Development 124, 2537-2552 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV1178

Gli1 is a target of Sonic hedgehog that induces ventral neural tube development

Jeffrey Lee*, Kenneth A. Platt*,†, Patricia Censullo and Ariel Ruiz i Altaba‡

The Skirball Institute, Developmental Genetics Program and Department of Cell Biology, NYU Medical Center, 540 First Avenue, New York, NY 10016, USA

*These two authors contributed equally to the work and are listed alphabetically
†Present address: Lexicon Genetics Inc., 4000 Research Forest Drive, The Woodlands, TX 77381, USA
‡Author for correspondence (e-mail: ria@saturn.med.nyu.edu)

SUMMARY

The vertebrate zinc finger genes of the *Gli* family are homologs of the *Drosophila* gene *cubitus interruptus*. In frog embryos, *Gli1* is expressed transiently in the prospective floor plate during gastrulation and in cells lateral to the midline during late gastrula and neurula stages. In contrast, *Gli2* and *Gli3* are absent from the neural plate midline with *Gli2* expressed widely and *Gli3* in a graded fashion with highest levels in lateral regions. In mouse embryos, the three *Gli* genes show a similar pattern of expression in the neural tube but are coexpressed throughout the early neural plate. Because *Gli1* is the only *Gli* gene expressed in prospective floor plate cells of frog embryos, we have investigated a possible involvement of this gene in ventral neural tube development. Here we show that Shh signaling activates *Gli1* transcription and that widespread expression of endogenous frog or human glioma *Gli1*, but not *Gli3*, in developing frog embryos results in the ectopic differentiation of floor plate cells and ventral neurons within the neural tube. Floor-plate-inducing ability is retained when cytoplasmic Gli1 proteins are forced into the nucleus or are fused to the VP16 transactivating domain. Thus, our results identify Gli1 as a midline target of Shh and suggest that it mediates the induction of floor plate cells and ventral neurons by Shh acting as a transcriptional regulator.

Key words: *cubitus interruptus*, *Gli*, Shh, sonic hedgehog, mouse neural tube, zinc finger, *Xenopus*, floor plate

INTRODUCTION

Great progress has been made in recent years in the identification of signaling molecules that mediate embryonic inductions. However, much less is known of the molecular mechanisms that interpret the inducing information in responding cells, including the identification of transcriptional regulators that orchestrate the induced differentiation programs.

Within the nervous system, the differentiation of the floor plate and other ventral neural tube cell types depends on induction from the underlying mesodermal cells of the notochord (van Straaten et al., 1988; van Straaten and Hekking, 1991; Placzek et al., 1990, 1993; Yamada et al., 1991; Ruiz i Altaba, 1992; Goulding et al., 1993). Sonic hedgehog (Shh) secreted from the notochord is necessary and sufficient to induce ventral cell types (Riddle et al., 1993; Krauss et al., 1993; Echelard et al., 1993; Roelink et al., 1994; Chang et al., 1994; Ruiz i Altaba et al., 1995a; Ekker et al., 1995; Martí et al., 1995a; Chiang et al., 1996). Floor plate cells and ventral neurons can be induced in vitro by different concentrations of Shh (Roelink et al., 1994, 1995; Martí et al., 1995a; Tanabe et al., 1995; Hynes et al., 1995; Ericson et al., 1996) and ectopic expression of *Shh* within the neural tube leads to the induction of floor plate cells (Krauss et al., 1993; Echelard et al., 1993; Roelink et al., 1994; Ruiz i Altaba et al., 1995a). In addition, absence of the notochord or loss of function of Shh leads to severe embryonic deficiencies including the lack of ventral neural tube cell types (Yamada et al., 1991; Ruiz i Altaba, 1992; Placzek et al., 1993; Martí et al., 1995a; Chiang et al., 1996; Erickson et al., 1996; Roessler et al., 1996; Belloni et al., 1996).

An important step in floor plate development in response to Shh appears to be the expression of the winged-helix transcription factor HNF-3β (Ruiz i Altaba et al., 1993a, 1995a; Monaghan et al., 1993; Strähle et al., 1993; Ang et al., 1993; Sasaki and Hogan, 1993). Ectopic expression of HNF-3β, like that of its early functional homolog in frogs Pintallavis (Ruiz i Altaba and Jessell, 1992), leads to ectopic floor plate differentiation within the neural tube (Ruiz i Altaba et al., 1993b, 1995a; Sasaki and Hogan, 1994). In frog embryos, ectopic HNF-3β expression induces the expression of floor plate markers including *F-spondin*, *Shh* and HNF-3β itself, and ectopic expression of Shh induces the expression of *F-spondin*, HNF-3β and *Shh* itself (Ruiz i Altaba et al., 1993b, 1995a; Roelink et al., 1994).

