

pitx3 defines an equivalence domain for lens and anterior pituitary placode

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Summary

Hedgehog signaling is required for formation and patterning of the anterior pituitary gland. However, the role of Hedgehog in pituitary precursor cell specification and subsequent placode formation is not well understood. We analyzed pituitary precursor cell lineages and find that *pitx3* and *distal-less3b* (*dlx3b*) expression domains define lens and pituitary precursor positions. We show that *pitx3* is required for pituitary pre-placode formation and cell specification, whereas *dlx3b* and *dlx4b* are required to restrict pituitary placode size. In *smoothened* mutant embryos that cannot transduce Hedgehog signals, median pituitary precursors are mis-specified and form an ectopic lens. Moreover, overexpression of *sonic hedgehog* (*shh*) blocks lens formation, and derivatives of lens precursors express genes characteristic of pituitary cells. However,

overexpression of *shh* does not increase median pituitary placode size nor does it upregulate *patched* (*ptc*) expression in pituitary precursors during early somitogenesis. Our study suggests that by the end of gastrulation, *pitx3*-expressing cells constitute an equivalence domain of cells that can form either pituitary or lens, and that a non-Hedgehog signal initially specifies this placodal field. During mid-somitogenesis, Hedgehog then acts on the established median placode as a necessary and sufficient signal to specify pituitary cell types.

Key words: Adenohypophysis, Anterior neural plate border, Cell fate specification, Cell lineage, Distal-less, Patched, Smoothened, Sonic hedgehog, Ventral ectoderm, Zebrafish

Introduction

The pituitary gland is a key regulator of hormone secretion in vertebrates. The anterior lobe of the pituitary contains several cell types distinguished by their morphologies, locations and secreted hormones (Bentley, 1998; Dasen and Rosenfeld, 1999a; Dasen and Rosenfeld, 1999b). In mammals and birds, the anterior pituitary forms from Rathke's pouch, an invagination of oral ectoderm beneath the ventral forebrain, whereas in zebrafish, the anterior pituitary derives from a placode that forms between the anterior hypothalamus and ventral ectoderm (Glasgow et al., 1997). Although several studies analyzed anterior pituitary precursor cells (Houart et al., 1998; Kozlowski et al., 1997; Whitlock and Westerfield, 2000), the precise origin of precursor cells is not well understood. Most studies suggest a dual origin for anterior pituitary; it derives either from the anterior neural ridge or from the non-neural ectoderm (Couly and Le Douarin, 1988; Dasen and Rosenfeld, 1999b; Eagleson et al., 1995; Eagleson and Harris, 1990; Kawamura and Kikuyama, 1992; Kondoh et al., 2000; Sheng and Westphal, 1999).

Several transcription factors demarcate the presumptive pituitary and lens placodal field. Presumptive lens ectoderm in *Xenopus* expresses *Otx2*, *Pax6* and *Sox3*, and in zebrafish,

expression of *dlx3b* demarcates olfactory (Whitlock and Westerfield, 2000; Zygari et al., 1998) and otic placode precursor cells (Akimenko et al., 1994; Liu et al., 2003; Solomon and Fritz, 2002). We recently showed that *dlx3b*, *dlx4b*, *sox9a* and fibroblast growth factor (Fgf) signaling interact to specify the zebrafish otic placode (Liu et al., 2003). Because *dlx3b* and *dlx4b* are also expressed around the anterior border of the neural plate, these factors might also play a role in formation of the anterior pituitary.

Analysis of zebrafish Hedgehog pathway mutants, *you-too* (*gli2*) (Karlstrom et al., 1999), *iguana* (Kondoh et al., 2000) and *smu* (*smoothened*) (Varga et al., 2001), suggests that Hedgehog signaling is required for anterior pituitary placode specification. The observations that zebrafish Hedgehog pathway mutants form ectopic lenses at the expense of pituitary and that early Rathke's pouch expresses lens Delta-crystallin in chick suggest that these apparently unrelated tissues might arise from common precursors and that Hedgehog may be required to specify the pituitary lineage (Karlstrom et al., 1999; Kondoh et al., 2000; Varga et al., 2001). Other studies suggest that pituitary and lens cells 'transdifferentiate' in Hedgehog pathway mutants (Kondoh et al., 2000). In zebrafish, overexpression of *Shh* suggests that Hedgehog signaling is sufficient to pattern the anterior pituitary and induce pituitary

specific gene expression (Herzog et al., 2003; Sbrogna et al., 2003). In mouse, *Shh* is thought to regulate cell proliferation and cell-type specification (Treier et al., 2001) indirectly (Takuma et al., 1998).

Several homeodomain transcription factors may control specification of pituitary cell types in a combinatorial manner and are likely targets of signaling interactions (Dasen and Rosenfeld, 1999a). *Lhx3*, for example, is expressed initially in all cells of Rathke's pouch, is required for proliferation of ventral cell types (Bach et al., 1995; Zhadanov et al., 1995), and controls gene expression in thyrotrope and gonadotrope cells in synergy with recently identified *Pitx* genes (Bach et al., 1997; Tremblay et al., 1998). The *Pitx* family members belong to a *Bicoid-related* subclass of homeobox genes. *Pitx1* binds to and transactivates a *cis*-acting element required for proopiomelanocortin (*Pomc*) (Lamonerie et al., 1996) and other pituitary-specific gene expression, including *Lhx3* and *prolactin* (Szeto et al., 1999; Tremblay et al., 1998). In mouse, lens and mesencephalon, but not anterior pituitary, express *Pitx3*. Mutations in *Pitx3* lead to dominant cataracts and malformations of the anterior eye mesenchyme and its derivatives (Nunes et al., 2003; Semina et al., 2000). In *Xenopus*, stomodeum, lens and pituitary express *Xpitx3* (Pommereit et al., 2001).

To understand whether Hedgehog acts directly on pituitary and lens placode precursor cells and whether Hedgehog is sufficient to induce placode formation, we used lineage tracers to define the locations and gene expression patterns of pituitary and lens precursor cells precisely. We then analyzed placode specification in Hedgehog gain- and loss-of-function experiments.

Our results indicate that by the end of gastrulation, *pitx3* expression demarcates an equivalence domain of cells that gives rise to lens and pituitary. This domain overlaps partially with the *dlx3b* and *dlx4b* expression domains at the anterior neural plate border. *pitx3* is required at bud stage for pituitary pre-placode formation, whereas *dlx3b* restricts pituitary placode size during somitogenesis. During mid-somitogenesis, Hedgehog signaling is necessary for pituitary placode specification and is sufficient to induce expression of pituitary genes in *pitx3* expressing pre-placode. Hedgehog signaling is also sufficient to block *pitx3* expression in presumptive lens precursors and subsequent lens tissue differentiation. We suggest that a non-Hedgehog signal initially induces an unspecified *pitx3* expressing pre-placodal field around the anterior border of the neural plate. Later, during mid-somitogenesis Hedgehog signaling specifies pituitary characteristics in the placode that forms from the midline of the pre-placode.

Materials and methods

Animals and embryonic staging

Wild-type and *smu*^{b641} mutant (Varga et al., 2001) zebrafish (*Danio rerio*) embryos were obtained and maintained with standard procedures (Westerfield, 2000) at 28.5°C. Developmental stages described by standard terms (Kimmel et al., 1995) or hours post fertilization (h). Gene symbols according to ZFIN (<http://zfin.org>).

Cloning of *pitx3* cDNA and phylogenetic analysis

Mouse and human *Pitx2* homeodomain sequences were used to design

degenerate PCR primers: *Pitx1*, 5' CYAGCCAGCAGCTSCASG-AGCTGGA 3'; *PitxR1*, 5' AGGCCCTTGGCDGCCCARTTGTTGTA 3'. These primers amplified an ~275 bp fragment from a 16- to 18-somite zebrafish cDNA library (B. Appel) that we cloned and used as probe to screen the cDNA library. A full-length cDNA was obtained and subcloned into pCS2+ vector (Accession Number AY525643). Parsimony and nearest neighbor joining phylogenetic analyses were performed with Paup (Sinauer Associates), ClustalX (Thompson et al., 1997) (<ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>) and NJPlot software (M. Gouy, UMR CNRS 5558, Université Lyon).

Single cell labeling, lineage tracing and fate mapping

To predict the locations of cells relative to morphological landmarks and gene expression domains in live, unlabeled embryos, we performed double in situ hybridization on 10-15 size-selected (590±5 µm diameter), staged embryos as described (Varga et al., 1999) and averaged gene expression domains of *pitx3* and *dlx3b*. Single-cell labeling with vital fluorescent dyes and cell fate mapping were conducted (Varga et al., 1999) using a 10×10 grid reticule (19 mm diameter, KR406; Zeiss) in the ocular (10×) of a fixed stage microscope (Zeiss Axioscope) and a differential amplifier (A-M Systems, Neuroprobe Amplifier 1600). At prim-5 stage (24 h), the descendants of the labeled progenitor cells were analyzed using fluorescence and confocal laser scanning microscopy (Zeiss LSM 510). Serial 1.6 µm optical sections (0.5 µm overlap between consecutive sections) were projected onto 2D images using Zeiss Software.

