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Zebrafish *smoothened* functions in ventral neural tube specification and axon tract formation

Zoltán M. Varga*,‡, Angel Amores, Katharine E. Lewis, Yi-Lin Yan, John H. Postlethwait, Judith S. Eisen and Monte Westerfield

Institute of Neuroscience, 1254 University of Oregon, Eugene, OR 97403, USA
*Present address: University Freiburg, Institute Biology 1 (Zoology), Department of Developmental Biology, Hauptstr. 1, D-79104 Freiburg, Germany
‡Author for correspondence (e-mail: varga@uni-freiburg.de)

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SUMMARY

Sonic hedgehog (Shh) signaling patterns many vertebrate tissues. *shh* mutations dramatically affect mouse ventral forebrain and floor plate but produce minor defects in zebrafish. Zebrafish have two mammalian *Shh* orthologs, *sonic hedgehog* and *tiggy-winkle hedgehog*, and another gene, *echidna hedgehog*, that could have overlapping functions. To examine the role of Hedgehog signaling in zebrafish, we have characterized *slow muscle omitted* (*smu*) mutants. We show that *smu* encodes a zebrafish ortholog of Smoothened that transduces Hedgehog signals. Zebrafish *smoothened* is expressed maternally and zygotically and supports specification of motoneurons, pituitary cells and ventral forebrain. We propose that *smoothened* is required for induction of lateral floor plate and a subpopulation of hypothalamic cells and for maintenance of medial floor plate and hypothalamic cells.

Key words: Hedgehog, Forebrain patterning, Spinal cord, Floor plate, Motoneurons, Pituitary, Ventral neural tube, Zebrafish

INTRODUCTION

Intercellular signaling is crucial for embryonic patterning, cell specification and tissue induction. *Hedgehog* (*Hh)* encodes a secreted signal originally identified in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). Vertebrate family members have been subsequently isolated (Echelard et al., 1993; Ekker et al., 1995; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). In vivo and in vitro studies indicate that Hh signaling is required for dorsoventral patterning, specification of cell fates and proliferation in vertebrate neural tissue (Hammerschmidt et al., 1997). Two transmembrane proteins, Patched and Smoothened, mediate Hh signaling intracellularly (Ingham, 1998; Denef et al., 2000). Patched is the Hh receptor, but silences signaling in the absence of Hh. Smoothened interacts with Patched, but not Hh, and is required for signal transduction. Hh binding to Patched may relieve Patched repression of Smoothened (Kalderon, 2000). In vertebrate development, however, the role of Smoothened remains unclear, because loss-of-function mutations in s*moothened* have not been reported. Constitutively active forms of Smoothened mimic Sonic hedgehog (Shh) function cell-autonomously in patterning neural tube, inducing Hh target genes and specifying ventral cells, supporting evidence that Smoothened mediates Hh signaling (Hynes et al., 2000). Moreover, gain-of-function smoothened mutations are implicated in human basal cell carcinoma (Xie et al., 1998), consistent with loss-of-function mutations of patched (Hahn et al., 1996; Johnson et al., 1996a; Johnson et al., 1996b).

Hh signaling is required for development of the vertebrate ventral neural tube. Hh conveys ventral characteristics to anterior diencephalic cells (Dale et al., 1997). Teratogens and mutations that affect Hh signaling in human embryos can cause severe ventral CNS developmental anomalies, including holoprosencephaly (Muenke and Beachy, 2000). Mice with *Shh* mutations are cyclopic and lack motoneurons, floor plate and ventral forebrain (Chiang et al., 1996). By contrast, zebrafish *sonic-you* (*syu,* an ortholog of mammalian *Shh*) mutants develop normal medial floor plate and motoneurons and have relatively normal ventral forebrain patterning (Schauerte et al., 1998), although they lack lateral floor plate. However, *shh* overexpression is sufficient to alter forebrain gene expression and morphology (Barth and Wilson, 1995; Macdonald et al., 1995). One explanation for this discrepancy is that development of zebrafish hypothalamus requires both Nodal and Hh signaling (Rohr et al., 2001; Varga et al., 1999). Thus, mutations in *syu* (*shh*) alone may have a relatively mild effect. Another possible explanation is that in zebrafish, additional Hh family members, Tiggy-winkle hedgehog (Twhh; Ekker et al., 1995) and Echidna hedgehog (Ehh; Currie and Ingham, 1996), may act redundantly with Shh to pattern ventral neural tube (Nasevicius and Ekker, 2000; Zardoya et al., 1996).

Several other zebrafish mutations affect Hh signaling and produce similar phenotypes. Some of these have been termed

you-type, because mutants form bulky, U-shaped somites (van-Eeden et al., 1996). Mutations in *you*-type genes affect formation of horizontal myoseptum (Barresi et al., 2000; Lewis et al., 1999; van Eeden et al., 1996), epidermis, nervous system, pectoral fins, cartilage (Brand et al., 1996; van Eeden et al., 1996) and axon projections (Karlstrom et al., 1997). Axon pathfinding defects may be due to abnormal ventral neural tube patterning (Macdonald et al., 1994) and absence of guidance cues such as netrins (Lauderdale et al., 1998; Strähle et al., 1997).

Hh signaling may also regulate formation of anterior pituitary, a key endocrine organ. Anterior pituitary derives from Rathke's pouch, in close proximity to the ventral forebrain floor. Analysis of zebrafish *you-too* (*yot*, *gli2*) gene mutations and *iguana* (*igu*) mutants has implicated Hh in anterior pituitary differentiation (Karlstrom et al., 1999; Kondoh et al., 2000), and mouse studies suggest that Hh supports pituitary cell proliferation and specification (Treier et al., 2001). In zebrafish, presumptive anterior pituitary forms in the anterior ventral midline by 24 hours of development, as a placode that expresses genes such as *six3*, *lim3* and *nkx2.2,* at least some of which are influenced by Hh signaling (Glasgow et al., 1997; Karlstrom et al., 1999; Kobayashi et al., 1998).

We have recently isolated a novel mutation in zebrafish, *slow muscle omitted* (*smu),* that affects Hh signaling. Pharmacological and RNA injection studies suggest that *smu* mutations act on the Hh receptor complex (Barresi et al., 2000). We now report characterization, molecular identification and cloning of the *smu* gene. Our results suggest that the smu^{b577} and smu^{b641} mutant alleles affect zebrafish *smoothened*. *smu* mutants lack posterior primary motoneurons, secondary motoneurons, lateral floor plate, parts of ventral forebrain, and pituitary placode and have defects in optic chiasm and ventral forebrain commissural axon tracts. We show that *smoothened* transcripts are present maternally and zygotically throughout the early embryo until after gastrulation, when they become more restricted to tissues that express Hh. We also show that wild-type *smoothened* DNA or mRNA rescues the *smu* mutant phenotype.

Our analysis suggests that the requirement for Hh signaling differs along the anteroposterior axis. Maternal and zygotic Smoothened, together with Nodal, is required for induction of ventral forebrain and lateral floor plate cells. However in the tail, other ventral cell types such as motoneurons are lost even when only zygotic Smoothened expression is disrupted. Thus, we reconcile an apparent discrepancy between zebrafish and other vertebrates. We suggest that, as in mammals, Hh signaling is required for ventral CNS development in zebrafish, specifically lateral floor plate and posterior primary motoneuron specification, and that overlapping signals from several Hh family members converge on Smoothened, a common signal transducer.

MATERIALS AND METHODS

Animals

Wild-type (AB, C32, WIK, SJD) and mutant zebrafish (*Danio rerio*) were obtained from the University of Oregon Zebrafish Facility. Mutations were induced in AB males using ethylnitrosourea (Riley and Grunwald, 1995). The smu^{b577} and smu^{b641} alleles were isolated in a screen for morphological defects. Mutants were obtained from intercrosses of heterozygous carriers (Westerfield, 1995). Embryos were maintained at 28.5°C and staged by hours (h) or days (d) post fertilization using standard morphological criteria (Kimmel et al., 1995).

Cloning *smoothened* genomic DNA

An arrayed zebrafish PAC library (Amemiya and Zon, 1999) was screened with redundant primers (F-5′ TTY AAY CAR GCN GAR TGG GA 3′, R-5′ GTC CAN ACC CAN GTN SWC AT 3′) designed to amplify approximately 190 nucleotides of conserved sequence. Products from positive clones (including 08M08, 37B06, 72O02, 92K02) were sequenced and gene-specific primers used to sequence directly from PAC DNA and for screening of a pooled cDNA library. Positive cDNA clones were sequenced to identify *smoothened* cDNA (GenBank: AF395809).

Genetic mapping

Single-strand conformation polymorphism analysis (SSCP) was performed on DNA from 96 haploid progeny from the MOP cross (Johnson et al., 1996a; Johnson et al., 1996b; Knapik et al., 1998; Postlethwait et al., 1998; Shimoda et al., 1999) and analyzed with MapManager (http://mcbio.med.buffalo.edu/mapmgr.html). For SSCP analysis of the *smoothened* gene, a 190 base pair fragment was amplified from genomic DNA of C32 and SJD parental strains and members of the MOP panel using primers specific to intron 7: forward 5′-AAGAAGCTTCAGGGAATATGT-3′ and reverse 5′-TGTTCT-TGATGGCACATTCTG-3′. One primer was end-labeled for autoradiography on acrylamide gels (Postlethwait et al., 1998). Orthologs were identified by reciprocal BLAST analysis (Woods et al., 2000) and HomoloGene (http://www.ncbi.nlm.nih.gov/HomoloGene), and map positions were found using OMIM (http://www.ncbi.nlm.nih.gov/Omim), LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink), GeneMap'99 (http://www.ncbi.nlm.nih.gov/genemap99) and MGD (http://www.informatics.jax.org).

Phylogenetic analysis

We identified Smoothened sequences with tblastx (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) from GenBank (http://www3.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=n&form=0), imported them into CLUSTALX (Thompson and Jeanmougin, ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) and trimmed ends that did not align well. Neighbor joining trees were drawn with NJPlot (http://pbil.univ-lyon1.fr/software/njplot.html) and bootstraps calculated (Efron and Gong, 1983; Felsenstein, 1985; Swofford et al., 1996).

Nomenclature is from http://zfin.org/zf_info/nomen.html; http://www.informatics.jax.org/support/nomen/ and http://www2.ncbi.nlm.nih.gov/LocusLink/LLnomen.html.

