediate lobes of the pituitary gland arise from Rathke's pouch, a primitive structure resulting from an invagination of the oral ectoderm beginning at embryonic day 9.0 (e9.0) in the mouse. The pouch pinches off from the remaining oral ectoderm at approximately e11.5 and is characterized by a domain of apoptosis at the separation point (Charles et al., 2005). In early

Address all correspondence to: Sally A. Camper, Department of Human Genetics, University of Michigan Medical School, 4909 Buhl Building, 1241 Catherine St., Ann Arbor, MI 48109-0618, scamper@umich.edu, Phone: (734) 763-0682, Fax: (734) 763-3784.

pituitary development, the dorsal aspect of the pouch undergoes extensive cell proliferation, and cells migrate out ventrally and rostrally from Rathke's pouch and form the anterior lobe (Ikeda and Yoshimoto, 1991; Ward et al., 2005).

Pituitary development is mediated by the temporal and spatial expression of transcription factors in the pouch in response to BMP and FGF signaling in the surrounding tissues of the developing gland (Ericson et al., 1998; Dasen et al., 2001; Davis and Camper, 2007). These factors include the POU-domain transcription factor *Pit1*, recently renamed *Pou1f1*, and its predecessor, *Prophet of Pit1* (*Prop1*), LIM homeodomain factors *Islet1* (*Isl1*), *Lhx3* and *Lhx4*, the pituitary homeobox genes *Pitx1* and *Pitx2*, and the Rathke's pouch homeobox gene *Rpx* (*Hesx1)* (Watkins-Chow and Camper, 1998; Cushman and Camper, 2001). Additionally, members of the SOX family of transcription factors have also been implicated in pituitary gland size and shape formation (Camper, 2004; Rizzoti and Lovell-Badge, 2005).

In addition to the various transcription factors involved in pituitary development, WNT signaling is emerging as an important contributor. Both the canonical and non-canonical WNT pathways are highly conserved throughout evolution and are essential for proper growth, development, and organogenesis in both vertebrate and invertebrate organisms (Rijsewijk et al., 1987; Cadigan and Nusse, 1997). In the canonical pathway, a core set of proteins respond to WNT and prevent CTNNB1 (β-catenin) from being proteolyzed, thus, allowing β-catenin to activate target genes that modulate cell fate, proliferation and apoptosis. In the non-canonical pathway, WNTs function independent of β-catenin and can activate CamKII and protein kinase C (PKC), GTP-binding proteins that in turn activate phospholipase C (PLC) and phosphodiesterase (PDE), and also the planar cell polarity (PCP) pathway that activates Jun-N-terminal kinase (JNK) (reviewed in Kohn and Moon, 2005).

Several lines of investigation support roles for WNT signaling in pituitary gland organogenesis. For example, β-catenin can regulate the activity of three transcription factors with roles in pituitary development, *Pitx2*, *Nr5a1* (*Sf1*) and *Tcf7l2* (*Tcf4*) (Kioussi et al., 2002; Brinkmeier et al., 2003; Gummow et al., 2003; Brinkmeier et al., 2007). Downstream factors in the WNT pathway are also important for proper pituitary development. *Tcf4*$^{-/-}$ embryos exhibit severe pituitary overgrowth, with a 3-fold increase in anterior lobe volume (Brinkmeier et al., 2003). The mechanism underlying the overgrowth appears to involve expanded BMP and FGF expression (Brinkmeier et al., 2007). *Pitx2* is expressed in many tissues where WNT signaling is active, and in the presence of LiCl, which artificially activates downstream WNT signaling, an increase in *Pitx2* expression is detected in the developing Rathke's pouch at e10.5, as well as in cultured pituitary cells at this time point (Kioussi et al., 2002). *Pitx2* activity is also increased in the presence of a constitutively active form of β-catenin expressed in the gonadotrope-like αT3-1 pituitary cell line. *Wnt11* has been implicated as a target of *Pitx2* and β-catenin in cardiac development (Zhou et al., 2007), but the WNT(s) responsible for the β-catenin-mediated activation of *Pitx2* in the pituitary have yet to be identified. Moreover, the presence of activated β-catenin has not been demonstrated in the developing anterior pituitary. In addition, nuclear accumulation of β-catenin and subsequent activation of TCF/LEF transcription factors can occur after gonadotropin-releasing hormone (GnRH) stimulation in mouse pituitary gonadotrope-like cells (Gardner et al., 2007). Because GnRH receptor, like other G-protein coupled receptors (GPCRs) can activate the canonical WNT signaling pathway, β-catenin activation of *Pitx2* or other critical transcription factors could be independent of a Wnt signal.

Direct evidence for WNT signaling in pituitary development stems from pituitary abnormalities arising from disruption of *Wnt5a* and *Wnt4* (Treier et al., 1998; Cha et al.,

2004). *Wnt5a* mRNA expression has been detected in the ventral diencephalon adjacent to the pituitary and in the pituitary primordium beginning at e9.5 (Treier et al., 1998). *Wnt5a* mutant embryos exhibit abnormal branching and looping of the developing pituitary, though all hormone-producing cell types are generated (Cha et al., 2004). *Wnt4* is expressed from e9.5 onwards in Rathke's pouch and in the oral ectoderm. Expression becomes restricted to the dorsal aspect of the pouch by e14.5. Mice deficient in *Wnt4* reportedly have a reduced population of cells producing GH, TSH, and the alpha subunit common to LH, FSH and TSH (**a**lpha **g**lycoprotein hormone **su**bunit = αGSU or **c**horionic **g**onadotropin **a**lpha = CGA) at e17.5 (Treier et al., 1998). The mechanisms underlying the defects in *Wnt5a* and *Wnt4* mutants have not been elucidated.

Here we examine the role of WNT signaling in modulating ventral diencephalon gene expression and pituitary gland organogenesis. In the absence of *Wnt5a*, we show that FGF and BMP expression patterns are perturbed in the ventral diencephalon, supporting the idea that WNT and FGF signaling pathways interact in pituitary development (Wang and Shackleford, 1996; Brinkmeier et al., 2007). We confirmed that mice deficient in *Wnt4* alone exhibit reduced pituitary growth, although the effect on cell type specification is less dramatic than previously suggested. Using a classical genetic double mutant analysis we tested for functional redundancy between *Wnt5a* and *Wnt4* and found evidence that the mutant phenotypes are additive in the pituitary gland. *Wnt6* is expressed near the pituitary gland during critical times in development; however, examination of embryos deficient in *Wnt6* showed no obvious pituitary malformation. Because the effects of deficiencies of *Wnt4*, *5a*, or *6* are unlikely to account for the consequences of deficiencies in the known, critical, β-catenin-regulated transcription factors in the pituitary gland, we conducted a gene expression survey and identified several WNTs, FZDs, and WNT pathway molecules expressed in the pituitary and/or neighboring tissues in early development (e12.5-e14.5). We catalogued their spatial and temporal expression in the developing and adult pituitary gland, producing several candidate genes for future studies. In conclusion, our data suggest that the Wnt signaling pathway regulates pituitary development, in part, through functional intersection with other signaling pathways. The identification of additional Wnt pathway components in the pituitary and ventral diencephalon provides additional targets for investigation in order to more fully understand pituitary development.

Results

Wnt5a mutant embryos exhibit altered expression of BMP and FGF in the ventral diencephalon

Wnt5a is required for normal pituitary morphology, but the expression pattern of the protein has not been reported and the mechanism of action has been elusive (Cha et al., 2004). WNT5A protein is detectable as early as e10.5 in Rathke's pouch in the cells lining the presumptive lumen (Figure 1A). WNT5A is also present in the ventral diencephalon, particularly in the basal cells lining the lumen, with little or no expression in the cells on the apical aspect. At e11.5, WNT5A is expressed in both the rostral domain and caudal domain of the ventral diencephalon, and in Rathke's pouch (Figure 1B). The rostral domain is demarcated by the normal expression of *Bmp4* and *Fgf10*, and the caudal domain includes diencephalon tissue that strongly expresses TCF4 (Brinkmeier et al., 2007; Davis and Camper, 2007). This pattern of protein immunoreactivity is consistent with the reported expression pattern of *Wnt5a* mRNA (Treier et al., 1998). Protein expression persists in the infundibulum and ventral diencephalon through e14.5, and it is detectable in the ventral region of the anterior lobe at e14.5 (Figure 1D). The major expression domain of WNT5A in the diencephalon corresponds with the major expression domain of TCF4 (Brinkmeier et al., 2007). Additionally, some protein expression is detected in the anterior and intermediate

lobes of the adult pituitary, but no expression is detected in the posterior lobe of the adult (Figure 1F).

