

Diminished Sonic hedgehog signaling and lack of floor plate differentiation in *Gli2* mutant mice

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This paper is dedicated to Professor Yoshiaki Suzuki on his retirement

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SUMMARY

Floor plate cells at the midline of the neural tube are specified by high-level activity of Sonic hedgehog (Shh) secreted by notochord, whereas motor neurons are thought to be specified by a lower level activity of Shh secreted in turn by floor plate cells. In *Drosophila*, the *Gli* zinc finger protein Cubitus interruptus functions as a transcription factor activating Hedgehog-responsive genes. We report that the expression of known Shh-responsive genes such as *Ptc* and *Gli1* is downregulated in mutant mice lacking *Gli2*

function. *Gli2* mutants fail to develop a floor plate yet still develop motor neurons, which occupy the ventral midline of the neural tube. Our results imply that *Gli2* is required to mediate high level but not low level Shh activity and show that the development of motor neurons can occur in the absence of floor plate induction.

Key words: Motor neuron, Patched, HNF3 β , Sonic hedgehog, Floor plate, *Gli2*, *Drosophila*

INTRODUCTION

The Hedgehog (Hh) family of signaling molecules mediate many inductive processes during invertebrate and vertebrate development (see review by Hammerschmidt et al., 1997). In *Drosophila*, *hh* plays a vital role in determining cell fate and patterning during embryonic and postembryonic development. In vertebrates, one of the Hh homologs Sonic hedgehog (Shh) has been shown to be involved in the control of cell fate determination in the central nervous system, dorsoventral patterning of somites, anteroposterior polarity of limb and left-right asymmetry (see Chiang et al., 1996 and references therein).

Genetic analysis in *Drosophila* has identified a number of proteins involved in the transduction of Hh signal (reviewed in Ruiz i Altaba, 1997). They include Patched (Ptc), Smoothed, Protein kinase A, Fused, Costal2, Suppressor of fused and Cubitus interruptus (Ci). Of the many proteins implicated in Hh signaling in *Drosophila*, the *Gli* zinc finger protein Ci appears to mediate the nuclear functions of Hh by appropriately activating the transcription of some specific target genes and repressing the transcription of others (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997; von Ohlen et al., 1997); the activator and repressor functions of Ci are thought to be mediated by distinct forms of

Ci derived by post-translational processing (Aza-Blanc et al., 1997). Multiple *Gli* zinc finger proteins have been identified in humans (Ruppert et al., 1988), mice (Schimmang et al., 1992; Hui and Joyner, 1993; Walterhouse et al., 1993; Hui et al., 1994), chick (Marigo et al., 1996) and *Xenopus* (Lee et al., 1997; Marine et al., 1997). In humans, *Gli* zinc finger proteins are implicated in development and cancer. *GLI1* is an oncogene amplified in a variety of tumors (see Ruppert et al., 1991) and mutations of *GLI3* have been found in a number of dominant genetic disorders (Vortkamp et al., 1991; Kang et al., 1997; Radhakrishna et al., 1997).

The precise roles played by the three *Gli* genes in vertebrates have not yet been defined. *Gli1* has been proposed to be a major mediator of the Shh signal since *Gli2* and *Gli3* are broadly expressed during gastrulation and neurulation, while *Gli1* is expressed in regions close to *Shh*-expressing cells (Hui et al., 1994; Lee et al., 1997; Platt et al., 1997; Sasaki et al., 1997), and since misexpression of *Gli1* protein in mouse and frog embryos can mimic the effect of ectopic Shh signaling in the induction of various markers (including *Shh* itself) and ventral cell types in the neural tube (Hynes et al., 1997; Lee et al., 1997). *Gli3*, in contrast, has been proposed to function as a repressor of *Shh*, since *Gli3* mutant limbs show ectopic expression of *Shh* (Masuya et al., 1997; Buscher et al., 1997), and since in the chick limb *Shh* has also been found to up-

regulate *Gli1* expression and downregulate *Gli3* expression (Marigo et al., 1996). The role of *Gli2* in Shh signaling has not been defined to date, though mutant analysis has indicated that *Gli2* and *Gli3* play partially redundant functions during skeletal development (Mo et al., 1997).

Patterning of the ventral neural tube is one of the best-defined Shh-dependent processes (see Tanabe and Jessell, 1996). Studies in warm-blooded vertebrates have provided evidence that floor plate cells at the ventral midline of the spinal cord are induced by Shh derived from notochord cells in a process that requires direct contact with notochord, apparently reflecting a requirement for a high concentration of Shh for this induction; it has been proposed that lower levels of Shh derived from floor plate then induce diverse cell types, including motor neurons, in the ventral spinal cord in a dose-dependent manner (see Ericson et al., 1997 and references therein). A requirement for floor-plate-derived Shh for patterning the ventral spinal cord in warm-blooded vertebrates has seemed somewhat at odds with the analysis of the *cyclops* (*cyc*) mutant in zebrafish, which fails to develop a floor plate yet apparently shows an otherwise largely normal patterning of the ventral spinal cord (Hatta et al., 1991). Here we show that mice lacking *Gli2* function have diminished response to the notochordal Shh signal; the expression of several known Shh-responsive genes is downregulated in the developing somites and neural tube of the mutants. *Gli2* mutant neural tube fails to develop a floor plate yet still develop motor neurons, which are now found in the ventral midline region. These results show that *Gli2* is essential for the induction of floor plate cells but not motor neurons, i.e. that *Gli2* is essential for the transduction of a high level Shh signal. They also suggest that a source of Shh other than floor plate – presumably the notochord – is sufficient for motor neuron development at least in the context of this mutant background, a result that parallels the observation of largely normal ventral patterning in *cyclops* mutants in zebrafish.

MATERIALS AND METHODS

Mice

Gli2^{td} mutant mice were maintained in a mixed background of 129/Sv and CD1 as described (Mo et al., 1997). Genotype of mutant embryos was determined by PCR analysis of yolk sac DNA. The GtC101 gene trap insertion was introduced into the *Gli2*^{td} mutant background and was genotyped by β -galactosidase staining and/or Southern blot analysis (Gasca et al., 1995). In all the experiments described here, between 4 and 10 embryos were examined for each genotype at each stage.

Histological analysis, northern blot and in situ hybridization

Midday of the day of the vaginal plug was considered as 0.5 dpc in the timing of embryo collection. Embryos were fixed overnight in 4% paraformaldehyde at 4°C, processed, embedded in wax and sectioned at 6 μ m. Slides were then dewaxed, rehydrated and stained with hematoxylin and eosin. Total RNA from individual 9.5 dpc embryos was used for northern blot analysis as described (Hui and Joyner, 1993). Northern blot signals were quantitated by using a Phosphorimager. Whole-mount RNA in situ hybridization was performed essentially as described (Conlon and Herrmann, 1993). After in situ hybridization, embryos were embedded in gelatin and sectioned with a cryostat. Section in situ hybridization was carried out

according to published procedures (Hui and Joyner, 1993; Schaeren-Wiemers and Gerfin-Moser, 1993). The probes used were *Gli1*, *Gli2*, *Gli3* (Hui et al., 1994), *Shh* (Echelard et al., 1993), *Pax3* (Goulding et al., 1991), *Pax6* (Walter and Gruss, 1991), *Nkx2.2* (Shimamura et al., 1995), *Bo* (B. Xiao, personal communication), *Islet-1*, *Islet-2* (*Isl1*, *Isl2*; C.-c. H., unpublished), *ChAT* (choline acetyltransferase; Ishii et al., 1990), and *Ptc* (Goodrich et al., 1996).

