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

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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 Smoothed, mediate Hh signaling intracellularly (Ingham, 1998; Denef et al., 2000). Patched is the Hh receptor, but silences signaling in the absence of Hh. Smoothed interacts with Patched, but not Hh, and is required for signal transduction. Hh binding to Patched may relieve Patched repression of Smoothed (Kalderon, 2000). In vertebrate development, however, the role of Smoothed remains unclear, because loss-of-function mutations in smoothened have not been reported. Constitutively active forms of Smoothed mimic Sonic hedgehog (Shh) function cell-autonomously in patterning neural tube, inducing Hh target genes and specifying ventral cells, supporting evidence that Smoothed 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 (Twh; 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 myosepta (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 (smo), that affects Hh signaling. Pharmacological and RNA injection studies suggest that smo mutations act on the Hh receptor complex (Barresi et al., 2000). We now report characterization, molecular identification and cloning of the smo gene. Our results suggest that the smo<sup>577</sup> and smo<sup>b641</sup> mutant alleles affect zebrafish smoothened. smo 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 smo mutant phenotype.

Our analysis suggests that the requirement for Hh signaling differs along the antero-posterior 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 smo<sup>577</sup> and smo<sup>b641</sup> 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, 72002, 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://nucbio.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’-AAGAAGCTTCAAGGATATGF-3’ and reverse 5’-TGTCTT-TGATGCGAATTGCT-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/HomoGene), 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


Immunohistochemistry and in situ hybridization

Embryos were labeled (Wilson et al., 1990) with monoclonal antibodies: zn-5 for DM-GRASP, zn-8 for DM-GRASP, zn-9 for DM-GRASP and for screening of a pooled cDNA library. Positive cDNA clones were sequenced to identify smoothened cDNA

mRNA, genomic DNA and morpholino injections

The smoothened pCS2+ vector was digested with NotI or XhoI 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 mRNA 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′-GCACAGCAGT-3′ and 5′-GGTTGTTCT-3′, 5′-CTGGGATCGCTTTGGTCTGG-3′ and 5′-AATATCGGCGGGTGTTGTCT-3′ and 5′-AAGAAGCTTCAGGGAATATGTG-3′ and 5′-AGCAGAAGCCCCGCAGACAGT-3′.

Microscopy
Embryos were mounted (Westerfield, 1995), observed with Zeiss Microscopy, 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, smu577 and smu641, 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 smu641 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 smu641 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

<table>
<thead>
<tr>
<th>Injection (pg/embryo)</th>
<th>Wild-type phenotype</th>
<th>Partial smu* mutant phenotype</th>
<th>smu mutant phenotype</th>
<th>Total number observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double distilled H2O/Phenol Red</td>
<td>76%</td>
<td>–</td>
<td>24%</td>
<td>154</td>
</tr>
<tr>
<td>shh mRNA (200) two-cell stage</td>
<td>74%</td>
<td>–</td>
<td>26%</td>
<td>309</td>
</tr>
<tr>
<td>dnPKA mRNA (10, 50) two-cell stage</td>
<td>79%</td>
<td>16%</td>
<td>5%</td>
<td>335</td>
</tr>
<tr>
<td>PAC7202 gDNA (50) one-cell stage</td>
<td>75%</td>
<td>19%</td>
<td>6%</td>
<td>290</td>
</tr>
<tr>
<td>smoothened mRNA (100) one-cell stage</td>
<td>96%</td>
<td>4%</td>
<td>–</td>
<td>315</td>
</tr>
<tr>
<td>smoothened mRNA (50, 100) two-cell stage</td>
<td>74%</td>
<td>24%</td>
<td>2%</td>
<td>317</td>
</tr>
</tbody>
</table>

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.

Zebrafish smoothened 3499
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 nkr2.2 (Barth and Wilson, 1995). In smu b641 mutants, nkr2.2 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, nkr2.2-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). Zebrasfish mutants lacking syu (shh) develop normal medial floor plate but lack lateral floor plate (Schauerte et al., 1998), whereas cyclops (udv2) mutants lack medial floor plate (Dentehal 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...
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 (smub577, 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).

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 nkkx2.2. However, exogenous smoothened mRNA did not induce ectopic islet1, patched1 or nkkx2.2, 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;
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, nklx2.2 (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 nklx2.2 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
3503

Zebrafish smoothened 3503

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. 6.** Hh signaling patterns the midline of the anterior neural plate. (A,B) expression of zic1 at tailbud stage in wild-type (A) and smub641 mutant (B) embryos. (C,D) expression of foxb1.2 at tailbud stage in wild-type (C) and smub641 mutant (D) embryos. Arrows indicate location of hypothalamic precursors demarcated by foxb1.2. (E,F) Head morphology of wild-type (E) and smub641 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.

**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 smub641 mutant (bottom) embryos. emx1 expression expanded into ventral regions. (B) Expression of dlx2 in wild-type (top) and smub641 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 smub641 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 smub641 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.
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, we compared formation of the primary axonal scaffold (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 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 twohh, are sufficient for normal development of these tissues (Nasevicius and Ekker, 2000).

Hedgehog signaling specifies anterior pituitary precursors

In the Hh pathway mutants, you-too (gli2) and iguana, 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 nkd2.2, 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 you-too (gli2) or iguana, 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...
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<sup>b577</sup> 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<sup>b641</sup> 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, affecting Hh signaling and disrupt protein function and structure. In human SMOOTHENED, a gain of function missense mutation changes arginine into serine in the seventh transmembrane domain, indicating the importance of single amino acid changes in mutations that affect transmembrane domains. Consistent with the smu<sup>b641</sup> mutation disrupting the second transmembrane domain, we saw loss of expression of downstream targets of Hh signaling in smu<sup>b641</sup> 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<sup>b641</sup> 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 (Roehr 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 (nrd2) 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 (Roehr 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 (nrd2) 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 suy (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**

Dorsalventral 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 (Litingting 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 derepressed 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 redirect 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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