We examined expression of several transcription factors in *Wnt5a* mutants in an effort to elucidate the mechanism underlying the dysmorphology. PITX2 protein expression is present in Rathke's pouch at e10.5, indicating that the cells comprising the characteristic dysmorphic oral ectoderm have committed to pituitary cell fate (Figure 1H). *Wnt5a* mutants show unaltered expression of TCF4 in the lower domain of the ventral diencephalon (Figure 1I-J). Unlike previous reports of TCF4 expression (Cha et al., 2004), immunoreactivity detected with this antibody correctly mimics *Tcf4* mRNA expression from e10.5-e18.5, and no protein is detected in the *Tcf4* knockout (Brinkmeier et al., 2007; Davis and Camper, 2007). Expression of an activated form of β-catenin, which should be detected in cells responding to canonical WNT signaling, is unaltered in the *Wnt5a* mutant (Figure1K-L).

Sox3 mutants and heterozygotes share a similar dysmorphic pituitary phenotype with the *Wnt5a* mutants at e10.5 and e11.5. The dysmorphology in *Sox3* mutants has been attributed to the expanded domains of BMP and FGF signaling and *Hesx1* expression (Rizzoti et al., 2004). *Hesx1* expression is unaltered in *Wnt5a* mutants at e11.5 (Figure 1M-N) or at e12.5 (Cha et al., 2004), suggesting that *Wnt5a* is not upstream of *Hesx1*. *Wnt5a* mutants exhibit truncated expression of another homeobox gene, LHX3, at the caudal aspect of the pouch, where multiple invaginations of oral ectoderm tissue occur. Expression of another LIM homeodomain factor, ISL1 is unaltered in the *Wnt5a* mutants (Figure 1O-R).

Signals emanating from the ventral diencephalon form distinct boundaries of expression around Rathke's pouch. Early in development, the transcription factor SIX3 and signaling molecules such as BMP4, FGF8 and FGF10 are expressed in the portion of the ventral diencephalon that evaginates to become the infundibulum, and later the posterior lobe of the pituitary gland (Treier et al., 1998; Cha et al., 2004; Davis and Camper, 2007). This domain of expression in the ventral diencephalon forms a distinct rostral-caudal boundary of expression with *Sonic hedgehog* (*Shh*) (Davis and Camper, 2007). Immunohistochemistry with an antibody that detects the phosphorylated form of SMAD1 (pSMAD1) was used as an indicator of active BMP signaling. In normal mice expression of pSMAD1 is detected in the caudal domain of the ventral diencephalon in the same area as *Fgf10* transcripts. In the absence of *Wnt5a*, pSMAD1 expression extends rostrally, beyond the normal border of the caudal domain (Figure 1S-T). *Fgf10* expression is mutually exclusive with TCF4 expression and demarcates the boundaries of the caudal and rostral domains of the ventral diencephalon in wild-type e10.5 embryos. In *Wnt5a* mutant embryos, however, *Fgf10* expression is no longer limited to the caudal domain. The expression domain is expanded along the ventral diencephalon into the area that would normally constitute the rostral domain (Figure 1U-V). Thus, *Wnt5a* deficiency causes expansion of both BMP and FGF signaling domains in the ventral diencephalon.

Wnt4 has a mild effect on pituitary cell specification

Previous studies report a dramatic decrease in levels of GH, TSHβ, and αGSU immunoreactivity at e17.5 in *Wnt4* deficient animals (Treier et al., 1998). To explore the mechanism that underlies the hormone reduction in these animals, we examined the pituitary phenotype of the *Wnt4* mutants. To ensure our analysis would be comparable to the previous study, we utilized the same mutant mice on the same genetic background (Treier et al., 1998). We confirmed that there is no reduction in pro-opiomelanocortin (POMC) immunoreactivity in the anterior or intermediate lobes of mutant embryos, where it marks differentiated corticotropes and melanotropes, respectively (Figure 2A-B). Equal SF1 expression at e16.5 in wild type and mutants suggests that the pre-gonadotrope population is unaffected by loss of *Wnt4* (Figure 2C-D). In contrast to previous reports, we observed no

appreciable reduction in chorionic gonadotropin alpha (CGA or αGSU) immunoreactivity at 16.5 or e18.5 (Treier et al., 1998). Furthermore, we observed no reduction in levels of FOXL2 (Figure 2I-J), a protein that is co-expressed with αGSU in pre-gonadotropes and pre-thyrotropes, and activates αGSU transcription in cell culture and transgenic mice (Ellsworth et al., 2006). At e16.5 and e18.5 we observed a slight reduction in GH and TSHβ immunoreactivity in *Wnt4* mutant pituitaries (Figure 2K-N). PIT1-positive cells were reduced in the mutant embryos relative to wild type at e16.5 and e18.5 (Figure 2O-R), which could underlie the reduced number of somatotropes and thyrotropes. The consistent reduction in PIT1 expression suggests that the differentiated cell populations are reduced and not simply developmentally delayed.

To confirm these qualitative results we quantified the immunoreactivity of each hormone at e18.5 (Figure 3). GH immunoreactivity is significantly reduced in the mutants with a 66% reduction in activity relative to wild type (P=0.03). TSH immunoreactivity in the anterior lobe of the *Wnt4* mutants is reduced 78%, which is significant (P=0.04). The difference in αGSU immunoreactivity at e18.5 is not obvious, but quantification of αGSU levels in mutants revealed a mild 27% reduction of immunoreactivity compared with wild type, with a borderline level of significance (P=0.0495), suggesting that there could be a subtle reduction. As expected, POMC immunoreactivity in the anterior lobe, which did not appear to be grossly altered in *Wnt4* mutants, exhibited no statistically significant change when quantified; the 9% difference between wild type and mutant yields a P value of 0.86.

Independent roles of Wnt4 and Wnt5a in development

The canonical and non-canonical Wnt signaling pathways may interact and influence one another in development (Topol et al., 2003; Zhou et al., 2007). *Wnt4* is frequently associated with the non-canonical class of WNT molecules, although some WNTs can activate different signaling pathways depending on context (Mikels and Nusse, 2006). To determine if loss of *Wnt4*-mediated signaling affects the canonical pathway in the pituitary and ventral diencephalon, we analyzed expression of the activated form of β-catenin in the rostral domain of the ventral diencephalon and found it was undisturbed. We also found no changes in TCF4 immunoreactivity in the ventral diencephalon at e12.5 or *Lef1* mRNA levels in Rathke's pouch at e16.5 in *Wnt4* mutants (data not shown).

Wnt5a mutant pituitaries begin to show signs of abnormal development at e10.5. In these mutants, Rathke's pouch tissue expands rostrally, with extra invaginations of pouch tissue on the caudal side (Figure 4A, c). This dysmorphology persists through e18.5 (Figure 4B, c, bracket), though hormone immunoreactivity remains largely unaffected. The dysmorphic region of *Wnt5a* mutants contains some somatotropes (Figure 4B, c) (Cha et al., 2004), and a portion of differentiated corticotropes and melanotropes as indicated by the POMC and prohormone convertase 2 (PC2) staining in the region (Figure 4B, s, w). This expression of PC2 was used to assess the cell specificity of POMC-positive cells in the dysmorphic intermediate lobe of the *Wnt5a* mutants. Patches of cells in the *Wnt5a* mutant are positive for PC2 immunoreactivity, suggesting that only part of the dysmorphic tissue is truly intermediate lobe. POMC staining appears normal in the intermediate lobes of all genotypes examined. These hormone-positive cell types cannot account for all of the cells in the dysmorphic region, however, suggesting some of the cells fail to complete a hormone-producing differentiation program.

Surviving Wnt4, Wnt5a double mutants reveal no overlapping function in pituitary development

To assess the potential genetic interaction of *Wnt4* and *Wnt5a* in the pituitary, we produced F1 double heterozygotes and intercrossed them to generate *Wnt4*, *Wnt5a* double mutants.