β -galactosidase staining, immunohistochemistry and BrdU labeling

Embryos were fixed in PBS containing 3.7% formaldehyde for approximately 5 to 30 minutes according to the stage of the embryos: 7.5 dpc (5 minutes), 8.5 dpc (10 minutes) and 9.5 dpc (15 minutes). Embryos were stained overnight in the dark at 37°C (Gasca et al., 1995). After dehydration, embryos were embedded in paraffin, sectioned at 6 μ m and counterstained with nuclear-fast red. For 14.5 dpc embryos, staining was performed on cryosections. Whole-mount immunohistochemistry was performed according to published procedures using an HNF3 β -specific antibody (Marti et al., 1995b). Section immunohistochemistry using mouse monoclonal antibodies against Pax6 and Pax7 was performed as described (Ericson et al., 1996, 1997). BrdU-positive DNA replicating cells were determined as described by Gratzner et al. (1982).

RESULTS

Gli2 is not essential for notochord development

Mutant analysis has indicated that *Shh* is required for the maintenance of the notochord as well as the development of floor plate and motor neurons (Chiang et al., 1996). All three mouse *Gli* genes are broadly expressed during gastrulation and neurulation (Hui et al., 1994; Sasaki et al., 1997; Lee et al., 1997). In 8.5 days post coitum (dpc) mouse embryos, *Gli2* is highly expressed in the developing neural tube and surrounding mesodermal tissues (Fig. 1A). At this stage (~8 somites), the notochord expresses high level of *Shh* and the floor plate expression of *Shh* can only be detected in the mid/hindbrain region (Echelard et al., 1993). While *Gli2* appears to be co-expressed with *Shh* in the developing notochord, its expression in the prospective floor plate region precedes that of *Shh* (Fig. 1B,C). At 10 dpc, *Gli2* transcripts are not detected in the

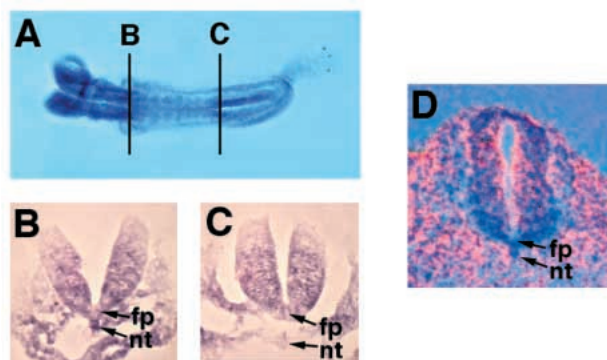


Fig. 1. *Gli2* expression in mouse embryos. (A) Expression in 7-somite-stage embryo. (B,C) Transverse sections at levels shown in A reveal *Gli2* expression in floor plate and notochord. (D) Expression of *Gli2* in a section of 10.0 dpc embryo showing high levels of *Gli2* transcripts in ventricular zone of the neural tube but not in floor plate and notochord.

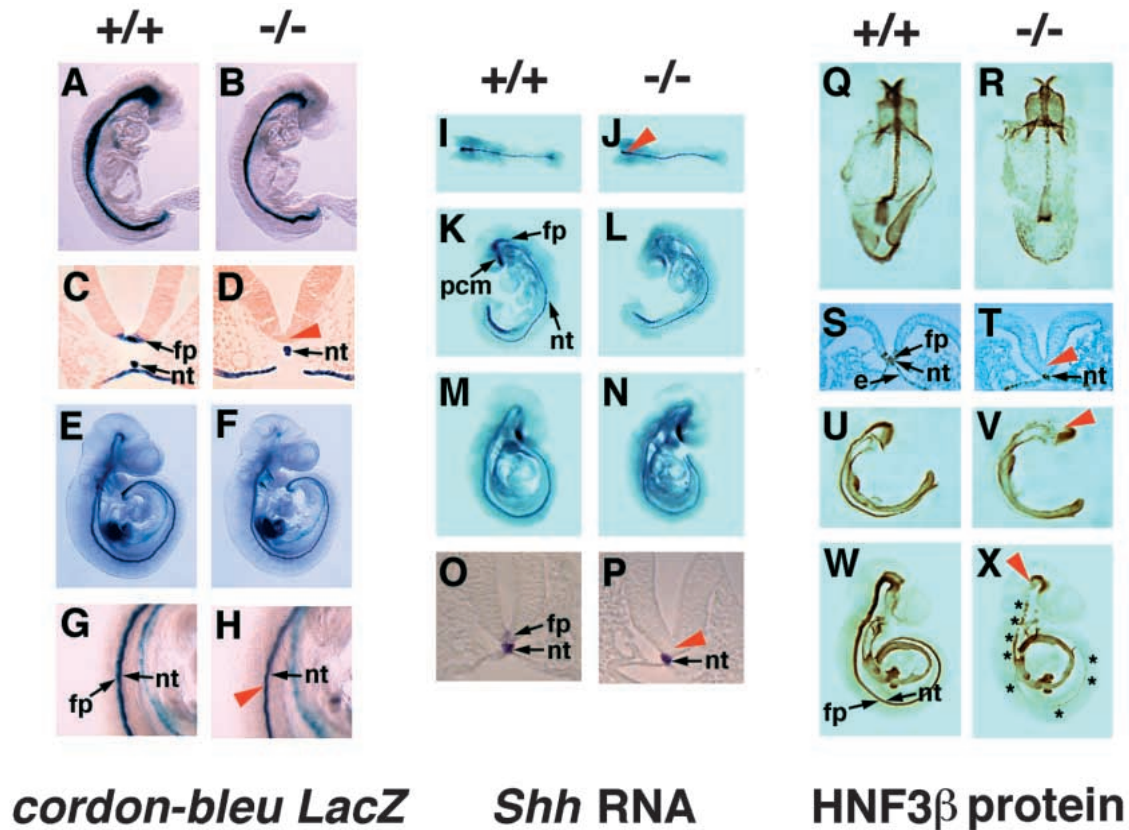


Fig. 2. Normal notochord development in *Gli2^{zfd}* mutants. (A-H) β -galactosidase staining of the GtC101 gene trap insertion in wild-type (A,C,E,G) and *Gli2^{zfd}* homozygote (B,D,F,H) embryos. (A,B) Whole-mount X-Gal staining of 8-somite-stage embryos. (C,D) Transverse sections of 10-somite-stage embryos. The arrowhead indicates no β -galactosidase activity in the mutant neural tube. (E, F) Whole-mount X-Gal staining of 25-somite-stage embryos. (G,H) Higher magnifications of the trunk regions in E and F. The arrowhead indicates no β -galactosidase activity in the mutant neural tube. (I-P) *Shh* expression determined by RNA in situ hybridization in wild-type (I,K,M,O) and *Gli2^{zfd}* homozygote (J,L,N,P) embryos. (I,J) Expression in 8-somite-stage embryos. The arrowhead indicates a weaker prechordal expression of *Shh* in the mutant. (K,L) Expression in 15-somite-stage embryos. (M,N) Expression in 25-somite-stage embryos. (O,P) Transverse sections of 15-somite-stage embryos reveal no *Shh* expression in the ventral midline of the mutant neural tube as indicated by the arrowhead. (Q-X) HNF3 β protein distribution determined by immunohistochemistry using a HNF3 β -specific antibody in wild-type (Q,S,U,W) and *Gli2^{zfd}* homozygote (R,T,V,X) embryos. (Q,R) Distribution of HNF3 β protein in 2-somite-stage embryos. (S,T) Transverse sections of Q and R reveal no HNF3 β protein in the mutant neural plate as indicated by the arrowhead. (U,V) HNF3 β protein distribution in 15-somite-stage embryos. (W,X) HNF3 β protein distribution in 25-somite-stage embryos. (V,X) Asterisks indicate patches of HNF3 β -positive cells in parts of the mutant hindbrain and spinal cord. The arrowheads indicate the midbrain/hindbrain junction. e, endoderm; fp, floor plate; nt, notochord; pcm, prechordal mesoderm.

notochord and floor plate (Fig. 1D). In both the notochord and floor plate, *Shh* expression persists until at least 14.5 dpc (Echelard et al., 1993).

