Multiple Roles for Hedgehog Signaling in Zebrafish Pituitary Development

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Abstract

The endocrine-secreting lobe of the pituitary gland, or adenohypophysis, forms from cells at the anterior margin of the neural plate through inductive interactions involving secreted morphogens of the Hedgehog (Hh), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) families. To better understand when and where Hh signaling influences pituitary development, we have analyzed the effects of blocking Hh signaling both pharmacologically (cyclopamine treatments) and genetically (zebrafish Hh pathway mutants). While current models state that Shh signaling from the oral ectoderm patterns the pituitary after placode induction, our data suggest that Shh plays a direct early role in both pituitary induction and patterning, and that early Hh signals comes from adjacent neural ectoderm. We report that Hh signaling is necessary between 10 and 15 h of development for induction of the zebrafish adenohypophysis, a time when *shh* is expressed only in neural tissue. We show that the Hh responsive genes *ptc1* and *nk2.2* are expressed in preplacodal cells at the anterior margin of the neural tube at this time, indicating that these cells are directly receiving Hh signals. Later (15–20 h) cyclopamine treatments disrupt anterior expression of *nk2.2* and Prolactin, showing that early functional patterning requires Hh signals. Consistent with a direct role for Hh signaling in pituitary induction and patterning, overexpression of Shh results in expanded adenohypophyseal expression of *lim3*, expansion of *nk2.2* into the posterior adenohypophysis, and an increase in Prolactin- and Somatolactin-secreting cells. We also use the zebrafish Hh pathway mutants to document the range of pituitary defects that occur when different elements of the Hh signaling pathway are mutated. These defects, ranging from a complete loss of the adenohypophysis (*smu/smo* and *yot/gli2* mutants) to more subtle patterning defects (*dtr/gli1* mutants), may correlate to human Hh signaling mutant phenotypes seen in Holoprosencephaly and other congenital disorders. Our results reveal multiple and distinct roles for Hh signaling in the formation of the vertebrate pituitary gland, and suggest that Hh signaling from neural ectoderm is necessary for induction and functional patterning of the vertebrate pituitary gland.
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Introduction

The vertebrate pituitary gland forms as a cranial placode through inductive interactions between two types of ectodermal tissues: head epidermis (classically called Rathke's pouch in mammals) and neural ectoderm of the ventral diencephalon. As a result of these interactions, two pituitary lobes form: epidermal tissue in the developing placode thickens to form the anterior lobe or adenohypophysis, while neural tissue grows ventrally to form the posterior lobe or neurohypophysis. Each of these two tissue types requires interactions with the other in order to form the appropriate pituitary structures (Baker and Bronner-Fraser, 2001; Dasen and Rosenfeld, 2001).

The origin of the pituitary placode now appears to be similar in all vertebrates examined. Historically, morphological studies in mammals pointed to epidermis of Rathke's pouch as the source of the adenohypophysis. However, fate mapping data from mouse (Osumi-Yamashita et al., 1994), rat (Kouki et al., 2001), chick (Couly and Le Douarin, 1985), and frogs (Eagleson et al., 1986; Knouff, 1935) indicate that Rathke's pouch/adenohypophyseal cells are derived from ectodermal cells at the anterior margin of the neural plate (reviewed in Baker and Bronner-Fraser, 2001;

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Rubenstein et al., 1998). Similarly, marker analysis and fate mapping in zebrafish suggest that the teleost adenohypophysis also originates at the anterior margin of the neural tube. More specifically, medial cells at the anterior margin of the zebrafish neural tube give rise to the pituitary placode, while more lateral/posterior cells give rise to the adjacent olfactory placodes (Whitlock and Westerfield, 2000).

Cell–cell signaling via the Hedgehog (Hh) signaling system has recently been shown to be required for adenohypophysis formation. Analyses of mice which lack Sonic Hedgehog (Shh) signaling only in developing adenohypophyseal cells show that Hh signaling is directly required for patterning and proliferation of cells in Rathke's pouch (Treier et al., 2001). Additionally, ectopic Shh leads to the expansion of ventral pituitary cell types in mice (Treier et al., 2001). Genetic approaches in zebrafish complement analyses in mice and have revealed a conserved role for Hh in teleost pituitary development. Mutations that severely block hedgehog signaling either at the level of the Hh receptor complex smoothened [the *slow-muscle-omitted (smu)* mutation] (Chen et al., 2001; Varga et al., 2001) or at the level of the Hh responsive transcription factor Gli2 [the *you-too (yot)* mutation] (Karlstrom et al., 1999) completely block formation of the adenohypophysis. In these mutants, an ectopic midline lens forms in place of the pituitary, suggesting transdifferentiation of placodal cells (Kondoh et al., 2000; Varga et al., 2001). Other identified zebrafish hedgehog pathway mutants include *sonic-you (syu),* which encodes Shh (Schauerte et al., 1998), and *detour (dtr),* which encodes zebrafish Gli1 (Karlstrom et al., in press). Based on shared phenotypes, *chameleon (con)* and *iguana (igu)* are also likely to affect some aspect of Hh signaling (Brand et al., 1996; Karlstrom et al., 1996; Odenthal et al., 2000; van Eeden et al., 1996). Together, these zebrafish mutants provide a unique tool for dissecting the roles played by different components of the Hh signaling system in adenohypophysis development.

Shh is clearly not the only important signaling molecule in the forming adenohypophysis. Other cell signaling molecules needed for pituitary development include members of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families (reviewed in Scully and Rosenfeld, 2002). Loss of BMP4 in mouse embryogenesis arrests early pituitary development and prevents the emergence of specific cell lineages (Takuma et al., 1998; Treier et al., 1998). FGF signaling is also needed early in pituitary induction and subsequently for differentiation of dorsal cell types (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998). In current models, it is proposed that Shh from ventral epidermal tissue induces a ventrodorsal gradient of BMP2 in oral ectoderm, which then acts to oppose dorsal FGF signals (Scully and Rosenfeld, 2002; Treier et al., 2001). These signaling interactions result in the activation of a large number of pituitary-specific transcription factors that include the lim homeodomain transcription factors *Lhx3* and *Lhx4* (reviewed in Dasen and Rosenfeld, 2001).

Lhx3 (*lim3* in zebrafish) and *Lhx4* are expressed in the developing adenohypophysis of mouse embryos, and mice lacking either of these genes have severe defects in adenohypophyseal induction and cell differentiation (Sheng et al., 1997). In zebrafish, *lim3* is expressed in the developing pituitary from early ages (Glasgow et al., 1997).

While previous genetic studies have shown that Hh signaling is directly needed for cell proliferation and patterning within the adenohypophysis (Treier et al., 2001), the source and timing of Hh signals is not well established. Published models suggest that epidermis adjacent to Rathke's pouch is the source of Hh signals in mammals (e.g., Scully and Rosenfeld, 2002; Treier et al., 2001). Since Shh is expressed at earlier times in neural tissue adjacent to the anterior neural ridge (Treier et al., 1998), and since fate mapping data show that this region is the source of Rathke's pouch (Osumi-Yamashita et al., 1994), it is equally likely that earlier neural expression plays a direct role in pituitary induction.

Understanding how and when Hh helps form the vertebrate pituitary is particularly important because defects in Hh signaling are implicated in a large number of human congenital disorders that result in hypopituitarism (Parks et al., 1999). These disorders include holoprosencephaly (HPE) and Pallister–Hall syndrome, which result in a large range of malformations from the complete loss of the pituitary gland to more subtle pituitary reductions and mislocations (Kang et al., 1997; Muenke and Cohen, 2000; Stevenson et al., 1993).

In this paper, we examine the temporal and spatial requirement for Hh signaling in zebrafish pituitary development. We show that Hh is needed during at least three stages for pituitary gland formation and that early Hh signals must originate from adjacent neural ectoderm. A careful analysis of the zebrafish Hh pathway mutants shows a range of pituitary phenotypes that result from mutations in different Hh pathway components. These combined genetic and experimental analyses of adenohypophysis development in zebrafish promise to shed light on human pituitary defects caused by mutations in different components of the Hh signaling system.

Materials and methods

Wild-type and mutant fish lines

Wild-type and mutant zebrafish are maintained at the University of Massachusetts-Amherst Fish Facility as described (Westerfield, 1993). Embryos were grown at 28.5°C and staged as described (Kimmel et al., 1995). Alleles used were *you-too, yot^{ty17}* (Karlstrom et al., 1996, 1999) *detour, dtr^{ts269}* (Karlstrom et al., 1996), *sonic you, syu^{tbx392}* (Schauerte et al., 1998), *slow-muscle-omitted, smu^{b641}* (Barresi et al., 2000; Varga et al., 2001), *iguana, igu^{ts294}* (Brand et al., 1996; Karlstrom et al., 1996), and *chameleon, con^{th6}* (Brand et al., 1996; Karlstrom et al., 1996).

In situ hybridization and antibody labeling

In situ labeling was performed as described (Karlstrom et al., 1999). Probes used were *dlx3* (Akimenko et al., 1994), *eyaB* (same as *eya1;* Sahly et al., 1999), *fgf8* (Furthauer et al., 1997), *isl1* (Inoue et al., 1994), *lim3* (Glasgow et al., 1997), *nk2.2* (Barth and Wilson, 1995), *ptc-1* (Concordet et al., 1996), *shh* (Krauss et al., 1993), and *twhh* (Ekker et al., 1995). Anti-sense digoxigenin-labeled probes were synthesized with the Dig RNA Labeling Kit (Sp6/T7) from Roche.

Antibody labeling was performed as described (Karlstrom et al., 1996). Briefly, embryos were fixed in 4% paraformaldehyde for either 2 h at room temperature or overnight at 4°C and, then dehydrated in MeOH. After rehydration, embryos were blocked for 1 h and incubated in polyclonal antibodies anti-PRL (1:2000) or anti-SL (1:2000) (generous gift of H. Kawauchi; Kawauchi et al., 1983; Rand-Weaver et al., 1992). For fluorescent labeling, embryos were incubated in Rhodamine-conjugated goat anti-rabbit antibody (1:1000; Sigma). For peroxidase labeling, embryos were incubated in goat anti-rabbit biotin (1: 200; Jackson) followed by avidin–HRP (1:500; Sigma). Peroxidase labeling was visualized with diaminobenzidine (DAB; Sigma). DAB-labeling reactions were stopped by incubation in 4% paraformaldehyde overnight. Embryos were cleared in 75% glycerol and examined by using DIC optics.

Cyclopamine treatments

Embryos were treated with 50 or 100 μM cyclopamine (gift from W. Gaffield, then purchased from Toronto Chemical) (Incardona et al., 1998) by adding 5 or 10 μL of 10 mM stock solution (in 95% EtOH) to 1 ml of egg water (0.3 g/L Instant Ocean Salt, 1 mg/L Methylene Blue) starting at the time points indicated. At early time points (up to 24 h), no difference was seen between 50 and 100 μM treatments. After the 24-h stage, treatment with 50 μM cyclopamine gave variable results, so 100 μM was used for older embryos. Control embryos were treated simultaneously with an equal volume (10 μL) of 95% EtOH (cyclopamine carrier) in 1 ml egg water. Treatments were carried out in 12-well plates in 1 mL egg water ($n = 30$ embryos/well) at 28.5°C. Embryos were dechorionated in egg water by using 0.2 mg/ml (final) pronase (Sigma) at 37°C and fixed at 21, 30, 36, or 48 h with 4% paraformaldehyde, dehydrated in MeOH, then processed for in situ hybridization and antibody labeling.