The progeny had a genetically heterogeneous background, which arose from the different mixed backgrounds that constituted the stocks for the *Wnt4* and *Wnt5a* mutants. Only two homozygous double mutant embryos were obtained at e10.5 and neither embryo appeared to be viable. *Wnt4, Wnt5a* double mutants appear to die by an unknown interaction of the two genes early in development. At e10.5 double mutant embryos appear dead or dying, but their pituitaries resemble the *Wnt5a* single mutant phenotypically at this age (Figure 4A, c, d). At e12.5 most *Wnt4,*$^{+/-}$*Wnt5a*$^{-/-}$ embryos are necrotic, with development arrested at or before e10.5 (n=6/8). One double mutant was obtained from 93 embryos at e18.5. Results of a χ^2 test show that this distribution of genotypes is not attributable to chance at either developmental stage; at e12.5 $P<0.05$ and at e18.5 $P<0.005$ (Table 1).

The *Wnt4,*$^{-/-}$*Wnt5a*$^{-/-}$ pituitary exhibits an additive phenotype. Somatotropes and thyrotropes appear to be slightly reduced in the double mutant relative to wild type, mimicking the findings for *Wnt4* (Figure 4B, d, h), while αGSU immunoreactivity does not appear drastically reduced. LHβ and POMC immunoreactivity appears normal at all genotypes examined. The dysmorphology evident around the lumen of the *Wnt5a* mutants is also apparent in the double mutant (Figure 4B, d, h, l, p, t, x). While hormone immunoreactivity remains largely unaffected, both *Wnt5a* single mutants and the *Wnt4, Wnt5a* double mutant contain a dysmorphic cleft crossing the lumen between the intermediate and anterior lobes. The intermediate lobe of the double mutant expresses PC2 in the same subset of cells as the single *Wnt5a* mutant (Figure 4B, w, x), suggesting the cell specification process in the double mutant is not altered beyond that of the single mutant. The combined phenotypes among the rare viable animals observed at either age do not appear more severe than either single mutant, which suggests separate roles for *Wnt4* and *Wnt5a* in pituitary development, despite the interaction in other developing organs that causes reduced viability.

Wnt6 does not affect pituitary gland development

A cDNA library generated from dissected *Prop1*$^{df/df}$ Rathke's pouch tissue at e14.5 contained *Wnt6* cDNA (Carninci et al., 2003). *Wnt6* transcripts are detected in tissues surrounding the developing pituitary beginning at e10.5. At this stage *Wnt6* is present in the pharyngeal arch. Expression in the oral ectoderm underlying the pituitary is detectable at e12.5 and to a lesser extent at e14.5 (Figure 5, arrowheads). No expression is detectable in Rathke's pouch or its derivatives. Expression is extinguished in the oral ectoderm by e18.5. Expression of POMC, GH, TSHβ, LHβ and αGSU is unchanged in *Wnt6*$^{-/-}$ embryos at e18.5. This suggests that *Wnt6* is dispensable for normal differentiation of pituitary cell lineages and morphogenesis.

Wnt pathway expression in the pituitary gland

Since *Wnt4, Wnt5a* and *Wnt6* were not critical for pituitary differentiation, we performed a PCR screen for the presence of other *Wnts* in the developing pituitary gland. Intron spanning primers were used to amplify *Wnt* and *Fzd* transcripts from Rathke's pouch RNA collected at e12.5 and e14.5, and from adult pituitaries. The cDNAs generated from the pituitary RNA, as well as from control tissues, were used as templates. Tissues for positive controls were selected based on previous reports of *Wnt* or *Fzd* expression in that tissue (www.informatics.jax.org). Adult testis, kidney, and lung, and e12.5 head, e12.5 body and e14.5 body RNA were used as positive controls for this assay. From this RT-PCR screening, transcripts from several *Wnt* and *Fzd* genes were identified in the pituitary with varying temporal expression patterns (Figure 6). Identities of the RT-PCR products were confirmed by DNA sequencing.

Wnt11 and *Wnt16* were expressed at all time points examined. Other *Wnt*s such as *Wnt2b, Wnt3* and *Wnt10b* were expressed only at one time point (e14.5, e12.5, adult, respectively).

Wnt5b, *Wnt7a*, and *Wnt7b* expression were not detected in the pituitary gland at any of the times examined (data not shown).

Several receptors of the WNT signaling pathway were also detected in the survey. *Fzd1* expression is detected in the embryonic cDNA, and *Fzd2*, *Fzd3*, and *Fzd4* expression is observed at all three times in pituitary development. *Fzd6* and *Fzd8* are detected in developing e12.5 and e14.5 pituitary cDNA, but not in the adult tissue. *Fzd9* is not detected in the pituitary at any of the times examined (data not shown).

Regulators of the WNT pathway are also present in the pituitary. *Wnt inhibitory factor-1* (*Wif1*) is detected throughout pituitary gland development and in the adult. *Wise*, the *Wnt inhibitor in the surface ectoderm*, and its highly related counterpart *Sclerostin*, *Sost*, were also detected during embryonic stages.

In situ hybridization analysis for expression of Wnt pathway members

In situ hybridization analysis of *Wnts* and *Frizzleds* identified in the initial RT-PCR survey reveal temporally and spatially restricted expression of these genes in the pituitary gland and ventral diencephalon (Figure 7). *Wnt11* and *Wnt16* are expressed in Rathke's pouch beginning at e10.5 and continuing through e16.5. Expression can also be detected in the rostral, lower domain of the ventral diencephalon early in development, from e10.5-e12.5. *Wnt11* and *Wnt16* transcripts, however, appear to be excluded from the forming infundibulum at all ages examined. Their expression within Rathke's pouch is concentrated dorsally, with less hybridization signal in the area of differentiating cells in the anterior lobe. Expression of *Fzd3* is detected in the lower domain of the ventral diencephalon with no detectable expression in the infundibulum. This is similar to *Wnt11* and *Wnt16* expression patterns. *Fzd6* expression is detectable throughout the ventral diencephalon and Rathke's pouch through e16.5. *Dvl2* and the negative regulator of WNT signaling, *Axin2*, are expressed in the ventral diencephalon with *Wnt11*, *Wnt16*, and *Fzd3*. *Dvl2* and *Axin2* expression are also concentrated in the dorsal aspect of Rathke's pouch.

Discussion

Wnt5a affects patterning of the ventral diencephalon

Wnt5a mutant animals exhibit pituitary dysmorphology (Cha et al., 2004). Our data implicate expanded FGF and BMP signaling as the underlying mechanism for the dysmorphology. *Bmp4* is required for the invagination of Rathke's pouch (Takuma et al., 1998), but *noggin* expression is required to attenuate this dorsal BMP activity (Davis and Camper, 2007). The excess BMP activity that we observed in *Wnt5a*$^{-/-}$ embryos may induce additional oral ectoderm to invaginate, contributing to the dysmorphology. *Sox3* and *Tcf4* deficient mice exhibit expanded BMP and FGF signaling and abnormalities in Rathke's pouch (Rizzoti et al., 2004; Brinkmeier et al., 2007). Taken together, these observations support the idea of cross talk between the signaling pathways.

Elevated levels or temporal expansion of *Hesx1* transcription can cause inappropriate induction of Rathke's pouch tissue from the oral ectoderm (Dattani et al., 1998). We examined *Hesx1* expression in the oral ectoderm of *Wnt5a* mutants at e11.5, but we noted no change, suggesting that *Hesx1* does not contribute to the dysmorphology in *Wnt5a* mutants. *Sox3* is expressed early in development in the ventral diencephalon and presumptive hypothalamus in a similar pattern to that of *Wnt5a* (Solomon et al., 2004), and mutations in both of these genes result in similar expansion of BMP and FGF signals. *Wnt5a* and *Sox3* may function in a similar pathway or parallel pathways because *Sox3* expression is unaltered in *Wnt5a* mutants (data not shown).

FGF signaling induces proliferation and expansion of pituitary cell types in pituitary explants, and overexpression of FGF in transgenic mice causes excess proliferation and pouch dysmorphology (Ericson et al., 1998; Treier et al., 1998). Based on these observations we suggest that the expansion of the *Fgf10* expression domain in *Wnt5a* mutants may induce additional oral ectoderm tissue to differentiate into Rathke's pouch. The excess tissue could result in extra folds along the lumen, causing the characteristic dysmorphology.