Previous studies have indicated a conservation in the cytoplasmic transduction of the hh signal from flies to vertebrates (Fan et al., 1995; Goodrich et al., 1996; Marigo et al., 1996a-d; Hahn et al., 1996; Johnson et al., 1996; Stone et al., 1996; Hammerschmidt et al., 1996; Epstein et al., 1996; Concordet et al., 1996). Genes involved in interpreting the Shh signal

could regulate HNF-3β function during floor plate induction. In *Drosophila*, *Cubitus interruptus* (*ci*) encodes a putative transcription factor with five zinc fingers that acts downstream of all other known components in the hh transduction pathway regulating the expression of hh target genes (Orenic et al., 1990; Eaton and Kornberg, 1990; Forbes et al., 1993; Slusarski et al., 1995; Perrimon, 1995; Sánchez-Herrero et al., 1996; Domínguez et al., 1996; Alexandre et al., 1996). Homologs of *ci* in other species include three vertebrate genes: *Gli* (hereafter referred as *Gli1*), *Gli2* and *Gli3* (Kinzler et al., 1987, 1988; Kinzler and Vogelstein, 1990; Ruppert et al., 1988, 1990; Walterhouse et al., 1993; Hui et al., 1994) and the nematode *tra-1* gene involved in sex determination (Zarkower and Hodgkin, 1992).

Gli1 was originally identified as an amplified nuclear oncogene in human gliomas and sarcomas (Kinzler et al., 1987; Roberts et al., 1989) and the mouse *Gli* genes are expressed in many embryonic cell types in overlapping domains (Walterhouse et al., 1993; Hui et al., 1994). Loss of function of *Gli3* in humans leads to a series of defects described as the Greig cephalopolysyndactyly syndrome (Vortkamp et al., 1991) and *Gli3* mutant mouse (extra-toes) embryos show defects in limb and forebrain development that mimic the human syndrome (Johnson, 1967; Schimmang et al., 1992; Hui and Joyner, 1993; Franz, 1994). However, it is not known whether the *Gli* genes participate in the induction of neural pattern by Shh.

In this paper we show that the *Gli* genes display distinct yet overlapping domains of expression during embryogenesis and have different functions. *Gli1* is normally expressed in midline neural plate cells during gastrulation, it is transcriptionally activated by Shh signaling and Gli1, but not Gli3, can mimic the inductive effects of Shh on neural tube pattern. Together, these results strongly support a role of Gli1 as a target and mediator of Shh signaling in floor plate and ventral neuronal differentiation.

MATERIALS AND METHODS

Animals, embryos, microinjection and transfections

Xenopus laevis embryos were obtained by standard methods (e.g. Ruiz i Altaba, 1993) and staged according to Nieuwkoop and Faber (1967). Exogastrulae were obtained as previously described (Ruiz i Altaba, 1992). Microinjection of plasmid DNA was performed as described (Ruiz i Altaba, 1993) introducing 20-200 pg of supercoiled DNA. The injections were localized to the animal-most region to deliver the plasmids to prospective neuroectodermal cells (Ruiz i Altaba et al., 1995a). Lineage tracing with rhodamine-lysine-dextran (M_r>70 000; Molecular Probes) was performed by coinjecting RLDx in water at 25 mg/ml with plasmid DNA. COS-7 cells (ATCC) were cultured and transfected by standard procedures with lipofectamine (GIBCO) and examined 24-48 hours after transfection. CD1 mouse embryos were obtained at the desired embryonic (E) day by natural matings from the Skirball Institute transgenic facility or Taconic Animal Farms. E0.5 was counted as the morning after conception.

cDNA library screens

A partial mouse Gli1 cDNA (Hui et al., 1994) was used as a probe to screen a frog neurula stage (stage 17) cDNA library (Kintner and Melton, 1987) at moderate stringency. Several positive clones were identified and classified by Southern blot and sequence analyses. The longest cDNA clones from the stage 17 library screen were cloned into the *Eco*RI site of pBluescript (Stratagene) yielding pf*Gli1* (3.6 kb), pf*Gli2* (4 kb) and pf*Gli3* (4 kb).To isolate the 5′ sequences missing from the *fGli1* clone, RACE reactions were performed with primer 5′AGAGATGGGCTGATAGTTCC3′ yielding 300 additional nucleotides. This product was then used to isolate a partial cDNA clone containing the entire 5′ end from the stage 17 cDNA library at a frequency of 3.5×10^{-6}. The full-length clone was obtained by joining the original fGli1 cDNA to the 5′ partial cDNA at their *Nru*I sites. Overlapping sequences showed that the two cDNAs derive from the same gene.

Plasmids and constructs

cDNAs for injection were subcloned either into pcDNA1-Amp (Invitrogen) yielding subclones named pCMV- followed by the name of the cDNA or into pCS2MT, a vector that contains 6 myc tags in frame (Turner and Weintraub, 1994; Rupp et al., 1994), kindly provided by D. Turner. Expression of the myc-tagged cDNA fragments is driven by CMV regulatory sequences as in the case of pcDNA 1-Amp.