RNA injections

Capped *shh* and *GFP* RNA were made by in vitro transcription using T7 polymerase (mMessage Machine; Ambion). The *shh*/T7TS plasmid was linearized with *Bam*HI, the *twhh*/T7TS plasmid was linearized with *Hin*dIII (Ekker et al., 1995) and our *GFP*/T7TS plasmid was linearized with *Xba*I. RNA was diluted in 1× "dani-eau" solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM Hepes, phenol red]. Embryos were injected through the chorion with approximately 100 pg RNA at the one- to two-cell stage by using a pressure injector, grown in egg water at 28.5°C, fixed at 30 h, and processed for in situ hybridization or antibody labeling.

Results

Functional regionalization in the early zebrafish adenohypophysis

To better understand the early formation and patterning of the zebrafish adenohypophysis, we first examined expression of the known pituitary marker *lim3* (Glasgow et al., 1997) in relation to the homeobox transcription factor *nk2.2,* which is expressed in the adenohypophysis and is positively regulated by Hh signals (Barth and Wilson, 1995; Karlstrom et al., 1999; Varga et al., 2001). As previously reported (Glasgow et al., 1997), *lim3* (*Lhx3* in mouse) is expressed throughout the visibly thickened placode at 36 h of development (Fig. 1A). In contrast, we found that *nk2.2* is expressed only in the anterior region of the adenohypophyseal placode (Fig 1B), uncovering previously unreported regional patterning within the placode that might be related to Hh signaling. To determine whether this regionalization of early gene expression is correlated with functional organization of the adenohypophysis, we examined the onset of hormone expression in the placode. We found that antibodies generated against the salmon pituitary hormones Somatolactin (SL) and Prolactin (PRL) (Kawauchi et al., 1983; Rand-Weaver et al., 1992) label the developing zebrafish adenohypophysis as early as 30 h of development. SL-secreting cells are present throughout the adenohypophysis, similar to the expression domain of *lim3* (compare Fig. 1A, C, and E), while PRL-secreting cells are located anteriorly in the region that expresses *nk2.2* (Fig. 1B, D, and F). The restricted expression of *nk2.2* and PRL indicates that cells in the developing adenohypophysis are not equivalent and that Hh signaling may play a role in this functional patterning of the zebrafish pituitary.

Hh signaling directs pituitary development in three distinct phases

Previous studies have shown that Hh signaling is necessary for pituitary development (Karlstrom et al., 1999; Kondoh et al., 2000; Treier et al., 2001). To directly test when Hh signaling is needed for adenohypophyseal induction and patterning, we assayed the effects of pharmacologically blocking Hh signaling at various developmental stages. Pituitary gene expression was assayed in embryos treated with the plant steroidal alkaloid, cyclopamine, that blocks Hh signaling at the level of the smoothened receptor complex protein (Taipale et al., 2000).
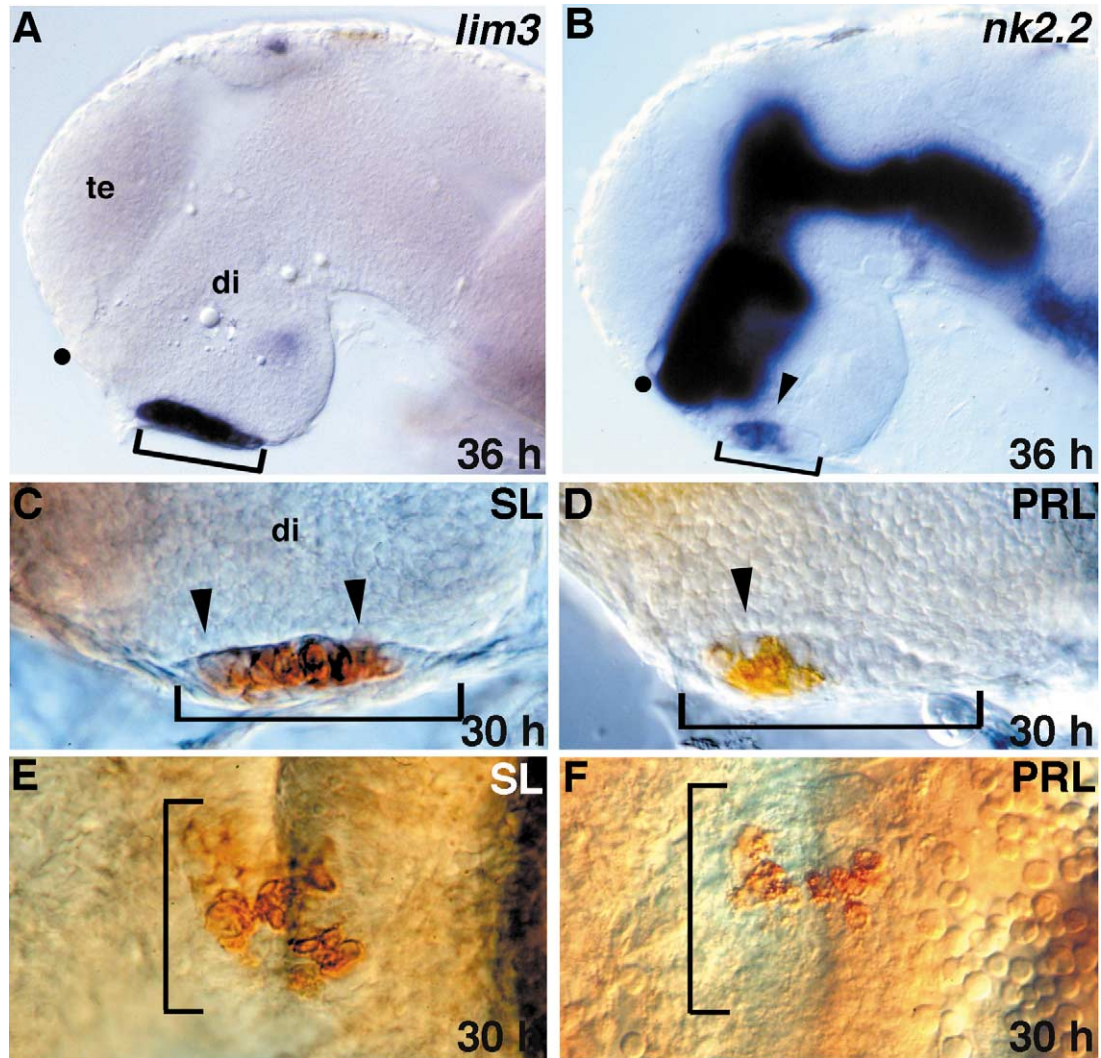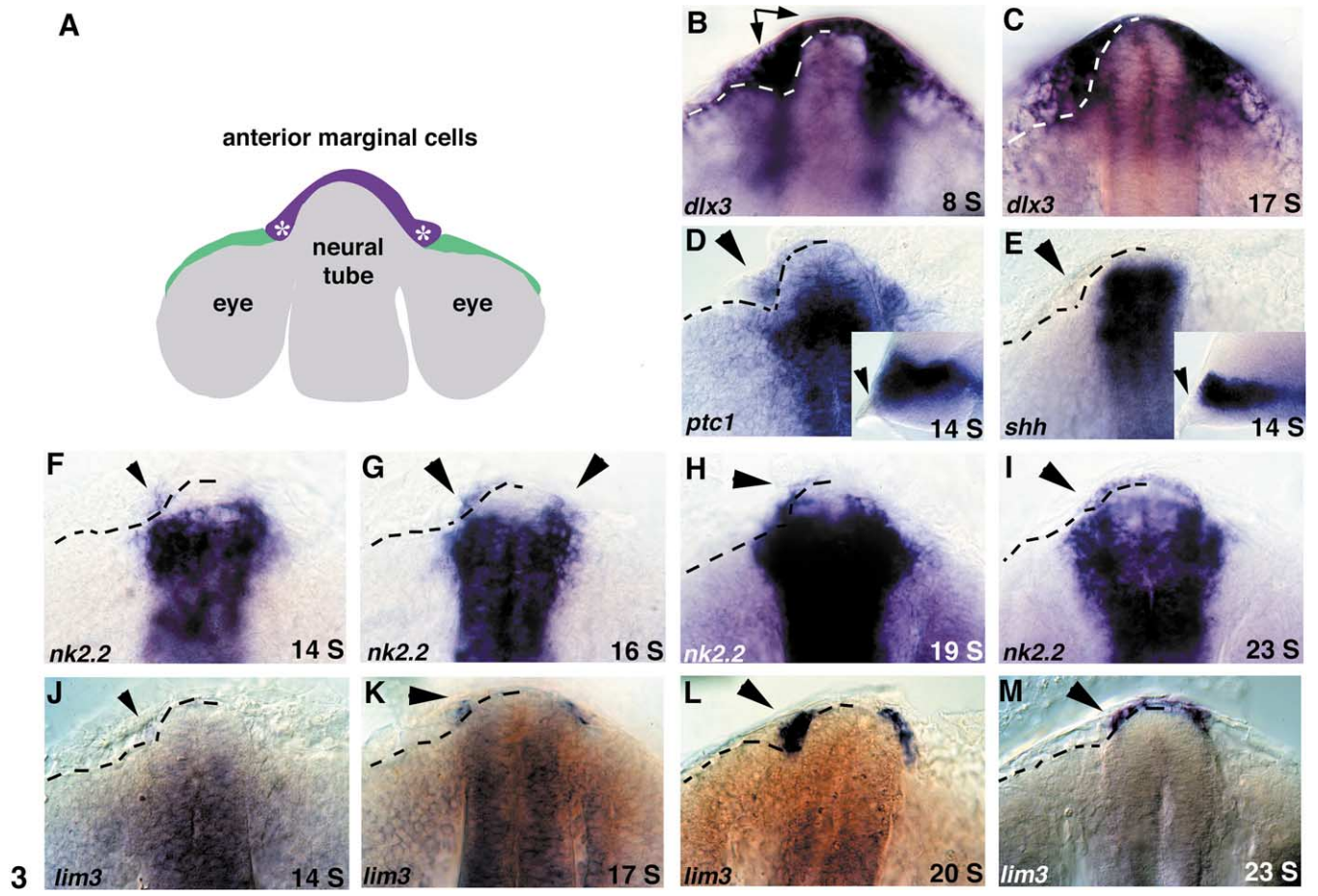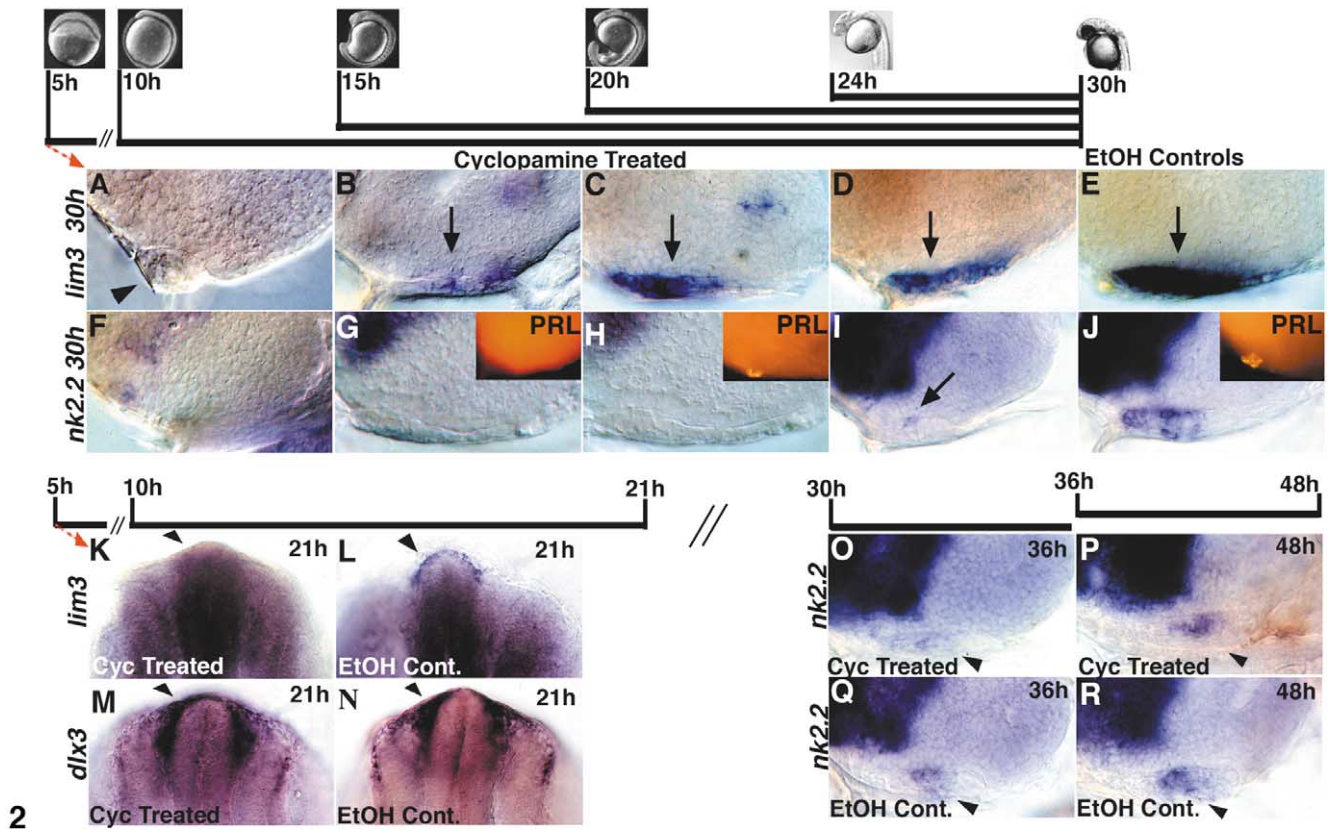
Fig. 1. Regionalization of the early adenohypophysis. (A) *lim3* is expressed in the entire adenohypophyseal placode. (B) *nk2.2* is expressed in the anterior region (arrowhead) of the placode. (C, E) Somatolactin (SL)-secreting cells are present throughout the adenohypophysis at 30 h (arrowheads). (D, F) Prolactin (PRL)-secreting cells are present only in the anterior region of the adenohypophysis at 30 h (arrowhead). (A–D) Lateral views, eyes removed, anterior to the left. (E, F) Ventral views, anterior up. Brackets show anterior/posterior extent of the morphologically visible placode. te, telencephalon; di, diencephalon; dot shows position of optic recess.