FGF overexpression interferes with cell specification and causes striking dysmorphology in transgenic mice, but *Wnt5a* mutants have normal cell specification and mild dysmorphology (Treier et al., 1998; Cha et al., 2004). This discrepancy could be due to the fact that expansion of FGF expression is transient and within the physiological range in *Wnt5a* mutants, and the overall level of excess FGF produced in transgenic mice is much higher and potentially non-physiological. *Wnt5a* can antagonize the canonical WNT signaling pathway (Topol et al., 2003), and it can stabilize β-catenin under certain circumstances (Mikels and Nusse, 2006). Though the spatial and temporal expression patterns of WNT5A and activated β-catenin overlap in tissues adjacent to the pituitary gland, we observe no difference in levels of activated β-catenin immunoreactivity in the *Wnt5a* mutant (Figure 1K-L). Thus, *Wnt5a* is not likely to be the activating signal for β-catenin in this context.

Lhx3 has been shown to be important for pituitary cell survival (Zhao et al., 2006; Ellsworth et al., 2008). For proper LHX3 expression, other pituitary factors such as *Lhx4*, or the combination of *Pitx1* and *Pitx2* transcripts are required (Raetzman et al., 2002; Charles et al., 2005). Additionally, inhibition of BMP signaling (*Noggin*) and Notch activation of transcriptional repressors (*Hes1*) are required for normal expression of LHX3 (Davis and Camper, 2007; Raetzman et al., 2007). We observe the same exclusion of LHX3 expression from the caudal side of Rathke's pouch in *Wnt5a* mutants as was observed in *Hes1* mutants. This implicates the interplay of several different signaling pathways in the activation of critical pituitary transcription factors.

The dysmorphology characteristic of *Wnt5a* mutants at e18.5 has a striking similarity to that of the *groucho*-related *Aes* mutant mice, which exhibit an abnormal connection of pituitary tissue to the intermediate lobe (Brinkmeier et al., 2003), and some phenotypic similarity to the *Prop1* mutant (Ward et al., 2005). The dysmorphic tissue of *Wnt5a* mutants is comprised of some GH-positive cells, and cells destined to become intermediate lobe, though the dysmorphic region contains some incompletely differentiated cells. These undifferentiated cells may result from an abnormal connection of neural tissue to the intermediate lobe, as seen in the *Uncx4.1* mutant mice at birth (Asbreuk et al., 2006).

Interaction between WNT5A, FGF, and BMP signaling pathways has been observed in other organs. *Fgf10* is upregulated in the *Wnt5a* mutant lung, and overexpression of *Wnt5a* in developing lung causes increased *Fgf10* expression, altered spatial pattern of *Bmp4* expression, and reduced epithelial branching (Li et al., 2002; Li et al., 2005). In addition, expression of *Fgf8* can reduce *Wnt5a* expression in the developing mouse cerebral cortex (Shimogori et al., 2004). Together, these findings support our hypothesis that *Wnt5a* regulates *Fgf10* in the ventral diencephalon, which has an indirect effect on Rathke's pouch. Thus, the balance between each signaling pathway is critical for normal pituitary gland organogenesis.

Wnt4 causes reduction in the PIT1 lineage

Wnt4 is expressed early in development, from e9.5 in Rathke's pouch, with limited expression in the dorsal aspect of the pouch through e14.5 (Treier et al., 1998; Olson et al., 2006). *Wnt4* is also expressed in the oral ectoderm throughout development. *Wnt4* was

implicated as a necessary element in anterior pituitary precursor cell expansion and αGSU expression (Treier et al., 1998).

Our analysis of *Wnt4* mutants at e16.5 and e18.5 reveals a mild reduction of anterior lobe size, although, in contrast to the previous report, we see no drastic reduction in αGSU cell number at either e16.5 or e18.5, and quantification of αGSU immunostaining results in a barely significant change in expression (P=0.0495).

The pituitary transcription factor PIT1 is responsible for differentiation and expansion of somatotropes, thyrotropes and lactotropes (Camper et al., 1990; Li et al., 1990). We have found *Wnt4* mice have reduced PIT1, which is likely the cause for the reduction in numbers of somatotropes and thyrotropes. *Pit1* deficiency does not alter the size of the pituitary gland until several days after birth, so the delay in *Pit1* expression does not account for the pituitary hypoplasia in *Wnt4* mutants during gestation (Ward et al., 2006).

Wnt4 and Wnt5a function independently in the pituitary gland

Currently, 19 WNT related genes have been identified in the mouse (http://www.stanford.edu/~rnusse/wntwindow.html). Due to the large number of mammalian WNT family members, WNTs are likely to compensate for one another in development. *Wnt4* and *Wnt5a* may both function in the non-canonical WNT/Ca^{2+} pathway (reviewed in Kuhl et al., 2000). Since *Wnt4* and *Wnt5a* are expressed in complementary regions in and surrounding the pituitary gland, and have overlapping temporal expression patterns, we conducted a classic double mutant analysis to test for interaction between the two genes.

Conditional mutants for *Wnt4* and *Wnt5a* would be ideal for studying genetic interaction in the pituitary gland because double mutants were underrepresented at e10.5 and e18.5, suggesting that *Wnt4* and *Wnt5a* are interacting early in development, resulting in lethality of some embryos before e10.5. Because pituitary hormones are not necessary for fetal growth or survival to term, the lethality must arise from a requirement for either *Wnt4* or *Wnt5a* in other organs. We do not observe a genetic interaction between *Wnt4* and *Wnt5a* in the pituitary glands of the surviving double mutants. *Wnt4* mutants do not have an observable morphological phenotype at e10.5, and a double mutant at this age exhibits the characteristic *Wnt5a* phenotype. At e18.5, an additive phenotype is observed in the double mutant. This suggests that the roles of *Wnt4* and *Wnt5a* in the pituitary gland are functionally distinct and not synergistic or overlapping.

Wnt6 is not required for pituitary gland development

Using *in situ* hybridization, expression of *Wnt6* was localized to the oral ectoderm, but *Wnt6* was not detected in Rathke's pouch tissue at any time examined throughout development. Expression of *Wnt6* was detected by RT-PCR in e12.5 laser-captured Rathke's pouch cDNA (Olson et al., 2006). This may indicate that there are low levels of *Wnt6* expression in Rathke's pouch that precluded detection by *in situ* hybridization, or the laser capture may have included oral ectoderm outside the pituitary anlage. Despite this discrepancy in expression patterns, it is clear that *Wnt6* mutants exhibit no obvious pituitary morphological abnormalities and undergo cell differentiation appropriately. Thus, the expression of *Wnt6* is not required for pituitary gland development.

Multiple opportunities for Wnt signaling to regulate pituitary growth and development

TCF4 was detected in Rathke's pouch, and expression was localized by immunohistochemistry to the rostral domain of the ventral diencephalon (Douglas et al., 2001; Brinkmeier et al., 2007). In the absence of *Tcf4*, the pituitary gland exhibits a

profound increase in anterior lobe size, demonstrating an important role for TCF4 in repressing pituitary gland growth (Brinkmeier et al., 2003).

It is not known which WNT, if any, regulates *Tcf4* expression. An RT-PCR survey performed on laser-captured e12.5 Rathke's pouch cDNA also reports expression of *Wnts 3, 11* and *16* at e12.5. Additionally, it was reported that Wnts *5b, 7a* and *7b* were present at e12.5 (Olson et al., 2006), though these transcripts were not detected in our survey. Our analysis suggests that *Wnt11* and *Wnt16* are expressed in a pattern that might permit them to activate *Tcf4* expression (Figure 8). Such a role has been suggested for *Wnt16* in synovial joint formation (Guo et al., 2004) where activated β-catenin is detected in the rostral domain concurrent with *Wnt11* and *Wnt16* expression. Furthermore, LEF/TCF reporter expression is activated in the caudal domain of the ventral diencephalon in an overlapping pattern with *Fgf10* and *Bmp4*, suggesting that canonical WNT signaling may modulate FGF and BMP signaling in that region (Maretto et al., 2003).