Immunohistochemistry and in situ hybridization

Embryos were labeled (Wilson et al., 1990) with monoclonal antibodies: zn-5 for DM-GRASP, zl-1 for lens fiber cells and anti-acetylated tubulin (Sigma) for axons. Gene expression was analyzed by mRNA in situ hybridization (Hauptmann and Gerster, 1994; Jowett and Lettice, 1994). We isolated full-length *smoothened* cDNA and cloned it into the *Stu*I-*Xho*I site of the pCS2+ Vector. The vector was digested using *Cla*I and dig-labeled full-length antisense probe was synthesized using T7 RNA Polymerase (Roche).

mRNA, genomic DNA and morpholino injections

The *smoothened* pCS2+ vector was digested with *Not*I or *Xho*I and SP6 RNA polymerase used for in vitro mRNA synthesis (Ambion). Single blastomeres were injected with 1 nl of *smoothened*, *shh*, or *dnPKA* mRNA or PAC DNA (PAC 7202). To assess maternal Smoothened function, one-cell stage embryos were injected with approximately 100 pg synthetic *shh* mRNA (Ambion mMessage Machine Kit) and fixed at tailbud stage for *patched1*, or at the two-

somite stage for *nkx2.2* in situ hybridization. To examine residual Hedgehog function in *smu* mutants, approximately 10 nl of a combination of *shh* (Nasevicius and Ekker, 2000) and *twhh* (Lewis and Eisen, 2001) morpholino antisense oligonucleotides (Gene Tools) was injected into one-cell stage embryos at 1 mg/ml each. Embryos were fixed at prim-5 stage and assayed for *nkx2.2* expression by in situ hybridization.

cDNA isolation and RT-PCR

We isolated total RNA, synthesized first strand cDNA with Superscript II (Gibco) and did PCR. RT-PCR primers: 5′-GCGCCCCTGCTCCATTGTT-3′ and 5′-AGCCGACAAAGCCAATGCCACTA-3′, 5′-TGGATGGAGCCCGCAATGAGATA-3′ and 5′-CTGGGATCGGCTTGTTGGTCTGG-3′; 5′-AATATCGGGCCGGGTTTGTTCT-3′ and 5′-CCCAGCCCACCGTACCTATCCT-3′; and 5′-AAGAAGCTTCAGGGAATATGT-3′ and 5′-AAGCAGAGCCGGCACAGCAGT-3′.

Microscopy

Embryos were mounted (Westerfield, 1995), observed with Zeiss Axiophot2 or LSM310 microscopes, and photographed. Photographs were adjusted (Adobe Photoshop) to match original brightness, contrast and hue.

RESULTS

slow muscle omitted is a *you*-type gene encoding zebrafish smoothened

We identified two recessive, lethal, Mendelian alleles, smu^{b577} and smu^{b641}, based on phenotypes (Fig. 1A; Barresi et al., 2000) reminiscent of *you*-type mutants (van Eeden et al., 1996) that affect the Hh pathway (Karlstrom et al., 1999; Schauerte et al., 1998). At 24 h, *smu* mutants have bulky U-shaped somites, ventral body curvature, and reduced ventral forebrain and interocular distance. The dorsoventral extent of posterior diencephalon and spinal cord are also reduced, and floor plate is poorly differentiated (Fig. 1B).

The similar appearance of *you*-type and *smu* mutants suggested that *smu* might encode a protein in the Hh pathway. Because protein kinase A acts downstream of the Hh receptor and dominant-negative PKA (dnPKA) activates the Hh pathway (Hammerschmidt et al., 1996), we injected dnPKA mRNA into single blastomeres of two-cell stage *smu* mutants. dnPKA injections caused partial rescue of the *smu* mutant phenotype (52/69; Table 1), strengthening the conclusion that *smu* encodes a Hh pathway member. *smu* heterozygotes complemented *syu*(*shh*), *you*, *igu*, *detour* (*dtr*), *yot*(*gli2*) and *chameleon*, and, thus, *smu* is a new *you*-type gene.

To learn where in the Hh pathway *smu* acts, we injected *shh* mRNA into *smu* mutants and found that even large amounts of *shh* mRNA did not affect the smu^{b641} mutant phenotype (0/82; Table 1). This result suggested that *smu* encodes a pathway component downstream of Shh and that this allele blocks Hh signaling effectively. By contrast, 203 out of 227 wild-type siblings injected with *shh* mRNA showed strong morphological defects and ectopic expression of *nkx2.2* and *pax2a*, which is attributable to Shh overexpression (Barth and Wilson, 1995; Macdonald et al., 1995). Therefore, we cloned the zebrafish *smoothened* gene (see Fig. 1) from a PAC genomic library and injected genomic DNA from the PAC into *smu* mutants. We observed partial rescue of the mutant phenotype (54/72; Table 1), suggesting that *smu* encodes zebrafish Smoothened or another gene product from the PAC clone.

To determine phylogenetic relationships of zebrafish Smoothened, we identified similar sequences from other organisms and constructed neighbor-joining trees. The resulting phylogenetic tree showed that chicken Smoothened is an outgroup to mammalian Smoothened, and that zebrafish Smoothened aligns outside tetrapods, as expected from the known phylogenetic relationships of ray-fin fish to tetrapods (Fig. 1C). As predicted, the tree shows *Drosophila* Smoothened as an outgroup to vertebrates. The very high bootstrap values strongly support the conclusion that zebrafish Smoothened is an ortholog of human *SMOOTHENED* (Fig. 1C). This conclusion further predicts that zebrafish *SMOOTHENED* should map in a genome region with conserved syntenies to human (Hsa) chromosome 7q32.3 that contains human *SMOOTHENED*. To test this prediction, we mapped a genetic polymorphism located in intron 7 of *smoothened* to linkage group 4 (LG4). Within a 20 cM region on LG4 that contains *smoothened*, there are at least five additional loci whose apparent orthologs map on Hsa7q (Fig. 1D). These conserved syntenies provide independent support that *smoothened* is a zebrafish ortholog of mammalian *SMOOTHENED*.

To learn whether *smoothened* was a candidate for *smu*, we tested linkage between *smu* and *smoothened* and found that *smoothened* maps very near both *smu* mutant alleles on LG4 (Fig.1E). To determine with greater accuracy the location of *smu* relative to *smoothened*, we collected haploid progeny from a heterozygous female bearing one LG4 from the WIK wild-type strain and the other LG4 carrying the smu^{b641} allele on an

Table 1. The *smu* mutant phenotype is rescued by dnPKA, *smoothened* mRNA and genomic *smoothened* DNA injections, but not by *shh* mRNA

Injection (pg/embryo)	Wild-type phenotype	Partial *smu** mutant phenotype	*smu* mutant phenotype	Total number observed
Double distilled H_2O/Phenol Red	76%	–	24%	154
shh mRNA (200) two-cell stage	74%	–	26%	309
dnPKA mRNA (10, 50) two-cell stage	79%	16%	5%	335
PAC72O2 gDNA (50) one-cell stage	75%	19%	6%	290
smoothened mRNA (100) one-cell stage	96%	4%	–	315
smoothened mRNA (50, 100) two-cell stage	74%	24%	2%	317

Single blastomeres of embryos from crosses of $smu^{+/-}$ heterozygotes were injected and phenotypes judged at 24 h. Rescue of *smu* mutant phenotype was assessed by tail movement and in situ hybridization with probes for *patched1, nkx2.2*, *islet1* (Fig. 3) and *pax2a* (data not shown).

*Partial rescue of *smu* mutant embryos was assessed by (partial) mutant morphology and gene expression phenotypes.

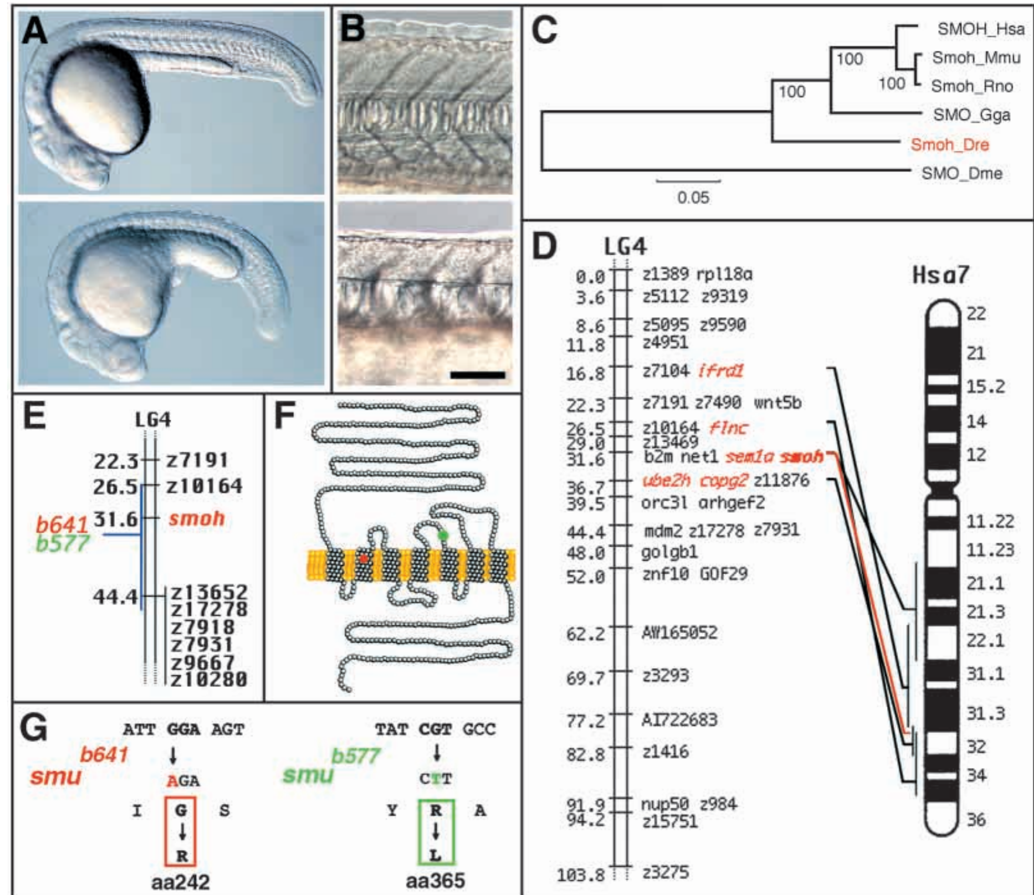

Fig. 1. The *smu* mutant phenotype is due to mutations in *smoothened*. (A) 24 h wild type (top); 24 h *smu*^{b641} mutant (bottom). (B) In *smu*^{b641} mutants (bottom), floor plate was poorly differentiated, somites lacked a horizontal myoseptum and were U-shaped compared with wild type (top). (A,B) Side views, dorsal is towards top, anterior is towards left. Scale bar: 300 μm in A; 50 μm in B. (C) Phylogenetic analysis. Statistical robustness was estimated by bootstrap, numbers indicate the percent of times each node was obtained. *Danio rerio* (Dre), *Drosophila melanogaster* (Dme, P91682), *Homo sapiens* (Hsa, NP_005622), *Gallus gallus* (Gga, O42224), *Mus musculus* (Mmu, P56726) and *Rattus norvegicus* (Rno, NP_036939). (D) Syntenic relationships. Conserved syntenies to Hsa 7q32.3 indicate that *smoothened* is a zebrafish ortholog of mammalian smoothened. (E) Mapping. Diagram of LG4 showing positions of *smoothened* (*smoh*) gene and *smu* mutations. (F) Model of protein encoded by *smoothened*. The red (*smu*^{b641}) and green (*smu*^{b577}) dots indicate amino acids altered in the two alleles. A seven-pass transmembrane protein is the predicted structure of zebrafish Smoothened (TMHMM1.0 software, Center Biol. Sequence Analysis, Department Biotech., Tech. University of Denmark). (G) Mutations. Point mutations in *smu*^{b641} (red) and *smu*^{b577} (green).