Fig. 2. Timed inhibition of Hh signaling with cyclopamine. Initiation and duration of cyclopamine treatment is indicated by black bars with images showing appropriately staged embryos (images from Karlstrom and Kane, 1996). Embryos in (A–J) were fixed and labeled at 30 h. (A) Treatments starting at 5 or 10 h result in the loss of a visible adenohypophyseal placode and loss of all *lim3* expression at 30 h. These embryos often develop an ectopic lens in place of an adenohypophysis (arrowhead in A). (B) Treatment at 15 h severely reduces *lim3* expression in a thin but visible placode. (C, D) Embryos treated starting at 20 or 24 h have smaller adenohypophyses with reduced *lim3* expression. (E) Control embryos treated with an equal volume of EtOH (cyclopamine carrier) have normal adenohypophyses that express *lim3*. (F, G) Cyclopamine treatments starting at 5, 10, or 15 h eliminate *nk2.2* expression and PRL cells (inset in G) at 30 h. (H) Treatment at 20 h eliminates *nk2.2* expression and severely reduces PRL cell numbers (inset). (I) Cyclopamine treatment starting at 24 h reduces *nk2.2* expression in the anterior adenohypophysis (arrow). (J) Normal *nk2.2* and PRL labeling in EtOH-treated control embryos. (K) Cyclopamine treatments at 5 or 10 h eliminate *lim3* expression at 21 h. (M) *dlx3* expression at 21 h is not affected by early cyclopamine treatments. (L, N) *lim3* and *dlx3* expression in EtOH-treated controls. (O, Q) Cyclopamine treatment starting at 30 h reduces *nk2.2* expression at 36 h relative to controls (arrowheads). (P, R) Embryos treated from 36–48 hours have *nk2.2* expression similar to that seen in control treated embryos (compare arrowheads). (A–J) and (O–R) show lateral views, anterior to the left, eyes removed; (K–N) show dorsal/anterior views, anterior up. Cyo, cyclopamine.

Fig. 3. *ptc1* and *nk2.2* expression precedes *lim3* expression in preplacodal cells at the anterior margin of the neural tube. (A) Schematic of the zebrafish forebrain during somitogenesis, dorsal view, anterior up. Based on expression of *lim3* and fate mapping experiments, the pituitary placode arises from medially located cells (purple) at the anterior edge of the neural tube (Glasgow et al., 1997; Whitlock and Westerfield, 2000). Asterisks show where the pituitary transcription factors *lim3* and *nk2.2* are first expressed. (B) At the 8-somite (S) stage, the early placodal marker *dlx3* labels marginal cells spanning the midline of the neural tube and more lateral cells that coalesce to form the olfactory placodes (arrows). (C) At 17 S, *dlx3* is expressed by a thinner band of preplacodal cells at the anterior margin. (D) The Hh receptor *ptc1* is expressed in neural tissue and in anterior marginal cells (arrowheads) at 14 S. Inset shows lateral view. (E) *shh* expression is restricted to neural tissue, no expression is seen in epidermal cells (arrowheads). Inset shows lateral view. (F) *nk2.2* expression begins anterior to the neural tube as early as 14 S and is often seen only on the left side (arrowhead). (G) By 16 S, *nk2.2* is expressed anterior to the neural tube on both sides of the embryo (arrowheads). (H, I) Between 19 and 23 S, *nk2.2* expression expands across the midline in anterior marginal cells (arrowheads). (J) At 14 S, no *lim3* expression is detectable in anterior marginal cells (arrowhead). (K) *lim3* expression is first detectable at approximately 17 S (arrowhead). (L, M) Between 20 and 23 S, *lim3* expression expands across the midline (arrowheads). All panels show dorsal/anterior views of the anterior edge of the nervous system, anterior up. Insets in (D) and (E) show lateral views, anterior to the left. Dotted lines on left side of embryo show position of the visible border between neural tissue and epidermal cells at the anterior margin of the nervous system. This border was established by careful examination of magnified images and multiple labeled embryos.
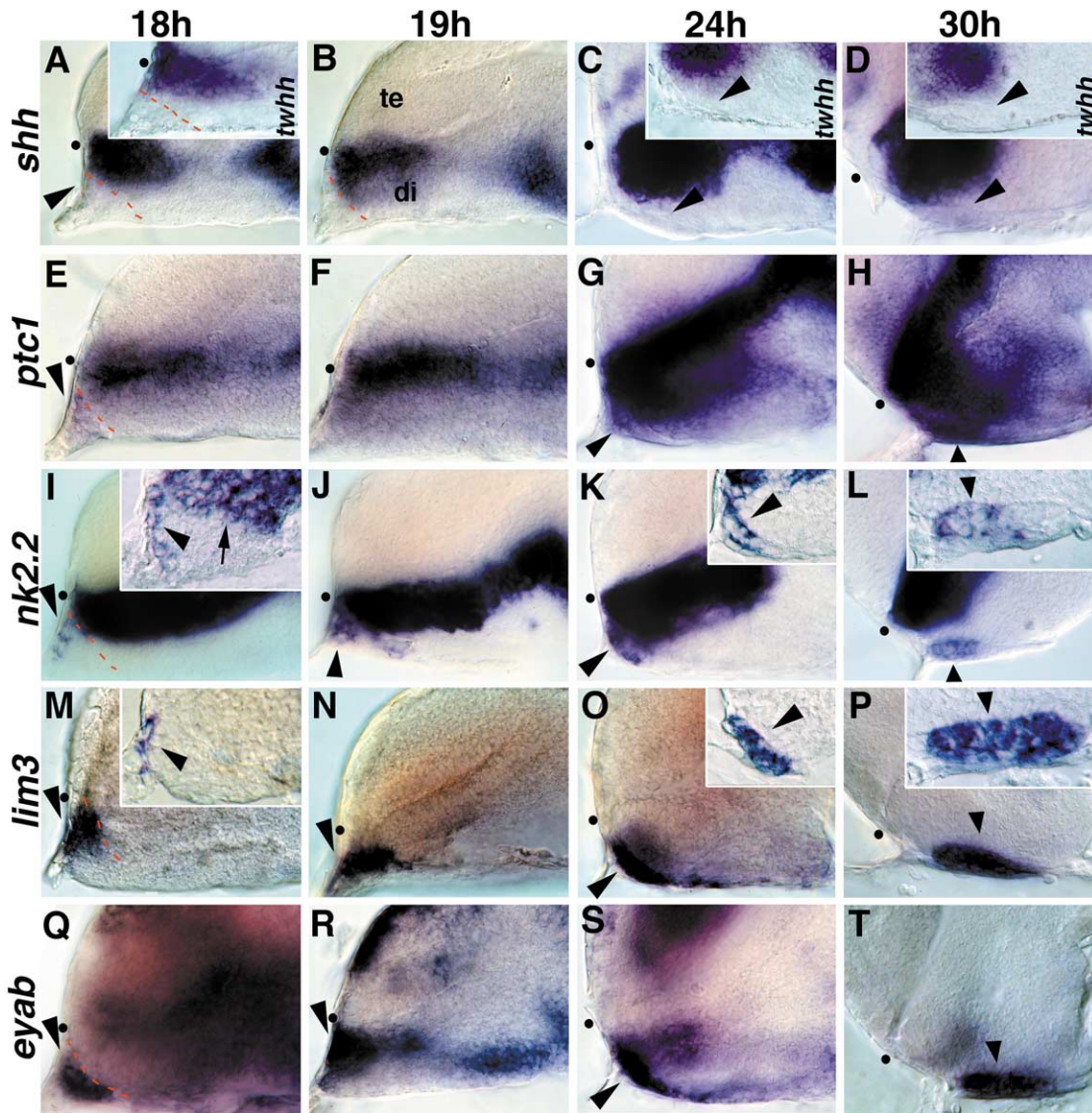
Fig. 2

