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# *Sonic hedgehog* is not required for the induction of medial floor plate cells in the zebrafish

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## SUMMARY

**Sonic hedgehog (Shh) is a secreted protein that is involved in the organization and patterning of several tissues in vertebrates. We show that the zebrafish *sonic-you* (*syu*) gene, a member of a group of five genes required for somite patterning, is encoding Shh. Embryos mutant for a deletion of *syu* display defects in patterning of the somites, the lateral floor plate cells, the pectoral fins, the axons of motorneurons and the retinal ganglion cells. In contrast to mouse embryos lacking Shh activity, *syu* mutant embryos do form medial floor plate cells and motorneurons. Since ectopic overexpression of *shh* in zebrafish embryos does not induce ectopic medial floor plate cells, we conclude that *shh* is neither required nor sufficient to induce this cell type in the zebrafish.**

Key words: *sonic hedgehog*, *sonic-you*, Zebrafish, Floor plate, Somitogenesis

## INTRODUCTION

In vertebrates two structures at the dorsal midline, the notochord and the floor plate, provide signals that play an important role in patterning the ventral neural tube and the somitic mesoderm. In response to signals from the notochord, distinct ventral cell types differentiate in the ventral neural tube, namely the floor plate cells immediately adjacent to the notochord and the motorneurons in a more lateral position (reviewed by Placzek, 1995). In chick and mouse, signaling from the notochord to the somites induces differentiation into sclerotome and suppresses dermomyotome development (reviewed by Bumcrot and McMahon, 1995).

In the zebrafish, a number of genes with defects in midline structures or signaling have been identified in mutant screens. They can be divided roughly into three classes with defects predominantly either in the notochord, the floor plate or the somites. At least four genes, *floating head* (*flh*), *momo* (*mom*), *no tail* (*ntl*) and *doc*, were found to be required for early notochord formation (Halpern et al., 1993; Odenthal et al., 1996; Stemple et al., 1996; Talbot et al., 1995). Mutations in all four genes result in embryos lacking muscle pioneer cells, which form following the induction of adaxial cells through signaling from the notochord, and a horizontal myoseptum, which separates the somites into a dorsal and a ventral part. Indeed, cell transplantation experiments carried out in *ntl* and *doc* have shown that the somite defects in these mutants can be rescued by clones of wild-type cells in the notochord (Halpern et al., 1993; Odenthal et al., 1996).

In the zebrafish, the floor plate can be subdivided into a single row of medial floor plate cells that are flanked by lateral floor plate cells (Odenthal and Nüsslein-Volhard, 1998; Bernhardt et al., 1992). We have adopted this broader definition since the cells lateral to the medial floor plate fulfil many roles that are similar to it. They possess a similar cuboidal shape, express many of the markers that are expressed in the medial floor plate including axonal attractants (Lauderdale et al., 1998) and play a role in axon guidance (Kuwada et al., 1990). As a further example, the lateral cells express *axial*, the zebrafish *HNF-3β* homologue (Strähle et al., 1996). This gene is considered to mark the floor plate in chick and mouse.

In addition to the notochord, mutations in *flh* and *mom* also affect the floor plate, (Talbot et al., 1995; Odenthal et al., 1996), whereas in embryos mutant for *ntl* or *doc* the floor plate is present. Mutations in three genes, *cyclops* (*cyc*), *one-eyed-pinhead* (*oep*) and *schmalspur* (*sur*), were found to affect the formation of the medial floor plate (Brand et al., 1996; Hatta et al., 1991b; Schier et al., 1996). At 24 hours of development, embryos that are homozygous mutant for any of these three genes lack the medial floor plate, although it does form later in embryogenesis in *cyc* and *oep* mutants (Strähle et al., 1997). The somites are normal in mutants for either of these genes supporting the notion that distinct mechanisms may underlay the induction of medial floor plate cells and somite patterning.

Mutants of five genes (the *you*-type genes), *you*, *you-too* (*yot*), *sonic-you* (*syu*), *chameleon* (*con*) and *u-boot*(*ubo*), affect somite patterning and lack the horizontal myoseptum, but no obvious notochord defect is seen (van Eeden et al., 1996). In *yot*, *you*, *con* and *syu*, the adaxial cells and the muscle pioneers are reduced or absent. The similarity of the somite defect observed in the notochord mutants and the *you*-type mutants suggests that the latter interfere with the transduction of a signal from the notochord to the somites (Odenthal et al., 1996), whereas the notochord mutants eliminate the source of the signal itself. Cell transplantation experiments have shown that in contrast to *ntl* and *doc*, *yot* and *ubo* are required in the somitic cells receiving the signal from the notochord (van Eeden et al., 1996). In addition to the somite patterning defect, mutations in *syu* and *con* also cause reduced pectoral fins (van Eeden et al., 1996).

*Sonic hedgehog* is a member of a gene family encoding signaling molecules related to the *hedgehog* gene of *Drosophila melanogaster* (Lee et al., 1992; Nüsslein-Volhard and Wieschaus, 1980). It has been cloned from a variety of vertebrate species from zebrafish to human (Chang et al., 1994; Echelard et al., 1993; Krauss et al., 1993; Marigo et al., 1995; Riddle et al., 1993; Roelink et al., 1994). It was found to be expressed in a number of structures that are known to pattern neighbouring tissues in the developing embryo (reviewed by Hammerschmidt et al., 1997). At the vertebrate midline, *shh* is expressed in the notochord and the floor plate, both of which are capable of inducing ventral cell fates in the neural tube. In the vertebrate limb bud, *shh* is expressed in the zone of polarizing activity (ZPA), which is involved in anteroposterior patterning of the limbs (Riddle et al., 1993). Furthermore, in the chicken embryo, asymmetric expression of *shh* in the node has been implicated in the development of left-right asymmetry of the heart (Levin et al., 1995).

In chick and mouse embryos, the involvement of *shh* in patterning processes at the midline has gained further evidence through ectopic expression studies and experiments, in which patterning of explanted embryonic tissues was achieved by treatment with purified Shh protein in vitro. Shh, when applied to neural plate explants, was found to be able to induce the floor plate markers *HNF-3β* and *Shh* in a contact-dependent fashion and the motorneuron markers *Isl-1* and *Isl-2* in a dose-dependent manner (Martí et al., 1995; Roelink et al., 1995). Similarly, Shh was able to induce sclerotome and repress dermomyotome development when applied to explants of presomitic mesoderm (Fan et al., 1995) and mimic the limb patterning activity of the ZPA in the developing chick limb (López-Martínez et al., 1995). Biochemical analysis and in vitro experiments revealed that the patterning activities reside within an N-terminal peptide generated by autoproteolytic cleavage of the Shh protein (Fan et al., 1995; Lai et al., 1995; López-Martínez et al., 1995; Martí et al., 1995; Porter et al., 1995; Roelink et al., 1995).

In the zebrafish, three *hedgehog* gene family members are expressed in the midline in partially overlapping patterns. Similar to the expression in other vertebrates, *shh* is expressed in the notochord, the medial floor plate, the ventral midline of the brain and the posterior fin bud of the zebrafish embryo (Krauss et al., 1993). The expression of *tiggy-winkle hedgehog* (*twhh*) is restricted to the medial floor plate and the ventral midline of the brain during early somitogenesis (Ekker et al., 1995), whereas *echidna hedgehog* (*ehh*) is transcribed in the notochord exclusively (Currie and Ingham, 1996).