Notochord forms in *Shh* mutants; however, it begins to degenerate in a rostral-to-caudal manner starting from 8.5 dpc (Chiang et al., 1996). Previously, we have shown that homozygous *Gli2^{zfd}* mutant mice, which contain a targeted deletion of the DNA-binding zinc finger motifs, exhibit severe skeletal defects indicating that *Gli2* is required for the normal patterning and development of skeletal elements (Mo et al., 1997). To address whether *Gli2* is required for the formation and/or maintenance of the notochord, we examined the expression of three notochord markers, *cordon-bleu* (*cbl*; Gasca et al., 1995), *Shh* and *HNF3 β* , in homozygous *Gli2^{zfd}* embryos from 8.0 dpc to 14.5 dpc (Figs 2, 3A,B, 8E-F; data not shown). A *lacZ* transgene insertion at the *cbl* locus, GtC101 (Gasca et al., 1995), was introduced into *Gli2^{zfd}* mutant background and β -galactosidase staining was used to

visualize *cbl* expression. Similar to wild-type littermates, *Gli2^{zfd}* homozygotes displayed a normal pattern of *lacZ* expression in the notochord (Fig. 2B,D,F,H). *Gli2^{zfd}* homozygotes also revealed normal expression of *Shh* and *HNF3 β* in the developing notochord (Fig. 2I-X; data not shown). Furthermore, 8.5-10.5 dpc mutant embryos showed normal expression of two other notochord markers, *T* and *HNF3 α* (data not shown). The expression of *Shh* could be clearly detected in the mutant notochord at 10.5 dpc (not shown), 12.5 dpc (Fig. 3B) and 14.5 dpc (not shown). Together, these observations suggest that *Gli2* is not essential for notochord development. However, instead of undergoing a ventral displacement, the mutant notochord retains close contact with the ventral part of the neural tube (Figs 3B,D, 8F). Sclerotomal cells were not found between the neural tube and notochord (see Fig. 8F) and the formation of vertebral bodies was mostly abolished in *Gli2^{zfd}* homozygotes (Mo et al., 1997).

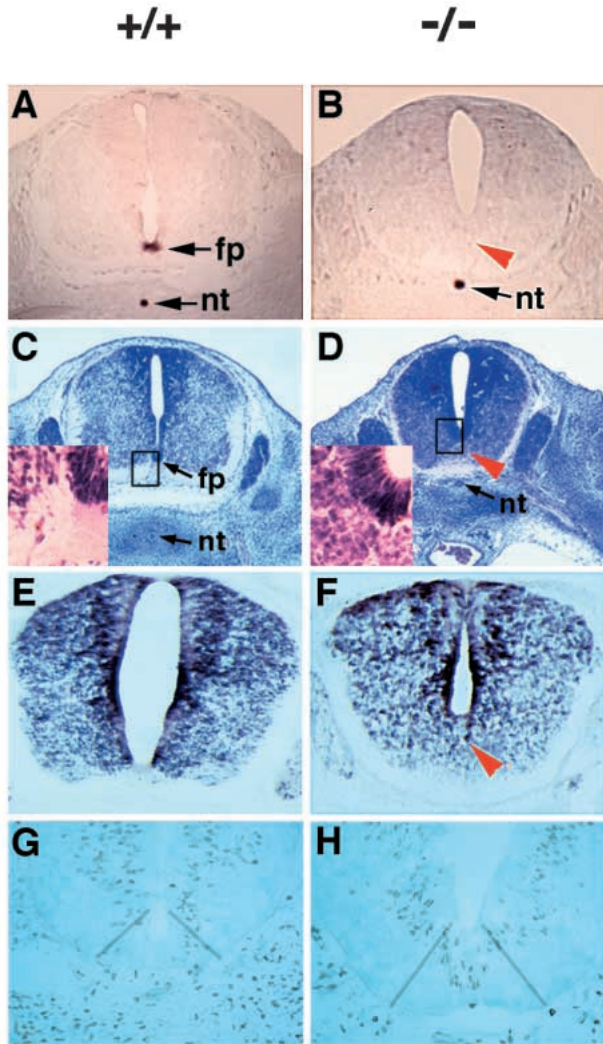


Fig. 3. Lack of floor plate differentiation in *Gli2^{zfd}* homozygotes. (A,C,E,G) Wild-type embryos. (B,D,F,H) *Gli2^{zfd}* homozygotes. (A,B) *Shh* expression in 12.5 dpc embryonic spinal cord. The arrowhead indicates the absence of *Shh* expression at the midline region of the neural tube in *Gli2^{zfd}* homozygotes. (C,D) Hematoxylin- and eosin-stained transverse sections of the trunk spinal cord of 14.5 dpc embryos. The arrowhead indicates the thickened midline region of the neural tube of *Gli2^{zfd}* mutant. Higher magnifications of ventral midline region are shown as inserts. (E,F) The expression of a neuronal marker *Bo* in 12.5 dpc embryonic spinal cord. The arrowhead indicates expression of *Bo* at the midline region of the mutant neural tube. (G,H) Sections of 10.5 dpc embryos showing mitotically active cells detected by BrdU incorporation (brown staining). Number of BrdU-positive cells within the ventral midline region (marked by the translucent lines) were counted and summarized in Table 1. fp, floor plate; nt, notochord.

Lack of floor plate differentiation in *Gli2^{zfd}* homozygotes

Neural explant studies indicate that Shh is required for the induction of floor plate differentiation by the notochord (see Ericson et al., 1996). Consistent with these *in vitro* observations, mutant mice lacking Shh signaling do not develop floor plate cells (Chiang et al., 1996). To address

Table 1. Mitotic cells in the floor plate region of 10.5 dpc wild-type and *Gli2^{zfd}* homozygous embryos

Tissue	Wild type	<i>Gli2^{zfd}</i> homozygotes
Thoracic spinal cord	10.8±2.5	31.6±2.8
Lumbar spinal cord	23.3±3.8	40.1±2.5

Numbers are mean ± s.e.m.; n=7-12 sections analyzed at each level.

whether *Gli2* functions as a downstream mediator of Shh signaling during floor plate induction, we analyzed the neural expression of *HNF3β*, *cbl* and *Shh* in *Gli2^{zfd}* homozygotes (Fig. 2).