Fig. 4. Regionalization within the developing adenohypophysis. (A–D) Expression of *shh* and *twhh* (insets) is restricted to the neural tube; no expression is seen in anterior epidermal cells (arrowhead in A). (E–H) *ptc1* expression extends into nonneural cells at the anterior edge of the embryo, anterior to the *shh* expression domain (arrowheads). (I, J) *nk2.2* is expressed anterior to the neural tube at 18 h (arrowhead), and this expression is distinct from the neural expression domain (arrow in inset). (K, L) At 24 and 30 h, *nk2.2* is expressed in the anterior region of the visibly thickened adenohypophyseal placode (arrowheads) with expression restricted to the anterior region at both ages (arrowheads in insets). (M, N) *lim3* expression anterior to the neural tube at 18 and 19 h (arrowhead). (O, P) *lim3* continues to be expressed in the entire thickened placodal region at 24 and 30 h (arrowheads). (Q–T) The placodal marker *eyaB* is expressed throughout the adenohypophyseal placode (arrowheads). All panels show lateral views, eyes removed, anterior to the left. Insets show parasagittal sections. te, telencephalon; di, diencephalon; dot shows position of optic recess.

Pre-somitogenesis: Hh signaling is required for pituitary induction

Incubation in cyclopamine starting prior to the onset of somitogenesis (5, 8, or 10 h) completely blocks adenohypophyseal development (Table 1), similar to the phenotype seen in the most severe Hh signaling mutants *smu/smo* (Varga et al., 2001) and *yot/gli2* (Karlstrom et al., 1999) (see below). Following these early treatments, no placode is visible at 30 h, and both *lim3* and *nk2.2* expression are completely absent in the epidermis ventral to the hypothalamus (Fig. 2A and F). Embryos treated before 10 h also often develop an ectopic lens in place of the adenohypoph-

ysis (Fig. 2A), as is seen in *smu/smo, yot/gli2,* and *igu* mutants (Kondoh et al., 2000; Varga et al., 2001). The lack of a pituitary in embryos treated with cyclopamine starting at 10 h shows that, while earlier Hh signaling may play a role in pituitary induction, it is not sufficient to induce *lim3* expression in the absence of later Hh signaling.

These results show that early Hh signaling is required for pituitary development but do not reveal whether this requirement is specific for induction of pituitary placode cells, or whether the loss of pituitary structures is due to a general loss of preplacodal cells at the anterior margin of the neural tube. To test for a pituitary specific role for Hh signaling and
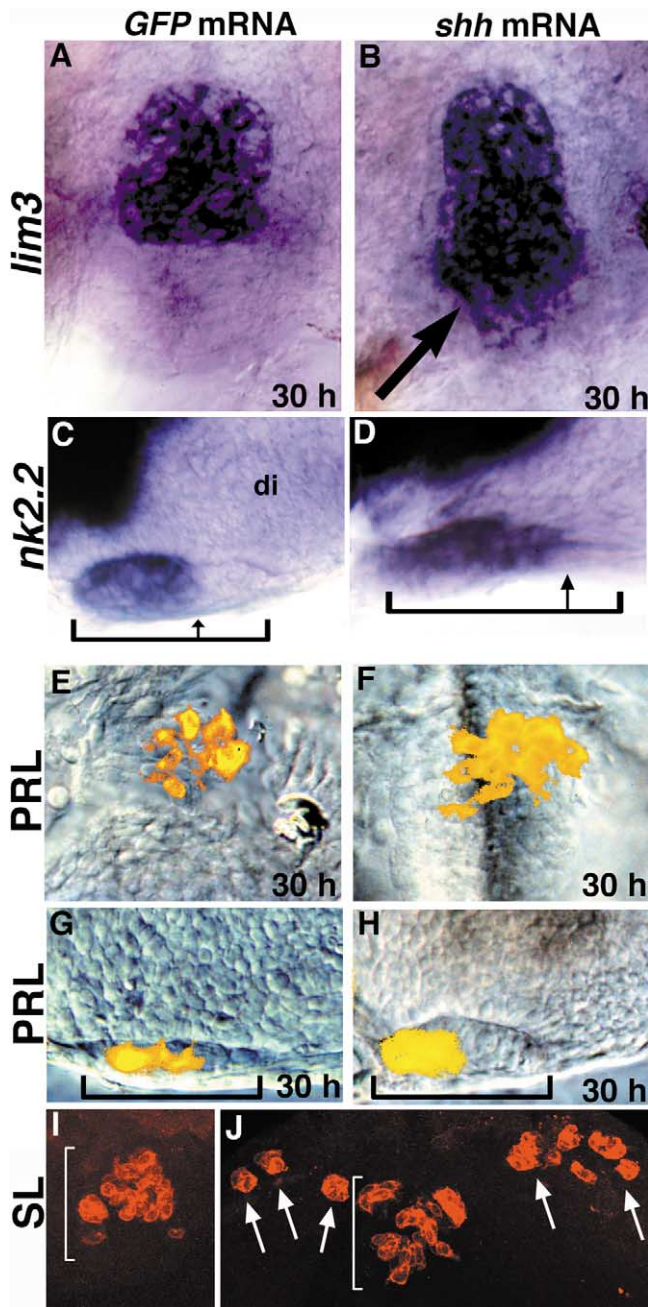
Fig. 5. Ectopic *shh* expands *lim3* and *nk2.2* expression and induces PRL and SL cell populations. (A, B) *lim3* expression in *GFP* mRNA-injected (A) and *shh* mRNA-injected (B) embryos. *shh* mRNA injections lead to posterior expansion of the *lim3* expression domain (arrow in B). (C, D) *nk2.2* expression in *GFP* mRNA-injected (C) and *shh* mRNA-injected (D) embryos. *nk2.2* expression is expanded within the developing placode (compare arrows in C and D). (E–H) PRL cell populations in *GFP* (E, G) and *shh* (F, H) mRNA-injected embryos. The number of PRL-producing cells is consistently expanded in the anterior region of the adenohypophysis. (I) SL-producing cells are present throughout the adenohypophysis (bracket) in control (GFP) RNA-injected embryos. (J) Ectopic SL cells (arrows) are induced after *shh* mRNA injection. All panels show 30-h embryos that were injected with 100 pg of the indicated mRNA at the two- to four-cell stage. (A, B, E, F, I, J) Ventral views, anterior up. (C, D, G, H) Lateral views, eyes removed, anterior to the left. Brackets show anterior/posterior extent of adenohypophysis. di, diencephalon.

determine whether competent placodal cells are present, we examined the expression of the anterior neural ridge marker *dlx3* in embryos treated with cyclopamine at early time points. Embryos were treated at 5 or 10 h of development, fixed at 21 h (23 S), and processed to determine *dlx3* and *lim3* expression. Early treatment with cyclopamine had no effect on *dlx3* expression at the anterior margin (Fig. 2M). This suggests that anterior marginal cells are present at early stages and that these cells can express markers appropriate for cranial placode development. In contrast, early cyclopamine treatments result in a loss of *lim3* expression in the same region (Fig. 2K), indicating that adenohypophysis development is specifically disrupted.

Since current models suggest that Hh signals originate from invaginated oral epidermis, we examined early pituitary-specific gene expression in relation to the invagination at the anterior end of the embryo that might correspond to the formation of oral ectoderm. At the times when cyclopamine blocks pituitary formation, we see no anterior invagination of oral ectoderm (see Fig. 4 below). In fact, *lim3* continues to be expressed superficially, ventral to the diencephalon, through 36 h of development (see Fig. 1). Thus, early Hh-dependent events are occurring prior to the invagination of oral ectoderm.

Late somitogenesis stages: Hh signaling patterns the pituitary

To test for later Hh roles in adenohypophysis development, we next used cyclopamine to block Hh signaling at later time points. Treatment starting at 15 h (12 S) and 20 h (21 S) leads to more subtle defects in the early pituitary placode. Treatment starting at 15 h (12 S) eliminates *nk2.2* expression while severely reducing *lim3* expression (Fig. 2B and G; Table 1). PRL cells are either eliminated (Fig. 2G, inset), or a few (1–6) cells are seen (Table 2). Treatment starting at 20 h eliminates *nk2.2* expression, but PRL-producing cells are present and *lim3* is expressed in a morphologically visible adenohypophysis (Fig. 2C and H; Tables 1 and 2). Blocking Hh signaling at these stages selectively blocks anterior gene expression, suggesting that Hh signaling is necessary for anterior cell fates. This indicates that Hh signaling may help pattern the pituitary along the anterior/posterior axis.

Post somitogenesis: Hh signaling maintains anterior pituitary gene expression

Cyclopamine treatments starting at 24 h lead to slightly reduced adenohypophyseal placodes with extremely reduced *nk2.2* expression at 30 h (Fig. 2D and I; Table 1). Since *nk2.2* is strongly expressed at 24 h (see Fig. 4K below), we conclude that Hh signaling is needed to maintain this expression after 24 h. Cyclopamine effects become weaker after 30 h. About one-third of the embryos treated at 30 h and examined at 36 h have adenohypophyses that appear normal morphologically but have moderately reduced *nk2.2* expression (Fig. 2O), while about two-thirds of

Table 1
Effect of cyclopamine treatment on gene expression in the adenohypophysis

Cyclopamine treatment (stage started)	Embryos with no adenohypophyses		Embryos with reduced adenohypophysis			Unaffected embryos	
	no *lim3*	no *nk2.2*	⇓ *lim3*	no *nk2.2*	⇓ *nk2.2*	wt *lim3*	wt *nk2.2*
5–30 h (pregastrulation)	91/91 (100%)	102/102 (100%)	0	0	0	0	0
8–30 h (80% epiboly)	80/80 (100%)	60/60 (100%)	0	0	0	0	0
10–30 h (1 somite)	55/55 (100%)	78/78 (100%)	0	0	0	0	0
15–30 h (12 somites)	0	0	31/31 (100%)	67/67 (100%)	0	0	0
20–30 h (21 somites)	0	0	43/43 (100%)	51/51 (100%)	0	0	0
24–30 h (prim 5)	0	0	108/108 (100%)	0	159/159 (100%)	0	0
30–36 h (prim 15)	n.d.	0	n.d.	0	11/34 (32%)	n.d.	23/34 (68%)
36–48 h (prim 22)	n.d.	0	n.d.	0	4/24 (17%)	n.d.	20/24 (83%)
EtOH (all experiments)	0	0	0	0	0	406/406 (100%)	417/417 (100%)

⇓, reduced expression; n.d., no data.

the embryos treated at this time point have wild-type levels of *nk2.2* expression (Table 1). This suggests that 30 h may be a transitional time point when Hh signaling is no longer required for maintenance of *nk2.2* expression in the anterior adenohypophysis. To test this, we treated embryos starting at 36 h and assayed *lim3* and *nk2.2* expression at 48 h. We observed no differences between treated embryos and controls (Fig. 2P and R), confirming that Hh signaling is not needed to maintain anterior gene expression at these later time points.