Whole-mount mRNA in situ hybridization, lineage tracer detection and immunofluorescence

Embryos were fixed and hybridized with one or two mRNA probes (Hauptmann and Gerster, 1994; Varga et al., 1999). We used mRNA probes for *pitx3* and *dlx3b* as landmarks for the prospective placodal field and mRNA probes for *pomc*, *ptc1*, *ptc2*, *nkx2.2*, *lhx3*, *bmp2b*, *shh*, *eyes absent1 (eya1)*, *foxb1.2* and *pax6a*. Fast Red (Boehringer, Sigma) was the substrate for alkaline phosphatase color reaction to detect one mRNA probe and the lineage tracer and NBT and BCIP (Boehringer Mannheim) were used to detect the other mRNA probe. This allowed us to detect fluorescein labeled mRNA probe and fluorescein-dextran injected cells at the same time. We labeled lens fiber cells with z1-1 (Trevarrow et al., 1990) monoclonal antibody (Zebrafish International Resource Center) as described (Varga et al., 2001).

Photoactivation of fluorescein

To label groups of precursor cells, we injected one-cell stage embryos with 1% DMNB-caged fluorescein (Molecular Probes) in 0.2 M KCl. At bud stage, we uncaged cells in regions of the *dlx3b* and *pitx3* expression domains that include lens or pituitary precursors. UV light directed through a pinhole diaphragm, ~40 µm diameter or larger, was used for photoactivation and we detected uncaged fluorescein at prim-5 stage using fluorescence and confocal microscopy (Zeiss LSM510), or antibody labeling in combination with in situ hybridization as described above.

Morpholino antisense oligonucleotide and mRNA injection

To study the roles of *pitx3*, *dlx3b* and *dlx4b* in pituitary and lens formation, we injected 5-8 ng of morpholino antisense oligonucleotides into one-cell stage embryos: *pitx3* morpholino, 5'-AGGTTAAAATCCATCACCTCTACCG-3'; *dlx3b* morpholino, 5'-ATGTCGGTCCACTCATCCTTAATAA-3'; *dlx4b* morpholino, 5'-GCCCCGATGATGGTCTGAGTGCTGC-3' (Liu et al., 2003). Per embryo, we injected ~8-10 ng of 8 µg/µl (in 0.2 M KCl) *pitx3* morpholino; 5-7 ng each *dlx3b* and *dlx4b* morpholinos; and 5 ng each *pitx3*, *dlx3b* and *dlx4b* morpholinos. Per embryo, we injected *pitx3:eGFP* (600 pg); *5Δpitx3:eGFP* (600 pg); *shh* (10-100 pg); and

dominant-negative Protein Kinase A (dnPKA:GFP, 1 ng). mRNAs were synthesized in vitro (mMessage Machine Kits, Ambion). The embryos were analyzed morphologically at bud (10 h), prim-5 and long-pec (48 h) stages, and fixed in 4% paraformaldehyde. We analyzed 16 μm cryosections to identify cells doubly labeled with photoactivated fluorescein and *lim3* mRNA probe.

To ensure that the *pitx3* morpholinos block translation of *pitx3* mRNA, we co-injected *pitx3* mRNA (see Fig. S1A-D in the supplementary material), *pitx3:eGFP* mRNA that encodes the normal 5' UTR of *pitx3* fused to the *eGFP* open reading frame (Fig. S1E-G), or a 5'UTR that contains five modified bases (*5Δpitx3:eGFP*) (see Fig. S1H,I in the supplementary material).

Cell transplantation

Wild-type embryos were injected with *dnPKA:GFP* mRNA and rhodamine-dextran (2.5%). The *dnPKA* mRNA and rhodamine-injected embryos were used as donors at dome stage and we transplanted 10 to 20 cells from the animal pole to wild-type host animal poles. Donor and host embryos were maintained as pairs in 24-well dishes in penicillin and streptomycin (1% each) in Danieau's solution. At prim-5 stage, donors were analyzed for loss of lens phenotype that resulted from *dnPKA* expression; hosts were analyzed only if the donor sibling lacked both lenses.

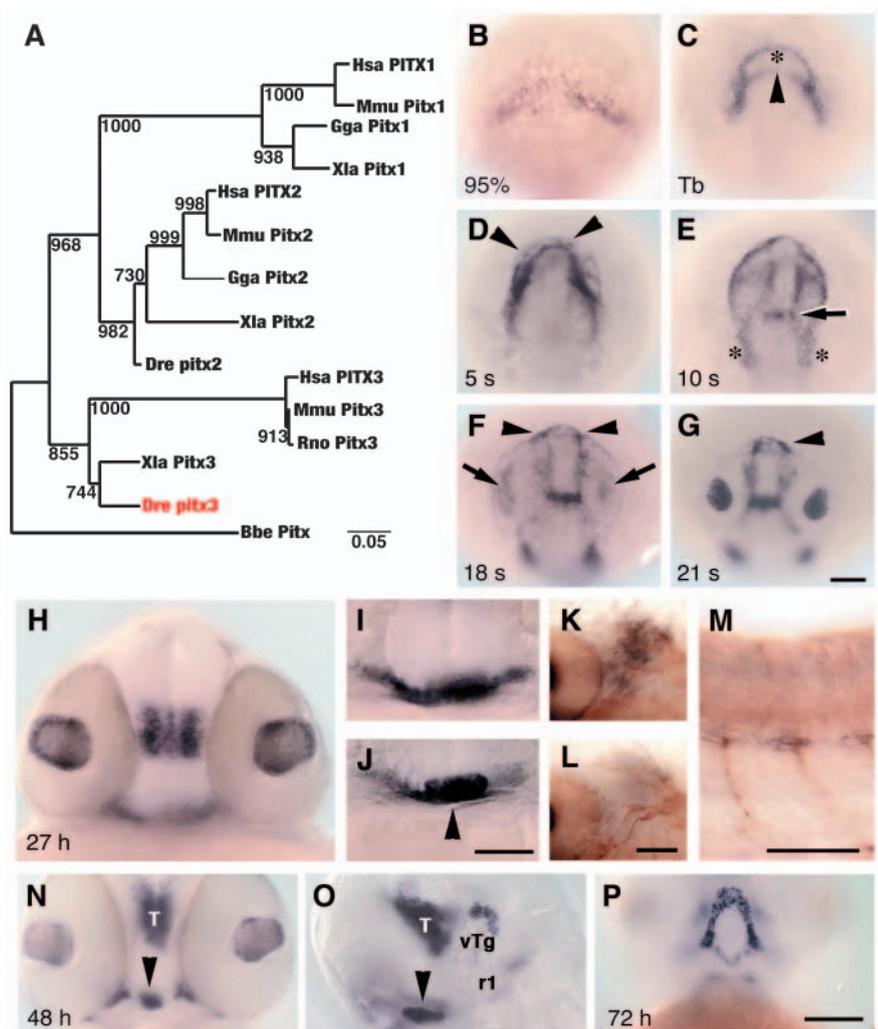
Results

Anterior neural plate border, lens and pituitary cells express *pitx3*

We cloned a novel zebrafish *Pitx* gene and studied its phylogenetic relationship, temporal and spatial expression patterns, and its role in pituitary and lens development. Nearest neighbor-joining analysis of the amino acid sequence of the full-length protein shows that the gene encodes zebrafish *Pitx3*. Like *Pitx2*, zebrafish *Pitx3* is more closely related to *Xenopus Pitx3* than to mammalian *Pitx3* orthologs (Fig. 1A). Analysis of the homeodomain amino-acid sequence further supports this assignment: zebrafish, *Xenopus* and mouse *Pitx3* homeodomains are 100% identical, the human homeodomain ortholog is 98% identical. The zebrafish *Pitx3* homeodomain is less similar to the *Pitx1* and *Pitx2* homeodomains of other vertebrates: 98% identical to *Xenopus Pitx1*, and 97% identical to mouse and human *Pitx1* homeodomains.

Cells at the anterior neural plate border and, later, lens and pituitary cells express *pitx3*. We detect zebrafish *pitx3* expression by 95% epiboly in anterior neural plate border cells

Fig. 1. Zebrafish lens and pituitary cells express *Pitx3*. (A) Nearest-neighbor joining tree of full-length *Pitx* amino acid sequences. Zebrafish *Pitx3* (red) groups with *Xenopus* and mammalian *Pitx3* proteins. Bootstrap analysis: random number seed, 111; numbers on branches indicate the number of times each node was obtained in 1000 runs. *Danio rerio* (*Dre*, AF181681), *Homo sapiens* (*Hsa*, P78337, AF238048, AF041339), *Mus musculus* (*Mmu*, NM_011097, NM_011098, NM_008852), *Rattus norvegicus* (*Rno*, NM_019334, NM_019247), *Gallus gallus* (*Gga*, AF069397, AF076640), *Xenopus laevis* (*Xla*, AF217647, AF077767, AF297713) and *Branchiostoma belcheri* (*Bbe*, AF195616). (B-J) In wild-type embryos, *pitx3* is expressed (black) at (B) 95% epiboly; (C) bud stage, in two domains that outline underlying polster (asterisk) and anterior neural plate border (arrowhead); (D) five-somite stage, in cells (arrowheads) around the anterior neural keel; (E) 10-somite stage, presumptive first branchial arch precursors (asterisks) and presumptive forebrain-midbrain border (arrow); (F,G) 18- and 21-somite stages, in prospective lens (arrows) and pituitary placodes (arrowheads); (H) 27 h, in lens and ventral posterior diencephalon; (I) 27 h, in ventral head mesenchyme; (J) 27 h, pituitary (arrowhead). (K-M) 27 h. Double labeling with mRNA probe for *pitx3* (black) and anti-acetylated tubulin (brown) indicates that branchial arch mesenchyme (K), which is posterior to eye and ventral to trigeminal placode (L), and trunk muscle pioneer cells (M) express *pitx3*. (N-P) At 48-72 h, cells in pituitary (arrowheads), thalamus (T), ventral tegmentum (vTg), first rhombomere (r1) and Meckel's cartilage (P) express *pitx3*. (B-G) Dorsal view of prospective head region, ventral towards the top; (H-J,N) frontal view, dorsal upwards; (K,L) dorsal view, anterior towards left, different focal planes of same region; (M,O) side view, anterior towards left and dorsal towards top; (P) ventral view, anterior towards top. Scale bars: 100 μm in B-G,H-J,N-P; 50 μm in K-M.