AB background. Among 32 phenotypically *smu*^{b641} segregants tested, none showed recombination with *smoothened* by SSCP analysis (data not shown). This placed *smoothened* less than 0±3 cM (P<0.05) from *smu* consistent with the *smu* mutations disrupting *smoothened*.

We confirmed that *smu* mutations altered *smoothened* by comparing sequences of *smoothened* cDNA extracted from wild-type and *smu* mutant embryos. Wild-type *smoothened* encodes 808 amino acids that form a predicted seven-pass transmembrane protein (Fig. 1F). In *smu*^{b641}, a point mutation changed glycine (codon 242, GGA corresponding to human codon 277) into arginine (AGA; Fig. 1G). In *smu*^{b577}, a point mutation changed arginine (codon 365, CGT corresponding to human codon 400) into leucine (CTT; Fig. 1G). In *smu*^{b641} the mutation occurred within the predicted second transmembrane domain, whereas in *smu*^{b577} the mutation occurred in the extracellular domain between the fourth and fifth transmembrane domains.

smu is required for expression of Hh target genes and induction of lateral floor plate and posterior motoneurons

If *smu* encodes Smoothened, homozygous mutants should lack expression of genes that depend on Hh signaling, such as *nkx2.2* (Barth and Wilson, 1995). In *smu*^{b641} mutants, *nkx2.2* was strongly reduced in the entire CNS (Fig. 2A); only a few cells expressed the gene at thalamic, interocular positions close to the presumptive zona limitans intrathalamica (Barth and Wilson, 1995). In *smu*^{b577}, however, *nkx2.2*-expressing cells were more frequent than in *smu*^{b641}, indicating allelic differences. Expression of *patched1* in medial somites and ventral spinal cord (Fig. 2B) is under direct control of Hh signaling (Concordet et al., 1996). In *smu* mutants, *patched1* expression was nearly absent (Fig. 2B).

Previous in vitro and in vivo studies in tetrapods suggested that Shh plays a key role in floor plate formation (Roelink et al., 1994; Roelink et al., 1995). Zebrafish mutants lacking *syu* (*shh*) develop normal medial floor plate but lack lateral floor plate (Schauerte et al., 1998), whereas *cyclops* (*ndr2*) mutants lack medial floor plate (Odenthal et al., 2000; Sampath et al., 1998). In *smu* mutants, medial floor plate was present, as indicated by expression of *col2a1* (Fig. 2C,D) and *shh* (Fig. 2E). Surprisingly, by 27 h, one to three cell diameter gaps appeared (Fig. 2E), revealing that *smu* is required for maintenance of medial floor plate. Lateral floor plate was absent from *smu* mutants as judged by absence of *foxa2* (formerly *axial*; Strähle et al., 1993) expression (Fig. 2F), consistent with the hypothesis that Shh signaling is required

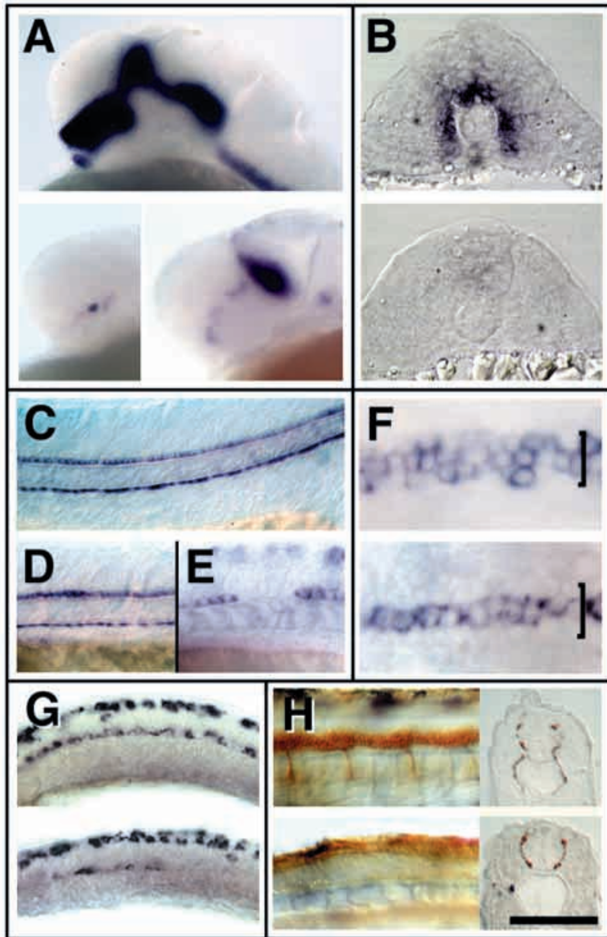

Fig. 2. *smu* mutants have defective Hh signaling. (A) In 24 h *smu*^{b641} mutants (bottom left), *nkx2.2* gene expression was reduced compared with wild type (top); *nkx2.2* gene expression was less severely affected in *smu*^{b577} mutants (bottom right). (B) At the 16-somite stage, *patched1* expression was essentially absent from *smu*^{b641} mutants (bottom) compared with wild type (top). (C-E) At 24 h, floor plate and hypochord were present in both mutant (D) and wild-type (C) embryos labeled for *col2a1* (Yan et al., 1995). By 27 h (E) gaps appeared in *smu* mutant floor plate indicated by *shh* expression. (F) At 20-somite stage, mutants (bottom) lacked lateral floor plate compared with wild type (top), indicated by *foxa2*. (G) At the 18-somite stage, *islet1*-expressing primary motoneurons were absent from tail and posterior trunk, and were reduced in anterior spinal cord in *smu*^{b641} mutants (bottom) compared with wild type (top). (H) In *smu*^{b641} mutants (bottom), secondary motoneurons were absent at 24 h, indicated by DM-GRASP labeling. (A,C-E,G,H) Side views, dorsal is towards the top, anterior is towards the left. (B,H, insets) Transverse sections of anterior trunk, dorsal is towards the top. (F) Dorsal views, anterior is towards the left. Scale bar:120 µm (A, G); 110 µm (B,H); 100 µm (C-E); 50 µm (F); 82 µm (H, insets).

for lateral floor plate formation (Schauerte et al., 1998). Because the *smu* mutations probably affect all Hh signaling, our results suggest either that development of medial floor plate is independent of Hh and requires other signals such as Nodal (Odenthal et al., 2000; Sampath et al., 1998) or that medial floor plate depends upon maternal *smoothened* message that supports early Hh signaling.

Shh signaling is required for motoneuron formation in mouse (Chiang et al., 1996). By contrast, deletion of *syu* (*shh*) function does not affect zebrafish motoneuron formation (Schauerte et al., 1998). To investigate this difference, we first examined motoneuron function in *smu* mutants. At 24 h, *smu* mutants were paralyzed and never moved spontaneously, although some responded to prodding with a single muscle contraction (*smu*^{b577}, 18/40; *smu*^{b641}, 13/45). Because zebrafish muscle contractions are neurogenic (Grunwald et al., 1988), this observation suggested that motoneurons were affected by the mutation. We tested this by labeling *smu* mutants with a probe for *islet1* mRNA, a marker, together with soma position, of motoneurons (Appel et al., 1995; Inoue et al., 1994). At the 18-somite stage and later, *smu* mutants lacked nearly all *islet1*-expressing primary motoneurons in posterior (somites 10-18; Fig. 2G) but not anterior spinal cord (somites 1-9). Secondary motoneurons that can be labeled with antibodies against DM-GRASP were also absent (Fig. 2H).

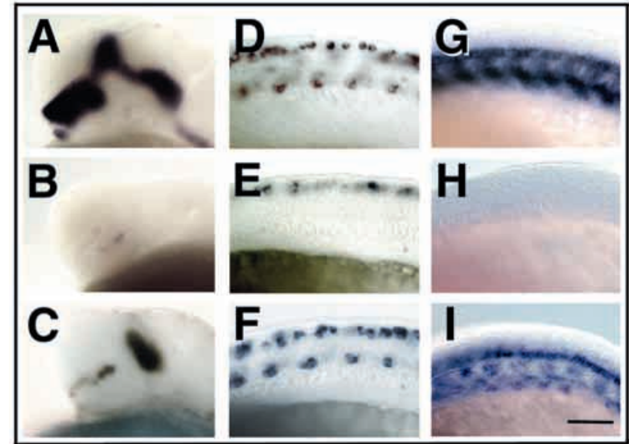

Fig. 3. The *smu* mutant phenotype is rescued by injection of *smoothened* RNA. (A,B) At 24 h, *smu*^{b641} embryos (B) had almost no *nkx2.2* expression compared with wild type (A). (C) Injection of 50 pg *smoothened* RNA at the two-cell stage partially rescued *nkx2.2* expression. (D,E) At 18 h, *smu*^{b641} embryos (E) lacked *islet1*-expressing primary motoneurons in posterior trunk and tail compared with wild type (D). (F) Injection of 100 pg *smoothened* RNA at the one-cell stage completely rescued primary motoneuron phenotype in *smu*^{b641} mutants. (G,H) At 24 h, *smu*^{b641} embryos (H) had almost no *patched1* expression compared with wild type (G). (I) Injection of 50 pg *smoothened* RNA at the two-cell stage almost completely rescued *patched1* expression. Side views, dorsal towards the top, anterior towards the left. Scale bar: 75 µm in A-C; 50 µm in D-F; 62 µm in G-I.