Hh response in the early pituitary placode

Our cyclopamine experiments indicate that Hh signaling is necessary for pituitary formation between 10 and 20 h of development but do not illuminate whether this role is direct or indirect. Since *ptc1* expression is directly regulated by Hh signals and is commonly used to indicate a direct cellular response to Hh signals (see Goodrich and Scott, 1998; Ingham and McMahon, 2001), we examined *ptc1* expression at the anterior margin of the embryo between 15 and 20 h of development. Consistent with a direct requirement for Hh signaling in early pituitary induction, *ptc1* is expressed in preplacodal cells anterior to the visible border of the neural tube as early as 16 h (Fig. 3D). Similar anterior *ptc1* expression was seen in mouse (Treier et al., 2001). To

Table 2
Effect of cyclopamine treatment on PRL secreting cells

Cyclopamine treatment	Embryos with no PRL cells	Embryos with 1–6 PRL cells	Embryos with 9–15 PRL cells (wt)
15–30 h (12 somites)	8/22 (36%)	14/22 (64%)	0
20–30 h (21 somites)	0	10/20 (50%)	10/20 (50%)
EtOH (all experiments)	0	0	30/30 (100%)

confirm that this expression is in preplacodal cells, we compared *ptc1* expression with that of the preplacodal marker *dlx3* (Whitlock and Westerfield, 2000). Similar to *ptc1, dlx3* is expressed anterior to this same visible border (Fig. 3B and C). Also consistent with our cyclopamine results, *shh* and the closely related molecule *twhh* are expressed at this age in the forebrain. In contrast to *ptc1*, expression of *shh* (Fig. 3E) and *twhh* (data not shown) is restricted to the adjacent neural tube. This indicates that neural tissue adjacent to the forming placode must be the source of Hh signals.

To understand how this early Hh signaling relates to pituitary gene expression, we next examined pituitary expression of *nk2.2* and *lim3*. Like *ptc1*, *nk2.2* expression is known to depend on Hh signaling (Barth and Wilson, 1995; Karlstrom et al., 1999; Varga et al., 2001). *nk2.2* expression begins in cells anterior to the neural tube by 15–16 h (12–14 S) on the left side of the embryo (Fig. 3F) and is present on both sides of the midline by 17 h (16 S; Fig. 3G). Expression of the more definitive pituitary marker *lim3* also begins on the left side of the neural plate (Glasgow et al., 1997; data not shown) but is not detectable in these cells until approximately 17.5 h (17 S; Fig. 3J and K), 1–2 h after *nk2.2* expression begins and slightly earlier than previously reported (Glasgow et al., 1997). By 19 h (19–20 S), *nk2.2* and *lim3* are both expressed at comparable levels in cells anterior to the neural tube (Fig. 3H and L). By 20.5 h (23 S), both *nk2.2* and *lim3* expression span the midline anterior to the neural tube (Fig. 3I and M).

To summarize, expression of the Hh responsive genes *ptc1* and *nk2.2* at the anterior margin of the neural tube indicates that these preplacodal cells are directly responding to Hh signals at a time when disruption of Hh signaling eliminates pituitary formation. At the same time, expression of *shh* and *twhh* is restricted to adjacent neural ectoderm, suggesting that the diencephalon is the source of these Hh signals. This Hh response precedes the expression of the pituitary-specific transcription factor *lim3* by 1–2 h.

Table 3
shh mRNA injections

Injected mRNA	Embryos with ⇑ *lim3*	Embryos with ⇑ *nk2.2*	Embryos with ⇑ PRL	Embryos with ⇑ SL
50 pg *shh*	n.d.	0/57	n.d.	n.d.
100 pg *shh*	65/75 (87%)	83/94 (88%)	14/14 (100%)	27/34 (79%)
500 pg *shh*	25/25 (100%)	43/43 (100%)	n.d.	n.d.
100 pg *GEP*	0/40	0/75	0/50	0/16

⇑, ≥50% increase in area of gene expression (*lim3, nk2.2*) or cell number (PRL, prolactin; SL, somatolactin); n.d., no data.

Hh signaling and patterning of the adenohypophysis

Our cyclopamine experiments indicate that Hh signaling between 20 and 25 h plays a role in functional patterning of the adenohypophysis. To learn when the adenohypophysis first becomes regionally patterned, and to determine whether Hh signaling might play a direct role in this patterning event, we examined the expression of *shh, twhh, ptc1, nk2.2, lim3,* and the general placodal marker *eyaB* (same as *eya1,* Sahly et al., 1999) in the adenohypophysis as it becomes morphologically distinct at the end of somitogenesis. At 18 h (18 S), 19 h (20 S), and 24 h, *shh* and *twhh* expression are restricted to neural tissue of the diencephalon (Fig. 4A–C). In contrast, *ptc1* and *nk2.2* expression extend into epidermal cells anterior to the developing forebrain, in the same region that expresses the pituitary marker *lim3* and the placodal marker *eyab* (Fig. 4). This shows that epidermal cells in the developing placode are directly responding to Hh signaling at these ages, and that the source of Hh signals is most likely the ventral diencephalon. Interestingly, the *nk2.2* expression domain is initially broader than the *lim3* expression domain (compare insets in Fig. 4I and M). However, by 24 h, *nk2.2* and *ptc1* expression becomes restricted to the anterior region of the *lim3-* and *eyaB-* expressing placode (Fig. 4G, K, O, and S), suggesting that only anterior cells continue to directly respond to Hh signals. *shh* and *twhh* are expressed exclusively in neural tissue dorsal to the developing adenohypophysis until 30–36 h, when expression begins in ectoderm and endoderm ventral to the diencephalon (not shown; see Fig. 9). Importantly, *ptc1* and *nk2.2* expression in the anterior region of the adenohypophysis indicates that these cells are directly responding to Hh signals at a time when cyclopamine blocks anterior cell fates.

Overexpression of Shh expands pituitary gene expression

To test whether Shh signaling can directly regulate pituitary cell fates, we ectopically expressed Shh by injecting *shh* encoding mRNA at the one- to two-cell stage and analyzed pituitary *lim3, nk2.2,* PRL, and SL expression at 30 h. Ectopic *shh* expression consistently leads to expanded *lim3* expression in epidermis ventral to the forebrain in close proximity to the normal expression domain (Fig. 5A and B; Table 3). Ectopic *shh* also consistently leads to

increased numbers of SL-secreting cells (see Fig. 7I), and these cells can be found in ectopic locations throughout the ventral forebrain (Fig. 5J), sometimes as far lateral as the eye.

In most *shh*-injected embryos, *nk2.2* expression expands to almost fill the entire, somewhat enlarged, adenohypophyseal placode (Fig. 5C and D; Table 3). PRL-expressing cells are increased by ectopic *shh* expression (see Fig. 7I), but these cells are only found in the anterior region of the adenohypophysis (Fig. 5E–H; Table 3). Thus, while ectopic Hh signals can expand *nk2.2* throughout the placode, differentiation of PRL cells seems to be confined to anterior domains, perhaps because of a requirement for another permissive factor in the anterior region, or alternatively because of the presence of a posterior repressor. These experiments show that Shh signaling is capable of inducing both the expression of pituitary-specific genes and the differentiation of specific populations of hormone-secreting cells.

Zebrafish Hh pathway mutants display a range of pituitary defects

Hh signaling mutations lead to a broad range of human phenotypes, from loss of ventral midline tissue in the forebrain and complete cyclopia to more minor cranial midline defects (Odent et al., 1999; Roessler and Muenke, 2001). Since this same range of cranial phenotypes is seen in a number of zebrafish mutations known to affect Hh signaling (Brand et al., 1996), we examined pituitary development in the zebrafish Hh pathway mutants to determine how forebrain midline defects correlate with pituitary deficiencies.

The most severe pituitary phenotype is seen in *smu/ smoothened* mutant embryos which have no detectable pituitary and generally lack ventral forebrain tissue (Varga et al., 2001). In *smu* mutants, pituitary expression of *lim3* and *nk2.2* is completely absent (Fig. 6C and D; Table 4; Varga et al., 2001), no Prolactin- and Somatolactin-secreting cells develop (Fig. 7), and an ectopic lens forms in the region of the adenohypophysis (Fig. 6D; Varga et al., 2001). These defects are identical to those seen upon early treatments with cyclopamine (starting at 10 h or before) and therefore seem to reflect an early and complete block of Hh signaling. Less severe ventral forebrain deficiencies in *igu* mutant
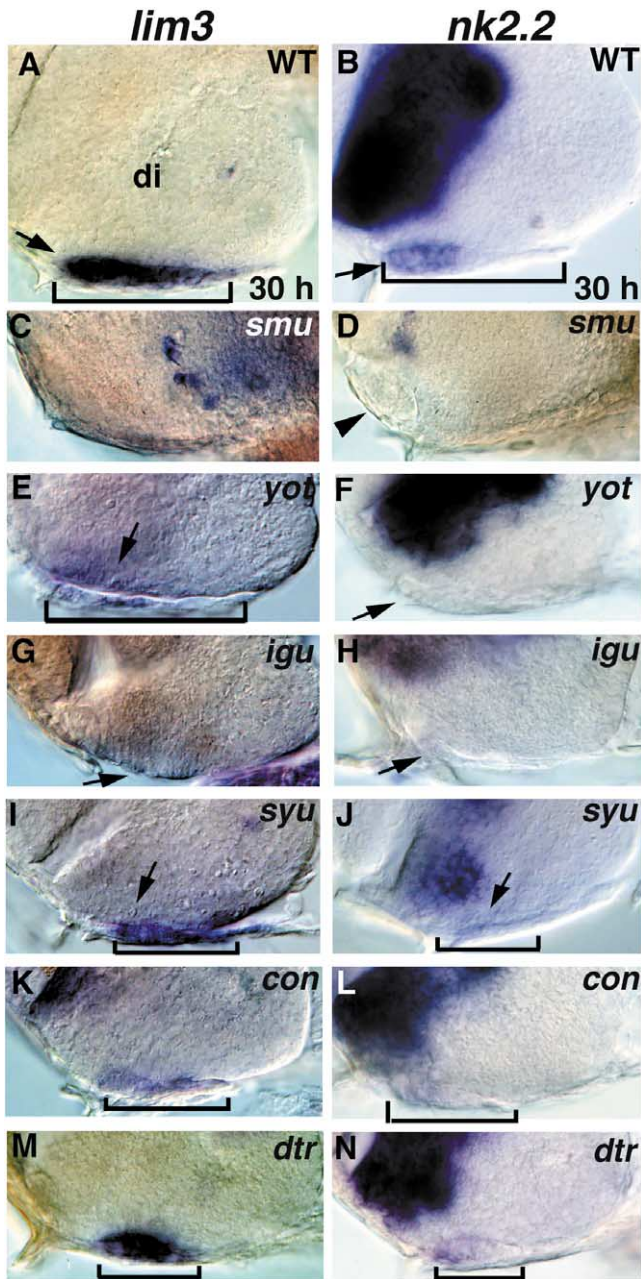
Fig. 6. Pituitary defects in Hh pathway mutants. (A, B) Wild-type pituitary expression of (A) *lim3* and (B) *nk2.2* (arrows). (C, D) *smu/smo* mutant embryos lack a discernible adenohypophysis and often develop an ectopic lens (arrowhead in D). *lim3* (C) and *nk2.2* (D) expression is absent in the region. (E, F) *yot/gli2-DR* mutants have severely reduced or absent *lim3* expression and no placodal *nk2.2* expression (arrow). (G, H) *igu* mutants also have reduced or absent *lim3* expression and no placodal *nk2.2* expression (arrows). (I, J) *syu/shh* mutants have reduced *lim3* expression and no placodal *nk2.2* expression (arrows). (K, L) *con* and (M, N) *dtr/gli1* mutants both have moderately reduced *lim3* expression and reduced *nk2.2* expression. Lateral views of ventral head, eyes removed, anterior to the left. Brackets show anterior/posterior extent of the adenohypophysis when visible. di, diencephalon.