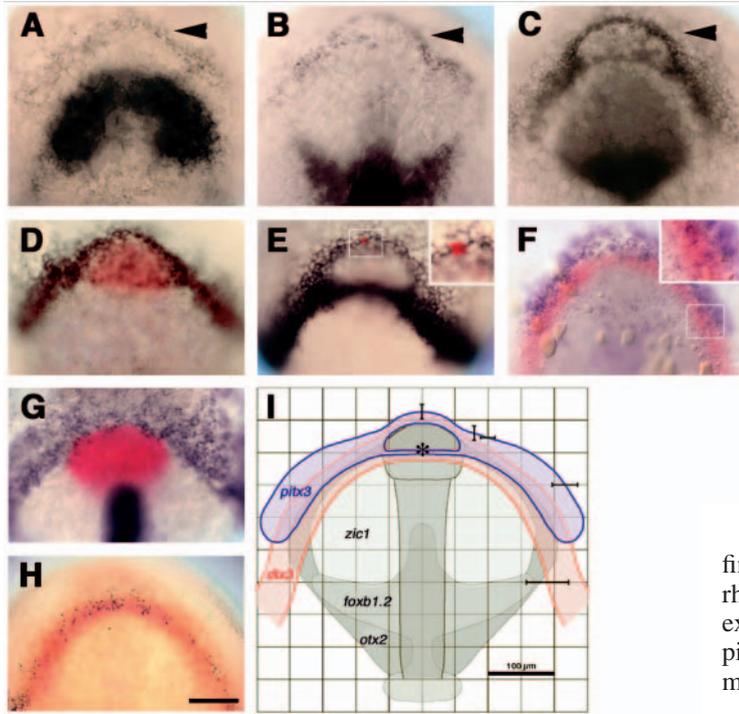


Fig. 2. *pitx3* and *dlx3b* form overlapping expression domains at the anterior neural plate border. (A–D) Embryos labeled with probes for *pitx3* (arrowheads) and (A) *zic1*, (B) *foxb1.2*; (C) *pax6a* or (D) *hgg1* (red). (E) Anterior neural plate border cells express *dlx3b*. (E, inset) Single cell injected with fluorescein-dextran, labeled for lineage tracer (red). (F–H) A subset of cells co-expresses: (F) *pitx3* (blue) and *dlx3b* (red); (G) *bmp2b* (blue, non-neural ectoderm), *shh* (blue, neural plate midline) and *hgg1* (red, polster); (H) *dlx3b* (red) and *eya1* (black). (I) Sketch of anterior neural plate gene expression domains (*pitx3*, blue, $n=12$; *dlx3b*, red, $n=15$; *zic1*, *foxb1.2*, *otx2*, grey) (Varga et al., 1999) to predict location of *pitx3*-expressing cells in live unlabeled embryos. Averaged gene expression domains; bars indicate standard deviations from the mean, relative to neural plate midline and center of polster (asterisk). All panels at bud stage, except C, which is at the one-somite stage. Dorsal views of prospective head region, ventral towards the top. Scale bars: 100 μm .

(Fig. 1B). At bud stage, the *pitx3* expression domain resembles two inverted u-shaped regions stacked on top of each other (Fig. 1C). These *pitx3*-expressing cells are located in a row that is two to four cells wide at the transition between non-neural ectoderm and prospective placode (anterior to the polster) and a row at the presumptive transition between prospective placode and neural plate (posterior to the polster). Farther posteriorly and laterally, presumptive lens placode precursors express *pitx3*. Ectodermal cells superficial to the polster (between the two *pitx3*-expressing rows) express *pitx3* at low levels (Fig. 1C, asterisk; Fig. 2C,D). At the five-somite stage, *pitx3* expression is downregulated in most of the anterior expression domain and only small patches of expression remain (Fig. 1D). At the ten-somite stage, *pitx3* is upregulated in a region that presumably corresponds to the border that later forms between forebrain and midbrain (Fig. 1E). In addition, *pitx3* is expressed at this stage in the border between non-neural ectoderm and prospective placodes, in presumptive first branchial arch, in ventral head mesenchyme, and between the eye primordia and diencephalon. We also find *pitx3* expression in prospective lens ectodermal cells on the surface of the eye primordia (Fig. 1E). At the 18-somite stage (18 h), *pitx3* expression becomes more prominent in lens epithelial precursors and in bilateral expression domains at the anterior end of the forebrain that correspond to previously described *lim3* expression in presumptive pituitary placode (Fig. 1F, arrowheads) (Glasgow et al., 1997; Herzog et al., 2003; Sbrogna et al., 2003). From the 21-somite stage (19.5 h) through prim-10 stage (27 h), lens, pituitary, head mesenchyme and ventral diencephalic cells express *pitx3* (Fig. 1G–J) as well as mesenchymal cells of the presumptive first branchial arch (Fig. 1K,L). In trunk somites, presumptive muscle pioneers express *pitx3* (Fig. 1M). At long-pec (48 h) and protruding-mouth (72 h) stages, ventral diencephalic cells and lens fiber cells downregulate *pitx3* expression (Fig. 1N). In addition, we

find that presumptive nucleus coeruleus cells in the first rhombomere (Holzschuh et al., 2001) and tegmental cells express *pitx3* (Fig. 1O). At protruding-mouth (72 h) stage, pituitary cells and the primordia of the first and second mandibular arches express *pitx3* (Fig. 1P).

***pitx3* expression at the neural plate border demarcates lens and pituitary cell fates**

To test whether *pitx3* expression demarcates pituitary and lens precursors at bud stage, we analyzed the fate of *pitx3*-expressing cells using embryonic morphology and the *pitx3* and *dlx3b* expression domains as landmarks for single cell lineage analysis. We correlated the expression domain of *pitx3* with *zic1*, *foxb1.2*, *pax6a* (Fig. 2A–C) and *otx2* (not shown) that we used previously to fate map the anterior neural plate (Varga et al., 1999). Because Pax6 has been shown to regulate both lens and eye development (Ashery-Padan and Gruss, 2001; Kondoh, 1999; Zygar et al., 1998), we did not use *pax6a* and *pax6b* as neural plate landmarks or in functional assays. However, because *dlx3b* has been shown to demarcate prospective olfactory (Kondoh, 1999; Whitlock and Westerfield, 2000; Zygar et al., 1998) and otic placode cells (Akimenko et al., 1994; Liu et al., 2003), we compared *pitx3* expression with *dlx3b* expression (Fig. 2E,F) and find that the expression domains overlap significantly (Fig. 2F).

To understand the spatial relationship between Hedgehog-producing cells and placodal precursors, we analyzed where *pitx3*-positive cells are located relative to median *shh*-expressing cells. Because polster cells express *hgg1* at bud stage just beneath the superficial *pitx3*-expressing ectoderm (Fig. 2D), we also used *hgg1* expression to correlate the locations of median neural plate border cells, ventral ectoderm and *shh* expression. At bud stage, we find that prechordal plate cells and cells in the midline of the neural plate express *shh* (Fig. 2G). Thus, the median ectodermal cells that express *pitx3* near the polster, are near Hedgehog-secreting cells in the median neural plate (Fig. 2G), suggesting that these cells are in a position to receive significant levels of Hedgehog signal. We also analyzed other genes that demarcate ventral ectoderm and the border of the neural plate such as *bmp2b* (Fig. 2G) and *eya1* (Fig. 2H). However, we did not use the *eya1* expression domain to establish the pituitary and lens fate map, because