In the developing neural tube, *HNF3β* expression is first detected at 2-somite stage and *cbl* and *Shh* are expressed later at around the 5- to 6-somite stage (Echelard et al., 1993; data not shown). While *cbl* and *Shh* are expressed in the floor plate (Fig. 2C,O), *HNF3β* is normally expressed in a broader domain including the floor plate and some ventral neurons (Fig. 2S; see Ruiz i Altaba, 1996). In *Gli2^{zfd}* homozygotes, *HNF3β* expression was not detectable in the ventral neural tube at 8.5 dpc (Fig. 2T). At later stages (9.0 and 9.5 dpc), patches of *HNF3β*-positive cells could be found along the ventral midline of the mutant neural tube (Fig. 2V,X). In hindbrain and anterior spinal cord regions, large patches of *HNF3β*-positive cells could be found (Fig. 2V,X). However, only few *HNF3β*-positive cells were observed in the caudal part of the mutant spinal cord. From 8.5 to 14.5 dpc, the floor plate expression of *cbl* and *Shh* is lost in *Gli2^{zfd}* homozygotes (Figs 2D,H,L,N,P, 3B; data not shown). Consistent with the notion that the *HNF3β*-positive cells found in the mutant neural tube are ventral neurons, there are more *HNF3β*-positive neuron-like cells in the hindbrain and anterior spinal cord than in the posterior spinal cord (Ruiz i Altaba, 1996). In 12.5-14.5 dpc wild-type embryos, floor plate cells appear as a thin layer of wedge-shaped cells at the ventral midline of the neural tube (Fig. 3C); these are epithelial cells that do not express terminal markers of neuronal differentiation (Fig. 3E) and have a lower rate of cell proliferation (Fig. 3G; see van Straaten et al., 1989). In *Gli2^{zfd}* homozygotes, the ventral midline of the neural tube instead showed an unusually high cellularity (Fig. 3D), expressed a pan-neural marker *Bo* (Fig. 3F) and showed 1.5- to 3-fold increase of cell proliferation when compared with the wild-type control (Fig. 3H; Table 1). Together, these observations indicated that floor plate differentiation does not occur in *Gli2^{zfd}* homozygotes.

Although floor plate differentiation is abolished in *Gli2^{zfd}* homozygotes, the development of more anterior neural structures seems to be less affected. In the mutants, some *lacZ*-positive *cbl*-expressing cells could be found in the rostral part of the midbrain (Fig. 2F). Furthermore, ventral forebrain expression of *Shh* and *HNF3β* could also be clearly detected in the mutants (Fig. 2N,V,X), although the activation of *Shh* expression was delayed (Fig. 2L). Unlike *Shh* mutant mice, which show severe forebrain defects including cyclopia (Chiang et al., 1996), *Gli2^{zfd}* homozygotes develop grossly normal forebrain and eyes (see Fig. 1 in Mo et al., 1997; data not shown).

Downregulation of *Ptc* expression in *Gli2^{zfd}* homozygotes

The observations that sclerotome and floor plate

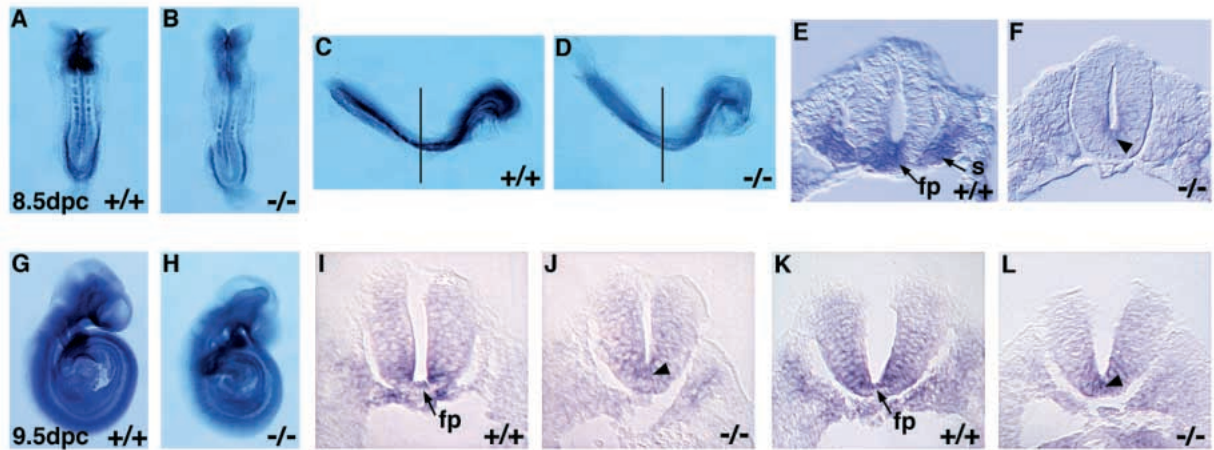


Fig. 4. Downregulation of *Ptc* expression in *Gli2^{zfd}* homozygotes. (A,C,E,G,I,K) Wild-type embryos. (B,D,F,H,J,L) *Gli2^{zfd}* homozygotes. *Ptc* expression detected by whole-mount RNA in situ hybridization (A-D and G-H) and sectioning after staining (E-F and I-L). (A,B) Dorsal view of 8.5 dpc embryos. (C,D) Lateral view of 8.5 dpc embryos. (E,F) Transverse sections of the embryos shown in C and D at the level indicated by the lines. (G,H) Lateral view of 9.5 dpc embryos. Transverse sections of 9.5 dpc embryos at rostral (I,J) and caudal (K,L) levels. Arrowheads indicate the midline expression of *Ptc* in the neural tube of *Gli2^{zfd}* homozygote. fp, floor plate; s, somite.

differentiation (both are Shh-dependent processes) are affected in *Gli2^{zfd}* homozygotes strongly suggest that loss of *Gli2* function reduces the response of the developing somite and neural tube to the notochordal Shh signal. *Ptc* expression is a diagnostic test for exposure of cells to Hh signaling in flies and vertebrates (see Perrimon, 1995; Goodrich et al., 1996). Consistent with the notion that *Gli2^{zfd}* homozygotes have diminished Shh signaling, *Ptc* expression was reduced in

8.5-10.5 dpc mutant embryos (Figs 4, 5K; data not shown). At 8.5 dpc, high levels of *Ptc* transcripts could be found in the ventral parts of the somites and neural tube, adjacent to the notochord (Fig. 4A,C,E; see Goodrich et al., 1996). In *Gli2^{zfd}* homozygotes, *Ptc* expression in these regions was clearly downregulated (Fig. 4B,D,F). In 9.5 dpc wild-type neural tube, ventral midline cells have begun to turn off *Ptc* expression (Figs 4I,K). Interestingly, although overall *Ptc*