If absence of motoneurons and changes in expression of Hh-regulated genes in *smu* mutants were due to lack of Smoothened function, then these phenotypes should be rescued by expression of exogenous Smoothened. We injected *smoothened* mRNA at the two-cell stage and saw rescue (Fig. 3; Table 1), demonstrating a requirement for Hh signaling in motoneuron development and expression of *patched1* and *nkx2.2*. However, exogenous *smoothened* mRNA did not induce ectopic *islet1*, *patched1* or *nkx2.2*, even at concentrations (up to 800 pg per embryo) more than sufficient for mutant phenotype rescue (100 pg per embryo), consistent with recent experiments in *Drosophila* (Alcedo et al., 2000;

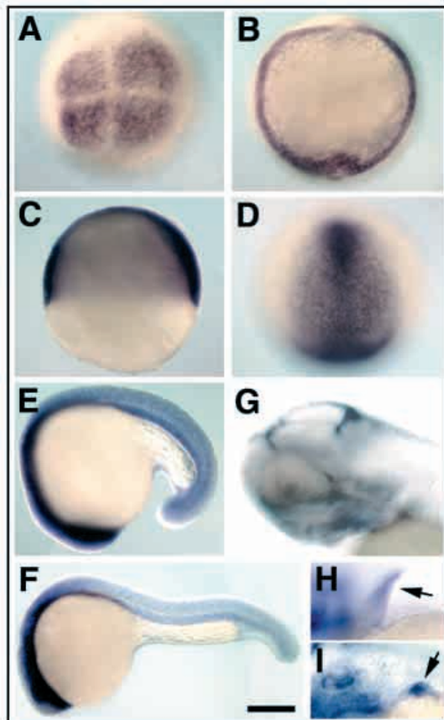

Fig. 4. *smoothened* is expressed both maternally and zygotically. (A) Maternal *smoothened* mRNA present at four-cell stage. (B,C) *smoothened* mRNA present throughout the embryo at shield (B) and 60% epiboly (C). (D) *smoothened* expression was downregulated in the non-neural ectoderm at tailbud stage. (E,F) *smoothened* was expressed at higher levels in the head than the rest of the body at 18-somites (E) and 24 h (F). (G) By 2 d, *smoothened* expression in the head was confined to dorsal brain nuclei and jaw cartilages. (H,I) At 2 d, *smoothened* was also expressed in the pectoral fin of wild-type (H) and *smu*^{b641} mutant (I) embryos. (A,B,D) Animal pole views. (B,D) Anterior is towards the top. (C,E-I) Side views, dorsal towards the top, anterior towards the left. Scale bar: 200 µm in A-E; 250 µm in F; 115 µm in G; 90 µm in H,I.

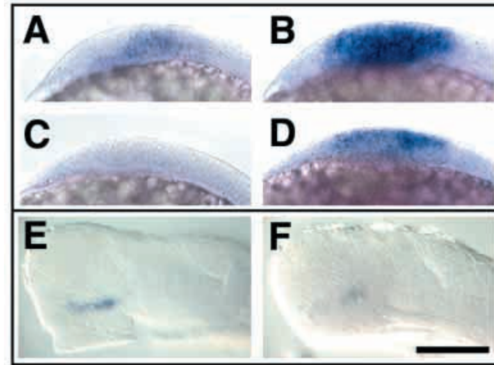

Fig. 5. *smu* mutant embryos have residual Hedgehog function provided by maternal Smoothened. *shh* mRNA increases expression of *nkx2.2* after injection into wild-type (B) and *smu* mutant (D) embryos compared with uninjected wild-type (A) or *smu* mutants (C). Injection of *shh* and *twhh* morpholinos into *smu* mutants decreases residual *nkx2.2* expression (F) compared with uninjected mutants (E). Side views, anterior towards the left; (A-D) two-somite stage; (E, F) Prim-6 stage. Scale bar: 100 µm.

Denef et al., 2000; Ingham et al., 2000) and suggesting that Smoothened is permissive for Hh signaling.

smoothened is expressed maternally and zygotically

The formation of primary motoneurons in anterior but not posterior spinal cord in *smu* mutants suggests that motoneuron specification might be mediated differently at different axial levels. For example, early anterior motoneuron formation might result from function of maternal Smoothened, whereas later posterior motoneuron formation might result from zygotic Smoothened. Therefore, we tested whether *smoothened* mRNA was present maternally in zebrafish. We found *smoothened* mRNA from the beginning of embryogenesis by RT-PCR (data not shown) and RNA in situ hybridization (Fig. 4A), well before midblastula transition when zygotic gene expression begins (Kane and Kimmel, 1993). At gastrula stages, cells throughout the embryo still expressed *smoothened* (Fig. 4B,C). By tailbud stage, ventral, non-neural ectoderm downregulated *smoothened* expression (Fig. 4D). At 18-somites, *smoothened* expression was widespread throughout the embryo but absent from ectodermal cells covering the yolk (Fig. 4E). At 24 h, ventral brain cells expressed highest levels of *smoothened*; posterior somite and spinal cord cells expressed very low levels of *smoothened* (Fig. 4F). Surprisingly at 48 h, cells in dorsal brain and posterior tectum at the mid-hindbrain boundary expressed *smoothened* in addition to regions of known Hh expression such as dorsal hindbrain, branchial arches, jaw cartilages and fin buds (Fig. 4G-I). Pectoral fin buds remained small and undifferentiated in *smu* mutants and degenerated almost completely by 48 h (Fig. 4H). In *smu* mutants, *smoothened* mRNA expression was virtually unaffected at all of these stages (Fig. 4) consistent with point mutations in the gene. Thus, as in fruit flies (van den Heuvel and Ingham, 1996), zebrafish embryos express *smoothened* maternally and zygotically.

The presence of maternal *smoothened* transcripts is consistent with the interpretation that the remaining anterior motoneurons in *smu* mutants also depend upon Smoothened function that is provided maternally. We attempted to remove maternal *smoothened* by rescuing homozygous mutant embryos to adulthood using wild-type *smoothened* mRNA injection to generate maternal-zygotic *smu* mutants. However, none of the homozygous mutants survived, probably because Smoothened is required late in development or even in the adult, long after the injected mRNA is degraded. We also attempted to block the function of maternal transcripts with morpholino antisense oligonucleotides (Nasevicius and Ekker, 2000); however, this also failed (Lewis and Eisen, 2001), probably because maternal protein is unaffected by morpholinos. Consistent with the presence of maternal Smoothened function, we found that expression of the Hh target genes, *nkx2.2* (8/23; Fig. 5A-D) and *patched1* (14/24, not shown), increased slightly after *shh* mRNA injection into *smu* mutants. This increase was apparent at the two-somite stage but not after the 18-somite stage, suggesting that maternal Smoothened function is lost during this period. We also injected morpholinos for *twhh* and *shh* and saw a reduction of the remaining *nkx2.2* expression in most *smu* mutant forebrains (8/11; Fig. 5E,F), suggesting that there is residual Hh signaling in *smu* mutant embryos. Furthermore, essentially all motoneurons were absent from triple mutants (*syu*(*shh*);*cyc*;*flh*) that lacked nearly all Hh signaling (Lewis and

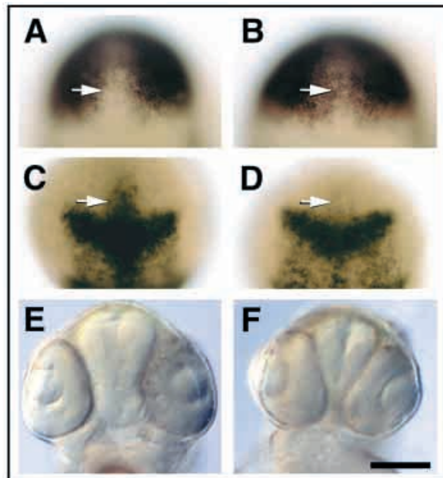

Fig. 6. Hh signaling patterns the midline of the anterior neural plate. (A,B) expression of *zic1* at tailbud stage in wild-type (A) and *smu*^{b641} mutant (B) embryos. (C,D) expression of *foxb1.2* at tailbud stage in wild-type (C) and *smu*^{b641} mutant (D) embryos. Arrows indicate location of hypothalamic precursors demarcated by *foxb1.2*. (E, F) Head morphology of wild-type (E) and *smu*^{b641} mutant (F) embryos. In 48 h *smu* mutants, the hypothalamus was reduced in size, consistent with reduced *foxb1.2* expression in neural plate precursors and subsequent progressive loss of hypothalamic tissue. (A-D) Dorsal views of prospective head region, anterior towards top. (E,F) Ventral views, anterior towards top. Scale bar: 200 µm in A-D; 135 µm in E,F.

Eisen, 2001). Together, these data support the interpretation that *smu* mutant embryos have a low level of Hh function mediated by maternally supplied Smoothened that is lost during early segmentation stages.