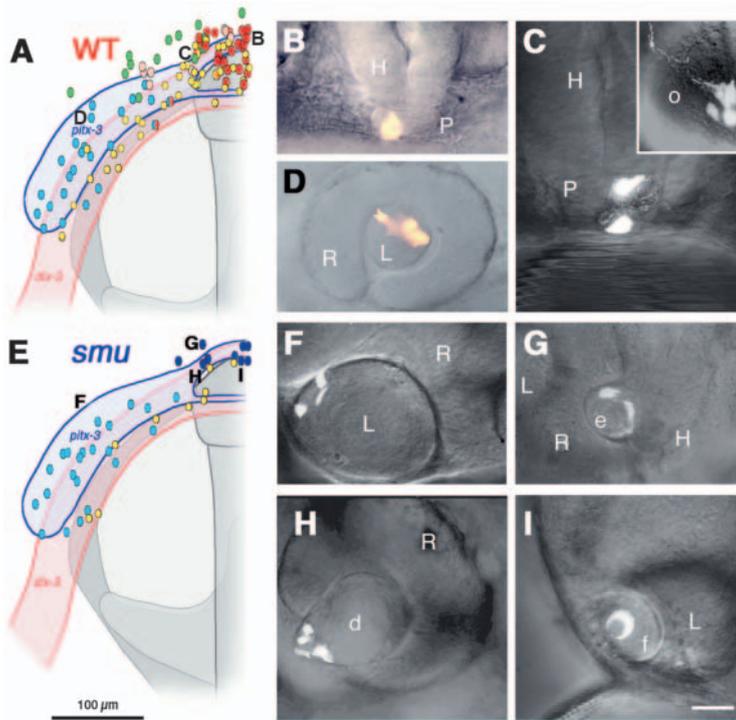


Fig. 3. Pituitary precursors form lens in *smoothened* mutant embryos. (A–D) Wild-type embryos, (E–I) *smu* mutant embryos. Colored dots and letters in A,E represent locations of injected precursor cells at bud stage and their fates at prim-5 stage (B–D,F–I). (A) Fate map of wild-type embryo ($n=105$) shows pituitary precursor cells (red) at anterior midline (B, $n=22$), lens precursor cells (blue) at lateral neural plate border (D, $n=21$) and olfactory placode precursors (yellow) closer to neural plate (C, inset, $n=37$). (C) Split fates from a single precursor cell (yellow/red dots in A) contribute to pituitary and olfactory placode. Green dots, epidermal precursors; pink dots, head mesenchymal precursors. (E) Fate map of *smoothened* mutant embryo ($n=33$). Precursor cells give rise to ectopic median (dark blue) or normal retinal lenses (light blue); yellow, olfactory placode precursors ($n=9$). (F) Precursor cells from lateral *pitx3* domain (light blue) give rise to lens epithelial and primary lens fiber cells. (G–I) Median placodal precursors give rise to ectopic (G), distorted (H) or fused (I) lens, and not to pituitary as expected from median positions in wild-type embryos (A, red). Ventral ectoderm and head mesenchyme cells not shown for clarity. d, distorted lens; e, ectopic lens; f, medially fused lens; H, hypothalamus; L, retina associated lens; o, olfactory placode; P, pituitary; R, retina. (A,B) Dorsal view, prospective head, anterior towards the top (compare with Fig. 2I). (B,C,G) Frontal view, dorsal towards the top. (D,F,H,I) Side view, anterior towards the left. Scale bar: 100 μm in A,E; 50 μm in B,D,E; 25 μm in C,F–I.

eyal expression at the anterior neural plate border overlaps with the *dlx3b* expression domain (Fig. 2H) that gives rise to olfactory placode (Whitlock and Westerfield, 2000). Although *eyal* is expressed in the olfactory placode at prim-5 (24 h) stage, it is downregulated in lens during late somitogenesis (Sahly et al., 1999).

Pituitary precursor cells are mis-specified in *smoothened* mutant embryos

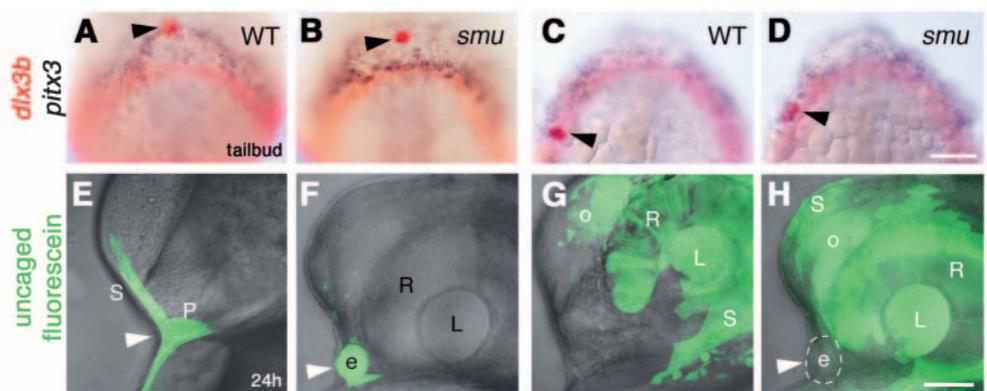
We have previously shown that *smoothened* mutant embryos that cannot transduce Hedgehog signals lack pituitary and instead form ectopic lenses (Varga et al., 2001) (see Fig. S2 in the supplementary material). This could occur because median

pituitary precursors are mis-specified and form lens at the expense of pituitary or, alternatively, because lens precursors aberrantly invade the midline.

To distinguish between these possibilities, we fate mapped the cells that give rise to pituitary and lens in wild-type and *smoothened* mutant embryos. We labeled cells at bud stage, using *pitx3* and *dlx3b* as landmarks for lens and pituitary precursors, and analyzed their progeny at prim-5 stage.

In wild-type embryos, we find that *pitx3*- and *dlx3b*-expressing cells at the midline of the anterior neural plate border contribute to pituitary (Fig. 3B, and red dots in 3A) or olfactory placodes (Fig. 3A, yellow dots). Precursors closer to non-neural ectoderm mainly form pituitary cells (Fig. 3A, red

Fig. 4. Ectopic lens formation in *smoothened* mutant embryos does not result from aberrant movement of lens precursors. (A–D) Bud stage embryos fixed immediately after photoactivation, then hybridized with probes for *pitx3* (blue) and *dlx3b* (red). Photoactivated median anterior cells (A,B; $n=14$) and lateral posterior cells (C,D; $n=11$) in wild-type (A,C) and *smoothened* mutant (B,D) embryos labeled with anti-fluorescein (red, black arrowheads). (E–H) Embryos at prim-5 stage, after uncaging at bud stage as indicated in A–D. (E) Wild-type; skin and anterior pituitary placode are labeled ($n=10$; white arrowhead). (F) *smoothened* mutant; ectopic lens (white arrowhead) and skin (F; $n=7$) are labeled (photoactivation as indicated in A,B). (G,H) Wild-type (G; $n=19$) and *smoothened* mutant (H; $n=7$), skin, lens, retina and olfactory placode labeled (photoactivation approximately as indicated in C,D). (H) Ectopic median lens (white arrowhead, circle) is not labeled in *smoothened* mutant embryos ($n=7$). More cells were labeled in G,H than indicated in control embryos (C,D). (E–H) Superimposed bright-field images and fluorescent confocal stacks. e, ectopic lens; L, retina-associated lens; o, olfactory placode; P, pituitary; R, retina; S, skin. (A–D) Dorsal views of prospective head region, anterior towards top; (E–H) side views, anterior towards the left, dorsal towards the top. Scale bars: 100 μm in A–D; 50 μm in E–H.



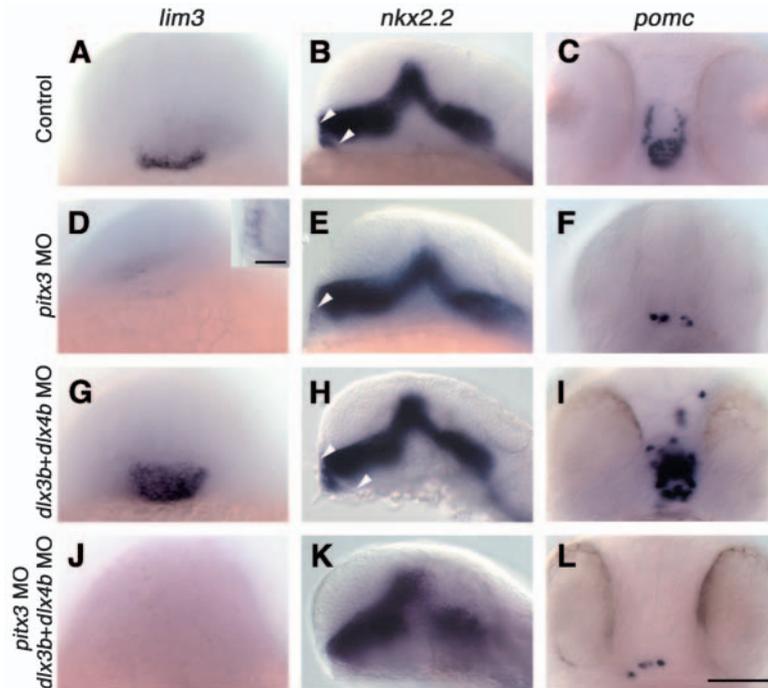


Fig. 5. *pitx3* is required for pituitary placode formation, whereas *dlx3b* and *dlx4b* control pituitary placode size. Wild-type (A-C) and morpholino (MO)-injected (D-L) embryos labeled with probes for *lim3* (A,D,G,J), *nkx2.2* (B,E,H,K) and *pomc* (C,F,I,L). (D-F) Injection of *pitx3* morpholinos ($n=94$) leads to loss of pituitary placode (20/94 embryos) or severe reduction in pituitary cell number (D, inset; $n=69/94$). (G-I) Injection of *dlx3b* and *dlx4b* morpholinos ($n=103$) leads to enlarged pituitary placodes and increased pituitary cell numbers ($n=78/103$), arrowheads in B,E,H. (J-L) Injection of *pitx3*, *dlx3b* and *dlx4b* morpholinos ($n=83$) leads to loss of pituitary placode (42/83 embryos) or severe reduction in pituitary cell number ($n=25/83$). (A,C,D,F,G,I,J,L) Frontal views, dorsal towards the top; (B,E,H,K) side views, anterior towards the left. Scale bars: 100 μm in A-L; 25 μm in D, inset.

dots) or ventral ectoderm (Fig. 3A, green dots), and cells closer to the neural plate have a greater tendency to contribute to olfactory placode (Fig. 3A, yellow dots).