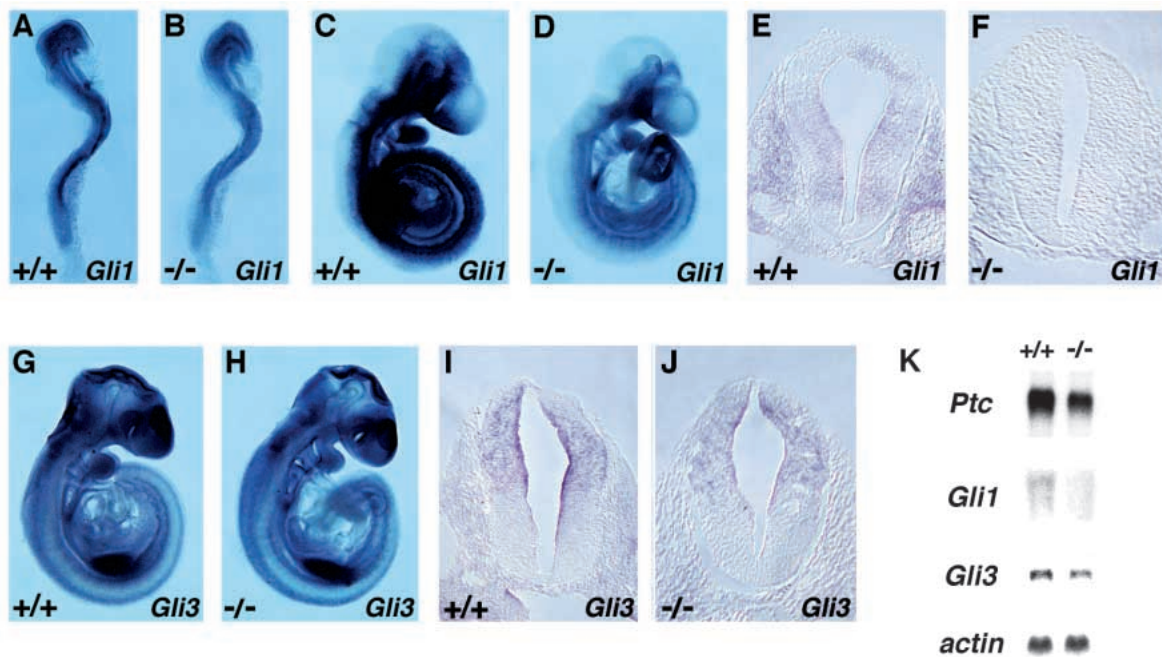


Fig. 5. Downregulation of *Gli1* expression in *Gli2^{zfd}* homozygotes. (A,C,E,G,I) Wild-type embryos; (B,D,F,H,J) *Gli2^{zfd}* homozygotes. *Gli1* expression detected by whole-mount RNA in situ hybridization (A-D) and sectioning after staining (E,F). (A,B) Lateral view of 8.5 dpc embryos. (C,D) Lateral view of 9.5 dpc embryos. (E,F) Transverse sections of 9.5 dpc embryos. *Gli3* expression in 9.5 dpc embryos detected by whole-mount RNA in situ hybridization (G,H) and sectioning after staining (I,J). (K) Northern blot analysis in 9.5 dpc embryos. Total RNA from individual 9.5 dpc wild-type or *Gli2^{zfd}* homozygote embryo was subjected to electrophoresis, blotted onto nylon membrane and hybridized with *Ptc*, *Gli1* and *Gli3* probes. β -actin mRNA was used to show the equal amount of RNA in both lanes.

expression was decreased in the mutants (Figs 4G,H, 5K), ventral midline cells of the mutant neural tube still expressed a fair amount of *Ptc* transcripts (Fig. 4J,L).

***Gli1* expression is downregulated in *Gli2^{zfd}* homozygotes**

Similar to *Ptc*, *Gli1* has been shown to be a Shh-responsive gene (Epstein et al., 1996; Hynes et al., 1997; Lee et al., 1997; Sasaki et al., 1997). We therefore examined whether *Gli1* expression is also downregulated in *Gli2^{zfd}* homozygotes. At both 8.5 and 9.5 dpc, *Gli1* expression was found to be significantly reduced in the mutants (Fig. 5A-D). At 8.5 dpc, the downregulation of *Gli1* in the ventral neural tube and somites appeared similar to that of *Ptc* (data not shown; see Fig. 4F). However, at 9.5 dpc, the reduction of *Gli1* transcripts was more drastic than that of *Ptc* (Compare Fig. 5C,D with Fig. 4G,H). Northern blot analysis of 9.5 dpc mutants revealed an ~80% reduction of *Gli1* transcripts but only about 50% reduction of *Ptc* transcripts (Fig. 5K). In both the neural tube and somites, few *Gli1* transcripts could be detected in the mutants (Fig. 5E,F and data not shown).

We also examined the expression of *Gli3* in *Gli2^{zfd}* homozygotes. No obvious difference in *Gli3* expression could be found in 8.5 dpc mutants (data not shown). Although we consistently detected a slight reduction of *Gli3* transcripts in 9.5 dpc *Gli2^{zfd}* homozygotes by both in situ hybridization (Fig. 5G-J) and northern blot analyses (Fig. 5K), its significance was not further investigated.

***Pax6* expression is ventrally shifted in *Gli2^{zfd}* mutant hindbrain and spinal cord**

Pax6 is involved in the control of progenitor cell identity in the hindbrain and spinal cord (Burrill et al., 1997; Ericson et al., 1997; Osumi et al., 1997). Its expression in the developing neural tube is regulated by Shh signaling (see Chiang et al., 1996; Ericson et al., 1997). In the embryonic hindbrain and spinal cord, *Pax6* is expressed at high levels in the dorsal basal plate and ventral alar plate, and at lower levels in the dorsal neural tube. In vitro studies indicated that *Pax6* expression in the neural explants could be induced at a low concentration of Shh (~0.4 nM) but was repressed at ~8-fold higher concentrations of Shh (Ericson et al., 1997). In *Shh* mutant mice, *Pax6* expression was greatly reduced and the expression domain extended across the ventral midline of the mutant neural tube (Chiang et al., 1996).

To examine whether *Pax6* expression is affected in *Gli2^{zfd}* homozygotes, we analyzed 10.5 dpc mutant neural tubes by both immunohistochemistry and in situ hybridization (Fig. 6A-E and data not shown). There was a ventral shift of the domain of *Pax6* expression in both the mutant hindbrain (Fig. 6B,D,E) and spinal cord (data not shown). A large population of Pax6-positive cells could be found at the ventral midline in the rostral part of the hindbrain (see Fig. 6B; r5 level). In the caudal part of the hindbrain (Fig. 6D; r7 level) and the spinal cord (data not shown), only few Pax6-positive cells were located at the ventral midline. A slight reduction of the number of Pax6-positive cells was consistently observed in the dorsal part of the mutant neural tube (compare Fig. 6B,D with Fig. 6A,C). By RNA in situ hybridization, a similar ventral shift of *Pax6* expression domain was also detected in the mutant neural tube (Fig. 6E and data not shown).