Smoothened is required for anterior neural plate patterning

We recently showed that a median anterior protrusion of the *foxb1.2* (previously *mariposa*; Moens et al., 1996) and *forkhead-3* (Odenthal and Nüsslein-Volhard, 1998) gene expression domain demarcates the location of hypothalamic precursors in neural plate (Varga et al., 1999). These cells are initially located posterior to retinal precursors that express the *odd-paired-like* gene *zic1* (previously *opl*; Grinblat et al., 1998), and they later shift anteriorly along the ventral midline to separate the eye primordia (Varga et al., 1999). In *smu* mutants, we observed a reduced indentation of the median *zic1* expression domain (Fig. 6A,B). Moreover, very few neural plate cells expressed *foxb1.2* in the region that normally protrudes into the *zic1* expression domain (Fig. 6C,D). These changes in gene expression in anterior neural plate are similar to, but not as severe as defects in *cyclops* (*ndr2*) mutants (Varga et al., 1999) and indicate that Hh signaling acts early in medial cell specification. Consistent with this result, we found that in *smu* mutants, the interocular distance was reduced at 24 h, although not as severely as in *cyclops* (*ndr2)* mutants (Hatta et al., 1991). By 2 d, the intraocular distance was more reduced (Fig. 6E,F) and at 3 d, hypothalamic tissue was entirely lost and the pigment epithelia of the two eyes contacted each other

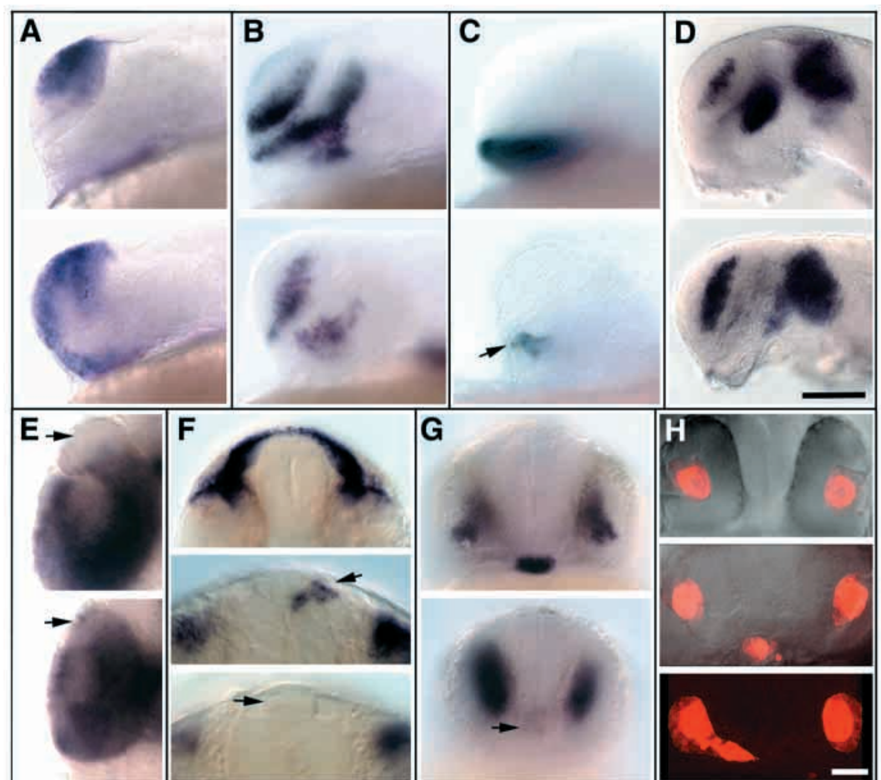

Fig. 7. *smu* mutations disrupt dorsoventral forebrain and retinal patterning, optic stalk formation and pituitary specification. (A) Expression of *emx1* in wild-type (top) and *smu*^{b641} mutant (bottom) embryos. *emx1* expression expanded into ventral regions. (B) Expression of *dlx2* in wild-type (top) and *smu*^{b641} mutant (bottom) embryos. Ventral *dlx2* expression was reduced in forebrain at the level of anterior and post-optic commissures; dorsal telencephalic expression was expanded. (C) Expression of *pax2a* in wild-type (top) and *smu*^{b641} mutant (bottom) embryos. In *smu* mutants, *pax2a* expression was lost in optic stalk and ectopic (arrow) in hypothalamus. (D) Expression of *pax6a* in wild-type (top) and *smu*^{b641} mutant (bottom) embryos. In *smu* mutants, *pax6a* expression was strongly reduced in thalamus. (E) Expression of *pax6a* expanded in ventral retina of *smu* mutants (bottom) compared with wild type (top). (F) Expression of *pax2a* in optic stalk (top, wild type) was lost in *smu* mutants (middle) and diencephalic cells expressed *pax2a* ectopically (arrow). An ectopic lens (arrow) developed in *smu* mutants, as shown in more ventral focal plane (bottom). (G) *smu* mutations affect specification of the pituitary. The anterior pituitary expressed *lim3* in wild-type embryos (top). In place of the pituitary, an ectopic lens (arrow) formed in *smu* mutants (bottom). (H) Lens fiber cells differentiated in wild type (top), *smu* mutant ectopic (middle) and retinal lenses (middle, bottom) indicated by zl-1 (red). (A-D) Side views, dorsal towards the top, anterior towards left. (E,F) Dorsal view, anterior towards top. (G,H) Anterior view, dorsal towards top. 24 h. Scale bar in D: 100 µm in A-D; 40 µm in E; 50 µm in F (top); 25 µm in F (bottom); 60 µm in G; in H, 50 µm.

(not shown). This suggests that, in addition to patterning anterior neural plate, Hh signaling is also required for maintenance of ventral forebrain cell populations that separate the eyes.

Smoothened is required for dorsoventral forebrain patterning

Previous studies suggested that Hh signaling is required for formation of ventral forebrain and specification of its cell types (Barth and Wilson, 1995; Dale et al., 1997). To test this hypothesis, we compared developmental regulatory gene expression domains within the forebrain (Hauptmann and Gerster, 2000; Rubenstein and Beachy, 1998) in wild-type and *smu* mutant embryos. Consistent with the patterning defect in neural plate and reduced interocular distance at 24 h, expression of several genes in ventral forebrain was affected in *smu* mutants. In wild-type embryos, cells of dorsal telencephalon and posterior tuberculum expressed *emx1* (Fig. 7A, top), but in *smu* mutants ventral telencephalic cells also expressed *emx1* and the hypothalamic expression domain extended to the anterior limit of the neural tube (Fig. 7A, bottom). Cells in ventral telencephalon and hypothalamus normally express *dlx2* (Fig. 7B, top), but in *smu* mutants, *dlx2* gene expression was reduced in ventral telencephalon and more pronounced in dorsal regions, close to the presumptive pallium. In hypothalamus, *dlx2* expression was lost in the prospective preoptic area and was reduced in other regions (Fig. 7B, bottom). The optic stalk and ventral retina of wild-type embryos express *pax2a* (Fig. 7C, top), but *pax2a* expression in these regions was strongly reduced in *smu* mutants. Surprisingly, we found ectopic *pax2a* expression in the preoptic area (Fig. 7C, bottom; 7F, middle). Similarly, *pax6a* expression was strongly reduced in thalamic regions, whereas its expression was upregulated in dorsal telencephalon (Fig. 7D) comparable with the changes we observed in telencephalic *dlx2* expression (Fig. 7B). Our observations strongly support the hypothesis that Hh signaling functions to specify ventral telencephalon (Gunhaga et al., 2000; Kohtz et al., 1998) and diencephalon (Dale et al., 1997), and further indicate that when Hh signaling is reduced or absent, forebrain cells lose ventral and gain dorsal characteristics.

Smoothened is required for dorsoventral retinal patterning and optic stalk development

Previous studies implicated midline signaling and *pax* gene expression in formation of optic stalk and retina. Overexpression of *shh* leads to upregulation of *pax2a* expression and hypertrophy of optic stalk, while *pax6a* expression downregulates and the resulting retina is smaller (Macdonald et al., 1995). Surprisingly however, loss of *shh* in *syu* (*shh*) mutants leaves *pax* expression and eye development relatively unaffected (Schauerte et al., 1998). By contrast, *cyclops* (*ndr2)* mutants that lack Nodal1 signaling have severe *pax* expression and retinal defects (Macdonald et al., 1995). Thus, the relative roles of Hh, Nodal and Pax in ventral forebrain patterning are still unclear.

To learn whether Hh signaling is required for optic stalk and retina formation, we analyzed *pax2a* and *pax6a* expression in *smu* mutants. In wild-type embryos at 24 h, cells throughout the retinal primordium, except presumptive ventral retina expressed *pax6a* (Fig. 7E, top), whereas presumptive ventral retina and optic stalk expressed *pax2a* (Fig. 7F, top). In *smu* mutants, however, the entire retina expressed *pax6a* (Fig. 7E, bottom), and *pax2a* expression was lost from both retina and optic stalk (Fig. 7F, middle). Instead, hypothalamic cells in the prospective preoptic region variably expressed *pax2a* ectopically in *smu* mutants (Fig. 7F, middle). These results indicate that Hh signaling is required for optic stalk formation and eye patterning. Lack of comparable phenotypes in *syu* (*shh*) mutants may indicate that other Hh family members, such as *twhh*, are sufficient for normal development of these tissues (Nasevicius and Ekker, 2000).

Hedgehog signaling specifies anterior pituitary precursors

In the Hh pathway mutants, *yot* (*gli2*) and *igu*, an ectopic lens develops in place of the anterior pituitary (Kondoh et al., 2000), suggesting that Hh signaling functions in adenohypophysis development. We also observed an ectopic lens in *smu* mutants, between anterior ventral epidermis and hypothalamus where anterior pituitary normally forms (Fig. 7F-H). Surprisingly, the ectopic lens was located close to the region where *pax2a* was inappropriately expressed in hypothalamic cells (Fig. 7F). In addition to the ectopic midline lens, we also observed that retinal lenses were mis-shapen and extended anteriorly (Fig. 7H).

These observations raised the possibility that lens precursor cells were incorrectly specified in *smu* mutants. We tested this idea by labeling *smu* mutants with antisense probes for *lim3* mRNA (Glasgow et al., 1997) that is normally expressed in anterior pituitary, epiphysis, retina and spinal cord neurons. *smu* mutants completely lacked *lim3* expression in the region of prospective anterior pituitary, although other regions, including the epiphysis (not shown) and retina expressed it (Fig. 7G). We obtained similar results with other genes such as *nkx2.2*, *six3* and *islet1*, which are normally also expressed in anterior pituitary (not shown). Our results show that *smu* mutations affect pituitary development more severely than *yot* (*gli2*) or *igu*, and support the view that overlapping Hh signals normally specify anterior pituitary precursors.