The spatial and temporal pattern of *Wnt11* expression mimics the expression of *Wnt16* (Figure 8). While *Wnt16* is thought to activate the canonical WNT/β-catenin pathway, *Wnt11* is often classified with *Wnt5a* in the non-canonical WNT pathway. It is possible that both canonical and non-canonical signaling pathways are actively participating in patterning of the ventral diencephalon and Rathke's pouch. *Wnt11* is proposed to have overlapping functions with FGF ligands and receptors and members of the BMP family in regulation of ureteric branching (Majumdar et al., 2003). *Wnt11* and *Wnt5a* regulate convergent extension movements in the zebrafish, and their activities are negatively regulated by a gradient of BMP signaling (Myers et al., 2002). This suggests that *Wnt11* and *Wnt5a* may similarly pattern the pituitary gland, in part by influencing BMP signaling, but may not be involved in specification of the hormone-producing cell types.

The expression patterns of *Axin2* and *Dvl2* are also similar to the expression patterns of *Wnt11* and *Wnt16* (Figure 8). AXIN2, known to be an inhibitor of canonical WNT signaling, was reportedly expressed in the ventral aspect of Rathke's pouch from e11.5 to e14.5 (Olson et al., 2006). Our *Axin2* probe was generated from a full-length cDNA that was completely sequenced. This probe revealed an expression pattern beginning at e10.5 in Rathke's pouch, and continuing through e16.5. The specific pattern of expression, including concentrated mRNA signal in the dorsal aspect of the pouch, as well as in the rostral domain of the ventral diencephalon, supports our hypothesis that Wnt signaling is active in the pituitary and adjacent ventral diencephalon. While Olsen et al. (2006) report no change in expression of *Axin2* in e14.5 *Prop1* null embryos, *Axin2* expression has been shown to be down-regulated in $Prop1^{df/df}$ P1 cDNA compared to wild type via gene expression microarray analysis, suggesting more than just a circumstantial connection between *Prop1* and WNT signaling (Mortensen and Camper, unpublished).

Wnt10b is unique among the WNTs we surveyed in that it is expressed only in the adult pituitary gland. *Wnt10b* expression is detected in a high fraction (11/14) of human pituitary adenomas (Howng et al., 2002). WNT expression in adult pituitary might affect BMP signaling in the same way that we observed in pituitary development. This could be significant because BMP4 promotes cell proliferation in the prolactinomas, the most common type of pituitary adenoma (Paez-Pereda et al., 2003).

In conclusion, we have clearly defined the roles of *Wnt5a, Wnt4*, and *Wnt6* in development of the pituitary gland, and implicate additional WNT family members that may play functional roles. The ability of WNTs to influence BMP and FGF signaling pathways emerges as a common theme in the pituitary gland (Camper, 2004; Rizzoti et al., 2004; Brinkmeier et al., 2007; Davis and Camper, 2007). Disruption of this balance in two WNT

mutants results in patterning defects, dysmorphology, and a developmental delay in cell specification. The discovery of numerous other WNT family members expressed in the pituitary gland and ventral diencephalon suggests that there may be multiple roles for WNT genes in pituitary development and a great deal of overlapping function among WNT family members.

Experimental Procedures

Mouse care and embryo preparation

Wnt5a mutant mice on a mixed 129Sv^{Brd} and C57BL/6 background were obtained from Stephen Jones (Yamaguchi et al., 1999) and maintained at the University of Michigan by heterozygote matings. 129-*Wnt4*^{tm1Amc}/J mutant mice were obtained from The Jackson Laboratory and maintained at the University of Michigan by heterozygote matings and matings with wild-type C57BL/6J females, also from The Jackson Laboratory. We will refer to these mice as *Wnt4*$^{-/-}$. *Wnt4, Wnt5a* double mutants were obtained by intercrossing double heterozygous animals obtained from a *Wnt4*$^{+/-}$ × *Wnt5a*$^{+/-}$ parental cross. A *Wnt6* null allele was generated by Andreas Kispert at the Institute for Molecular Biology at Hannover Medical School, Germany, where e18.5 embryos were generated by heterozygous matings. Exon 3 and exon 4 were deleted from the *Wnt6* locus to assure generation of a null allele. Integrity of the null allele was ascertained by RFLP analysis. The generation of the *Wnt6* allele will be fully reported elsewhere. Mice were housed under the supervision of the Unit for Laboratory Animal Medicine and the University Committee for Usage and Care of Animals, and all procedures were in compliance with the principles outlined in the NIH Guidelines for the Care and Use of Experimental Animals. Genotyping was performed as previously described for *Wnt4* (Stark et al., 1994) and *Wnt5a* (Cha et al., 2004). Noon of the day of the vaginal plug is designated as embryonic day 0.5. Embryos were dissected and fixed 30 min-overnight in 3.7% formaldehyde in PBS at 4°C. Embryos were dehydrated to 100% ethanol, embedded in a Citadel 1000 (Thermo Electric, Chesire, England) paraffin embedding machine and sectioned sagittally or coronally at 6μm thickness for immunohistochemistry and *in situ* hybridization.

Immunohistochemistry, in situ hybridizations and histology

Immunohistochemistry for the pituitary hormones was performed on paraffin sections as previously described (Kendall et al., 1994), and visualized with diaminobenzadine (DAB) chromogen. Antibodies used in fluorescent immunohistochemistry were incubated at 4°C overnight. Rabbit anti-phosphorylated SMAD1 (pSMAD1) (Cell Signaling Technology, Inc., Danvers, MA) was used at 1:200 dilution overnight, and goat anti-WNT5A (R&D Systems Inc., Minneapolis, MN) at a 1:100 dilution overnight. Mouse anti-Lim3 (LHX3) monoclonal antibody (Developmental Studies Hybridoma Bank, U of Iowa) was used at a 1:200 dilution and mouse anti-TCF4 (Upstate Cell Signaling, Charlottesville, VA) was applied at 1:100. Mouse anti-activated β-CATENIN monoclonal antibody (Millipore, Billerica, MA) was used at a 1:100 dilution. The purified antibody for PITX2 was a gift from Dr. Phil Gage (University of Michigan), and was generated by Dr. Tord Hjalt, (Lund University, Sweden). Antibody staining was performed after treating slides with 3% H_2O_2:methanol 1:1 for 20 minutes followed by boiling 10 minutes in 0.1M citric acid pH 6.0. Primary antibodies were added after a 30 minute block in TSA blocking reagent. For pSMAD1 and PITX2, biotin-conjugated anti-rabbit secondary antibody at 1:200 (Jackson Immunoresearch, West Grove, PA) was used and for WNT5A, biotin-conjugated anti-goat secondary antibody at 1:200 was used (Vector Labs, Burlingame, CA). For LHX3, TCF4, and β-CATENIN, biotin-conjugated anti-mouse secondary antibody from the M.O.M Kit (Vector Labs, Burlingame, CA) was used. Amplification and detection were carried out with

the TSA Fluorescein System (Perkin-Elmer, Wellesley, MA) according to the manufacturer's directions.

Quantification of immunostained pituitary sections was performed using the ImagePro Plus 6.2 software program (Media Cybernetics, In., Bethesda, MD) (Meynen et al., 2007; Mitchell et al., 2007). Three sagittal slides were taken from each of three *Wnt4* mutant and three wild type or heterozygous embryos at e18.5. The anterior lobes of these sections were selected as the area of interest (AOI) and immunoreactive sites exclusively in the anterior lobes were identified using the program's histogram based color selection, which was set to recognize the DAB stained cells. For GH, TSHβ, αGSU and ACTH, the ImagePro Plus integrated optimal density (IOD) was chosen to quantify the amount of DAB staining for each slide, and the sums of optical density for each section were recorded, resulting in a calculation in arbitrary units for the amount of each hormone present. Each section was analyzed three separate times to ensure the software program was reading the samples consistently, and therefore the optical density for each slide was averaged. The averaged IOD values for the three slides for each of the six embryos examined were analyzed for significance using a repeated measures ANOVA test in StatView 5.0.1 from SAS Institute, Inc. (Cary, NC), and P-values were generated using $P<0.05$ as significant.