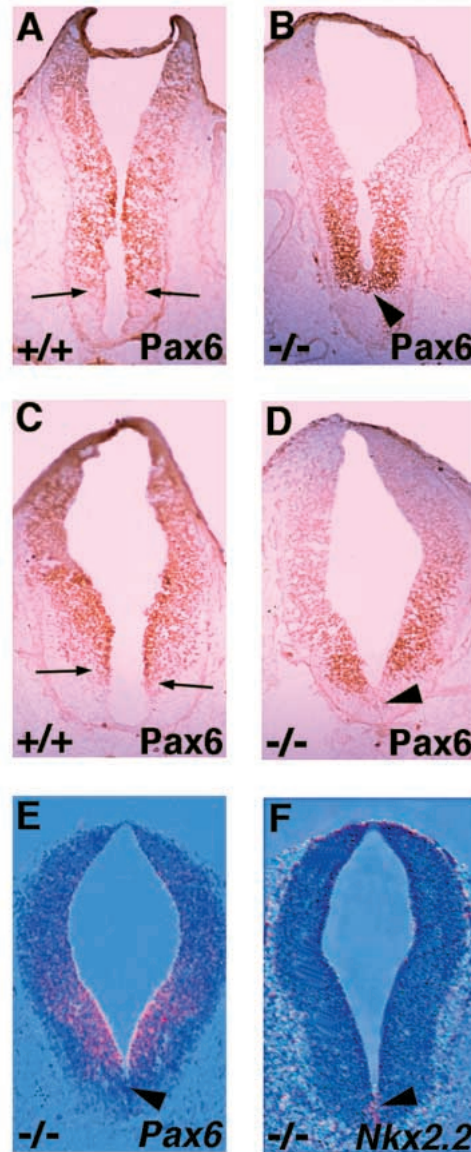


Fig. 6. Ventral shift of *Pax6* expression in the neural tube of 10.5 dpc *Gli2^{zfd}* homozygotes. (A,C) Wild-type embryos; (B,D,E,F) *Gli2^{zfd}* homozygotes. (A-D) Pax6 immunohistochemistry detected by HRP-conjugated secondary antibodies. (A,B) Sections at the r5 level. (C,D) Sections at the r7 level. Arrows indicate the ventral limit of Pax6-positive cells in the wild-type hindbrain (A,C). *Pax6* expression (E) and *Nkx2.2* expression (F) in the hindbrain (r7 level) detected by ³³P RNA in situ hybridization. Dark-field views of hybridization signals (red) were overlaid onto bright-field views (blue) of the same section. Arrowheads indicate the ventral midline of the mutant neural tube.

The expression of *Nkx2.2* is normally found in cells flanking the floor plate (Shimamura et al., 1995). In neural explants, *Nkx2.2* expression was induced at a Shh concentration that could repress *Pax6* expression (Ericson et al., 1997). In 10.5 dpc mutant neural tube, low level of *Nkx2.2* expression could be detected at the ventral midline (Fig. 6F and data not shown). Together, these observations indicated that diminished Shh signaling in the mutant neural tube results in a ventral shift of *Pax6*- and *Nkx2.2*-expressing cells.

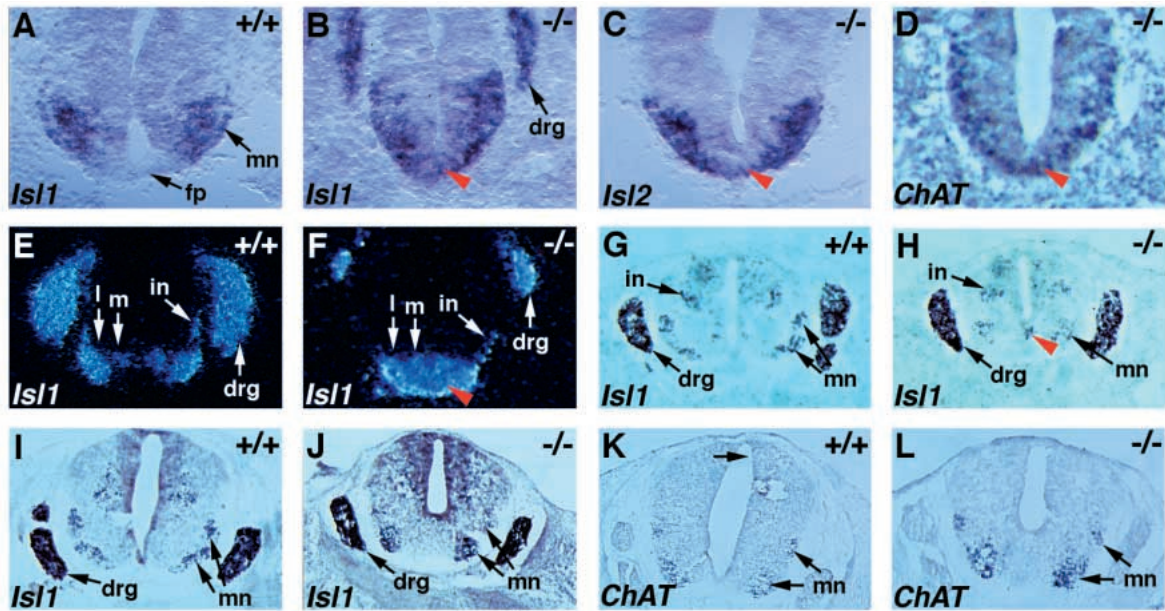


Fig. 7. Motor neuron development in *Gli2^{zfd}* mutants. Transverse sections of anterior spinal cord (A-D and G-L) and lumbar region of the spinal cord (E,F) were hybridized with various probes using wild-type and *Gli2^{zfd}* homozygote embryos, respectively. (A,B) *Isl1* expression at 9.5 dpc. (C,D) *Isl2* and *ChAT* expression in *Gli2^{zfd}* mutants at 9.5 dpc. (E,F) *Isl1* expression at 11.5 dpc. (G,H) *Isl1* expression at 12.5 dpc. (I,J) *Isl1* expression at 14.5 dpc. (K,L) *ChAT* expression at 14.5 dpc. drg, dorsal root ganglion; fp, floor plate; in, interneurons; l, lateral motor neurons; m, medial motor neurons; mn, motor neurons. Arrowheads indicate the expression of *Isl1*, *Isl2* or *ChAT* at the midline region of the neural tube in *Gli2^{zfd}* homozygotes.

Generation of motor neurons in the absence of floor plate

Shh signaling is required for the generation of motor neurons (Chiang et al., 1996). Neural explant studies have indicated that two critical periods of Shh signaling are involved in the generation of motor neurons (Ericson et al., 1996). During the early period, Shh derived from the notochord converts naive neural plate cells into a ventralized progenitor state (*Pax3/7*-negative). These ventral progenitors require a second period of Shh signaling, late in their final division cycle, to develop into motor neurons. Floor plate cells have been suggested to be the likely source of later Shh signaling (Ericson et al., 1996).