In general, we find that single precursor cells at bud stage give rise to progeny with a single cell fate (e.g. lens, pituitary or olfactory cells), indicating a high degree of cell specification (Fig. 3B). However, we find that these separate populations of precursor cells intermingle even though they are individually specified for one fate (Fig. 3A; single-colored dots). In only a few cases, did we observe dual (or split) cell fates of daughter cells (for example olfactory and pituitary) derived from a single precursor (Fig. 3A,C; two-colored dots).

Lens cells derive from lateral positions in the *pitx3* and *dlx3b* expression domains (Fig. 3A, blue dots). They intermingle with precursors of non-neural ectoderm on the ventral side (Fig. 3A, green dots) and with olfactory placode precursors closer to the neural plate (Fig. 3A, yellow dots). Thus, we find that lens precursors (Fig. 3A, blue dots) are usually farther from the neural plate and express *pitx3*, whereas *dlx3b*-expressing cells closer to the neural plate mainly contribute to olfactory placode (Fig. 3A, yellow dots). The large degree of intermingling of these two placodal cell fates is consistent with the overlap of the two gene expression domains and indicates that placodal

fields have not yet resolved by this developmental stage (Whitlock and Westerfield, 2000).

In *smoothened* mutant embryos, lateral *pitx3*- and *dlx3b*-expressing cells also give rise to lens (Fig. 3E,F). In the midline, however, in the region that corresponds to the location of pituitary precursors in wild-type embryos (Fig. 3A,B), precursors (dark blue dots) give rise to daughter cells in ectopic (Fig. 3G), distorted (Fig. 3H) and fused lenses (Fig. 3I). Thus, in the absence of Hedgehog signal transduction, median placode precursors are mis-specified and form lens instead of pituitary.

Lens precursor cells do not invade the midline in *smoothened* mutant embryos

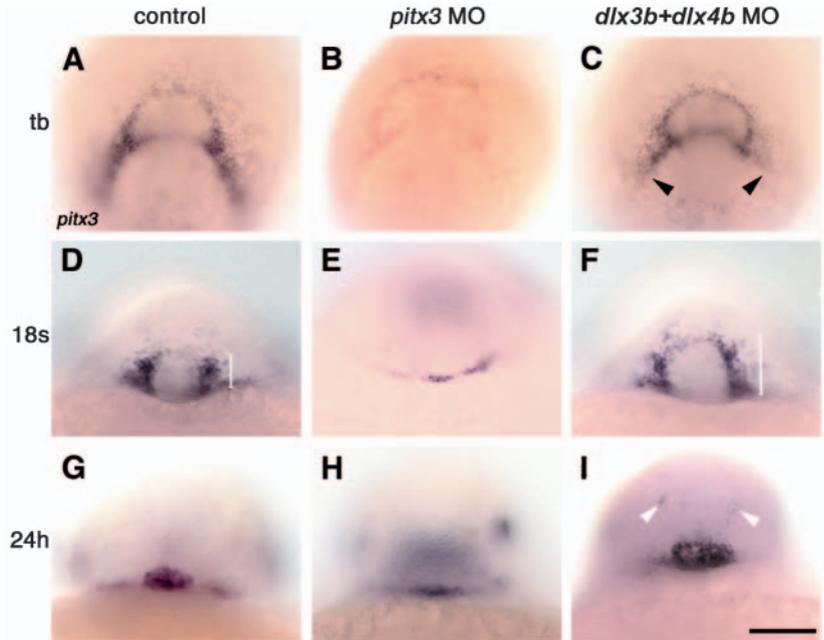
We tested whether lens precursors invade the midline of the anterior neural plate border in *smoothened* mutant embryos by photoactivating DMNB-caged fluorescein in lens precursors in wild types and mutants. Because large numbers of cells are labeled, this approach allows us to track cell movements that might have been missed previously with single cell labeling. When we uncage fluorescein in the anterior midline of the *pitx3* expression domain at bud stage (Fig. 4A,B), we find that pituitary and skin are labeled at prim-5 stage in wild-type embryos (Fig. 4E; arrowhead). In *smoothened* mutant embryos, however, when we uncage in the anterior midline, the ectopic median lens (Fig. 4F; arrowhead) and skin are later labeled (Fig. 4B,F). When we uncage in cells farther lateral in the *pitx3* and *dlx3b* expression domain (Fig. 4C,D), we find that in both wild-type and *smoothened* mutant embryos, skin, lens, olfactory placode, telencephalon and retina are labeled later (Fig. 4G,H). Typically, almost the entire retina associated lens is labeled in wild-type and mutant embryos, whereas in *smoothened* mutant embryos, the ectopic median lens is never labeled (Fig. 4H; arrowhead). Our results

indicate that in *smoothened* mutant embryos, lens precursors do not invade the midline aberrantly and that apparently the entire *pitx3* expression domain gives rise to lens. Thus, lateral cells contribute to the retinal lens and median cells that form pituitary in wild-type embryos are mis-specified and form ectopic lens in *smoothened* mutants.

Pitx3 is required for pituitary cell specification, whereas Dlx3b controls pituitary placode size

To analyze the roles of *pitx3*, *dlx3b* and *dlx4b* in pituitary development, we injected *pitx3*, *dlx3b* and *dlx4b* morpholinos and tested for expression of pituitary marker genes such as *lim3*, a pan-pituitary marker (Glasgow et al., 1997), the Hedgehog target gene *nkx2.2* and *pomc* (Herzog et al., 2003), a gene expressed in corticotropes and melanotropes. We find that 'knock-down' of *pitx3* causes reduction or complete loss of *lim3*, *nkx2.2* and *pomc* expression in pituitary (Fig. 5A-F), indicating that *pitx3* is required for pituitary cell specification. We find that *pitx3* is required in a dose-dependent manner; high doses of morpholinos (8-10 ng/embryo) lead to complete loss of pituitary placode (Fig. 5D-F), whereas less morpholino (2-4 ng/embryo) leads to a smaller placode, and variably reduced or lost expression of *lim3*, *nkx2.2* and *pomc* (Fig. 5D, inset).

Fig. 6. *Pitx3* is required for *pitx3* expression at bud stage, and *Dlx3b* and *Dlx4b* restrict pituitary pre-placode size during mid-somitogenesis. (A-I) Control and morpholino injected embryos, labeled with *pitx3* probe at bud stage (tb, A-C), 18-somite stage (D-F) and prim-5 stage (24 h; G-I). (B,E,H) *pitx3* morpholino injection leads to reduced *pitx3* expression in placodal field (B; $n=23/30$), in pituitary pre-placode (E; $n=33/46$) and in pituitary placode (H; $n=43/68$). (C,F,I) Injection of *dlx3b* and *dlx4b* morpholinos leads to reduction of *pitx3* expression in prospective lens precursors (arrowheads in C; $n=29/38$), and an expansion of the pituitary pre-placode (F, white bar; $n=23/30$) and pituitary placode size (I; $n=32/40$; arrowheads: ectopic *pitx3* expression). (A-C) Dorsal view, ventral towards the top. (D-I) Frontal view, dorsal towards the top. Scale bar: 100 μm .



By contrast, injection of *dlx3b* and *dlx4b* morpholinos increases both pituitary placode size and cell number (Fig. 5G-I), indicating that *dlx3b* and *dlx4b* function as negative regulators in pituitary placode development. To test epistatic relationships between *pitx3* and *dlx3b-dlx4b*, we injected embryos with all three morpholinos. We find that expression of *lim3*, *nkx2.2* and *pomc* is reduced or entirely lost at prim-5 stage (Fig. 5J-L), comparable with embryos injected with *pitx3* morpholinos alone. Together, these observations suggest that *pitx3* is epistatic to *dlx3b* and *dlx4b*, and that *Dlx3b-Dlx4b* is required to restrict pituitary cell number and placode size. By contrast, *Pitx3* is required for pituitary placode formation and cell type specification.

To learn when *pitx3*, *dlx3b* and *dlx4b* are required, we injected embryos with *pitx3* or *dlx3b* and *dlx4b* morpholinos and analyzed *pitx3* and *dlx3b* expression at bud, 18-somite and prim-5 stages. Injection of *pitx3* morpholino strongly reduces *pitx3* expression in prospective lens and pituitary precursors at bud stage (Fig. 6B), and at 18-somite and prim-5 stages (Fig. 6E,H), *pitx3* expression is reduced in pituitary pre-placode and placode, but not in head mesenchyme or ventral diencephalon. *dlx3b* and *dlx4b* morpholino injection reduces *pitx3* expression in lateral, prospective lens precursors at bud stage (Fig. 6C, arrowheads), but not in prospective pituitary precursors. In older, *dlx3b* and *dlx4b* morpholino-injected embryos, however, we find expanded *pitx3* expression in pituitary pre-placode (Fig. 6F), pituitary placode and in ectopic cells close to the olfactory placode (Fig. 6I, arrowheads). By contrast, injection of *pitx3* morpholinos did not affect *dlx3b* expression between bud and prim-5 stages (not shown). Injection of *dlx3b* and *dlx4b* morpholinos changes *dlx3b* expression, however, consistent with previous results (Solomon and Fritz, 2002). Thus, our results suggest that at the end of gastrulation, pituitary precursors require *Pitx3* function for *pitx3* expression and subsequent pituitary pre-placode formation, whereas *Dlx3b* and *Dlx4b* act on lens precursors at bud stage and on the median pre-placode later, during somitogenesis.