embryos (Brand et al., 1996) are accompanied by ectopic midline lenses (Kondoh et al., 2000) and severely reduced adenohypophyses (Fig. 6G and H; Table 4).

Loss of *shh* gene function in *syu* mutants (Schauerte et al., 1998), or the production of dominant repressor (DR) forms of the Hh-responsive transcription factor *gli2* found in *yot* mutants (Karlstrom et al., 1999; Karlstrom et al., in press), leads to ventral forebrain deficiencies that are less severe than those seen in *smu* and *igu,* and the eyes in *syu/shh* and *yot/gli2-DR* mutants are well separated (Brand et al., 1996; Schauerte et al., 1998). Despite these less severe visible cranial defects, the adenohypophysis is extremely reduced (60–70% of embryos) or absent (30–40% of embryos) in *syu/shh* and *yot/gli2-DR* mutants (Fig. 6; Table 4). A few hormone-producing cells are present in most *syu/shh* embryos (Fig. 7), suggesting that signaling molecules other than Shh (perhaps Twhh, FGFs, and/or BMPs) are sufficient to induce some pituitary tissue. Unlike *yot/gli2-DR* and the other mutants with severe pituitary defects, *syu* mutants do not have ectopic lenses, again suggesting that Twhh (or some other midline signaling molecule) can block lens induction at the midline in the absence of Shh signals.

The least severe cranial defects are seen in the Hh pathway mutants *con* (Brand et al., 1996; Karlstrom et al., 1996; Odenthal et al., 2000; Schauerte et al., 1998; van Eeden et al., 1996) and *dtr/gli1* (Brand et al., 1996; Karlstrom et al., in press; Karlstrom et al., 1996; Odenthal et al., 2000), which have well-separated eyes and no ectopic midline lenses (Kondoh et al., 2000). *con* and *dtr/gli1* mutants have morphologically visible adenohypophyses with reduced *lim3* and *nk2.2* expression (Fig. 6; Table 4) and reduced SL and PRL cell populations (Fig. 7). Thus, while the milder *con* and *dtr/gli1* pituitary phenotypes have no visible cranial defects, both mutants have significant defects in hormone cell populations.

To summarize, mutations that affect different components of the Hh signaling pathway affect the developing adenohypophysis to different degrees (Table 5). Even the mild ventral forebrain deficiencies seen in *yot* and *igu* are correlated with a near complete loss of adenohypophysis, while mild forebrain defects seen in *con* and *dtr* are correlated with significant reductions in certain hormone cell populations. Consistent with our *nk2.2* and *lim3* gene expression analysis, more anteriorly located PRL cells are generally more affected by Hh signaling defects than are the more broadly distributed SL cells. This suggests that Hh signaling defects in humans might lead to specific hormone deficiencies and that anterior populations might be more affected than more posterior cell types.

Specificity of pituitary defects in Hh pathway mutants

A critical question remains regarding the specificity of the pituitary defects seen in the Hh pathway mutants and cyclopamine treated embryos. Other signals from the ventral forebrain, most notably *fgf-8* and *bmp4,* are clearly needed for normal pituitary development (Ericson et al., 1998; Kimura et al., 1996). Thus, the pituitary defects seen
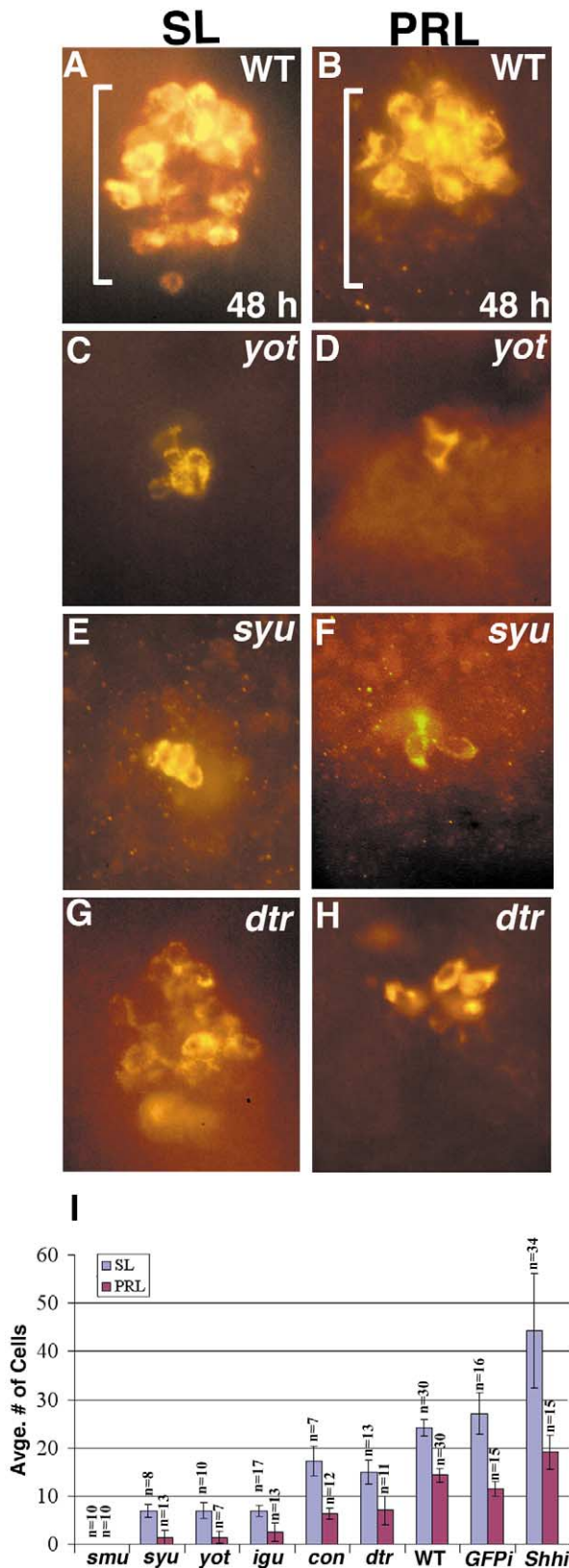
Fig. 7. Reduction of Prolactin (PRL)- and Somatolactin (SL)-secreting cells in Hh pathway mutants. (A, B) In wild-type embryos, SL-secreting cells are present throughout the adenohypophysis, while PRL-secreting cells are only present anteriorly. (C, D) *yot/gli2-DR* and (E, F) *syu/shh* mutants have severely reduced numbers of SL- and PRL-secreting cells.

in Hh pathway mutant embryos could be indirectly due to ventral forebrain defects that lead to a general loss of signaling molecule expression. We therefore examined cell differentiation in diencephalic tissue adjacent to the developing pituitary in *yot* and *syu* mutants which have ventral forebrain tissue and well-separated eyes, but lack a pituitary. The secreted factor *fgf8,* which is expressed in the anterior forebrain in mice and fish (Reifers et al., 1998; Shimamura and Rubenstein, 1997), is known to synergize with Hh signaling during mouse pituitary development (Treier et al., 2001). In *yot/gli2* and *syu/shh* mutants, *fgf8* is expressed in the appropriate region adjacent and dorsal to the developing adenohypophysis at 15 and 20 h of development (Fig. 8A–F), the times when we show that Hh signaling is required for pituitary induction (see Fig. 2). In addition, the transcription factor *isl1,* a target of BMP signaling in the adenohypophysis (Takuma et al., 1998) is expressed in the adjacent diencephalon, and in ectodermal cells continuous with the developing placode, in both *yot/ gli2* and *syu/shh* mutants (Fig. 8G–L). *shh* itself (Fig. 8M and N) is also expressed in this region in both *yot/gli2* and *syu/shh* mutant embryos, indicating that cells adjacent to the adenohypophysis have differentiated and can express signaling molecules at these ages. Finally, our previous studies showed that the transcription factor *six3* is also expressed in the anterior diencephalon in *yot* mutants at 24 h (Kondoh et al., 2000), indicating that this region of the brain forms in these mutants. Thus, while we cannot eliminate the possibility of indirect effects, the normal expression of several markers adjacent to the adenohypophysis, in combination with the expression of Hh responsive genes in preplacodal tissue, strongly argues that Hh signaling plays a direct and early role in pituitary induction.

Discussion

Our results show that Hh signaling plays an important role in several aspects of pituitary formation. While Hh is clearly not the only signaling molecule guiding pituitary induction, several lines of evidence suggest that Hh signaling *directly* contributes to pituitary induction and patterning. First, timed cyclopamine-mediated blockage of Hh signaling at early somitogenesis stages eliminates all adenohypophysis development, while later treatments interfere specifically with anterior gene expression. Second, expression of the Hh target genes *ptc1* and *nk2.2* indicates that cells at the anterior margin actively transduce Hh signals at

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(G, H) SL and PRL cells are more moderately reduced in *dtr/gli1* mutant embryos. (I) Bar graph showing average numbers of SL and PRL cells in all of the mutants examined ± s.d., numbers of embryos indicated by *n.* Rightmost bars show numbers of cells present at 30 h following injection of 100 pg of *shh* mRNA at the two- to four-cell stage. (A–H) Ventral views of 48-h embryos. Brackets show anterior/posterior extent of the adenohypophysis in wildtype embryos.