An apparent target gene of the Shh signaling pathway is the zebrafish homologue of *HNF-3β*, *axial* (*axl*), which is expressed in the ventral neural tube in a 3- to 5-cell-wide band of cells encompassing medial and lateral floor plate cells (Hammerschmidt et al., 1996a; Odenthal and Nüsslein-Volhard, 1998; Strähle et al., 1993, 1996). Protein kinase A (PKA) is a negative regulator of Shh signaling (Hammerschmidt et al., 1996a). Injection of mRNA encoding *shh* or dnPKA, a dominant negative form of PKA, into zebrafish embryos results in localized expansion of *axial* (*axl*) in the midbrain, hindbrain and anterior spinal chord (Hammerschmidt et al., 1996a; Krauss et al., 1993). Ectopic overexpression of dnPKA also leads to a substantial increase in the expression of *isl1* (Hammerschmidt et al., 1996a; Concordet et al., 1996), which is a marker for primary motorneurons in the zebrafish (Inoue et al., 1994; Korzh et al., 1993). No effects on the expression of either *axl* or *isl1*, however, were found in more posterior regions of the spinal cord. Injection of mRNA encoding a constitutively active form of PKA leads to the repression of structures that are promoted by the injection of dnPKA (Concordet et al., 1996; Hammerschmidt et al., 1996a). Interestingly, ectopic overexpression of PKA or dnPKA have no effect on *shh* expression in the floor plate and the midbrain and hindbrain of the zebrafish (Hammerschmidt et al., 1996a). Injection of mRNA encoding mouse *Shh* or *Indian hedgehog* (*Ihh*) was also shown to induce supernumerary muscle pioneers in the somites of wild-type zebrafish embryos (Hammerschmidt et al., 1996a). Contradictory to this result, it was reported that injection of mRNA encoding zebrafish *shh* alone was insufficient and coinjection of mRNA encoding *ehh* was required for the efficient induction of supernumerary muscle pioneers (Currie and Ingham, 1996).

Targeted disruption of *shh* in the mouse confirmed the role of Shh protein in patterning of embryonic tissues (Chiang et al., 1996). Mouse embryos homozygous for the *shh* mutation display a variety of midline patterning defects. Most notably, they lack a floor plate and motorneurons in the neural tube and, instead of bilateral eyes, only one cyclopic optic vesicle is formed. In the ventral neural tube, expression of *HNF-3β* is never initiated and, in the paraxial mesoderm, the expression of the sclerotomal marker *Pax1* is strongly reduced, whereas the expression domain of the dorsal dermomyotomal marker *Pax3* is expanded. Mouse embryos lacking Shh function also lack distal limb structures indicating that, in addition to anteroposterior patterning of the limb, Shh also plays an important role in distal limb outgrowth. An unexpected phenotype was that Shh is required to maintain an intact notochord.

Here we show that *syu* is the zebrafish homologue of the *shh* gene. We present evidence that the *you*-type genes encode components of the Shh signaling pathway from the notochord for patterning the somites and the ventral neural tube. Surprisingly we found that *shh* is neither required nor sufficient to induce medial floor plate cells in the ventral neural tube of the zebrafish.

## MATERIALS AND METHODS

### Fish stocks

Fish maintenance and mating were done as described in Mullins et al. (1994). The zebrafish strains used were Tübingen (Haffter et al., 1996) and WIK (Rauch et al., 1997). The following alleles were used: *yot$^{ty119}$*, *syu$^{tq252}$*, *con$^{tm15a}$*, *you$^{ty97}$* and *ubo$^{tp39}$* (van Eeden et al., 1996), *syu$^{t4}$*, *syu$^{tbq70}$* and *syu$^{tbx392}$* (this work).

### Fish mutagenesis, allele screen and complementation

For the allele screen, mutagenesis was done as described in Haffter et al. (1996). Carriers for new alleles of *syu* were identified among the $F_1$s by single pair matings to fish that were *syu$^{tq252}$* heterozygous carriers. 6960 resulting egg lays were screened around 72 hpf for noncomplementation of the *syu* mutation.

### Whole-mount antibody staining and in situ hybridization

In situ hybridization and antibody stainings were done as described in Hammerschmidt et al. (1996b). Antisense probes were made from *shh* (Krauss et al., 1993), *twhh* (Ekker et al., 1995), *collagen2a1* (Yan et al., 1995), *F-spondin2* (Higashijima et al., 1997; Klar et al., 1992), *myoD* (Weinberg et al., 1996), *twist* (B. Riggleman unpubl., Morin-Kensicki and Eisen, 1997), *fkd4* (Odenthal and Nüsslein-Volhard, 1998), *pax-2* and *pax-6* (Krauss et al., 1991). Antibodies used: 4D9 (Patel et al., 1989), znp1 and zn5 (Trevarrow et al., 1990).

### Southern hybridization, RT-PCR, PCR (also CA repeats) and DNA sequence analysis

DNA isolation from adult fish and embryos was done as described in Westerfield (1994). Plasmid DNA preparation, restriction digestion, PCR and Southern hybridization were done according to standard methods (Sambrook et al., 1989). Probes were labeled using the Amersham megaprime kit. Total RNA was prepared from embryos using the TriStar Reagent (AGS), reverse transcription was done using the MMLV reverse transcriptase (Life Technologies). The oligonucleotide primers used for the RT-PCR analysis were as follows: forward primer 5′ to intron 1 (A): CCGGGATCC-GTAACGTTGTGATTTCGAGGTC; reverse primer within intron 1 (B): CGCGCCTGACGCGTCTCAACGC; reverse primer 3′ to intron 1 (C): CCGGAATTCTGCTTTGACAGAGCAATGAATG.

DNA sequencing of mutant alleles of *syu* and founder fish was done on both strands using a Pharmacia ALFexpress, the templates were prepared by PCR amplification of individual exons from total genomic DNA of *syu* homozygous mutant embryos. Putative changes were confirmed by sequencing a clone from an independent PCR performed on template DNA prepared from a separate pool of embryos homozygous for *syu*.

### In vitro transcription and mRNA injection

*shh* and dnPKA mRNA for injection was prepared using the Ambion mMessage mMachine kit using *shh* and dnPKA expression constructs in pSP64T (Hammerschmidt et al., 1996a; Krauss et al., 1993). Embryos were injected in the blastomeres at the 1- to 8-cell stage. Up to 500 pl was injected at a concentration of 0.3 mg/ml to test medial floor plate-inducing capabilities of *shh* containing phenol red as a tracer.

### HeLa cell cotransfection and CAT activity assay

–563shhCAT-M was generated by replacing the wild-type shh fragment from –563 to + 216 in –563shhCAT (Chang et al., 1998) with the corresponding fragment amplified by PCR from *syu$^{tq252}$* genomic DNA. Plasmids were sequenced to verify correct amplification of *syu$^{tq252}$* sequence. HeLa cells were transfected by the calcium phosphate coprecipitation method and CAT assays using constant amounts of extract protein were performed as described (Gorman et al., 1982; Webster et al., 1989). Reaction products were separated by thin layer chromatography and conversion of chloramphenicol to its acetylated forms was determined with a Fuji BAS 2000 phosphoimager. Each transfection experiment was repeated at least two times with different $CsCl_2$ plasmid preparations.