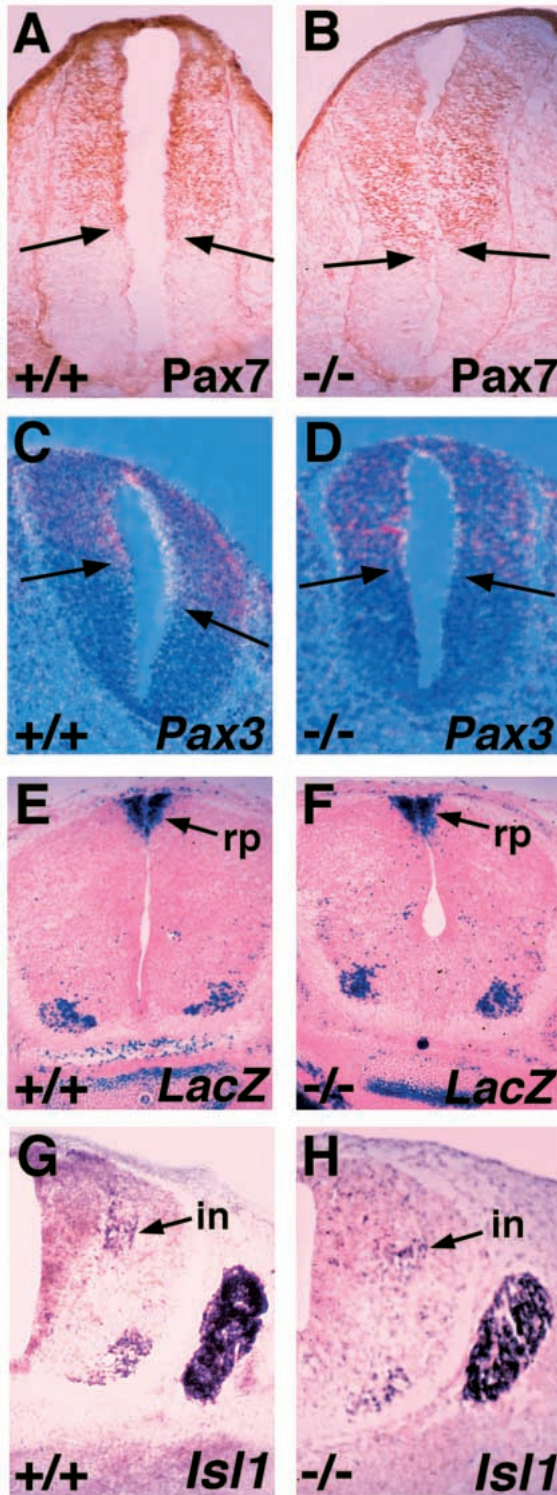
At 10.5 dpc, three markers of motor neuron differentiation, *Isl1*, *Isl2* and *ChAT*, could be detected in the ventral neural tube of *Gli2^{zfd}* homozygotes (Fig. 7B-D). In wild-type embryos, their expression was detected in two regions lateral to the floor plate (Fig. 7A; data not shown). The fact that motor neurons are induced in *Gli2^{zfd}* homozygotes is consistent with the notion that motor neuron differentiation can take place in the absence of floor plate differentiation (Tanabe and Jessell, 1996). To address whether motor neurons can develop further in the absence of floor plate differentiation, we analyzed the expression of *Isl1*, *Isl2* and *ChAT* in *Gli2^{zfd}* homozygotes from 10.5 to 14.5 dpc (Fig. 7 and data not shown). At 11.5 dpc, *Isl1* is expressed in two groups of motor neurons at the lumbar region of the spinal cord (Fig. 7E; see Fig. 6J in Tsuchida et al., 1994). In *Gli2^{zfd}* homozygotes, these two groups of *Isl1*-positive motor neurons could be clearly detected at the ventral midline (Fig. 7F). Later at 12.5 dpc, some motor neurons could still be found at the ventral midline of the mutant neural tube (see arrowhead in Fig. 7H). In the mutant neural tube, some

motor neurons could be found in their normal lateral destinations (compare Fig. 7H with 7G). At 14.5 dpc, although the mutant spinal cord appeared quite malformed, motor neurons could clearly be found (Fig. 7J,L). These results indicate that, although floor plate differentiation is absent in *Gli2^{zfd}* homozygotes, the development of motor neurons is grossly normal and suggest that motor neuron differentiation does not require additional factors from floor plate cells.

Development of dorsal cells in *Gli2^{zfd}* mutant neural tube

Along the dorsoventral axis of the developing neural tube, ventralizing signals from the notochord and floor plate are thought to counteract the dorsalizing signals from the surface ectoderm and roof plate (Tanabe and Jessell, 1996). The expression of *Pax3* and *Pax7* is broadly expressed in naive neural plate and it has been suggested that a low grade of Shh signaling is responsible for the repression of *Pax3/7* expression in the ventral neural tube (see Ericson et al., 1997). Consistent with this hypothesis, high levels of *Pax3* expression extended ventrally across the ventral midline of the neural tube in *Shh* mutant mice, which lack a floor plate and have a defective notochord (Chiang et al., 1996).

Since the neural tube of *Gli2^{zfd}* homozygotes have diminished Shh signaling and lacks a floor plate, we examined whether the expression of *Pax3* and *Pax7* is altered. At 10.5 dpc, *Pax7*-positive cells were found to be restricted to the dorsal half of the mutant hindbrain (data not shown) and spinal cord (Fig. 8A,B). In situ hybridization analysis also revealed a similar dorsal restriction of *Pax3* expression in the mutant neural tube (Fig. 8C,D; data not shown). Our analysis did not



indicate any significant changes in both the expression levels and dorsoventral limit along the neural tube of these two genes in the mutants. The development of the roof plate also appeared normal in *Gli2^{zfd}* homozygotes. At 14.5 dpc, *cbl* expression could be clearly detected by β-galactosidase staining in the roof plate of both the wild-type and mutant neural tubes (Fig. 8E,F). A distinct class of dorsal interneurons, termed D2 neurons, expresses *Isl1* (Liem et al., 1997). Consistent with the

Fig. 8. Development of dorsal cells in the spinal cord of *Gli2^{zfd}* homozygotes. (A,C,E,G) Wild-type embryos; (B,D,F,H) *Gli2^{zfd}* homozygotes. (A,B) Pax7 immunohistochemistry in 10.5 dpc embryonic spinal cord detected by HRP-conjugated secondary antibodies. (C,D) *Pax3* expression in 10.5 dpc embryonic spinal cord detected by ³³P RNA in situ hybridization. Dark-field views of hybridization signals (red) are overlaid onto bright-field views (blue) of the same section. The arrows indicate the ventral limits of *Pax3* and *Pax7* expression. (E,F) β-galactosidase staining of *cbl* expression in 14.5 dpc embryonic spinal cord. Arrows indicate the roof plate (rp). (G,H) *Isl1* expression in 12.5 dpc embryonic spinal cord detected by DIG in situ hybridization. The arrows indicate the D2 interneurons (in).

observations that the development of D2 neurons is not affected by the removal of notochord (i.e. ventralizing/Shh signal) in chick embryos (Liem et al., 1997), we also found that the expression of *Isl1* in the dorsal neural tube is grossly normal in *Gli2^{zfd}* homozygotes (Fig. 8G,H; see also Fig. 7F,H). These observations suggest that diminished Shh signaling and lack of floor plate differentiation do not significantly affect the development of dorsal cells in the *Gli2* mutant neural tube.

DISCUSSION

The role of vertebrate Gli zinc finger proteins in Hh signaling/neural patterning is not well understood (see Ruiz i Altaba, 1997). Here we show that the expression of Shh-responsive genes such as *Ptc* (Goodrich et al., 1996), *Gli1* (Epstein et al., 1996; Hynes et al., 1997) and *HNF3β* (Ruiz i Altaba et al., 1993, 1995a; Sasaki and Hogan, 1994; Sasaki et al., 1997) is greatly reduced in the neural tube of *Gli2^{zfd}* homozygotes despite the fact that the mutant notochord expresses a normal level of *Shh*. In addition, *Gli2^{zfd}* homozygotes lack floor plate differentiation and develop motor neurons at the ventral midline of the neural tube. These observations suggest that *Gli2^{zfd}* homozygotes show diminished Shh signaling in the neural tube and that *Gli2* is a downstream mediator of Shh signaling.

Gli2 is essential for floor plate differentiation

Previous work has shown that mutant mice with defects in notochord development fail to initiate floor plate differentiation (see Ang and Rossant, 1994; Weinstein et al., 1994 and their references). Although *Gli2* is transiently expressed in the developing notochord (see Fig. 1) and Shh signaling is required for the maintenance of notochord (Chiang et al., 1996), several observations suggest that the notochord of *Gli2^{zfd}* mutants is normal. The developmental expression of all notochord markers tested is similar in the mutants and their wild-type littermates (see Results). In particular, the mutant notochord expresses a normal level of *Shh*. The fact that motor neuron differentiation can be induced in the mutants and that overtly normal dorsoventral patterning of the mutant neural tube occurs further suggest that the failure of floor plate differentiation in *Gli2^{zfd}* homozygotes is not due to defects in the mutant notochord and that *Gli2* function is specifically required for floor plate differentiation.