In situ hybridizations were performed on paraffin sections using digoxygenin-labeled antisense riboprobes (Roche, Indianapolis, IN) as previously described (Douglas et al., 2001). Probes for *Wnt11, Wnt16* and *Fzd3* were generated from RT-PCR products as described below, cloned into pGEM-T Easy cloning vector (Promega, Madison, WI), and verified by DNA sequencing. *Wnt11* was subcloned into pBluescript (Stratagene, La Jolla, CA) and the antisense probe was linearized with *Kpn*I and labeled with T3 polymerase. *Wnt16* was linearized with *Nco*I and labeled with SP6 polymerase, and *Fzd3* was linearized with *Sph*I and labeled with SP6 polymerase. Probes were diluted 1:50 and hybridized at 50°C overnight. Probes for *Wnt6, Fzd6, Axin2* and *Dvl2* were obtained from embryonic pituitary cDNA libraries (Carninci et al., 2003). *Wnt6* was linearized with *Not*I and labeled with T3 polymerase and hybridized at 55°C. *Fzd6, Axin2*, and *Dvl2* were linearized with *Sal*I, labeled with T3, and hybridized at 53°C. A plasmid containing *Fgf10* was a gift from Brigid Hogan (Duke University Medical Center) and a probe was generated using *Bam*HI to linearize and T3 polymerase to label. A plasmid containing *Hesx1* was a gift from Paul Q. Thomas (University of Adelaide, Australia). An anti-sense probe was generated by *Bam*HI linearization and labeling with T3 polymerase, and sense probe generated with *Eco*RI and T7. For negative controls, sense probes were generated or no probe was added for hybridization. Hematoxylin and eosin staining were performed as previously described (Cha et al., 2004).

RT-PCR

RNA was isolated from Rathke's pouches dissected from wild-type embryos at e12.5 and e14.5, and from wild-type adult pituitaries using the Trizol method per manufacturer directions (Invitrogen, Carlsbad, CA). 5μg total RNA was treated for 30 minutes with DNaseI (Promega) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was generated using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as suggested by the manufacturer. 1μl of resulting cDNA was used as template in a 25μl PCR reaction. Intron-spanning primers were used to amplify PCR products on a Mastercycler gradient PCR machine (Eppendorf, New York, NY). The PCR was performed under the following conditions: 92°C for 3 minutes, followed by 40 cycles of 92°C for 30 seconds, annealing temperature for 45 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. Primer sets are listed 5′-3′ as follows: *Wnt2b* GAGGAGGCGATATGATGG, AGTCAGAGGCTTGAAGTG; *Wnt3* GCTGCCAAGAGTGTATTCG, CCTGTTCTGTTGCGGTAG; *Wnt10b*

CTGTTCTTGGCTTTGTTCAGTCG, CAGAGTTGCGGTTGTGGGTATC; *Wnt11* AAGGACTCAGAACTTGTGTATC, CCTGGTGTGGTGTCTTCC; *Wnt16* GACCGAATGTTCCTGTGAC, CGTAGCAGCACCAGATAAAC; *Fzd1* CCGGCCGGCTGAGCTTGGAACT, CAGGCGCGTACATGGAGCACAGGA; *Fzd2* TCGCCTGCTACTTCTATGAG, ACCTGGGAGAGGGGAAAG; *Fzd3* GGATGACCAAAGAAGCAAAGC, GGATGACCAAAGAAGCAAAGC; *Fzd4* TACATCTGGGTGAAGAGGAGCCTG, CTGCCAAAAACCAAGTGAGTGTC; *Fzd6* CGGAATGGCAGGGAAAGC, TGTACCACTGGGCTACTCTC; *Fzd8* TGCCCTGCCACAACCCCTTCTTTA, CAGCGCGGGGCCAGTGGTCTCATA. *Wif1* primers were as described (Heller et al., 2002). *Wise* and *Sost* primers were as described (Yanagita et al., 2006). PCR products were purified using a QiaExII Gel Extraction Kit (Qiagen) and sequenced to confirm their identity. Positive control cDNA was generated from e12.5 head, e12.5 body, e14.5 head, e14.5 body, liver, kidney, lung and testis and used as positive controls for each primer set. HPRT primers were used to determine quality of the cDNA and as a negative control on non-transcribed RNA.

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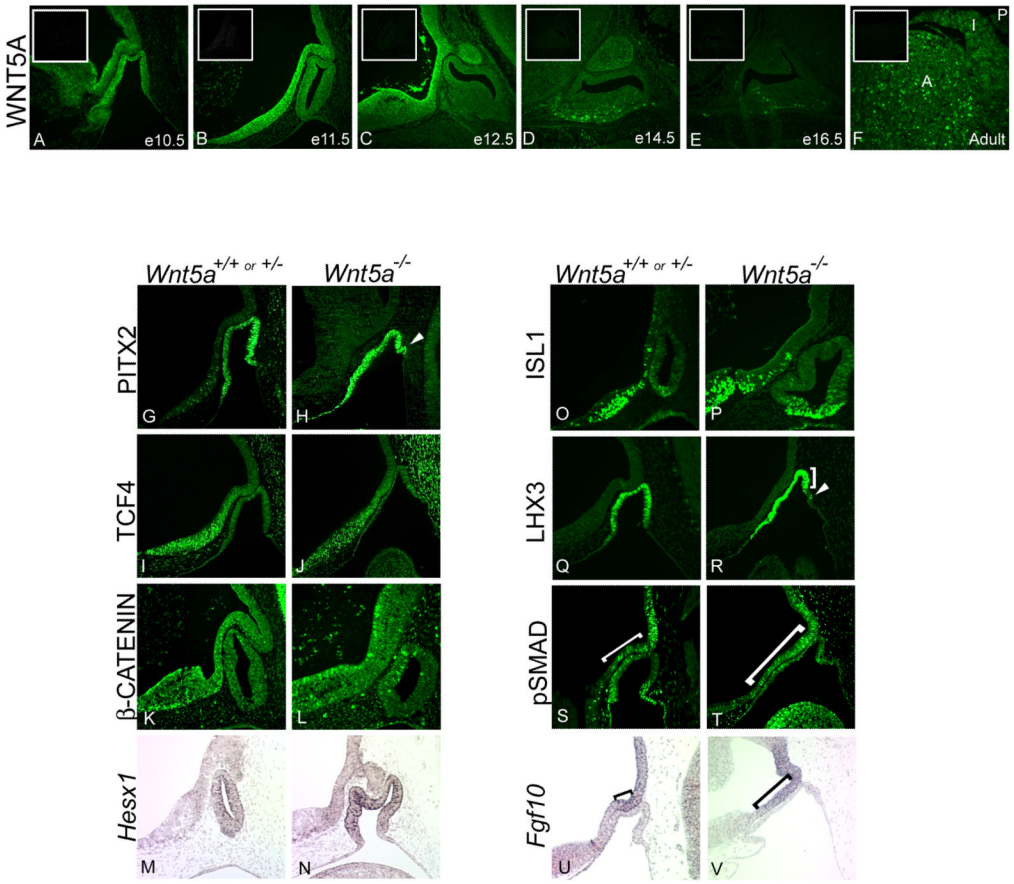

Figure 1. Loss of *Wnt5a* may alter pituitary gland patterning in early development

Immunohistochemical staining for WNT5A protein was performed on wild-type paraffin sections from e10.5 to e16.5 and adult pituitary glands (A-F). Expression is detected throughout the caudal and rostral domains of the ventral diencephalon from e10.5-e12.5. WNT5A immunostaining is also present in Rathke's pouch at e10.5 and e11.5, but the signal is no longer evident in the pouch by e12.5. At e14.5, WNT5A is expressed in the developing posterior lobe, as well as in the ventral anterior lobe, extending into the rostral tip. WNT5A expression is waning by e16.5, but is evident again in the adult pituitary in the anterior and intermediate lobes (A,I), but not in the mature posterior lobe (P). Insets are slides at each time point without primary antibody. Expression of signaling molecules and transcription factors was performed at e10.5 on *Wnt5a* wild type and mutant embryos (G-V). PITX2 antibody staining is detected throughout Rathke's pouch at e10.5, and in extra oral ectoderm invaginations (G-H, arrows). TCF4 and β-CATENIN are expressed normally in the ventral diencephalon at this time (I-L). *Hesx1* mRNA expression in the oral ectoderm is unaffected (M-N), as is ISL1 protein (O-P). LHX3 expression is truncated on the caudal side compared to wild type (Q-R, bracket). Extra invaginations of oral ectoderm do not express LHX3 (R, arrow). Phosphorylated SMAD1 (pSMAD1) is detected by immunohistochemistry (S-T). Brackets are used to mark the boundary of pSMAD1 expression from the infundibulum to where expression extends into the ventral diencephalon. *Fgf10* is detected in the developing ventral diencephalon in e10.5 embryos by *in situ* hybridization, with brackets used to demarcate its domain of expression (U-V).