## RESULTS

### *shh* and *syu* are genetically linked

The reduced pectoral fins displayed by *sonic-you* (*syu*) mutant embryos and the somite patterning defect suggested that *syu* might be a good candidate for the zebrafish *sonic hedgehog* (*shh*) gene (van Eeden et al., 1996). To test whether *shh* and *syu* are linked, we carried out a segregation analysis using a restriction fragment length polymorphism (RFLP) physically linked to the *shh* gene (Fig. 1A). Using *shh* as a probe for Southern blot analysis, we identified RFLPs for *Stu*I and *Sph*I between the Tübingen strain, the line in which the *syu* mutation was originally induced, and an unrelated wild-type strain WIK. In a linkage cross between a heterozygous *syu$^{tq252}$* carrier and a WIK fish, we then followed the segregation of these RFLPs into $F_2$ progeny of $F_1$ fish that were heterozygous for both, *syu$^{tq252}$* and the RFLPs. The $F_2$ offspring were sorted by mutant phenotype into pools of 50 embryos each. By Southern blot analysis we found that only the mutant fragment segregated into the mutant pool of embryos, whereas both fragments were found in the sibling pool at an expected ratio of 1:2 (mutant:wild-type; Fig. 1B). In a sensitivity control, we found that we could detect the DNA equivalent of one wild-type embryo in a pool of 50 mutant embryos (data not shown). Since we did not find any recombinants in two pools of 50 *syu* mutant embryos, we estimate the genetic distance between *syu* and *shh* to be less than 1 cM. We have confirmed this linkage independently by using a CA repeat polymorphism in the 3′ UTR of *shh* (data not shown).

### A deletion covering *shh* fails to complement *syu*

We fortuitously identified a spontaneous deletion covering the *shh* gene. DNA of embryos homozygous for this deletion failed to give a signal on Southern blots when probed with a cDNA for *shh*, whereas control hybridizations using *her1* (Müller et al., 1996) as a probe showed the same band as the wild-type siblings (Fig. 1C). Further Southern analysis of this deletion using genomic probes surrounding the *shh* gene revealed that this deletion extends beyond 4.0 kb 5′ to the start codon and 2.2 kb 3′ to the stop codon of the *shh* open reading frame (data not shown). As heterozygous carriers for this deletion failed to complement *syu$^{tq252}$*, we conclude that the *shh* gene and the *syu* mutation are under the same deletion. We termed this deletion allele *syu$^{t4}$*.

To identify additional ENU-induced alleles of *syu*, we carried out an allele screen by non-complementation of *syu$^{tq252}$*. By screening an estimated 3480 ENU-mutagenized genomes in single pair matings between $F_1$ progeny of ENU-mutagenized founder fish and *syu$^{tq252}$* heterozygous carriers, we identified two new ENU-induced alleles, *syu$^{tbq70}$* and *syu$^{tbx392}$*.

### DNA sequence of *syu* alleles

To identify the molecular lesion underlying the phenotype in the ENU-induced alleles of *syu*, we determined the DNA

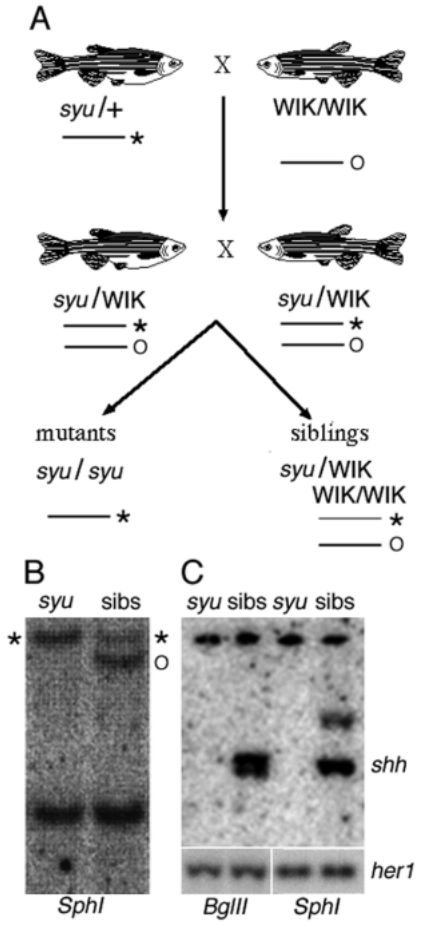


**Fig. 1.** (A) Cosegregation analysis between *shh* and *syu*. A *syu* heterozygous carrier was crossed to a wild-type reference fish WIK. Heterozygous $F_1$s were identified and mated to obtain pools of phenotypically mutant and sibling $F_2$ embryos. (B) Southern blot analysis using *shh* as a probe was carried out to follow the segregation of an *Sph*I RFLP linked to the *shh* gene into *syu* mutant and sibling $F_2$ embryos. Only the *syu* specific fragment (*) segregates into the mutant pool of embryos, whereas both, the *syu* specific and the WIK specific fragment (o), are found in the sibling pool at an expected ratio of 1:2. (C) The *shh* gene and *syu* are covered by the same deletion. Southern blot analysis using *shh* as a probe was carried out on DNA prepared from pools of $syu^{t4}$ homozygous mutant embryos and siblings. No signal is detected with DNA from the *syu* embryos, whereas a control hybridization using *her1* shows the same band with the *syu* mutant and the sibling embryos.