Table 4
Pituitary marker expression in zebrafish Hh pathway mutants

Mutant	*nk2.2* absent	*nk2.2* reduced	*lim3* absent	*lim3* reduced
smu	32/32 (100%)	0	27/27 (100%)	0
yot	22/22 (100%)	0	20/51 (39%)	31 (61%)
igu	18/18 (100%)	0	10/39 (26%)	29/39 (74%)
syu	33/33 (100%)	0	9/29 (31%)	20/29 (69%)
con	0	11/11 (100%)	0	9/9 (100%)
dtr	0	35/35 (100%)	0	20/20 (100%)

these cyclopamine-sensitive time points. Third, normal anterior expression of *dlx3* in cyclopamine-treated and Hh mutant embryos shows that early preplacodal differentiation is unaffected. Fourth, normal expression of *fgf8* and diencephalic markers adjacent to the adenohypophysis in Hh mutants that lack a pituitary indicates that ventral forebrain structures are present and appropriately express signaling molecules. Our gene expression analysis strongly suggests that early Hh signals emanate from adjacent neural tissue to induce and pattern the adenohypophysis.

Hh signaling and the origin of the zebrafish adenohypophysis

Our analysis of pituitary and placodal gene expression strongly supports an origin for the adenohypophysis at the anterior margin of the neural tube, consistent with one zebrafish fate mapping experiment (Whitlock and Westerfield, 2000) and similar to other vertebrates (reviewed in Baker and Bronner-Fraser, 2001). We found that *nk2.2* is initially expressed unilaterally on the left side of the neural plate (Fig. 3F) in the same left–anterior region as the pituitary-specific marker *lim3* (Glasgow et al., 1997; data not shown). This early asymmetric expression is consistent with other asymmetrically expressed molecules (reviewed in Burdine and Schier, 2000) and may point to an early role for nodal signaling in the earliest stages of pituitary induction. The epidermal expression of *nk2.2* precedes the expression of the pituitary marker *lim3* by at least 2 h, suggesting a very early role for Hh signaling in adenohypophysis induction. At the time that epidermal *nk2.2* expression begins, the Hh receptor *ptc1* is expressed at high levels in the same region,

further indicating that these cells are receiving Hh signals. *shh* and *twhh* are expressed only in adjacent diencephalic cells at this time. These data, combined with zebrafish fate map data (Whitlock and Westerfield, 2000), support a model for the origin of the zebrafish adenohypophysis in which (1) cells at the anterior and lateral margins of the neural tube first become competent to form placodal structures and then (2) Hh signaling from the diencephalon acts directly (likely in combination with other signals) to induce differentiation of medial preplacodal cells as adenohypophysis (Fig. 9).

Mouse experiments employing pituitary-specific expression of the Hh inhibitor Hip have also shown that Hh signaling plays a direct role in early pituitary induction (Treier et al., 2001). *pitx-1* regulatory elements were used to drive Hip expression at early stages in preadenohypophyseal cells, preventing Hh signaling only in this tissue. These mice lack an adenohypophysis, indicating that Hh signaling is directly needed for adenohypophysis induction. Since *pitx-1* is expressed at the anterior neural tube border very early in adenohypophyseal development and prior to the onset of *lim3* expression (Lamonerie et al., 1996; Szeto et al., 1996), these data are consistent with an early and direct role for Hh signaling in pituitary induction. Despite this, current models only show a later role for Hh signaling, and show the source of Hh as oral ectoderm surrounding Rathke's pouch (Scully and Rosenfeld, 2002; Treier et al., 2001). The idea that Hh signaling from within oral ectoderm helps pattern the adenohypophysis may derive from gene expression data showing dynamic Shh expression in oral ectoderm that becomes excluded from Rathke's pouch as it invaginates (Treier et al., 1998). Our timed inhibition of Hh

Table 5
Comparison of adenohypophyseal (adeno.) defects in Hh pathway mutants

	Gene		Ectopic midline lens	Ant. adeno. (*nk2.2*)	Entire adeno. (*lim3*)	Prolactin	Somatolactin
smu	*slow-muscle-omitted*	*smoothened*	yes	Absent	Absent	Absent	Absent
yot	*you-too*	*gli2*	yes	Absent	Abs./reduced	Abs./reduced	Reduced
igu	*iguana*	*unknown*	yes	Absent	Abs./reduced	Abs./reduced	Reduced
syu	*sonic you*	*sonic hedgehog*	no	Absent	Abs./reduced	Abs./reduced	Reduced
con	*chameleon*	*unknown*	no	Reduced	Reduced	Reduced	Reduced
dtr	*detour*	*gli1*	no	Reduced	Reduced	Reduced	Reduced

signaling in zebrafish indicates that Hh-dependent adenohypophyseal induction occurs very early in development at a time when *shh* and *twhh* are expressed only in neural tissue. Given the similarity of early *shh* and *ptc* expression at the anterior margin of the neural plate in both mice and zebrafish, these data suggest that, in both species, early Hh signals from the diencephalon may directly influence pituitary induction at the anterior margin of the neural tube.

Two roles for Hh signaling: midline lens inhibition and pituitary induction

The fact that an ectopic midline lens is seen in place of the adenohypophysis in the severe Hh signaling mutants *smu/smo* and *yot/gli2* suggests that an early role for Hh signaling is to inhibit midline lens formation in the developing placode (Kondoh et al., 2000; Varga et al., 2001). This raises the possibility that adenohypophyseal defects seen in these mutants may result from early misspecification of midline placodal cells as lens, and that Hh signaling does not actively induce pituitary differentiation. Our extensive Hh pathway mutant analysis argues against this model, as several Hh pathway mutants have pituitary defects in the absence of a midline lens. Most notably, adenohypophysis development is severely disrupted in *syu/shh* mutants, but no midline lenses have been seen (Fig. 6I and J; Kondoh et al., 2000). The presence of severe pituitary defects in *syu/shh* mutants also indicates that Shh, rather than the closely related molecule Twhh, is needed for pituitary induction. In fact, Twhh signals may be sufficient to block midline lens formation in *syu/shh* mutants, but are clearly unable to support normal pituitary development. The milder pituitary defects seen in *dtr/gli1* and *con* mutants also occur in the absence of midline lens formation, further supporting an active role for Hh signaling in the pituitary.

Functional patterning within the early adenohypophysis

Our gene expression analysis suggests that the adenohypophysis is regionally organized from the earliest stages of its development. By 24 h, *nk2.2* and *ptc1* expression are clearly restricted to the anterior portion of the adenohypophysis. This regional expression is maintained through at least 48 h of development. The selective anterior/dorsal activation of the Hh responsive genes *ptc1* and *nk2.2* is consistent with a direct role for Hh signaling in this patterning event (Fig. 4E–L, summarized in Fig. 9). Functional hormone-secreting cells differentiate before 30 h of development, with PRL-secreting cells located only in the anterior region and SL-secreting cells located throughout the adenohypophysis (summarized in Fig. 9). The functions of PRL and SL at such early stages of embryogenesis are unknown, but these hormones are known to regulate growth and osmotic balance in older fish and may be performing the same functions in the early embryo.

Mutant analyses often give a limited picture of gene function because later requirements for a gene can be masked by early, more severe phenotypes. The ability to block Hh signaling at defined points in embryogenesis allowed us to show that Hh signaling is needed later in development to establish and maintain regional gene expression in the anterior adenohypophysis. Our mutant analysis is consistent with this patterning role. Both *dtr/gli1* and *con* pituitary defects are more subtle than those seen in other Hh pathway mutants and anterior *nk2.2* expression appears to be more affected than *lim3* expression.

Shh overexpression studies and mutant analyses in mouse embryos also point to a role for Hh signaling in regional cell differentiation in the adenohypophysis. Ectopic Shh expression in Rathke's pouch in mouse embryos results in expanded ventral cell types (gonadotropes and thyrotropes) which are normally positioned close to a source of Shh in oral ectoderm (Treier et al., 2001). Similarly, our ectopic expression and zebrafish mutant analyses suggest a role for Hh signaling in differentiation of dorsal/anterior cell types (lactotropes and somatotropes) that normally form adjacent to a source of Hh in the diencephalon. This apparent difference in position of Hh-sensitive cell types (anterior in teleosts, ventral in mammals) could be explained by the different topologies of the two developing embryos, or might be the result of differences in timing of initial hormone cell differentiation. Alternatively, the final position of terminally differentiated cell types in the zebrafish adenohypophysis may be determined by cell migration that occurs after the period of Hh signaling.