Smoothened is required for formation of the forebrain primary axonal scaffold

Several of the gene expression domains affected in *smu* mutants are thought to demarcate borders along which axon tracts form (Macdonald et al., 1994). To test this hypothesis further, we compared formation of the primary axonal scaffold in the forebrains of wild-type and *smu* mutant embryos. Because *smu* mutations affect patterning of the ventral midline and *pax* gene expression, we analyzed commissure formation and formation of the optic chiasm, using DM-GRASP antibodies that label retinal ganglion cells and their axons (Fashena and Westerfield, 1999). Unlike in wild type (Fig. 8A), we observed that retinal ganglion cell axons were unable to form an optic chiasm or to cross to the contralateral side in *smu* mutants (Fig. 8B). As with other mutations that affect the Hedgehog signaling pathway (*detour*, *you-too*, *iguana*; Karlstrom et al., 1996) retinal ganglion cell axons turned ipsilaterally and rostrally as soon as they exited the retina. Observations obtained using anti-acetylated tubulin antibodies confirmed absence of the optic chiasm (Fig. 8D). We observed similar defects in forebrain commissures. In wild type, anterior

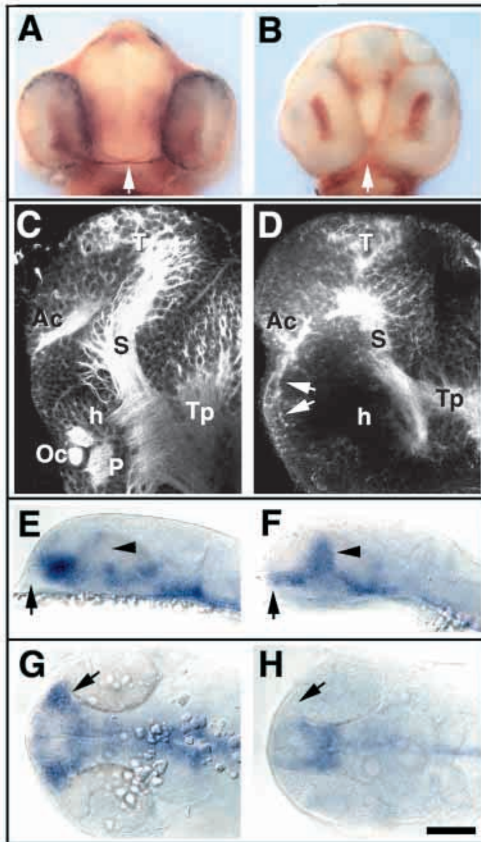

Fig. 8. *smu* mutations disrupt formation of forebrain axon tracts and commissures. (A,B) An optic chiasm (arrow) was formed in wild-type (A) but not *smu* mutant (B) embryos. In *smu* mutants (D), anterior commissure axons (arrows) projected into hypothalamus (h), optic chiasm was absent and supra-optic tract axons (S) extended toward the ventral midline (compare with wild type in C). (E-H) Expression of *netrin1* is altered in *smu* mutant embryos. In *smu* mutant embryos (F) *netrin1* expression is upregulated in the midline of the neural tube at the level of the post optic commissure (E,F; arrows) and near the presumptive zona limitans intrathalamica (E,F; arrowheads). The *netrin1* expression domain close to the presumptive exit point of ganglion cell axons from the retina (G, arrow) is absent in *smu* mutants (H, arrow). Ac, anterior commissure; h, hypothalamus; Oc, optic chiasm; P, post-optic commissure; S, supra-optic tract; T, telencephalon; Tp, tract of the post-optic commissure. (A,B) Ventral views, dorsal towards the top, DM-GRASP label, 36 h. (C-F) Side views, dorsal towards the top, anterior towards the left, 48 h; (C,D) acetylated tubulin label. (G,H) Dorsal views, anterior towards left. Scale bar: 100 μm in A,B; 22.5 μm in C,D; 85 μm in E-H.

commissure axons are restricted to ventral telencephalon and do not extend into diencephalon. Telencephalic neurons that extend axons into the supraoptic tract form connections between telencephalon and diencephalon (Chitnis and Kuwada, 1990; Wilson et al., 1990). In *smu* mutants, however, anterior commissure axons grew across the telencephalic-diencephalic border, much like supraoptic tract axons, but splayed out across the diencephalon, rather than forming fascicles (Fig. 8D). The supraoptic tract also developed aberrantly; it formed bifurcated tracts and failed to extend to its normal target territory near the nucleus of the tract of the post-optic commissure (nTPOC). Although hypothalamic neurons developed in the nTPOC, the post-optic commissure did not form in *smu* mutants. In some embryos, however, we observed a few axons that extended from the nTPOC to the contralateral side, where they intermingled with ectopically projecting anterior commissure axons. These results indicate that Smoothened-mediated Hh signaling is required for patterning of forebrain commissures and axons tracts.

These defects in axon tracts suggested that axon pathfinding is disrupted in forebrain of *smu* mutants. Analysis of *noi* (*pax2a*) mutants has suggested that Netrins promote growth toward the midline (Macdonald et al., 1997). Consistent with this idea, we found a marked reduction in *netrin1* expression in the region where axons exit the retina and project to the optic stalk (Fig. 8E-H). Thus, retinal ganglion cell axons may be misguided in *smu* mutants, owing to the absence of Netrin1 and optic stalk. We also found a reduction in *netrin1* expression in the region of the anterior commissure (Fig. 8G,H) and, surprisingly, an increase in expression in the presumptive zona limitans intrathalamica (Fig. 8E,F), changes that could underlie aberrant pathfinding of these axons.

DISCUSSION

Hh is a key signaling molecule for embryonic patterning and tissue polarization (Ingham, 1998). In this study, we identify *smu* (Barresi et al., 2000) as the locus encoding zebrafish Smoothened, a protein required for Hh signal transduction. Two independent lines of evidence, phylogenetic analysis and conserved syntenies, strongly support the conclusion that zebrafish *smoothened* is the ortholog of tetrapod *SMOOTHENED*. We show that mutations in *smoothened* affect dorsoventral patterning, cell specification and axon tract formation in regions of the embryonic CNS that express Hh proteins, and that synthetic *smoothened* mRNA can rescue embryonic morphology and expression of Hh target genes in *smu* mutants. Our analysis suggests that signaling by Hh family members mediates both overlapping and separate specification and patterning functions during CNS development.

Transduction of Hh signaling is affected in *smu* mutants

We characterized the molecular defects in two *smu* mutant alleles and found that single nucleotide changes led to missense mutations in both cases. The smu^{b577} point mutation changed an amino acid from charged to hydrophobic in an extracellular domain, which would be expected to have dramatic effects on protein folding. Similarly, point mutations, such as the one in smu^{b641} that change a residue from uncharged to charged in the middle of a transmembrane domain, can also severely disrupt protein structure and function. In human *SMOOTHENED*, a gain of function missense mutation changes arginine into serine in the seventh transmembrane domain in primitive neuroectodermal tumors and basal cell carcinomas (Booth, 1999; Reifenberger et al., 1998) indicating the importance of single amino acid changes in mutations that affect transmembrane domains. Consistent with the smu^{b641} mutation disrupting the second transmembrane domain, we saw loss of expression of downstream targets of Hh signaling in smu^{b641} mutants, and even high doses of *shh* did not rescue the mutant

phenotype. These results suggest that all zygotic Smoothened function is lost in homozygous *smu*^{b641} mutants.

In contrast to mouse (Chiang et al., 1996), mutations in most zebrafish Hh pathway genes affect only a subset of tissues thought to require Hh signaling and many mutants have mild or variable phenotypes (Brand et al., 1996; Karlstrom et al., 1996; Lewis et al., 1999; Odenthal et al., 2000; Schier et al., 1996; van Eeden et al., 1996). However, the *smu* mutant alleles we describe are fully penetrant and show no phenotypic variability. In addition, these mutations affect at least most tissues known to require Hh signaling for normal development in vertebrates (Hammerschmidt et al., 1997). Our observations, are consistent with the suggestion that Hh signals converge on a single Smoothened in zebrafish. However, the *smu* mutant phenotype is less profound than the phenotype of mouse *Shh* deletions; *smu* mutants form anterior primary motoneurons and are synophthalmic rather than cyclopic. These observations raise the possibility that another *smoothened* might mediate these functions, perhaps a duplicate arising at the time of the genome duplication that occurred in the teleost lineage leading to zebrafish (Amores et al., 1998; Postlethwait et al., 1998). Alternatively, maternal transcripts and protein may provide enough Smoothened function to mediate early Hh signaling in *smu* mutants (Lewis and Eisen, 2001). Additionally, zebrafish Nodal may subsume some functions of Hh in tetrapods (Rohr et al., 2001; Sampath et al., 1998); thus, defects in Hh signaling alone may lead to milder defects in zebrafish than targeted *shh* deletions in mouse.

Hh signaling is required for formation of a subset of floor plate cells

We show that lateral floor plate and parts of ventral forebrain fail to form in *smu* mutants. Consistent with our previous fate map analysis of ventral forebrain (Varga et al., 1999), we found that patterning defects in anterior neural plate of *smu* mutants at the end of gastrulation correlated with reduced hypothalamic tissue and interocular distance at 24 h. In comparison, *cyclops* (*ndr2)* mutants lack medial floor plate and ventral forebrain and form cyclopic eyes (Hatta et al., 1991; Sampath et al., 1998; Varga et al., 1999). Recent studies have suggested that in hypothalamus, both Nodal and Hh signaling are required for normal ventral forebrain specification (Rohr et al., 2001). Our results support this interpretation, because Nodal is thought to act upstream of Hh (Müller et al., 2000), and Hh expression is lost from the anterior neural plate in Nodal pathway mutants such as *cyclops* (*ndr2)* and *one-eyed-pinhead* (*oep*). Thus, in Nodal mutants, both medial floorplate, as well as its presumptive anterior extension into hypothalamus, is lost, whereas in the Hh pathway mutants, only lateral floor plate is affected (Odenthal et al., 2000) and the hypothalamus initially forms. We therefore suggest that the less severe forebrain defects in *smu* mutants are due to loss of the anterior extension of lateral floor plate in the forebrain. Furthermore, although medial floor plate may initially be induced in a Hh-independent manner by Nodal signals (Odenthal et al., 2000), Hh signaling is required much later for its maintenance.

smu is required for zebrafish motoneuron formation

Our data are consistent with the idea that, as in mouse (Placzek et al., 2000), Hh signaling is required for zebrafish motoneuron formation. In *smu* mutants, primary motoneurons form in anterior spinal cord, but not posteriorly, and later-developing secondary motoneurons completely fail to form. *smoothened* is expressed both maternally and zygotically, suggesting a dual role: maternal expression supports anterior primary motoneuron formation, whereas zygotic expression is required for primary motoneuron induction in posterior trunk and tail, as well as for later-developing secondary motoneurons. Our results also suggest that induction of primary motoneurons and secondary motoneurons in *syu* (*shh*) mutants probably results from other Hh proteins acting in place of Shh, reinforcing the idea that these signals have overlapping functions (Lewis and Eisen, 2001).