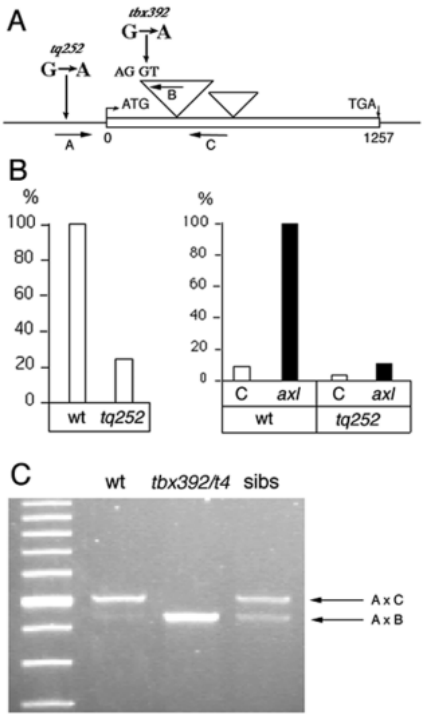


**Fig. 2.** (A) DNA alterations in ENU alleles of *syu*. The $syu^{tq252}$ mutation causes a G-to-A transition at a position 226 bp upstream of the *shh* start codon, the $syu^{tbx392}$ causes a G-to-A transition of the first nucleotide of the first intron of *shh* in the conserved splice donor junction. The two introns are indicated as inverted triangles. The positions and orientations of the oligonucleotide primers used for RT-PCR analysis are also indicated and labeled A-C. (B) The 5′ region of the sonic hedgehog gene in $syu^{tq252}$ directs reduced expression of chloramphenicol acetyl transferase activity in HeLa cells. Chimeric genes (–563shhCAT) containing wild-type *shh* sequences (wt) or the $syu^{tq252}$ mutant sequence (*tq252*) from –563 bp to +216 bp upstream of the CAT-coding region were transfected into HeLa cells either alone (left histogram) or together with CMVaxl (Axl) encoding zebrafish Axial, a transactivator of the *shh* promoter (right histogram). In controls of coexpression experiments (C), CMVβ encoding β-galactosidase was cotransfected in place of CMVaxl. The CAT activity obtained with the wild-type construct –563shhCAT alone (wt, left histogram) or when cotransfected with CMVaxl (Axl, wt; right histogram) were set to 100% and other CAT results were normalized accordingly (relative CAT activity). (C) RT-PCR analysis of total RNA extracted from wild-type, embryos that carried $syu^{tbx392}$ over the *shh* deletion ($syu^{t4}$) and siblings embryos containing wild-type and heterozygous $syu^{tbx392}$ carriers. The upper band specific for the spliced *shh* mRNA (A×C) was observed with RNA from wild types, whereas only the lower band specific for the *shh* mRNA, from which the first intron is not spliced (A×B), was observed with total RNA from $syu^{tbx392}/syu^{t4}$ mutant embryos. The faint lower band visible in the wild-type lane could be due to genomic DNA or small amounts of unspliced *shh* mRNA. Both bands were observed using RNA from sibling embryos. All three oligonucleotide primers were present in all three RT-PCR reactions indicating that both products can be amplified simultaneously and that the $syu^{tbx392}$ mutation inhibits splicing of the first intron of *shh*.

sequence of the entire coding regions and some flanking noncoding regions of the *shh* gene of $syu^{tq252}$, $syu^{tbq70}$ and $syu^{tbx392}$ (Fig. 2A). We could not identify any mutation in the *shh*-coding region of any allele of *syu* that would result in an amino acid change in the encoded protein. However, we did identify a G-to-A change in the conserved splice donor junction of the first intron of $syu^{tbx392}$. In $syu^{tq252}$, we identified a G-to-A change in the noncoding leader (Fig. 2A), at a position 226 bp upstream of the start codon and 27 bp downstream of the proximal transcription start site of the *shh* promoter (Chang et al., 1998). Sequencing of the corresponding regions of the originally mutagenized founder fish of $syu^{tbx392}$ and $syu^{tq252}$ showed that these sequence alterations were not present before the mutagenesis and were probably induced by the treatment with ENU. The molecular defect for $shh^{tbq70}$ was not identified.

### The mutation in the untranslated leader of *syu$^{tq252}$* reduces the expression of *shh*

The *shh* promoter is confined within the region between –563 and +216 relative to the proximal transcription start site (Chang et al., 1998). To test whether the transition at position +27 affects the function of the *shh* promoter, chimeric genes were constructed by placing either wild-type or *syu$^{tq252}$* sequences (from position –563 to +216) upstream of the coding region for chloramphenicol acetyl transferase (CAT). When transfected into HeLa cells, the mutant construct produced 5- to 10-fold less CAT activity in comparison to the wild-type construct (Fig. 2B). In addition, transactivation of CAT expression from the *shh* promoter by the winged-helix transcription factor Axial (Axl) (Strähle et al., 1993) is strongly reduced, when the mutant *shh-CAT* fusion construct is cotransfected with a plasmid encoding Axial (Fig. 2B). These data are consistent with the observed reduction of *shh* mRNA levels in *syu$^{tq252}$* mutants in vivo (see below). The transition from G to A at position +27 in the *shh* promoter region may affect the basal activity of the *shh* promoter. Alternatively, as the mutation resides within the *shh* transcript, the mutation might affect message stability.

### The mutation in *syu$^{tbx392}$* alters splicing of the *shh* mRNA

We performed an RT-PCR analysis using oligonucleotide primers that specifically amplify products from *shh* mRNA either containing or lacking the first intron (Fig. 2C). Using total RNA extracted from wild-type embryos, we only observed the RT-PCR product specific for the spliced form of *shh* mRNA, whereas with total RNA from embryos that carried *syu$^{tbx392}$* over the *shh* deletion (*syu$^{t4}$*), we only observed the RT-PCR product specific for *shh* mRNA from which the first intron was not spliced. Using total RNA from sibling embryos containing wild-type and heterozygous *syu$^{tbx392}$* carriers, we observed both forms, indicating that both products can be amplified simultaneously. Since all three oligonucleotide primers were present in all three reactions, this demonstrates that the *syu$^{tbx392}$* mutation impairs splicing of the first intron of *shh*. The aminoterminal portion of the Shh protein is thought to extend from codon 22 to 198. The failure to remove this intron causes the *shh* open reading frame to be truncated after codon 100 and extend into the first intron by another 8 codons until it hits a stop codon.

### The phenotypic strength of *syu* alleles correlates with *shh* expression

Embryos homozygous for *syu* fail to form a horizontal myoseptum and form U-shaped instead of V-shaped somites (Fig. 3). 3-day-old *syu* embryos are generally smaller with small eyes, reduced spacing of the eyes, a small head and a thinner tail. They have a circulation defect which is probably due to the absence of a functional dorsal aorta. The deletion allele *syu$^{t4}$* in addition shows a brain necrosis phenotype (data not shown). This additional phenotype may be due to (an)other gene(s) affected by the deletion. Mutations in *syu* cause reduced pectoral fins such that the length of the pectoral fins decreases with increasing phenotypic strength of the alleles (Fig. 4A). The two weak alleles, *syu$^{tq252}$* and *syu$^{tbq70}$*, show a variable reduction in the length of the pectoral fins which frequently varies between the two pectoral fins of one individual. In the strong ENU allele *syu$^{tbx392/t4}$*, both pectoral fins are very tiny and in the deletion allele pectoral finbuds are established but fail to grow out.

We examined the expression of *shh* in all four alleles of *syu* by whole-mount in situ analysis at 36 and 22 hpf (Fig. 4B,C). *shh* expression in the pectoral fin buds and the floor plate is reduced in *syu$^{tq252}$* and *syu$^{tbq70}$*, barely detectable in embryos that carried *syu$^{tbx392}$* over *syu$^{t4}$* and absent in embryos homozygous for *syu$^{t4}$*. The varying degrees of reduction of *shh* mRNA levels in the different *syu* alleles correlate with the increasing allele strengths as judged by the pectoral fin phenotypes.

In chick embryos, expression of *Shh* in the node is asymmetric and appears to be involved in left-right asymmetry of the heart. In the zebrafish, left-right asymmetry of the heart is manifested by a ‘jogging’ movement of the midline heart tube to the left followed by looping to the right and several mutations affecting this process have been described (Chen et al., 1997). Injection of mRNA encoding *shh* disturbs left-right asymmetry of the zebrafish heart in a dose-dependent manner (Chen et al., 1997). In *syu* mutant embryos, however, left-right asymmetry of the heart appears normal (data not shown).

### Somite patterning in *syu* is defective

The muscle pioneers are a subset of muscle cells in the region of the horizontal myoseptum. They are recognized by the 4D9 antibody, which labels an Engrailed-epitope in 2-6 nuclei of such cells in each segment (Fig. 5G; Hatta et al., 1991a; Patel et al., 1989). In the somites of *syu$^{t4}$* mutant embryos, Engrailed-positive cells are absent (Fig. 5H), whereas in *syu$^{tq252}$* a few Engrailed-positive nuclei are occasionally seen in a few segments (data not shown). The lack or strong reduction of Engrailed-positive muscle pioneers correlates with the absence of a horizontal myoseptum in these mutants.