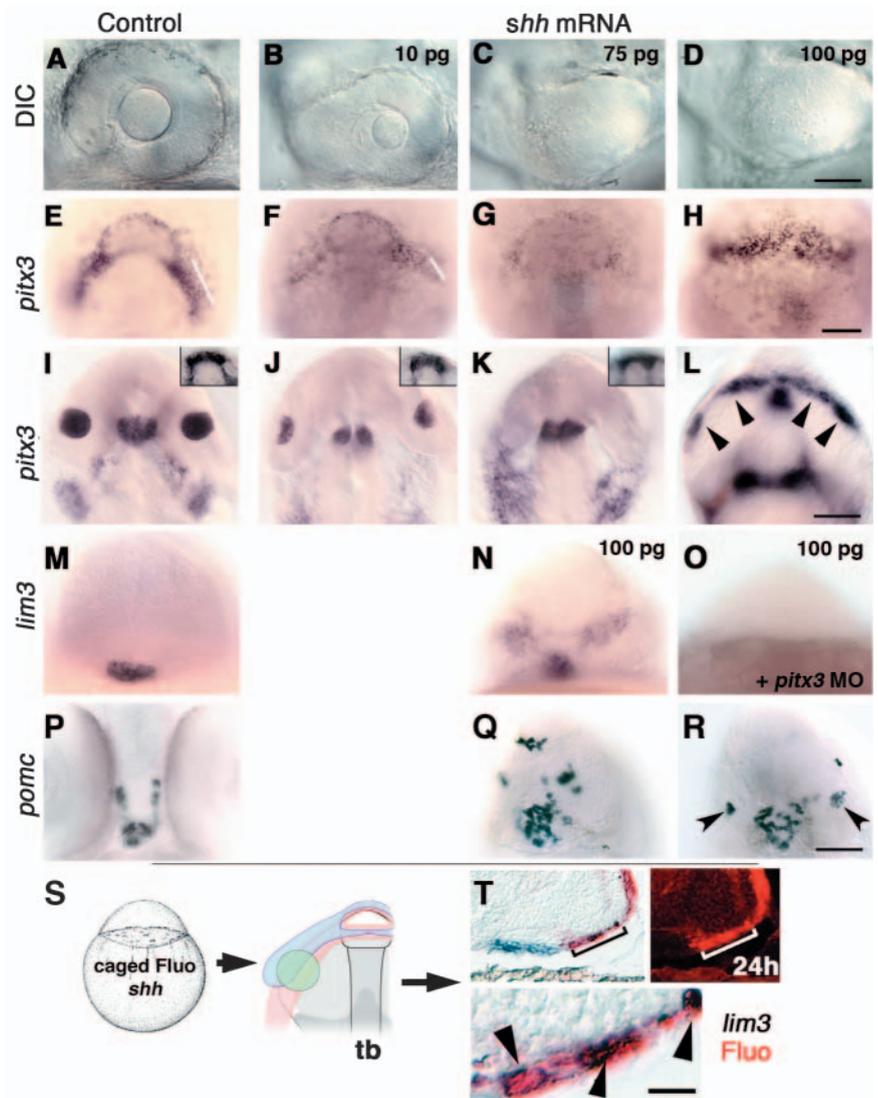
Because partially overlapping domains of cells express *pitx3* and *dlx3b* at bud stage, we also tested whether these genes function in lens formation. Our results indicate that *pitx3* and *dlx3b* (and *dlx4b*) are required for primary lens fiber cell specification but not for lens placode formation (see Fig. S3 in the supplementary material).

Hedgehog overexpression blocks lens formation and induces ectopic expression of pituitary genes

Loss of Hedgehog signal transduction results in mis-specification of pituitary precursor cells and subsequent ectopic lens formation (Fig. 3) (Karlstrom et al., 1999; Varga et al., 2001). Conversely, overexpression of *shh* suppresses lens formation (Barth and Wilson, 1995) and increases the number of ventral, anterior pituitary cell types (Herzog et al., 2003; Sbrogna et al., 2003). Consistent with these previous observations, we find that increasing amounts (10-100 pg/embryo) of synthetic *shh* mRNA block lens tissue formation (Fig. 7A-D) and, in parallel, downregulate lens *pitx3* expression (Fig. 7E-G,I-K). Although *pitx3* expression expands in branchial arches (Fig. 7K) and into the epithalamus (not shown), the size of the pituitary placode as such is unaffected (Fig. 7I-K, insets). Interestingly, at high levels of *shh* mRNA (100 pg/embryo), prospective ventral ectoderm at bud stage (Fig. 7H) and cells covering the retina at prim-5 stage (Fig. 7L) upregulate *pitx3* expression in a pattern reminiscent of normal bud stage *pitx3* expression (Fig. 7E, compare with Fig. 7H,L). This observation suggests that *pitx3*-expressing cells respond to Hedgehog differentially, depending on their position in the embryo and the source of Hedgehog signal. During normal development, median cells might acquire pituitary characteristics because of their proximity to Hedgehog signal, whereas in lateral regions absence of Hedgehog signal might favor lens specification. Thus, at the end of gastrulation, *pitx3*-expressing precursor cells might be competent to respond to Hedgehog signal directly, or by Hh induced, secondary signaling interactions.

Because highest *shh* concentrations caused ectopic *pitx3* expression at prim-5 stage, we examined whether Hedgehog also induces pituitary genes in *pitx3*-expressing precursors. In embryos injected only with high amounts of *shh* mRNA (100 pg/embryo), we find that both *lim3* and *pomc* are expressed ectopically between retina and epidermis (Fig. 7N,Q,R), as previously reported in similar studies (Herzog et al., 2003;

Fig. 7. Hedgehog can block lens formation and induce gene expression characteristic of pituitary cell types. (A-L) Increasing amounts (10, 75 and 100 pg) of injected *shh* mRNA affect lens size at prim-5 stage (A-D) and *pitx3* gene expression at bud (E-H) and prim-5 (I-L) stages in a dose-dependent manner. (B,J) Reduced lens size ($n=54/60$). (C,K; $n=52/60$; D,L; $n=93/99$) Absence of lens tissue. (I-K) Pituitary placode size at prim-5 stage is unaffected by hedgehog mRNA (I-K insets; $n=93/99$). (E-G) At bud stage, progressive loss of *pitx3* expression in presumptive lens precursors (bars; F, $n=10/14$; G, $n=11/12$) correlates with loss of lens tissue at prim-5 stage (B,C,J,K). (L) Ectopic *pitx3* expression following injections of 100 pg *shh* mRNA per embryo at bud stage in anterior ventral ectoderm (H, 8/8) and at prim-5 stage (L, arrowheads; $n=15/23$). (M,N;P-R) *shh* mRNA induces ectopic *lim3* (N; $n=30/36$) and *pomc* (Q,R; $n=26/32$). (Q) Ectopic *pomc*-expressing cells are scattered around forebrain ($n=19/26$) or (R, $n=7/26$) form small lens like structures (arrowheads). (O) Co-injection of *pitx3* morpholino (8 ng/embryo) and *shh* mRNA (100 pg) leads to loss of *lim3* expression in pituitary and ectopic *lim3*-expressing regions ($n=28/30$). (S) One-cell stage embryos injected with *shh* mRNA and caged fluorescein ($n=27$). Photoactivated spot of cells (green) at bud stage in the lens-forming region. (T) In *shh* mRNA-injected embryos, ectopic *lim3* ($n=9$)-expressing cells (blue) were also labeled with uncaged fluorescein (red, arrowheads). Upper left and lower (magnification of upper left) panels, Nomarski images; upper right panel, fluorescence micrograph of same region showing fast red fluorescence in ectopic *lim3*-expressing cell. (A-D) Side views, anterior towards the left, dorsal towards the top. (I-L, insets) Dorsal view, anterior towards the top. (E-H) View of prospective head region (animal pole), ventral towards the top. (M-R) Frontal views, dorsal towards the top. (T) prim-5 stage embryo, transverse cryosection (16 μm thickness) through ventral forebrain. Scale bars: 50 μm in A-D; 100 μm in E-L, insets, M-R; 12.5 μm in T, lower panel.



Sbrogna et al., 2003). Co-injection of *shh* mRNA (100 pg per embryo) and *pitx3* MO (8 ng per embryo) completely eliminates *lim3* expression from median and ectopic locations (Fig. 7O) suggesting that *pitx3* is required for pituitary cell specification when Hedgehog is overexpressed. In response to high levels of Hedgehog, ectopic *pomc*-expressing cells are scattered on top of the forebrain and retina (Fig. 7Q,R) and in some embryos, form small, flattened clusters of cells that resemble miniature lenses (Fig. 7R, arrowheads). This observation supports the idea that lens precursors form in their normal positions but express *pomc* in response to *shh*.