Hh signaling is required for ventral forebrain patterning and axon tract formation

Dorsoventral forebrain patterning is disrupted in *smu* mutants. Our analysis suggests that diencephalon and telencephalon lose ventral character. Hh signaling leads to induction of genes expressed ventrally, such as *nkx* (Barth and Wilson, 1995) and *dlx* (Moreno and Morata, 1999). In *smu* mutants, we found reduced expression of genes that normally demarcate ventral regions like *nkx2.2*, *dlx2* and *pax6a*, and expansion of dorsally expressed *emx1* into ventral regions, consistent with other studies indicating a role of Hh signaling for specification of ventral forebrain fates (Gunhaga et al., 2000; Kohtz et al., 1998). Recently, Hh signaling was shown to antagonize Gli3, a *cubitus interruptus* homolog that suppresses ventral fates (Litingtung and Chiang, 2000). Gli3 could mediate dorsal restriction of telencephalic genes such as *emx1* and, in the absence of Hh signaling, dorsal telencephalic genes may be de-repressed ventrally.

In addition to gene expression defects, axon tract formation is severely affected in the *smu* mutant ventral forebrain. In wild type, anterior commissure axons project to ventral telencephalon and not diencephalon. In *smu* mutants, however, anterior commissure axons grew aberrantly into diencephalon, which correlates with the expansion of *emx1* expression into ventral telencephalon and anterior diencephalon. Aberrant growth of anterior commissure axons into diencephalon in *smu* mutants may result from expression of dorsal genes like *emx1* in ventral telencephalic and anterior diencephalic cells that redirects these cells to adopt fates of more dorsal cells including normal targets of these axons. Additionally, Hh signaling may regulate directly expression of Netrin1 and other extracellular guidance cues in ventral neural tube (Lauderdale et al., 1998; Strähle et al., 1997). Thus, changes in the distributions of these extracellular cues in Hh pathway mutants, such as we have demonstrated for Netrin1 in *smu* mutants, may lead to the aberrant growth of axons, including loss of commissures.

Hh signaling promotes pituitary and suppresses lens development

Hh signaling has been implicated in pituitary development. In chick, the oral ectoderm thought to be responsible for anterior pituitary induction expresses Shh before Rathke's pouch forms (Dasen and Rosenfeld, 1999), and recent analysis suggests that Shh is required for pituitary formation in mouse (Treier et al., 2001). Analysis of zebrafish *yot* (*gli2*) and *igu* mutants showed that an ectopic lens forms in place of the anterior pituitary placode (Karlstrom et al., 1999; Kondoh et al., 2000). These ectopic lenses are morphologically indistinguishable from

normal lenses and express β-crystallins, suggesting that disruption of Hh signaling leads to transdifferentiation of anterior pituitary cells into lens (Kondoh et al., 2000).

We found that *smu* mutants completely lack a pituitary and form ectopic or expanded lenses. Tissue explants in *Xenopus* showed that a large domain of non-neural ectoderm is transiently competent to form lens (Henry and Grainger, 1990; Servetnick and Grainger, 1991). This domain forms at the lateral edge of the neural plate, some distance from midline Hh signaling. Overexpression of *shh* in wild-type zebrafish embryos suppresses lens formation (Barth and Wilson, 1995), and our analysis of *smu* mutants demonstrates that reduced Hh signaling induces ectopic lenses. These results indicate that Hh signaling influences specification of cells in non-neural ectoderm.

Recent fate map analyses indicate that pituitary precursors arise from the anterior neural ridge in tetrapods (Rubenstein et al., 1998) and in zebrafish from the corresponding region, demarcated by *dlx3* expression (Whitlock and Westerfield, 2000). In this median position, unspecified precursors could be exposed to Hh signals emanating from ventral midline. Hh may suppress lens fate in these precursors or instruct them to differentiate as pituitary placode. Alternatively, anterior pituitary precursor cells might not be mis-specified in *smu* mutants, rather lens precursors might be incorrectly allocated to median positions, and pituitary precursors might contribute to other, yet unidentified fates, or die. Lens differentiation requires signals from neural plate (Henry and Grainger, 1990). Our observation that cells close to the ectopic lens in the anterior hypothalamus ectopically express *pax2a*, suggests that lens may also influence gene expression in the neural tube.

Our results resolve the controversy of whether Shh functions differently during cell fate specification in zebrafish and tetrapods. Zebrafish have two orthologs of human *SHH*, *shh* and *twhh* (Krauss et al., 1993; Ekker et al., 1995; Zardoya et al., 1996), but our results suggest that all zebrafish Hh signaling apparently acts through a single Smoothened protein. Thus, Hh-dependent specification of some early forming cell types may occur in zebrafish *smu* mutants but not in mouse *Shh* mutants, because Smoothened function is provided maternally. In addition, other signals such as Nodal family members act in concert with Hedgehogs in zebrafish (Odenthal et al., 2000; Rohr et al., 2001). It will be important to learn whether this is also the case during mammalian development. Finally, if all Hh signaling acts through a single transmembrane mediator, we need to understand how and at which level of the signaling cascade tissue and cell specificity is achieved. At the transcriptional level, combinations of different Gli proteins have been implicated in tissue induction and cell-type specification (Ruiz i Altaba, 1998). We suggest that other modulating factors function at the cell membrane. They could convey tissue and cell specificity, either by interacting directly with Hh (Chuang and McMahon, 1999) or the transmembrane proteins Patched and Smoothened.

Note added in proof

Chen et al. (Chen et al., 2001) report similar observations of a retroviral induced mutation in *smoothened*.

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REFERENCES

Alcedo, J., Zou, Y. and Noll, M. (2000). Posttranscriptional regulation of smoothened is part of a self-correcting mechanism in the Hedgehog signaling system. *Mol. Cell* **6**, 457-465.

Amemiya, C. T. and Zon, L. I. (1999). Generation of a zebrafish P1 artificial chromosome library. *Genomics* **58**, 211-213.

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., Ho, R. K., Langeland, J., Prince, V., Wang, Y. L. et al. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science* **282**, 1711-1714.

Appel, B., Korzh, V., Glasgow, E., Thor, S., Edlund, T., Dawid B., I. and Eisen S., J. (1995). Motoneuron fate specification revealed by patterened LIM homeobox gene expression in embryonic zebrafish. *Development* **121**, 4117-4125.

Barresi, M. J., Stickney, H. L. and Devoto, S. H. (2000). The zebrafish slow-muscle-omitted gene product is required for hedgehog signal transduction and the development of slow muscle identity. *Development* **127**, 2189-2199.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

Booth, D. R. (1999). The hedgehog signalling pathway and its role in basal cell carcinoma. *Cancer Metastasis Rev.* **18**, 261-284.

Brand, M., Heisenberg, C.-P., Warga, R. M., Pelegri, F., Karlstrom, R. O., Beuchle, D., Picker, A., Jiang, Y. J., Furutani-Seiki, M., van-Eeden, F. J. M. et al. (1996). Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* **123**, 129-412.

Chen, W., Burgess, S. and Hopkins, N. (2001). Analysis of the zebrafish *smoothened* mutant reveals conserved and divergent functions of hedgehog activity. *Development* **128**, 2385-2396.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature* **383**, 407-413.

Chitnis, A. B. and Kuwada, J. Y. (1990). Axonogenesis in the brain of zebrafish embryos. *J. Neurosci.* **10**, 1892-1905.

Chuang, P. T. and McMahon, A. P. (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397**, 617-621.

Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P. and Ingham, P. W. (1996). Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* **122**, 2835-2846.

Currie, P. D. and Ingham, P. W. (1996). Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* **382**, 452-455.

Dale, J. K., Vesque, C., Lints, T. J., Sampath, T. K., Furley, A., Dodd, J. and Placzek, M. (1997). Cooperation of *BMP7* and *SHH* in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* **90**, 257-269.

Dasen, J. S. and Rosenfeld, M. G. (1999). Signaling mechanisms in pituitary morphogenesis and cell fate determination. *Curr. Opin. Cell Biol.* **11**, 669-677.

Denef, N., Neubuser, D., Perez, L. and Cohen, S. M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* **102**, 521-31.

Echelard, Y., Epstein, D. J., St. Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Efron, B. and Gong, G. (1983). A leisurely look at the bootstrap, the jacknife, and cross-validation. *Am. Stat.* **37**, 36-48.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate *hedgehog* proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

Fashena, D. and Westerfield, M. (1999). Secondary motoneuron axons localize DM-GRASP on their fasciculated segments. *J. Comp. Neurol.* **406**, 415-424.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791.

Glasgow, E., Karavanov, A. A. and Dawid, I. B. (1997). Neuronal and neuroendocrine expression of Lim3, a Lim class homeobox gene, is altered in mutant zebrafish with axial signaling defects. *Dev. Biol.* **192**, 405-419.

Grinblat, Y., Gamse, J., Patel, M. and Sive, H. (1998). Determination of the zebrafish forebrain: induction and patterning. *Development* **125**, 4403-4416.

Grunwald, D. J., Kimmel, C. B., Westerfield, M., Walker, C. and Streisinger, G. (1988). A neural degeneration mutation that spares primary neurons in the zebrafish. *Dev. Biol.* **126**, 115-128.

Gunhaga, L., Jessell, T. M. and Edlund, T. (2000). Sonic hedgehog signaling at gastrula stages specifies ventral telencephalic cells in the chick embryo. *Development* **127**, 3283-3293.

Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S. et al. (1996). Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell* **85**, 841-851.

Hammerschmidt, M., Bitgood, M. J. and Mcmahon, A. P. (1996). Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. *Gene Dev.* **10**, 647-658.

Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to *hedgehog*. *Trends Genet.* **13**, 14-21.

Hatta, K., Kimmel, C. B., Ho, R. K. and Walker, C. (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339-341.

Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. *Trends Genet.* **10**, 266.

Hauptmann, G. and Gerster, T. (2000). Regulatory gene expression patterns reveal transverse and longitudinal subdivisions of the embryonic zebrafish forebrain. *Mech. Dev.* **91**, 105-118.

Henry, J. J. and Grainger, R. M. (1990). Early tissue interactions leading to embryonic lens formation in *Xenopus laevis*. *Dev. Biol.* **141**, 149-163.

Hynes, M., Ye, W., Wang, K., Stone, D., Murone, M., Sauvage, F. and Rosenthal, A. (2000). The seven-transmembrane receptor smoothened cell-autonomously induces multiple ventral cell types. *Nat. Neurosci.* **3**, 41-46.

Ingham, P. W. (1998). Transducing Hedgehog: the story so far. *EMBO J.* **17**, 3505-3511.