The muscle pioneers originate from a subset of the adaxial cells that express *myoD* just adjacent to the notochord (Fig. 5A; Felsenfeld et al., 1991; Weinberg et al., 1996). In *syu* mutant embryos, *myoD* expression in the adaxial cells is strongly reduced (Fig. 5B). However, the reduction of *myoD* even in the strongest allele of *syu* is less pronounced than in *you-too*, another member of the *you*-type class of mutants (van Eeden et al., 1996 and data not shown).

Shh protein was shown to induce sclerotome when applied to explants of presomitic mesoderm in vitro (Fan and Tessier-Lavigne, 1994). At late somite stages of zebrafish development, *twist* is expressed in the posteriormost notochord and in sclerotome cells in the ventral part of the somites (Fig. 5C; Hammerschmidt et al., 1996; Morin-Kensicki and Eisen, 1997). We investigated *twist* expression in the somites of *syu* mutant embryos and found only a minor reduction in the level of *twist* staining even in embryos of the strongest allele of *syu* (Fig. 5D). Shh is therefore not required to accomplish at least some level of *twist* expression in the sclerotome of the zebrafish.

### *syu* affects patterning of the ventral neural tube

In the zebrafish, the medial floor plate is a single row of cells in the ventralmost part of the neural tube (Hatta et al., 1991b), which express several genes including *shh*, *F-spondin2* and *collagen2a1* (*col2a1*) (Fig. 5E; Higashijima et al., 1997; Klar

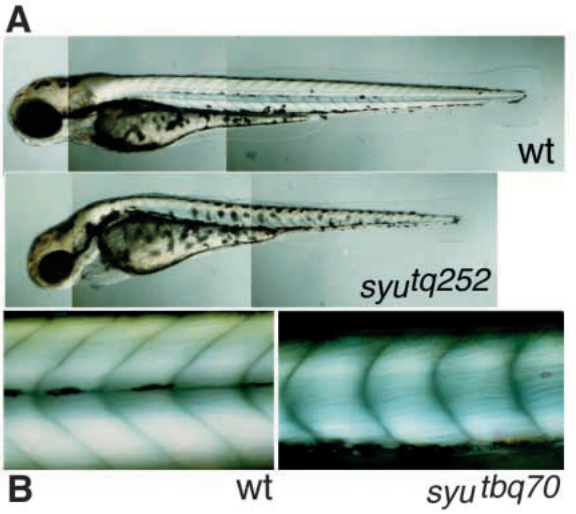


**Fig. 3.** (A) Nomarski picture showing wild-type and *syu*$^{tq252}$ mutant embryos at 72 hpf in lateral view. The somites are clearly U-shaped and the horizontal myoseptum is missing in the *syu* mutant embryo, causing the failure of melanophores to assemble in a lateral stripe. (B) Dark-field picture showing the somites of wild-type and *syu*$^{tbq70}$ mutant embryos at 72 hpf in lateral view. Striated muscle shows a bright birefringence and somite boundaries show as dark lines. Wild-type somites are V-shaped and separated into a dorsal and a ventral part by the horizontal myoseptum. The somites in the *syu* mutant embryo are reduced in size, clearly U-shaped and lack a horizontal myoseptum.

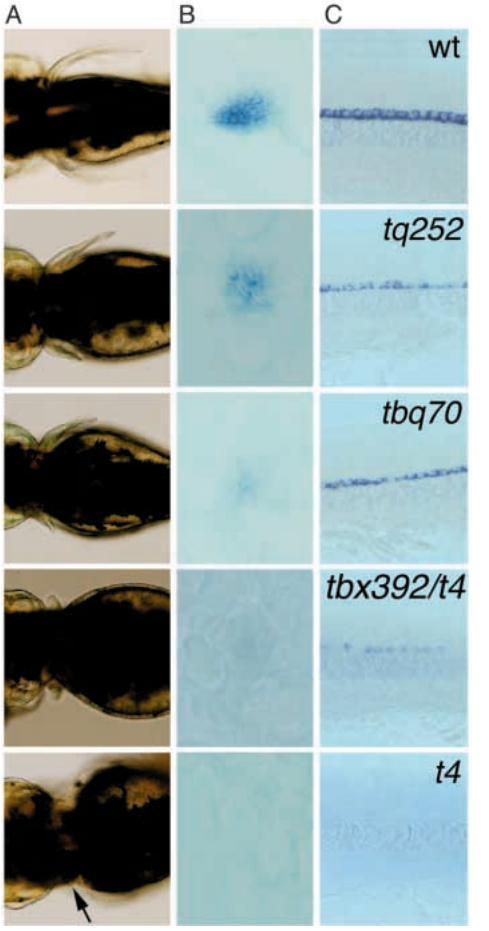


**Fig. 4.** (A) Nomarski pictures showing pectoral fins of wt and *syu* alleles in dorsal views. In the weak alleles, *syu*$^{tq252}$ and *syu*$^{tbq70}$, the pectoral fin phenotype is variable and frequently only the pectoral fin on one side of the embryo is reduced in size. In the strong allele, *syu*$^{tbx392/t4}$, the pectoral fins on both sides are reproducibly tiny and in the deletion allele *syu*$^{t4}$ finbuds are established (arrow) but fail to grow out. (B) Pectoral fins; dorsal views of *shh* whole-mount in situ hybridizations on *syu* alleles at 36 hpf. (C) Lateral views of *shh* whole-mount in situ hybridizations on *syu* alleles at 22 hpf. In the weak alleles, *syu*$^{tq252}$ and *syu*$^{tbq70}$, expression of *shh* is reduced, in embryos that carry *syu*$^{tbx392}$ over the *shh* deletion (*syu*$^{t4}$), it is barely detectable and no *shh* expression is seen in *syu*$^{t4}$ mutant embryos.

et al., 1992; Krauss et al., 1993; Yan et al., 1995). The residual expression of *shh* in the ventral neural tube of ENU-induced *syu* alleles at 22 hpf indicates that *shh*-expressing floor plate cells are still present in these mutants (Fig. 4C). In agreement with these data, *col2a1* expression at 28 hpf is normal in all four *syu* alleles including the deletion, indicating that the medial floor plate develops normally in *syu* (Fig. 5F and data not shown). The expression of another medial floor plate marker, *F-spondin2* (Higashijima et al., 1997; Klar et al., 1992) is also unaffected in *syu* mutant embryos at the 18-somite stage (data not shown).

In the zebrafish, two members of the *fork head* gene family, *axl* and *fkd4*, are not only expressed in the medial floor plate but also in lateral floor plate cells flanking the single row of *shh* expression (Fig. 5G; Odenthal and Nüsslein-Volhard, 1998; Strähle et al., 1996). In *syu*$^{t4}$ mutant embryos, expression of *fkd4* in lateral floor plate cells is absent and the expression is limited to the single row of medial floor plate cells normally expressing *shh* (Fig. 5H). Thus *shh* is required for development of these *fkd4*-expressing lateral floor plate cells, but not for development of the medial floor plate itself.