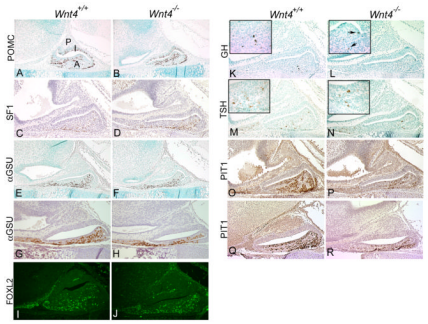

Figure 2. *Wnt4* has a mild effect on pituitary cell specification

Immunostaining for the pituitary hormones reveals that each of the major cell types have begun to differentiate properly by e16.5 (n=2) and e18.5 (n≥3). Sagittal sections of embryos were stained with antibodies that recognize the pituitary hormones. Pro-opiomelanocortin (POMC) and its cleavage product adrenocorticotropic hormone (ACTH) are unchanged in the mutant at e16.5 (A-B). *Steroidogenic factor 1* (SF1), marking pre-gonadotropes, is also unchanged at e16.5 (C-D). The alpha subunit shared by thyrotropes and gonadotropes, αGSU, appears unchanged both at e16.5 (E-F) and e18.5 (G-H), as does FOXL2 at e18.5 (I-J). Growth hormone (GH) is present at e16.5 in the wild type and mutant (K-L). Arrowheads indicate positive growth hormone cells. Thyroid-stimulating hormone β subunit (TSHβ) is also present by e16.5 (M-N). Insets show enlarged picture of anterior lobe region. PIT1 staining identifies pre-somatotropes, pre-lactotropes, and pre-thyrotropes in the wild type and mutant at e16.5 (O-P) and e18.5 (Q-R). A slight dysmorphology is seen in anterior lobe tissue surrounding the lumen in mutants at e16.5 (B,D,L,P) and e18.5 (H,R). Immunostaining was developed with FITC or diaminobenzidine (DAB), and sections are counterstained with methyl green or hematoxylin.

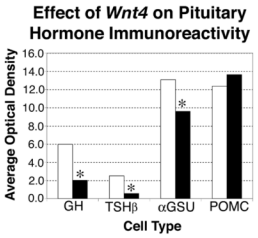

Figure 3. Effect of *Wnt4* on pituitary hormone immunoreactivity

Sagittal sections taken at e18.5 from three wild type and three mutant e18.5 *Wnt4* embryos were stained for GH, TSHβ, αGSU and POMC. White bars represent wild types and black bars represent mutants. Average optical density (OD) for each genotype was obtained from three slides and repeated three times using ImagePro Plus software. Optical density parameters were set independently for each hormone, and therefore OD levels are not comparable between the different hormones. OD units are arbitrary and represented in the graph as 1×10E6. Repeated measures ANOVA analyses of the average optical density for each hormone were performed to determine statistical significance. For GH, P=0.0317; for TSHβ, P=0.0402; for αGSU, P=0.0495; for POMC, P=0.8635. * Indicates significant P-values at 0.05.

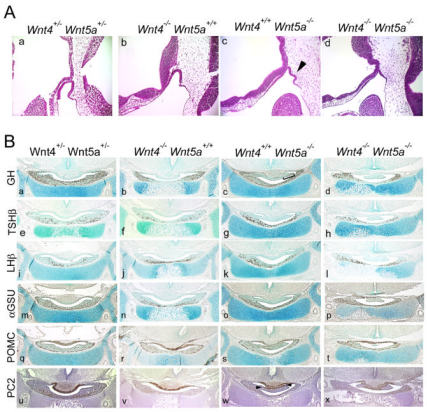

Figure 4. A mild additive pituitary phenotype in a *Wnt4, Wnt5a* double mutant

A. Hematoxylin and eosin stained paraffin sections of a *Wnt4*, *Wnt5a* double mutant at e10.5. Sections are oriented sagittally, with rostral to the left and caudal to the right. Arrowhead indicates dysmorphology of *Wnt5a* mutant. B. Coronal sections of embryos at day e18.5 were stained with antibodies to anterior pituitary hormones to examine cell specification. The dysmorphology of the *Wnt5a* mutant (c) is indicated by the bracket. Prohormone convertase 2 (PC2) immunostaining shows differentiation of parts of the dysmorphology as of *Wnt5a* mutants into corticotropes (w, arrows). A *Wnt4*,$^{+/-}$*Wnt5a*$^{+/-}$ embryo is shown for comparison. Sections are developed with DAB and counterstained with methyl green or hematoxylin.

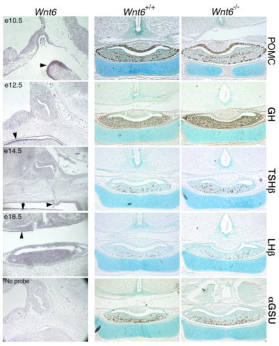

Figure 5. *Wnt6* is expressed near the pituitary during formation of Rathke's pouch, but is not required for pituitary development

Expression of *Wnt6* at critical time points of pituitary organogenesis is detected by *in situ* hybridization using NBT and BCIP for development of the purple precipitate. Sagittal sections of e10.5-e14.5 embryos are oriented with dorsal at the top and rostral at the left. Overnight hybridization without a riboprobe served as a negative control. Coronal sections of normal and *Wnt6* mutant embryos at e18.5 were immunostained and developed with DAB to assess cell specification with antibodies to POMC, GH, TSHβ, luteinizing hormone β subunit (LHβ), and αGSU. Sections are counterstained with methyl green.

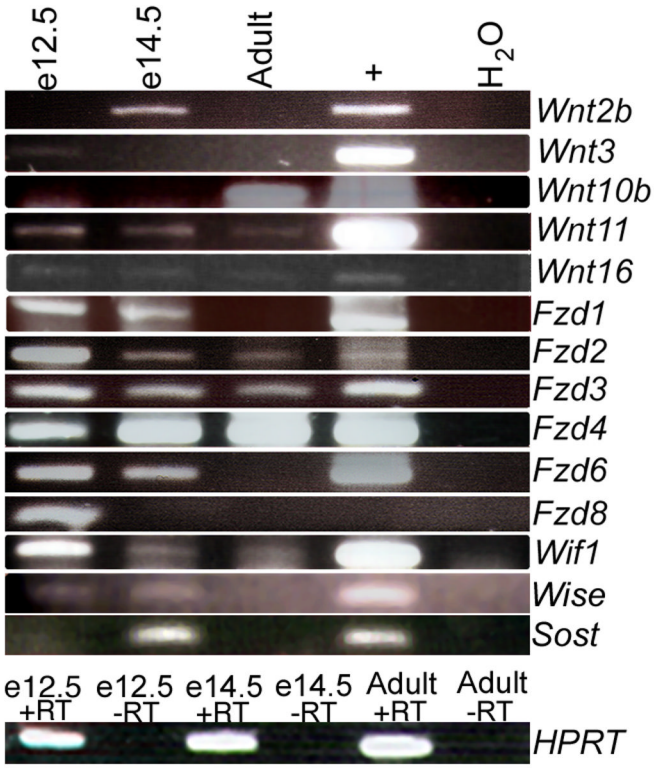

Figure 6. Many Wnt pathway genes are expressed in the developing pituitary

RT-PCR was used to detect expression of Wnt signaling pathway members and regulators during various times of pituitary gland development. RNA from the specified ages was analyzed using intron-spanning primers specific to each *Wnt* and *Frizzled* gene. For positive controls, cDNAs generated from adult and embryonic tissues were chosen that had previously been reported to express each *Wnt* or *Fzd* gene. Water was used as a negative control. HPRT PCR products showing +RT and −RT reactions confirm no genomic contamination in the e12.5, e14.5 or adult pituitary cDNA. PCR products were sequenced to confirm the identity of each gene.