Our *shh* and *ptc1* expression analyses show that cells in the anterior/dorsal adenohypophysis are closest to the source of *shh* in the diencephalon, and thus are likely to be exposed to the highest concentrations of Shh. Shh has been shown to act as a classical morphogen in the differentiation of ventral spinal cord neurons, with distinct neural cell fates being induced by different concentrations of Shh (Ericson et al., 1997). We hypothesize that Shh might act similarly as a morphogen to guide differentiation along the dorsal/anterior–ventral/posterior axis in the adenohypophysis (Fig. 9). Alternatively, or perhaps in combination, Hh signaling may function as a mitogen causing proliferation of cells in the anterior region, a role that has been shown in other systems (Rowitch et al., 1999). The fact that *shh*-injected embryos have, on average, two times the number of PRL-producing cells in the anterior adenohypophysis does not help answer this question, as increased cell numbers could be due to induction of more precursor cell types or to the increased proliferation of the same number of precursor cells. In addition, the failure of PRL cells to differentiate in more posterior regions could be because our generalized overexpression of Shh was insufficient to reach the appropriate threshold level posteriorly, or because additional factors are needed for PRL cell differentiation. We are currently investigating these possibilities.
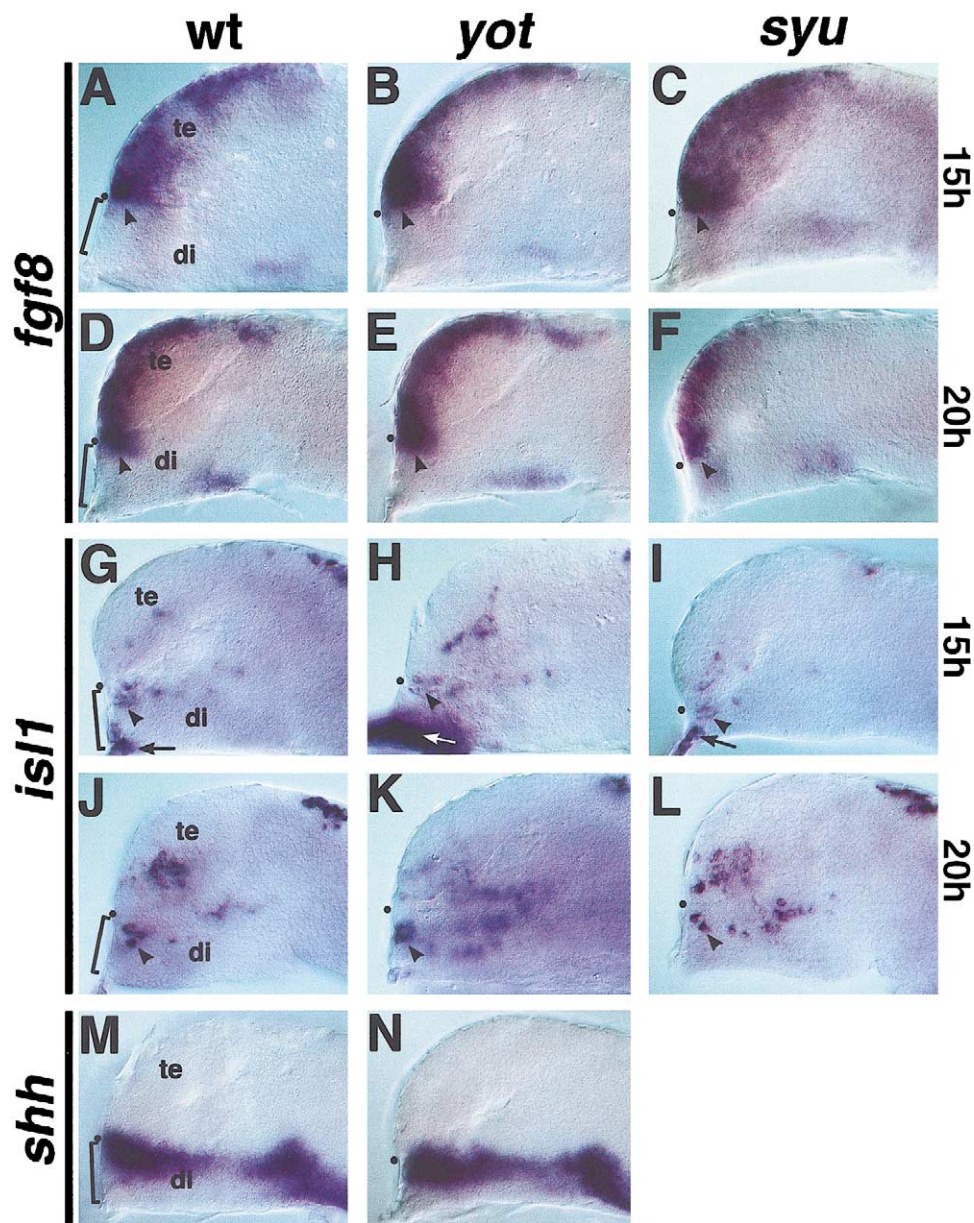
Fig. 8. Forebrain marker expression in *yot/gli2* and *syu/shh* mutant embryos. (A) The signaling molecule *fgf8* is expressed in the telencephalon and in dorsal diencephalic cells adjacent to the developing adenohypophysis (bracket) at 15 h of development. (B, C) In *yot/gli2* and *syu/shh* mutants, *fgf8* is expressed normally in cells adjacent to the developing adenohypophysis (arrowheads), suggesting that *fgf8* signaling defects do not account for the lack of pituitary development seen in these mutants. (D–F) At 20 h, *fgf8* is still expressed adjacent and dorsal to the adenohypophysis in *yot/gli2* and *syu/shh* mutants (arrowheads). (G) The transcription factor *isl1* is expressed in adjacent diencephalic cells (arrowheads), as well as in epidermal cells (arrows) that are continuous with and overlap the developing adenohypophysis (bracket) at 15 h of development. (H, I) *isl1* is expressed in these same adjacent cells in *yot/gli2* and *syu/shh* mutants (compare arrowheads and arrows). Increased labeling in (H) is due to the presence of additional extraembryonic membrane. (J–L) *isl1* is expressed in the diencephalon of both *yot/gli2* and *syu/shh* mutants at 20 h (arrowheads), indicating this diencephalic cell type can differentiate in the absence of Shh signals. Epidermal *isl1* expression is largely absent by 20 h in both wildtype and mutant embryos. (M, N) *shh* is appropriately expressed adjacent to the developing adenohypophysis at 15 h in *yot/gli2* mutant embryos (arrowheads), showing that this region of the brain is capable of signaling to adjacent epidermal tissue. In all panels, a black dot shows the position of the optic recess, the anterior border between the telencephalon (te) and diencephalon (di).

Hh signaling from neural to endocrine tissues

The most thoroughly described role for Hh signaling during organogenesis is in the establishment of ventral cell fates within the neural tube (reviewed in Ingham and Mc-

Mahon, 2001). Shh from axial mesoderm of the notochord directs differentiation of the floor plate and various ventral neural cell types in the overlying neural tube. As patterning occurs, *shh* expression is lost in the notochord and the floor plate becomes the source of *shh* (Placzek, 1995). Our results
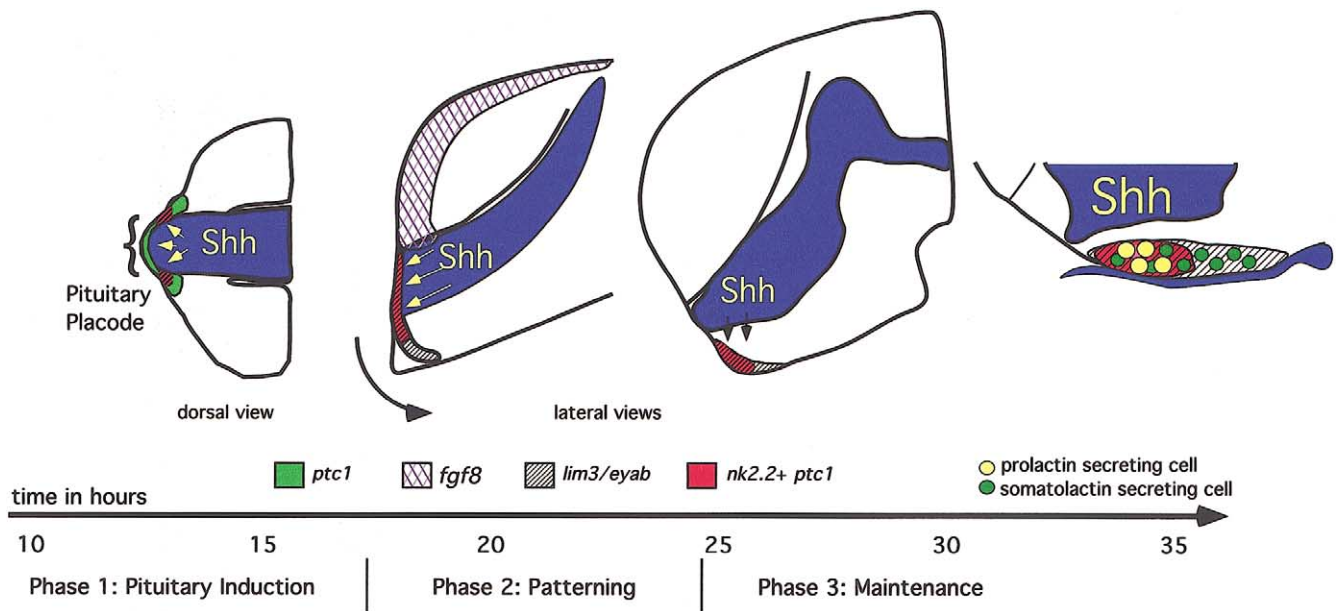
Fig. 9. Model summarizing requirements for Hh signaling in early pituitary development. Left diagram shows a dorsal anterior view while other schematics show lateral views. Phase 1: Hh signaling is required during early somitogenesis to induce the pituitary placode. *dlx3* expression at the anterior and lateral margins of the neural tube is thought to indicate the region competent to form placodal structures and this expression remains in anterior epidermal tissue at least through the 17-somite stage (17 h). Hh signaling induces *nk2.2* and *lim3* expression in medial cells at the anterior margin of the neural tube, while *fgf8* is expressed more dorsally in this same anterior region (not shown, see Fig. 8). Phase 2: Hh signaling is required for anterior expression of *nk2.2* during later somitogenesis stages. Anterior margin cells expressing *nk2.2* and *lim3* are initially adjacent to *shh*-expressing neural cells and become separated from the region of *shh* expression (arrow). *fgf8* is expressed in the anterior telencephalon, with expression adjacent to the dorsalmost region of the forming adenohypophysis. Phase 3: Hh signaling is required to maintain the expression of *nk2.2* in the anterior region of the adenohypophysis between 24 and 35 h of development. By 30 h, Prolactin (PRL)-secreting cells arise within the *nk2.2*-expressing anterior region, while Somatolactin (SL)-secreting cells are present throughout the *lim3*-expressing adenohypophysis. *shh* is expressed in overlying neural tissue and in endodermal tissue ventral to the adenohypophysis at these stages. All drawings are tracings of *shh*-labeled wild-type embryos.

indicate that expression of *shh* in anterior neural tissue also induces and patterns adjacent nonneural tissue, the developing epidermal cells in the adenohypophysis. A similar role for Shh has recently been described in the formation of pancreatic endocrine cells that arise from a distinct cell type, the endoderm (diIorio et al., 2002; Roy et al., 2001). Shh was shown to play multiple roles in pancreas islet cell development, being necessary both for initial induction and for proper cell positioning (diIorio et al., 2002). Interestingly, many of the transcription factors that respond to Hh signaling in neural tissues (*nk2.2, pax,* and *lim* genes) are similarly regulated in the nonneuronal cells of the pituitary and pancreas as they differentiate in response to Hh signals. The similar response in these distinct tissues (epidermis, endoderm, and neural ectoderm) suggests that Hh signaling helps convey positional information (distance from the source of Hh) to diverse endocrine precursor cells.

Zebrafish Hh mutants as a model for human holoprosencephaly

There is a broad spectrum of developmental phenotypes associated with mutations that disrupt Hh signaling in humans. Holoprosencephaly (HPE) is a major human congenital disorder and approximately 17% of human familial HPE is associated with SHH mutations (Muenke and Cohen, 2000). HPE phenotypes range from the most severe, including cyclopia and loss of the pituitary gland, to less severe forms that include closely spaced eyes, a single midline incisor, and hypopituitarism (Cameron et al., 1999; Johnson, 1989; Kjaer and Fischer-Hansen, 1995). In these less severe cases, pituitary deficiencies lead to decreased levels of Adrenocorticotropin (ACTH) or Growth Hormone (GH) (Cameron et al., 1999; Rappaport et al., 1977), which result in adrenal hypoplasia and short stature, respectively. Our panel of zebrafish Hh signaling mutants carry mutations in different components of the Shh pathway and have a range of pituitary phenotypes (see Figs. 6 and 7; Table 5) that may correlate to reported human pituitary defects, including specific hormone deficiencies (Cameron et al., 1999; Rappaport et al., 1977). In particular, our analysis shows that mutations in the Gli transcription factors may form the molecular basis of subtle forms of HPE that would be associated with specific pituitary patterning defects. Continued analysis of the zebrafish Hh pathway mutants thus promises to help explain how milder, nonlethal mutations in Hh signaling can lead to subtle pituitary defects that might have profound consequences for human development. The genetic and experimental accessibility of the zebrafish thus make it a powerful model system to better understand the molecular

nature of HPE and other disorders affecting pituitary development in humans.

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