We injected mRNA encoding *shh* into wild-type zebrafish embryos to test whether ectopic expression of *shh* was sufficient to induce medial floor plate cells. Injection of *shh* mRNA to a level at which somitic tissue shows a very strong response (Hammerschmidt et al., 1996a) and at which the lateral floor plate marker, *fkd4*, is ectopically induced did not lead to ectopic expression of either *col2a1* or *F-spondin2* (data not shown). We conclude that *shh* is neither required nor sufficient to induce medial floor plate cells in the zebrafish.

In the midline of the zebrafish, two additional *hedgehog* homologs, *ehh* and *twhh* are expressed (Currie and Ingham, 1996; Ekker et al., 1995), which could compensate for the lack of *shh* function in certain aspects of development. We analyzed the expression of *ehh* and *twhh* in *syu* mutant embryos and found that the expression of both *hedgehog* homologs is unaffected by the lack of *shh* (Fig. 5I,J).

### Axon patterning is abnormal in *syu* mutants

Shh protein is known to induce motorneurons in explants of neural plate from the chick (Martí et al., 1995; Roelink et al., 1995). In mouse embryos homozygous for the *shh* mutation, motorneurons are absent, as shown by the absence of the expression of the *Islet-1* gene (Chiang et al., 1996). In *syu*

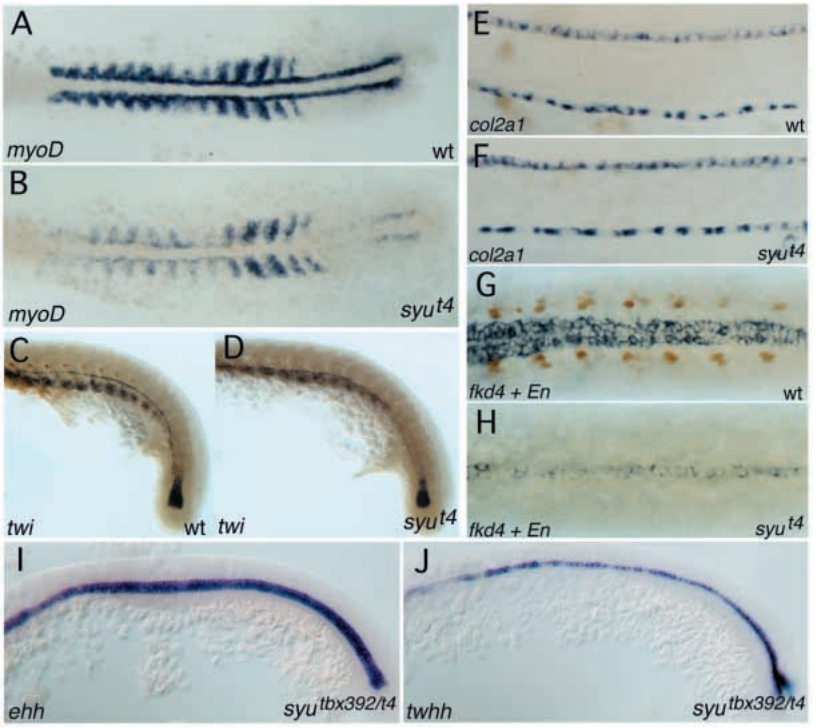


**Fig. 5.** Expression of myoD in a wild-type (A) and a *syu*$^{t4}$ mutant embryo (B), 14-somite stage, dorsal view on flat-mounted embryo. Expression of *myoD* is strongly reduced in the adaxial cells, also more lateral expression in the somitic mesoderm is lower. Residual levels of *myoD* remain detectable in the adaxial cells, especially in the tailbud region. Double staining for *twist* (in situ hybridization) and Engrailed (4D9 antibody labeling) in wild-type (C) and *syu*$^{t4}$ mutant (D) embryo, side view on tail, 22 hpf. *twist* labels the early notochord in the tailbud, the hypochord and the sclerotome visible in the ventral part of the somites (all blue staining). In the *syu* mutant, *twist* staining is only slightly reduced and shifted dorsally relative to the notochord due to the abnormal shape of the somites. Engrailed positive muscle pioneers (brown staining) are absent from *syu* mutant embryos. Expression of *col2a1* in wild-type sibling (E) and *syu*$^{t4}$ mutant embryo (F) at 29 hpf, side view. *col2a1* labels both the medial floor plate and the hypochord. *syu*$^{t4}$ has no effect on medial floor plate staining. Whole-mount antibody and in situ staining for Engrailed (4D9) and *fkd4* , respectively, of wild-type sibling (G) and *syu*$^{t4}$ mutant (H) embryo at 24 hpf, dorsal view. Computer composite of two focal planes. One plane shows the nuclei of the muscle pioneers (brown). The second plane shows the cytoplasmic *fkd4* staining in the ventral neural tube encompassing medial and lateral floor plate. In *syu* mutant embryos, muscle pioneers are absent and *fkd4* expression is restricted to the medial floor plate itself. Expression of *ehh* (I) and *twhh* (J) at 15- to 17-somite stage *syu*$^{tbx392/t4}$ mutant embryos. The expression of both *hedgehog* homologs in *syu* mutant embryos is indistinguishable from wild-type siblings.

mutant embryos, primary and secondary motorneurons are present, but axon patterning of these neurons is abnormal (Fig. 6B and data not shown). Primary motorneurons were stained by the znp-1 monoclonal antibody (Trevarrow et al., 1990). CaP and MiP axons, which normally project to the ventral and dorsal myotome, were found to run along the neural tube horizontally in *syu* mutant embryos at a high frequency (van Eeden et al., 1996, data not shown). Secondary motorneurons were stained using the zn5 antibody (Trevarrow et al., 1990). In *syu* mutant embryos, the axons of the secondary motorneurons fail to branch and instead cease to extend or grow further ventrally in an abnormal pattern (Fig. 6B).

The zn5 antibody also labels the retinal axons, which normally cross the midline to connect the retinal ganglion cells (RGCs) to the tectum on the contralateral side (Fig. 6C; Karlstrom et al., 1996). In *syu* mutant embryos, these RGC axons frequently fail to cross the midline and remain on the ipsilateral side or, if they do cross the midline, frequently grow aberrantly (Fig. 6D-F; Table 1).

## Lack of *shh* has no effect on the expression pattern of either *pax-2* or *pax-6* in the developing eye

It has been reported that ectopic expression of either *shh* or *twhh* alters proximodistal fates in the developing eye as manifested by an alteration of the expression patterns of *pax-2* and *pax-6* (Ekker et al., 1995; Hammerschmidt et al., 1996a; Macdonald et al., 1995). We did not observe any obvious abnormality of *pax-2* or *pax-6* expression in the developing eye of *syu*$^{t4}$ mutant embryos (data not shown). This indicates that the lack of *shh* alone does not affect proximodistal fates in the developing eye of the zebrafish.