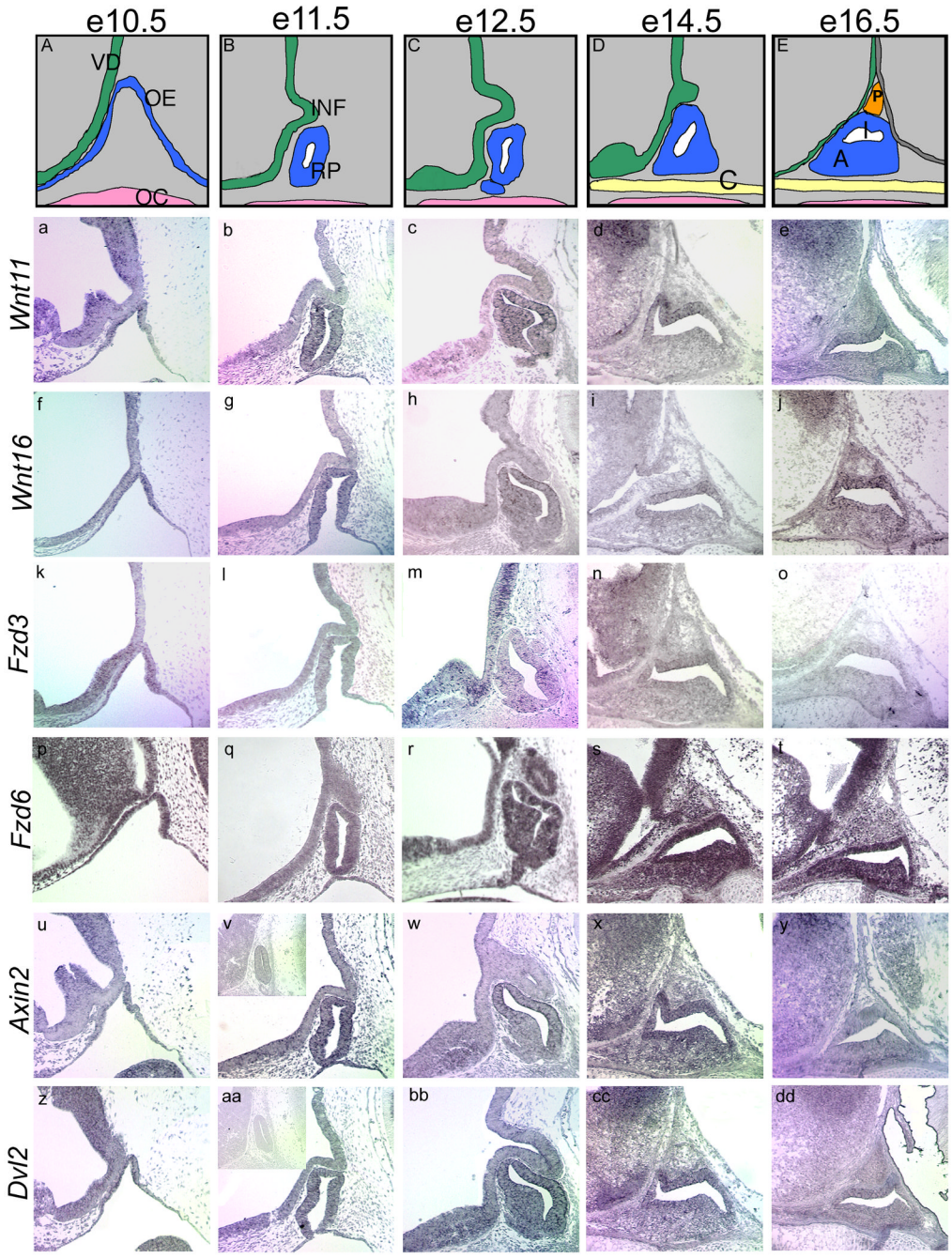

Figure 7. Spatial expression patterns of Wnt pathway members in the developing pituitary and ventral diencephalon

Formation of Rathke's pouch is illustrated from e10.5 to e16.5 (A-E). At e10.5, the oral ectoderm begins to invaginate and pinches off around e11.5 to form Rathke's pouch. Meanwhile, the ventral diencephalon evaginates to form the infundibulum. From e12.5 to e14.5, the anterior lobe begins to form as cells surrounding the lumen rapidly divide and migrate out of the pouch. By e16.5, the three distinct lobes of the developing pituitary are evident, with the posterior lobe arising from the infundibulum. *In situ* hybridizations were performed on wild-type sagittal sections from e10.5 to e16.5 to determine temporal and spatial patterns of expression. Slides are oriented with dorsal to the top and rostral to the left.

Inset panels (v, aa) show sense slides for negative controls. VD, ventral diencephalon; OE, oral ectoderm; OC, oral cavity; INF, infundibulum; RP, Rathke's pouch; C, cartilage plate of hard palate; P, developing posterior lobe; I, developing intermediate lobe; A, developing anterior lobe.

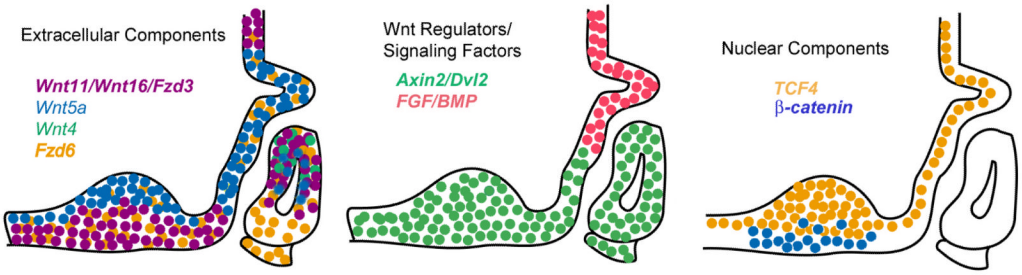

Figure 8. Gene expression summary of BMP, FGF and WNT signaling molecules

Signals from the ventral diencephalon and from Rathke's pouch early in development are important for proper patterning of the pituitary gland. These signals are expressed in a spatially restricted manner that is important for regulating pituitary shape and growth. *Wnt4* has a limited window of expression in Rathke's pouch (Treier et al., 1998). WNT5A is expressed throughout the ventral diencephalon and in Rathke's pouch early in development. *Wnt11*, *Wnt16*, and *Fzd3* are expressed in the rostral domain of the ventral diencephalon, and excluded from the caudal domain and infundibulum. Their expression is also seen in Rathke's pouch, where it becomes dorsally concentrated by e14.5. These factors, along with *Axin2* and *Dvl2*, are expressed in a mutually exclusive pattern relative to FGF and BMP, suggestive of an antagonistic regulation. *Fzd6* has no such restriction and is expressed throughout the pouch and ventral diencephalon. Downstream effecter TCF4 is also expressed throughout the ventral diencephalon, particularly on the apical side. Activated β-CATENIN expression is seen in the rostral domain of the ventral diencephalon. Taken together, these expression studies suggest extensive Wnt activity in the developing pituitary gland, with potential for overlapping functions among different members of the family.

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Table 1

***Wnt4, Wnt5a* double mutants are underrepresented**[1]

			E10.5		E12.5		E18.5	
Ratios	**Wnt5a**	**Wnt4**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
1/16	+/+	+/+	2	5	1	3	11	6
2/16	+/-	+/+	6	10	8	6	13	12
1/16	-/-	+/+	5	5	1	3	1	6
2/16	+/+	+/-	15	10	8	6	15	12
4/16	+/-	+/-	24	20	18	12	36	23
2/16	-/-	+/-	17	10	2*	6	7	12
1/16	+/+	-/-	4	5	5	3	5	6
2/16	+/-	-/-	5	10	5	6	4	12
1/16	-/-	-/-	2	5	0	3	1	6
Total			**80**	P < 0.9	**48**	P < 0.05	**93**	P < 0.005

[1]Results of a χ^2 test show that the distribution of these genotypes is likely not due to chance at e12.5 (P<0.05) and at e18.5 (P<0.005).

*Six severely necrotic embryos were also found of this genotype.