We further tested the possibility that Hedgehog induces pituitary gene expression in lens precursors by co-injecting caged-fluorescein and *shh* mRNA (100 pg per embryo) into one-cell stage embryos. We uncaged fluorescein in prospective lens and skin precursors (Fig. 7S) and analyzed these embryos by in situ hybridization with probes for *lim3* and by antibody labeling to detect caged-fluorescein. We find that *shh* mRNA-

injected embryos lack lens and have ectopic *lim3*-expressing cells near the eyes doubly labeled with fluorescein (16/16 embryos; Fig. 7T, arrowheads). Because lens is entirely absent in these embryos, doubly labeled cells indicate that (at least some) lens/nasal precursors express pituitary genes in response to *shh* mRNA injection. Thus, loss of lens tissue and ectopic *pitx3*, *lim3* and *pomc* expression suggest that Hedgehog signaling suppresses lens development and induces gene expression characteristic of the pituitary cell lineage in derivatives of *pitx3* expressing lens precursors.

Hedgehog acts indirectly to regulate pituitary and lens fates

To test whether *pitx3*-expressing cells are competent to respond to Hedgehog and, as a result, express genes characteristic of pituitary, we analyzed whether *shh* induces *ptc* genes in pituitary or lens precursors. At bud stage, *ptc1* is expressed in median neural plate cells (Fig. 8A,C), but not in *pitx3*-

expressing cells at the anterior neural plate border that give rise to lens or pituitary. In embryos injected with *shh* mRNA (100 pg/embryo), pituitary and lens precursors that express *pitx3*, but not *dlx3b*, do not upregulate *ptc1* (Fig. 8B,D). By contrast, cells throughout the neural plate and *dlx3b*-expressing neural plate border cells, upregulate *ptc1* in response to *shh* injection (Fig. 8D). Thus, even though Hedgehog induces pituitary gene expression, is required for pituitary specification, and is sufficient to block lens formation, placodal precursor cells that express only *pitx3*, do not express *ptc1* and *ptc2* (data not shown) in response to *shh* mRNA injection.

Hedgehog patterns the pituitary placode (Herzog et al., 2003; Sbrogna et al., 2003) during mid-somitogenesis when pituitary and lens placodes form. However, the observations that *shh* overexpression fails to upregulate *ptc* expression in pituitary precursors at bud stage or to affect median placode size suggest that Hedgehog acts indirectly on pituitary and lens precursors at bud stage. To test this idea, we injected one-cell stage donor embryos with biotin-rhodamine dextran and *dnPKA* that activates the Hedgehog signaling cascade cell autonomously. We then transplanted groups of cells from these donor embryos into uninjected blastula stage hosts. We find that *ptc1* is upregulated before the 18-somite stage in transplanted cells on top of the eye vesicle (Fig. 8E). However, at prim-5 stage, we find that donor derived cells that express *dnPKA* contribute to lens (Fig. 8F) and do not express pituitary genes (not shown), even though all corresponding donor embryos completely lack lens. We also find that *dnPKA*-expressing cells contribute to lens, regardless of whether large groups (Fig. 8F) or only a few cells (not shown) have been transplanted. Thus, even though overexpression of Hedgehog blocks lens formation and induces ectopic pituitary gene expression (Fig. 7), cell-autonomous activation of the Hedgehog signaling cascade does not induce pituitary marker genes or prevent cells from contributing to lens. Because injected mRNA is degraded during the first day of development, this result supports the hypothesis that Hedgehog signaling acts indirectly on lens precursors during early stages of development. Presumably, block of lens formation and induction of ectopic pituitary cells in *shh* mRNA-injected embryos is an event downstream of Hedgehog signaling due to changes in other cellular interactions and signaling systems regulated by Hedgehog.

Discussion

We suggest a new model for specification and patterning of the anterior cranial placodes that form the pituitary, nose and lens. Initially, precursor cells for these placodes occupy overlapping fields demarcated by overlapping *pitx3*- and *dlx3b*-expression domains at the neural plate border (Fig. 2). Our results indicate that Pitx3-expressing, non-neural ectoderm cells at bud stage later give rise mainly to lens and anterior pituitary placodes (Fig. 3). Pitx3 is required for its own expression and also for pituitary placode formation (Figs 5, 6). By contrast, *Dlx3b* and *Dlx4b* are mainly expressed in olfactory precursor cells in this region (Whitlock and Westerfield, 2000) and function to limit the size of the pituitary placode (Figs 5, 6). Both Pitx3 and *Dlx3b*-*Dlx4b* are required for pituitary and lens specification and differentiation (Fig. 5; see Fig. S3 in the supplementary material). Our results

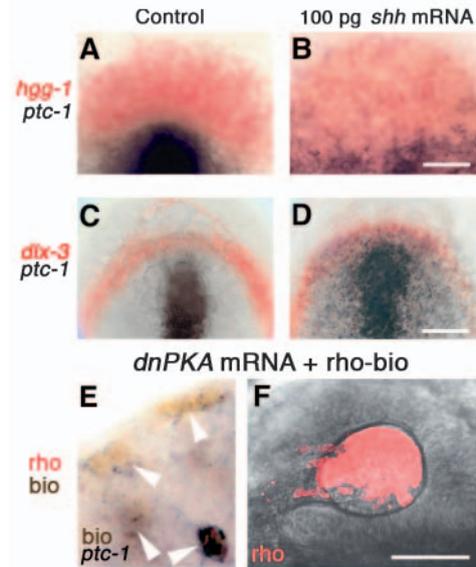


Fig. 8. At bud stage, lens and pituitary precursor cells do not upregulate *ptc1* in response to *shh* mRNA. (A-D) Bud stage embryos labeled with probes for (A,B) *hgg1* (red) and *ptc1* (black), (C,D) *dlx3b* (red) and *ptc1* (black). (E) At the 18-somite stage, *dnPKA* mRNA and rhodamine-biotin injected, transplanted cells are *ptc1* positive (arrowheads) in ectoderm of eye vesicle in unlabeled host ($n=11$). (F) At prim-5 stage, transplanted cells (rho, red) contribute to lens ($n=27$). (A-D) Dorsal view of prospective head region, ventral towards the top. (E) Dorsal view, anterior towards the top, midline towards the right. (F) Side view, anterior towards the left, dorsal towards the top; confocal fluorescence micrograph reconstructed from 90 optical sections (optical slice 1.6 μm ; 0.25 μm overlap between consecutive sections). Scale bars: 100 μm in C,D; 50 μm in A,B,F; 12.5 μm in E.

support the idea that *pitx3* demarcates an equivalence domain, because in the absence of Hedgehog signaling, median Pitx3-expressing cells are mis-specified and contribute to ectopic or medially fused lenses (Kondo et al., 2000; Sbrogna et al., 2003; Varga et al., 2001). In addition, we show that Hedgehog signaling induces genes in lens precursors characteristic of pituitary placode, further supporting the view that *pitx3*-expressing precursor cells have equivalent potential to form lens or pituitary (Figs 7, 8). Our results also indicate that cell fate depends on the level of Hedgehog signal, even though lens and pituitary precursors are not competent to respond to Hedgehog at the end of gastrulation (Figs 7, 8). Pituitary precursors do not express Ptc genes at bud stage, and lens precursors form lens in spite of cell-autonomous activation of the Hedgehog signaling pathway. Thus, overexpression of Hedgehog might lead to activation of other mechanisms that affect patterning and specification of the placodal field. We suggest that initially an unspecified placodal field is formed, in a Hedgehog independent manner, mediated by Pitx3, *Dlx3b* and *Dlx4b* interaction. The placodal field is subsequently subdivided into an outer, *pitx3*-expressing pre-placode with lens epithelial cell character and an inner, *dlx3b*-*dlx4b*-expressing olfactory pre-placode (Fig. 9). During mid-somitogenesis, Hedgehog is sufficient and required to induce pituitary genes in the outer pre-placode, and thus directs it to form median pituitary and lateral lens placodes.