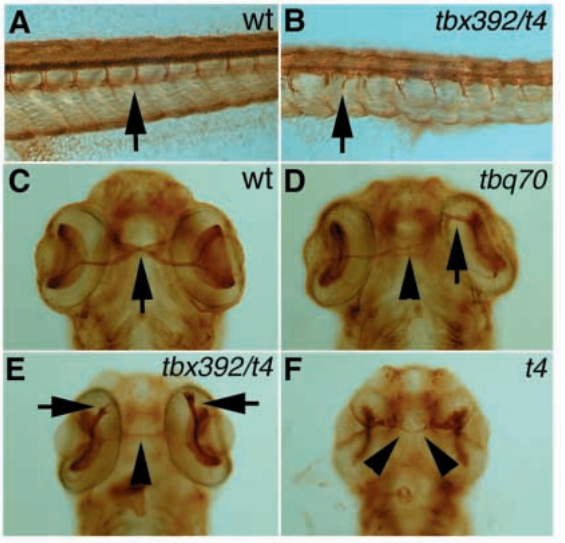


**Fig. 6.** Zn5 antibody staining of secondary motorneurons in a wild-type (A) and a *syu*$^{tbx392}$/*syu*$^{t4}$ mutant (B) embryo at 52 hpf. In *syu* mutant embryos the axons of the secondary motorneurons (indicated by arrows) fail to branch and instead cease to extend or grow further ventrally in an abnormal pattern. Zn5 staining of retinal axon projections in wild-type (C) and *syu* mutant alleles (D-F) at 52 hpf. In wild-type embryos, the retinal ganglion cell axons have crossed the midline where the optic chiasm is formed (arrow) and grow towards the tectum on the contralateral side. In *syu* mutant embryos, these retinal axons frequently fail to cross the midline and remain on the ipsilateral side (arrows) or, if they do cross the midline, frequently grow abnormally and in many cases only form very thin axon bundles (arrowheads).

**Table 1. Frequency of normal and abnormal retinal projections in different alleles of *syu***

| Allele | % of mutant embryos with abnormal retinal projections (cases) | % of sibling embryos with abnormal retinal projections (cases) |
|---|---|---|
| $syu^{tq252}$ | 4 (1/24) | 0 (0/21) |
| $syu^{tbq70}$ | 67 (29/43) | 0 (0/37) |
| $syu^{tbx392}/syu^{t4}$ | 96 (26/27) | 0 (0/22) |
| $syu^{t4}$ | 100 (21/21) | 0 (0/23) |

### Ectopic expression of *shh* or dnPKA rescues Engrailed-positive muscle pioneer cells in *syu*

To establish whether the somite defect of *syu* can be rescued by transient expression of *shh*, we injected *shh* mRNA and assayed for the induction of Engrailed-positive muscle pioneers (Table 2). It was previously shown that injection of in vitro transcribed *shh* mRNA can induce supernumerary Engrailed-positive muscle pioneers when injected into wild-type embryos (Hammerschmidt et al., 1996a). We identified *syu* mutant embryos in which supernumerary Engrailed-positive muscle pioneers were induced on one side, whereas the other side showed no Engrailed-positive cells demonstrating that we can rescue muscle pioneers in *syu* embryos (data not shown). Injection of mRNA encoding a dominant negative form of protein kinase A, dnPKA, was also shown to induce supernumerary Engrailed-positive muscle pioneers in wild-type embryos (Hammerschmidt et al., 1996a). We could also rescue *syu* by the injection of mRNA encoding dnPKA as shown by a one-sided induction of supernumerary Engrailed-expressing muscle pioneers (Table 2).

### The *you*-type genes encode a single signaling pathway

To investigate whether *syu*, *you-too* (*yot*), *chameleon* (*con*) and *you* encode components of a single signaling pathway, we constructed the following double mutants: $yot^{ty119}/con^{tm15a}$, $yot^{ty119}/syu^{tq252}$, $syu^{tq252}/you^{ty97}$ and $syu^{t4}/con^{tm15a}$. The progeny of parents that were double heterozygous carriers for any of these combinations did not contain embryos displaying stronger phenotypes than either of the single mutants alone. Whole-mount in situ hybridization with *col2a1* (for *syu*/*con* double mutants) or *shh* (for *yot*/*con*, *yot*/*syu* and *syu*/*you* double mutants) as medial floor plate markers revealed that neither of the double mutants had any obvious effect on the medial floor plate (data not shown). Since none of these double mutants uncovered any overlapping function, we conclude that these genes are likely to encode components of a single signaling pathway. Since none of the double mutant combinations with *syu* affected the medial floor plate, we conclude that neither of the three other genes (*yot*, *con* or *you*) encodes a possible signaling function required for medial floor plate induction in the absence of normal Shh activity.

**Table 2. Rescue of muscle pioneer cells in different *you*-type mutants by overexpression of *shh* and dnPKA**

| Gene | Allele | Injected mRNA | % response in mutants (cases) | % response in siblings (cases) |
|---|---|---|---|---|
| *syu* | *t4* | *shh* | 84 (21/25) | 69 (46/67) |
| *yot* | *ty119* | *shh* | 0 (0/19) | 35 (22/63) |
| *con* | *tm15a* | *shh* | 92 (11/12) | 81 (21/26) |
| *you* | *ty97* | *shh* | 86 (6/7) | 35 (8/23) |
| *ubo* | *tp39* | *shh* | 62 (8/13) | 79 (26/33) |
| *syu* | *t4* | dnPKA | 85 (17/20) | 70 (52/74) |
| *yot* | *ty119* | dnPKA | 0 (0/39) | 28 (37/134) |

### Order of the *you*-type genes in the pathway

To order other *you*-type genes with respect to the Shh signaling pathway, we injected *shh* mRNA into embryos mutant for *u-boot* (*ubo*), *yot*, *con* or *you*. We found induction of supernumerary muscle pioneers with injection of *shh* mRNA in *ubo*, *con* and *you* similar to *syu*. However, in *yot*, we never obtained induction of Engrailed-positive cells with either *shh* mRNA or with mRNA encoding dnPKA, indicating that *yot* is downstream of *shh* and protein kinase A in the Shh signaling cascade.

## DISCUSSION

In this paper, we describe several lines of evidence that the phenotype of *syu* mutant embryos is due to mutations in the *shh* gene. We show that *syu* and *shh* are both genetically and physically linked and a deletion, which fails to complement *syu* mutations, uncovers the *shh* gene. Sequencing of ENU-induced alleles revealed that one of these alleles, $syu^{tbx392}$, is due to a mutation in a conserved region at the splice donor junction leading to the failure of splicing the first intron of the *shh* mRNA. A point mutation in the noncoding 5′-leader of $syu^{tq252}$ reduces *shh* expression in the embryo. These in vivo observations are paralleled by in vitro results; CAT expression driven by the $syu^{tq252}$ mutant sequence in a HeLa cell expression system is strongly reduced in comparison to the CAT expression obtained with the wild-type sequence. In summary, we take this evidence as sufficient to conclude that *syu* is identical to the *shh* gene of the zebrafish.

The phenotype displayed by *syu* mutant embryos is in many respects similar to that found in mice lacking *shh* gene function (Chiang et al., 1996). Similar to the mouse phenotype *syu* mutant embryos show patterning defects in the somites and lack the distal outgrowth of the anterior limbs. As was reported for mice lacking *shh*, left-right asymmetry of the heart is unaffected in *syu* mutant embryos, indicating that *shh* is not essential for the induction of left-right asymmetry of the heart in either organism.

In mouse embryos, the lack of Shh function causes cyclopia and the expansion of *pax-6* expression throughout the optic cup at the expense of *pax-2*. In the zebrafish, it was shown that ectopic expression of *shh* or *twhh* alters the expression pattern of these two early markers for proximodistal cell identity in the eye towards more proximal fates (Ekker et al., 1995). Since *syu* mutant embryos form two bilateral eyes and show a normal proximodistal expression pattern of both *pax* genes in the eye, we suggest that *twhh* expression in the ventral brain is responsible for this patterning alone or can at least partially compensate for the lack of *shh* in the head. Nevertheless, the pathfinding errors of RGC axons in the brain demonstrate a specific function for *shh* in the ventral midline of the brain.