Ingham, P. W., Nystedt, S., Nakano, Y., Brown, W., Stark, D., van den Heuvel, M. and Taylor, A. M. (2000). Patched represses the Hedgehog signalling pathway by promoting modification of the Smoothened protein. *Curr. Biol.* **10**, 1315-1318.

Inoue, A., Takahashi, M., Hatta, K., Hotta, Y. and Okamoto, H. (1994). Developmental regulation of Isl-1 mRNA expression during neuronal differentiation in embryonic zebrafish. *Dev. Dyn.* **199**, 1-11.

Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr et al. (1996a). Human homolog of *patched*, a candidate gene for the basal cell nevus syndrome. *Science* **272**, 1668-1671.

Johnson, S. L., Gates, M. A., Johnson, M., Talbot, W. S., Horne, S., Baik, K., Rude, S., Wong, J. R. and Postlethwait, J. H. (1996b). Half-tetrad analysis in zebrafish II: centromere-linkage analysis and consolidation of the zebrafish genetic map. *Genetics* **142**, 1277-1288.

Jowett, T. and Lettice, L. (1994). Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. *Trends Genet.* **10**, 73-74.

Kalderon, D. (2000). Transducing the hedgehog signal. *Cell* **103**, 371-374.

Kane, D. A. and Kimmel, C. B. (1993). The zebrafish midblastula transition. *Development* **119**, 447-456.

Karlstrom, R. O., Trowe, T., Klostermann, S., Baier, H., Brand, M., Crawford, A. D., Grunewald, B., Haffter, P., Hoffman, H., Meyer, S. U. et al. (1996) Zebrafish mutations affecting retinotectal axon pathfinding. *Development* **123**, 427-438.

Karlstrom, R. O., Trowe, T. and Bonhoeffer, F. (1997). Genetic analysis of axon guidance and mapping in the zebrafish. *Trends Neurosci.* **20**, 3-8.

Karlstrom, R. O., Talbot, W. S. and Schier, A. F. (1999). Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target gli2 affect ventral forebrain patterning. *Genes Dev.* **13**, 388-393.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Knapik, E., Goodman, A., Ekker, M., Chevrette, M., Delgado, J., Neuhauss, S., Shimoda, N., Driever, W., Fishman, M. and Jacob, H. (1998). A microsatellite genetic linkage map for zebrafish (Danio rerio). *Nat. Genet.* **18**, 338-343.

Kobayashi, M., Toyama, R., Takeda, H., Dawid, I. B. and Kawakami, K. (1998). Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125**, 2973-2982.

Kohtz, J. D., Baker, D. P., Corte, G. and Fishell, G. (1998). Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. *Development* **125**, 5079-5089.

Kondoh, H., Uchikawa, M., Yoda, H., Takeda, H., Furutani-Seiki, M. and Karlstrom, R. O. (2000). Zebrafish mutations in gli-mediated hedgehog signaling lead to lens transdifferentiation from the adenohypophysis anlage. *Mech. Dev.* **96**, 165-174.

Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.

Lauderdale, J. D., Pasquali, S. K., Fazel, R., van Eeden, F. J., Schauerte, H. E., Haffter, P. and Kuwada, J. Y. (1998). Regulation of netrin-1a expression by hedgehog proteins. *Mol. Cell. Neurosci.* **11**, 194-205.

Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.

Lewis, K. E., Currie, P. D., Roy, S., Schauerte, H., Haffter, P. and Ingham, P. W. (1999). Control of muscle cell-type specification in the zebrafish embryo by hedgehog signalling. *Dev. Biol.* **216**, 469-480.

Litingtung, Y. and Chiang, C. (2000). Specification of ventral neuron types is mediated by an antagonistic interaction between shh and gli3. *Nat Neurosci* **3**, 979-985.

Macdonald, R., Xu, Q., Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S. and Wilson, S. W. (1994). Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron* **13**, 1039-1053.

Macdonald, R., Barth, A. K., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

Macdonald, R., Scholes, J., Strähle, U., Brennan, C., Holder, N., Brand, M. and Wilson, S.W. (1997). The Pax protein Noi is required for commissural axon pathway formation in the rostral forebrain. *Development* **124**, 2397-2408.

Moens, C. B., Yan, Y.-L., Appel, B., Force, A. G. and Kimmel, C. B. (1996). *valentino*: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-3990.

Moreno, E. and Morata, G. (1999). Caudal is the Hox gene that specifies the most posterior Drosophile segment. *Nature* **400**, 873-877.

Muenke, M. and Beachy, P. A. (2000). Genetics of ventral forebrain development and holoprosencephaly. *Curr. Opin. Genet. Dev.* **10**, 262-269.

Müller, F., Albert, S., Blader, P., Fischer, N., Hallonet, M. and Strähle, U. (2000). Direct action of the Nodal-related signal Cyclops in induction of sonic hedgehog in the ventral midline of the CNS. *Development* **127**, 3889-3897.

Nasevicius, A. and Ekker, S. C. (2000). Effective targeted gene '*knockdown*' in zebrafish. *Nat. Genet.* **26**, 216-220.

Nüsslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* **287**, 795-801.

Odenthal, J. and Nusslein-Volhard, C. (1998). fork head domain genes in zebrafish. *Dev. Genes Evol.* **208**, 245-258.

Odenthal, J., van Eeden, F. J., Haffter, P., Ingham, P. W. and Nüsslein-Volhard, C. (2000). Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways. *Dev. Biol.* **219**, 350-363.

Placzek, M., Dodd, J. and Jessell, T. M. (2000). Discussion point. the case for floor plate induction by the notochord. *Curr. Opin. Neurobiol.* **10**, 15-22.

Postlethwait, J. H., Yan, Y. L., Gates, M. A., Horne, S., Amores, A., Brownlie, A., Donovan, A., Egan, E. S., Force, A., Gong, Z. et al. (1998). Vertebrate genome evolution and the zebrafish gene map. *Nat. Genet.* **18**, 345-349.

Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P. and Reifenberger, G. (1998). Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.* **58**, 1798-1803.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Riley, B. B. and Grunwald, D. J. (1995). Efficient induction of point

mutations allowing recovery of specific locus mutations in zebrafish. *Proc. Natl. Acad. Sci. USA* **92**, 5997-6001.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. et al. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of Sonic Hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rohr, K. B., Barth, K. A., Varga, Z. M. and Wilson, S. W. (2001). The Nodal pathway acts upstream of Hedgehog signalling to specify ventral telencephalic identity. *Neuron* **29**, 341-351.

Rubenstein, J. L., Shimamura, K., Martinez, S. and Puelles, L. (1998). Regionalization of the prosencephalic neural plate. *Annu. Rev. Neurosci.* **21**, 445-477.

Rubenstein, J. L. R. and Beachy, P. A. (1998). Patterning of the embryonic forebrain. *Curr. Opin. Neurobiol.* **8**, 18-26.

Ruiz i Altaba, A. (1998). Combinatorial Gli gene function in floor plate and neuronal inductions by *Sonic hedgehog*. *Development* **125**, 2203-2212.

Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-189.

Schauerte, H. E., van Eeden, F. J., Fricke, C., Odenthal, J., Strahle, U. and Haffter, P. (1998). Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125**, 2983-2993.

Schier, A. F., Neuhauss, S. C. F., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z. et al. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123**, 165-178.

Servetnick, M. and Grainger, R. M. (1991). Homeogenetic neural induction in *Xenopus*. *Dev. Biol.* **147**, 73-82.

Shimoda, N., Knapik, E. W., Ziniti, J., Sim, C., Yamada, E., Kaplan, S., Jackson, D., de Sauvage, F., Jacob, H. and Fishman, M. C. (1999). Zebrafish genetic map with 2000 microsatellite markers. *Genomics* **58**, 219-232.

Strähle, U., Blader, P., Henrique, D. and Ingham, P. W. (1993). Axial, a zebrafish gene expressed along the developing body axis, shows altered expression in cyclops mutant embryos. *Genes Dev.* **7**, 1436-1446.

Strähle, U., Fischer, N. and Blader, P. (1997). Expression and regulation of a netrin homologue in the zebrafish embryo. *Mech. Dev.* **62**, 147-160.

Swofford, D. L., Olsen, G. J., Waddell, P. J. and Hillis, D. M. (1996). Phylogenetic inference. In *Molecular Systematics*. Sunderland, USA: Sinauer Associates,.

Treier, M., O'Connell, S., Gleiberman, A., Price, J., Szeto, D. P., Burgess, R., Chuang, P. T., McMahon, A. P. and Rosenfeld, M. G. (2001). Hedgehog signaling is required for pituitary gland development. *Development* **128**, 377-386.

van den Heuvel, M. and Ingham, P. W. (1996). *smoothened* encodes a receptor-like serpentine protein requied for *hedgehog* signalling. *Nature* **382**, 547-551.

van Eeden, F. J. M., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A. et al. (1996). Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* **123**, 153-164.

van-Eeden, F. J. M., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y.-J., Kane, D. A. et al. (1996). Mutations affecting somite formation and patterning in the zebrafish, Danio rerio. *Development* **123**, 153-164.

Varga, Z. M., Wegner, J. and Westerfield, M. (1999). Anterior movement of ventral diencephalic precursors separates the primordial eye field in the neural plate and requires *cyclops*. *Development* **126**, 5533-5546.

Westerfield, M. (1995). The Zebrafish Book; A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). Eugene: University of Oregon Press.

Whitlock, K. E. and Westerfield, M. (2000). The olfactory placodes of the zebrafish form by convergence of cellular fields at the edge of the neural plate. *Development* **127**, 3645-3653.

Wilson, S. W., Ross, L. S., Parrett, T. and Easter, S. S., Jr (1990). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, Brachydanio rerio. *Development* **108**, 121-145.

Woods, I. G., Kelly, P. D., Chu, F., Ngo-Hazelett, P., Yan, Y. L., Huang, H., Postlethwait, J. H. and Talbot, W. S. (2000). A comparative map of the zebrafish genome. *Genome Res.* **10**, 1903-1914.

Xie, J., Murone, M., Luoh, S. M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. W., Hynes, M., Goddard, A. et al. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* **391**, 90-92.

Yan, Y. L., Hatta, K., Riggleman, B. and Postlethwait, J. H. (1995). Expression of a type II collagen gene in the zebrafish embryonic axis. *Dev. Dyn.* **203**, 363-376.

Zardoya, R., Abouheif, E. and Meyer, A. (1996). Evolution and orthology of hedgehog genes. *Trends Genet.* **12**, 496-497.