HNF3β is a direct target of Gli proteins

Misexpression of *HNF3β* in mouse and frog embryos leads to

the induction of ectopic floor plate suggesting that *HNF3 β* expression can trigger floor plate differentiation (Hynes et al., 1995; Ruiz i Altaba et al., 1993, 1995a; Sasaki and Hogan, 1994). In response to Shh signal, *HNF3 β* expression can be rapidly induced in neural explants (Marti et al., 1995a; Roelink et al., 1995; Ruiz i Altaba et al., 1995b; Tanabe et al., 1995). Since this induction can take place in the presence of cycloheximide, *HNF3 β* appears to be an immediate target of Shh signaling (Ruiz i Altaba et al., 1995b). By transgenic analysis, a floor-plate-specific enhancer has been identified in the 3' end of the *HNF3 β* gene (Sasaki and Hogan, 1996). A Gli-binding site within the enhancer is required for the floor-plate-specific activity and the Gli-binding site can function as a Shh-responsive element in a rat forebrain cell line (Sasaki et al., 1997). Furthermore, a human Gli1 protein can act as a transcriptional activator in the absence of any exogenous Shh signals. Recently, it has also been reported that misexpression of Gli1 protein induces ectopic *HNF3 β* expression in frog and mouse embryos (Hynes et al., 1997; Lee et al., 1997). Together, these observations suggest that Gli transcription factors are responsible for the Shh-dependent induction of *HNF3 β* during floor plate differentiation. Our results here provide genetic evidence supporting the hypothesis that *HNF3 β* is a direct target of Gli proteins.

In mice, all three *Gli* genes are transiently expressed in midline neural plate cells prior to floor plate differentiation (Hui et al., 1994; Lee et al., 1997; Sasaki et al., 1997; this report). In *Gli2^{zfd}* homozygotes, *Gli1* expression in the developing neural tube is drastically downregulated. It is possible that the failure of floor plate differentiation in *Gli2^{zfd}* homozygotes is caused by the reduction of *Gli1/2* function. Interestingly, mice homozygous for a targeted deletion of the *Gli1* DNA-binding zinc finger domain region are viable with no obvious abnormalities (C.-c. H., unpublished data). These observations suggest that *Gli2* and/or *Gli3* might be able to fully compensate for the loss of *Gli1* function during development. Similarly, *Gli3* is not essential for floor plate differentiation since *Gli3* null mutant neural tube also develops a normal floor plate (Q. D., unpublished data). Future experiments using double or triple mutant analysis should allow us to test the potential functional redundancy of the *Gli* genes in Shh signaling and neural patterning. In particular, it will be interesting to analyze the development of ventral neurons in the compound mutants.

Motor neurons develop in the absence of floor plate differentiation

Neural explants studies indicate that two critical periods of Shh signaling are required for the specification of motor neuron identity (Ericson et al., 1996). In higher vertebrates such as mice, the notochord gradually undergoes a ventral displacement and the floor plate seems to be the source of Shh signal for the second phase of signaling. Although our analysis cannot eliminate the possibility that *Gli2^{zfd}* homozygotes have some subtle defects in motor neuron development, our results clearly indicate that floor plate is not required for the induction of *Isl1* expression and motor neuron differentiation. This is reminiscent to the phenotype of the zebrafish *cyc* mutants, which exhibit a strikingly similar floor plate defect (Hatta et al., 1991; Hatta, 1992). Homozygous *cyc* mutants develop a morphologically normal

notochord but lack a floor plate and motor neurons develop at the ventral midline of the spinal cord. In *Gli2^{zfd}* homozygotes, the notochord retains contact with the ventral neural tube throughout development. Our results indicate that Shh signal from the mutant notochord is sufficient for the generation of motor neurons. In other words, the Shh signal from the notochord can substitute for the Shh signal from the floor plate, provided the notochord remains in close contact with the ventral neural tube. It is worth noting that the notochord is in close contact with the neural tube in lower vertebrates such as zebrafish (see Hatta, 1992).

Role of *Gli2* in Shh signaling

Both *Gli1* and *Gli3* have been implicated in Shh signaling (see Ruiz i Altaba, 1997). Here we have provided genetic and molecular evidence illustrating an essential role of *Gli2* in Shh signaling. Previous studies have shown that *Ptc* and *Gli1* expression can be induced by Shh signaling (Epstein et al., 1996; Goodrich et al., 1996; Hynes et al., 1997; Lee et al., 1997; Marigo et al., 1996). Consistent with the notion that Gli2 protein is a transcription factor mediating Shh signaling and *Gli2^{zfd}* homozygotes have diminished Shh signaling, the expression of Shh-responsive genes such as *Ptc* and *Gli1* is reduced. Graded Shh signaling has been shown to induce the expression of *HNF3 β* , *Nkx2.2* and *Pax6* in different cell populations in the ventral neural tube (see Ericson et al., 1997). As expected, diminished Shh signaling results in a ventral shift of *Nkx2.2* and *Pax6* expression domain in the mutant neural tube. Our results thus suggest that Gli2 protein functions as a transcriptional activator, directly or indirectly, regulating the expression of Shh-responsive genes.

Gli transcription factors in vertebrate Hh pathway

In vertebrates, there are multiple Hh signals and Gli proteins. In contrast, Ci is the only known *Drosophila* Gli transcription factor that mediates the Hh signal. In order to implement different functions, Ci is known to be modified post-transcriptionally. A truncated form produced by proteolysis functions as a direct repressor of *hh* and *dpp* expression (Aza-Blanc et al., 1997). A nuclear form of Ci probably acts together with CBP in the activation of Hh-responsive gene expression (Akimaru et al., 1997). Extensive sequence similarities between Ci and vertebrate Gli proteins in the DNA-binding zinc finger domain, CBP-binding domain and putative PKA phosphorylation sites (see Marine et al., 1997) suggest that some of the modifications found in Ci may be evolutionarily conserved. Similar to Ci, vertebrate Gli proteins also appear to be mostly cytoplasmic (see Lee et al., 1997).

There are several observations indicating that Gli proteins may have different roles in different vertebrate species. For example, all three *Gli* genes are expressed in midline neural plate cells during mouse embryogenesis (Hui et al., 1994; Lee et al., 1997; Sasaki et al., 1997) and, as shown here, *Gli2* is essential for floor plate differentiation. However, in *Xenopus*, only *Gli1* is expressed in midline neural plate cells prior to floor plate differentiation (Lee et al., 1997). It is worth noting that there are at least four *Xenopus* Hh genes and expression of three of them overlaps in the entire neural plate (Ekker et al., 1995). It is quite possible that different Gli proteins can respond differentially to various Hh signals. In support of this, two different Hh signals are known to regulate the development

of distinct muscle cell populations in zebrafish embryos (Currie and Ingham, 1996).

It remains unclear whether any individual Gli proteins can perform multiple functions as Ci does. Mutations of *GLI3* in dominant genetic disorders support the notion that Gli3 protein can perform multiple functions. In Pallister-Hall syndrome, an expected truncation in Gli3 protein similar to the repressor form of Ci results in a much more severe phenotype than those of Greig cephalopolysyndactyly syndrome which has a loss of *GLI3* function (Kang et al., 1997). These observations raise the possibility that, similar to Ci, Gli3 protein may also be regulated by proteolysis and mutations in Pallister-Hall syndrome result in the production of a constitutively active repressor. However, it remains to be tested whether proteolysis of the Gli proteins is an evolutionarily conserved mechanism in Hh signaling.

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