A surprising finding is that *syu* embryos do form medial floor plate cells and motorneurons and therefore *shh* is not required to induce these ventral cells in the neural tube of the

zebrafish. This is clearly different from the phenotype observed in the mouse, where neither floor plate nor motorneurons are formed. The presence of medial floor plate in embryos homozygous for the deletion of *shh* and the absence of an effect on medial floor plate cells by ectopic overexpression of *shh* shows that, in the zebrafish, *shh* is neither required nor sufficient to induce medial floor plate cells. This is in apparent contradiction to the induction of floor plate markers observed after the injection of *shh* homologs into *Xenopus laevis* embryos (Ruiz i Altaba et al., 1995). However, in these experiments, ectopic floor plate induction was never observed at the stages at which endogenous floor plate is being induced, indicating that the ectopic induction of floor plate achieved at later stages of development may be of little physiological relevance.

Mutations in genes that act in different steps in a signal transduction pathway should give similar phenotypes. The *you*-type mutants have been grouped by their similarity to the *syu* mutant phenotype. The double mutant analysis carried out between *syu*, *con*, *yot* and *you* has shown that none of the double mutants affects the medial floor plate and none of the double mutants displays any obvious phenotype exceeding that observed with embryos homozygous for $yot^{ty119}$. This suggests that these genes encode components of a single signaling pathway and that none of the other *you*-type genes encodes a redundant signal involved in the induction of medial floor plate cells.

Nevertheless, the absence of *fkd4* expression in lateral floor plate cells demonstrates specific requirements for *shh* in patterning the ventral neural tube. This is also in agreement with *shh* injection studies, leading to the ectopic expression of *axial*, which similarly to *fkd4* is expressed in lateral and medial floor plate cells (Hammerschmidt et al., 1996a; Krauss et al., 1993). It has been reported that *syu* mutants have morphological defects in the floor plate (Brand et al., 1996). We speculate that the reduced visibility of the floor plate in *syu* mutants may well be due to the absence of the lateral floor plate cells. The motorneuron axon defects of *syu* mutant embryos also indicates a specific requirement for *shh* in patterning these axons. It is, however, possible that the motorneuron axon defect is a secondary consequence of somite patterning defects in this mutant. Ectopic overexpression of dnPKA causes a substantial increase of *isl1*-expressing cells in the neural tube, indicating that Hedgehog signaling is able to induce primary motorneurons in the zebrafish embryo (Hammerschmidt et al., 1996a). Since *syu* mutant embryos do form primary and secondary motorneurons, it is conceivable that *twhh* expressed in the floor plate is responsible for the induction of these more lateral cell types in *syu* mutant embryos.

Injection studies have suggested that both *shh* and *ehh* may be required for efficient induction of supernumerary muscle pioneers in wild-type zebrafish embryos (Currie and Ingham, 1996). The reduction of *myoD* expression in the adaxial cells even in the strongest allele of *syu* is less pronounced than in *yot* (van Eeden et al., 1996 and data not shown). This suggests that the lack of *shh* function in the zebrafish does not completely block signaling from the notochord and that *ehh* may be responsible for the residual expression of *myoD* in the adaxial cells of *syu*. Cell transplantation experiments have shown that *yot* is required in somitic cells receiving the Shh signal (van Eeden et al., 1996) and, since the phenotype of embryos mutant for $yot^{ty119}$ is even stronger than that of embryos homozygous for a deletion of *shh*, we speculate that *yot* is also involved in transducing signals encoded by other *hedgehog* homologues such as *ehh*.

The positive response of *you*, *con* and *ubo* mutants to *shh* injection would suggest that the corresponding genes are upstream of *shh* or act in parallel. However, it is conceivable that artificially high levels of *shh* used in the injection assay could bypass the requirement for an otherwise essential component of the *shh* signaling cascade. Particularly this may be the case for *ubo*, which was shown to be required in the somitic cells receiving the Shh signal (van Eeden et al., 1996).

Although we cannot formally exclude the possibility that either *ehh* or *twhh* could compensate for the lack of *shh* in medial floor plate induction, we favor an alternative model by which medial floor plate cells are not induced by any Hedgehog molecule, but rather arise through a cell fate decision in a common precursor of notochord and medial floor plate. Such a cell fate decision was recently proposed by Halpern et al. (1997) through the analysis of double mutants for *ntl* and *cyc*. The finding that, in these double mutants, the *ntl* mutation suppresses the floor plate deficiency in *cyc* mutants suggested that the Ntl protein promotes notochord development and suppresses floor plate fate in common precursor cells (Halpern et al., 1997). Substantial but incomplete rescue of floor plate deficiency was also observed in double mutants of *ntl* and *oep* (Strähle et al., 1997). A cell fate selection model also explains the finding that, in embryos mutant for strong alleles of *ntl*, the band of *shh*-expressing floor plate cells is more than one single row of cells wide (Odenthal et al., 1996; Strähle et al., 1996). Fate mapping studies have indicated that floor plate cells and notochord cells in the avian embryo arise from a common progenitor population in Hensen's node (Catala et al., 1996). Floor plate and notochord precursors also occupy overlapping areas in the shield fate map of the zebrafish embryo (Shih and Fraser, 1995).

The existence of distinct pathways functioning in the induction of medial floor plate cells and somite patterning is further supported by the fact that mutations in genes required for notochord formation have different effects on the floor plate (Odenthal et al., 1996). Whereas mutations in *flh* or *mom* result in floor plate defects, mutations in *doc* or *ntl* do not cause any deficiency of floor plate cells, but rather promote floor plate fate as is the case for strong alleles of *ntl*. Following the argument of a cell fate decision, it seems plausible that *flh* and *mom* may affect a common precursor for notochord and floor plate.

A genetic pathway specifically leading to the formation of medial floor plate is defined by mutations that do affect the medial floor plate such as *cyc*, *oep* and *sur*. None of these genes affects somite patterning or the *fkd4*-expressing lateral floor plate cells (J. O., unpublished data), which implies that they do not encode part of the Shh signaling pathway. Mosaic analyses by cell transplantation have indicated that mutations in either *oep* or *sur* block medial floor plate development autonomously (Odenthal and Nüsslein-Volhard, 1998; Strähle et al., 1997; J. O. and C. F., unpublished data). Neither of these genes is required for notochord formation and since they seem to be involved in promoting medial floor plate cell fate in a cell autonomous fashion, this precludes their function as an inductive signal from the notochord. Finally, the *oep* and *cyc*

genes have recently been identified molecularly and neither of them encodes a known component of the *shh* signal transduction pathway (Zhang et al., 1998; M. R. Rebagliati, R. Toyama, P. H. and I. Dawid, unpublished data).

In addition to the *you*-type mutants, a number of mutants that show phenotypes partially overlapping with that of *syu* have been described. We are investigating whether these mutations may be affecting *ehh* or *twhh*, respectively, or other components of a complex network of Hedgehog signaling pathways. Further characterization of these groups of mutants should permit dissection of the overlapping functions and specific requirements of Hedgehog molecules in patterning the midline, the CNS and the limbs of the zebrafish embryo.

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