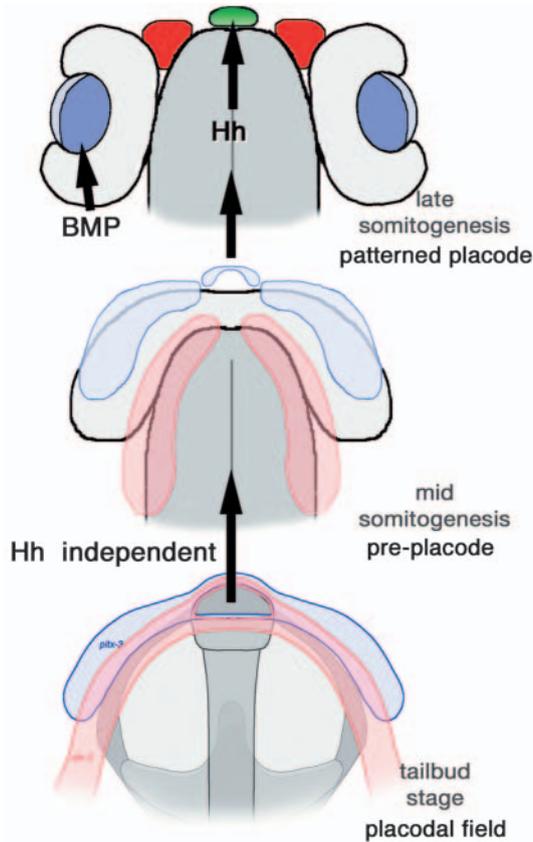


Fig. 9. Pre-placodes form from an unspecified placodal field independent of Hedgehog. (Bottom) At neural plate stages, *pitx3* (blue) and *dlx3b-dlx4b* (red) expression domains demarcate placodal fields (Figs 2, 3). (Middle) Lens and pituitary pre-placode forms in a Hedgehog-independent process and has lens epithelial character (light blue). (Top) In a Hedgehog-dependent process, pituitary tissue (green) is specified (Figs 7, 8) (Herzog et al., 2003; Karlstrom et al., 1999; Kondoh et al., 2000; Sbrogna et al., 2003; Treier et al., 2001; Varga et al., 2001). Similarly, lens epithelial cells (light blue) differentiate into lens fiber cells (dark blue) under influence of BMPs (Belecky-Adams et al., 2002; Faber et al., 2002).

Sequence and functional homology of Pitx3 in vertebrates

Zebrafish *pitx3* may have subsumed the functions of murine *Pitx1* and *Pitx3*. In mice, non-neural ectoderm and its derivatives, including olfactory placodes, Rathke's pouch and subsequently all endocrine adenohypophyseal cells, express *Pitx1* (Lancôt et al., 1997; Gage et al., 1999). *Pitx1* expression is required for development of Rathke's pouch and activation of pituitary *pomc* and *prl* expression (Szeto et al., 1999; Tremblay et al., 1998). However, midbrain dopaminergic neurons, eye mesenchyme and lens, but not pituitary, express *Pitx3*. Mouse *aphakia* mutants and mice lacking functional *Pitx3* promoter elements fail to develop differentiated lenses (Nunes et al., 2003; Semina et al., 2000) but have no apparent pituitary defects. Consistent with these studies, human fetal mesencephalon, but not anterior pituitary, expresses *PITX3* (Pellegrini-Bouiller et al., 1999). By contrast, the *Xenopus* ortholog, *Xpitx3*, is expressed in presumptive stomodeum, lens and pituitary (Pommereit et al., 2001); and our results show

that, in zebrafish, *pitx3* is also expressed in prospective lens and pituitary and that embryos injected with *pitx3* morpholinos fail to differentiate primary lens fiber cells or pituitary placode (Figs 5, 6).

pitx3 expression demarcates an equivalence domain with transient lens character in non-neural ectoderm

Our results provide a genetic mechanism to explain the placode forming competence of non-neural ectoderm. In *Xenopus*, apparently all non-neural head ectoderm, including the midline, has a transient lens-forming bias (Henry and Grainger, 1987; Zygar et al., 1998). We show that median placodal cells are mis-specified and give rise to lens in *smoothened* mutant embryos (Fig. 3). This suggests that the *pitx3* expression domain demarcates an equivalence domain at the end of gastrulation, because the placodal field gives rise to a single (fused) placode with lens character. Formation of ectopic and fused lenses in *smoothened* mutants further indicates that Hedgehog signaling from neural plate puts an end to the lens-forming capacity of median non-neural ectoderm and specifies pituitary fate instead. Consistent with this idea, overexpression of *Shh* suppresses lens formation (Barth and Wilson, 1995) and induces pituitary genes in lens derivatives (Figs 7, 8).

We also show that loss of Hedgehog signal transduction leads to increased olfactory placode size (see Fig. S1 in the supplementary material). In lateral regions of the placodal field, we find that lens and olfactory precursor cell populations intermingle only where the *pitx3* and *dlx3b* expression domains overlap. By contrast, median *pitx3* and *dlx3b* domains overlap significantly and olfactory and pituitary precursors intermingle to a high degree in this region, presumably because the placodal fields of olfactory and pituitary placode have not segregated from each other by bud stage (Whitlock, 2004). In *smu* mutant embryos, olfactory and ectopic lens precursor cells intermingle near the midline. Although we do not find increased numbers of olfactory precursor cells in *smu* mutants (Fig. 3B), which might provide an explanation for the larger olfactory placodes, we can not exclude the possibility that median precursor cells have an equivalent potential to contribute to lens, pituitary and olfactory placode before segregation of median placodal fields. Nevertheless, our lineage analysis of median, *pitx3*-positive precursor cells strongly supports an equivalence domain for at least lens and pituitary fates.

Pitx3, Dlx3b and Dlx4b regulate pituitary placode size at different stages of development

Our observations provide evidence for a novel mechanism that regulates the size of the pituitary placode. We find that *Pitx3* is required for pituitary placode specification and formation (Fig. 5D-F) at the end of gastrulation (Figs 5, 6). Previous studies have implicated *Dlx3b* and *Dlx4b* in olfactory and otic placode formation (Whitlock and Westerfield, 1998; Whitlock and Westerfield, 2000; Solomon and Fritz, 2002; Liu et al., 2003; Hans et al., 2004). We find that at bud stage, *Pitx3*, *Dlx3b* and *Dlx4b* are expressed in partially overlapping domains, and that reducing *Dlx3b* and *Dlx4b* increases the size of the pituitary placode and prevents lens fiber cell specification (Fig. 5; see Fig. S3 in the supplementary material). Our results indicate that *Dlx3b-Dlx4b* and *Pitx3* may not interact directly, because loss of *Dlx3b* and *Dlx4b* functions leads to

downregulation of *pitx3* expression in lens precursors at bud stage, but does not affect pituitary *pitx3* expression. Similarly, the size of the pituitary pre-placode increases during mid-somitogenesis when *Dlx3b* and *Dlx4b* function is reduced (Fig. 6). Because *Dlx3b* and *Dlx4b* are required for olfactory placode formation, the enlargement of the pituitary pre-placode in their absence further supports the idea that intermingled olfactory and pituitary precursors at bud stage might have equivalent potential to form nose or pituitary. This possibility is also supported by split pituitary and olfactory fates that arise from single precursor cells that express both *pitx3* and *dlx3b* (Fig. 3).

We suggest that the initial overlap between the *pitx3*, *dlx3b* and *dlx4b* expression domains in the placodal field may eventually result in an outer pre-placodal domain, demarcated by *pitx3* expression (Fig. 9) and fated to give rise to pituitary and lens, and in an inner *dlx3b* and *dlx4b* positive domain, fated to give rise to olfactory placode (Whitlock and Westerfield, 2000). Initially, the entire outer pre-placodal domain may have potential to form lens (Kamachi et al., 1998; Ueda and Okada, 1986). Observations in chick that the early anterior pituitary anlage expresses the lens protein, δ -crystalline (Kamachi et al., 1998; Ueda and Okada, 1986) are consistent with this interpretation. Pax6 and other transcription factors are thought to mediate the competence of head ectoderm to form lens in response to inductive signals (Kondoh, 1999). Our results show that *pitx3* is required for specification of lens (see Fig. S3 in the supplementary material) and pituitary placodes (Fig. 5). Moreover, *pitx3* is required for *lim3* gene expression during normal development. Therefore, we suggest that Pitx3, in addition to Pax6 and, presumably, other factors, mediates the response of placode precursor cells and placodal cells to signaling interactions.

Non-Hedgehog signaling establishes the lens-pituitary placodal field, but Hedgehog induces pituitary character in the median placode

Previous studies showed that loss of Hedgehog signaling results in a conversion of pituitary to lens (Karlstrom et al., 1999; Kondoh et al., 2000; Varga et al., 2001) and have led to the generally accepted hypothesis that a major function of anterior midline Hedgehog expression is to specify the pituitary placode and prevent it from forming a median lens. The observation that ectopic, cell non-autonomous activation of Hedgehog signaling induces pituitary gene expression in at least some lens precursors is consistent with this interpretation. However, we find that overexpression of Shh does not increase pituitary placode size and that in *smoothened* mutants, a median placode (albeit of lens character) still forms. This indicates that Hedgehog signaling is neither sufficient nor required for median placode formation. Nevertheless, changes in Hedgehog signaling affect pituitary cell proliferation and specification in mouse and zebrafish (Herzog et al., 2003; Sbrogna et al., 2003; Treier et al., 2001). We suggest (Fig. 9) that formation of the lens and pituitary pre-placode is independent of Hedgehog signaling; however, once the placode forms, Hedgehog from ventral forebrain specifies pituitary character in the median placode (Herzog et al., 2003; Sbrogna et al., 2003; Treier et al., 2001). We further suggest that non-Hedgehog signaling interactions from neighboring tissues specify or block lens character in the lateral placodes, because

cell-autonomous activation of the Hedgehog pathway does not prevent lens formation (Fig. 8). Several lines of evidence suggest that BMP is required and sufficient to promote lens fiber cell differentiation in lens epithelial cells (Belecky-Adams et al., 2002; Faber et al., 2002). Thus, localized BMP, together with Hedgehog signaling, may mediate the subsequent differentiation of unspecified pre-placodal cells into lens fiber cells or hormone-producing pituitary cells.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/7/1579/DC1>

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