The full-length frog Gli1 cDNA was myc-tagged by cloning a sequenced 480 bp Pwo PCR *Eco*RI-*Xho*I product of the 5′ end of the coding sequence into pCS2MT followed by the insertion of the rest of the cDNA as a *Bcl*I-*Xho*I fragment, yielding pmyc-fGli1.

A myc tag deletion of pmyc-fGli1 was obtained by digesting this plasmid with *Eco*RI and *Cla*I, blunt ending and religation. In this clone, the start codon is the original one of Gli1.

A truncated (5′ deletion) version of frog Gli1 missing the first 224 amino acids was myc-tagged by cloning the *Eco*RI partial cDNA (clone 16) into the *Eco*RI site of pCS2MT yielding pmyc-fGli1-5′Δ.

The VP16->fGli1 clone was made by joining a sequenced PCR product of the VP16 transactivator domain containing an ATG (kindly provided by Dr Ed Ziff) using primers (5′AACGGATCCACCATGGTCGCGTACAGCCGCGCGCGTACG3′ and 5′AACGGATCCCCCACCGTACTCGTCAATTCC3′) to the *Bam*HI site in the polylinker of pcDNA1-Amp upstream of the fGli1 partial cDNA cloned into the *Eco*RI site. The ribosome binding site and initiator ATG in primer 1 are underlined. The nucleotides in between the *Bam*HI and the *Eco*RI sites in the polylinker of the vector added the aa sequence GST-SNGRQCAG upstream of the start of the fGli1 sequence.

The human Gli1 (Kinzler et al., 1987) and Gli3 (Ruppert et al., 1990) cDNAs (kindly provided by Dr Bert Vogelstein) yielded pCMV-hGli1 and pCMV-hGli3.

Human Gli3 was myc tagged by cloning the coding region of this cDNA from aa 18 to the end into the pCS2-MT vector yiedling pmyc-hGli3.

Cubitus interruptus cDNAs (Orenic et al., 1990) were kindly provided by Dr Robert Holmgren and Dr Tom Kornberg. The intronless cDNA clone (kindly provided by Dr Kornberg) yielded pCMV-Ci.

In situ hybridization, histology and RNA probes

Whole-mount in situ hybridization with digoxigenin-labeled antisense RNA probes was carried out in frog embryos according to Harland (1991) using maleic acid buffer and in mouse embryos according to Conlon and Herrmann (1993). Labeled embryos were sectioned in a microtome at 10-14 μm after embedding in paraplast/tissue prep (Fisher). Labeled mouse embryos were embedded in tissue tek and frozen after equilibrating in 30% sucrose in 0.1 M phosphate buffer. 50 μm sections were made in a cryostat and collected in glass slides (Superfrost; Fisher), briefly rinsed in PBS (~0.5-1 minute) and mounted in Aquamount (Lerner Labs.). In situ hybridization in tissue sections with radioactively labeled RNA probes was performed as described (Hui and Joyner, 1993). Photographs were taken with a Zeiss Axiophot microscope and images transferred to Photoshop.

Antisense RNA probes were made as follows: pCMV-*fGli1* (clone #16) was cut with *Bam*HI and transcribed with SP6 RNA polymerase. pCMV-*fGli2* (clone #21) was cut with *Bam*HI and transcribed with SP6 RNA polymerase. pBluescript-*fGli3* (clone #10) was cut with *Not*I and transcribed with T3 RNA polymerase. Frog

Shh (pfhh4 clone; Ruiz i Altaba et al., 1995a) was cut with *Not*I and transcribed with T3 RNA polymerase. A 1.4 kb PCR subclone of the frog *F-spondin* cDNA (Ruiz i Altaba et al., 1993a) in pCRII (Invitrogen) was cut and transcribed with SP6 polymerase. Frog neural-specific *β-tubulin* (*N-tubulin*) clone (Richter et al., 1988) was cut with *Bam*HI and transcribed with T3 RNA polymerase. *Pintallavis* pF5 clone (Ruiz i Altaba and Jessell, 1992) was cut with *Hin*dIII and transcribed with T7 RNA polymerase. Mouse *Gli1* clone (Hui et al., 1994) was cut with *Not*I and transcribed with T3 RNA polymerase. Mouse *Gli2* clone (Hui et al., 1994) was cut with *Hin*dIII and transcribed with T3 RNA polymerase. Mouse *Gli3* clone (Hui et al., 1994) was cut with *Hin*dIII and transcribed with T7 RNA polymerase. Mouse *Shh* clone (Echelard et al., 1993) was cut with *Hin*dIII and transcribed with T3 RNA polymerase. A mouse *HNF-3β* clone (Sasaki and Hogan, 1993) was cut with *Hin*dIII and transcribed with T7 RNA polymerase.

Antibodies

Primary antibodies were used in PBS with 0.1% Triton and 10% heat-inactivated goat serum. Antibodies against human Gli1 were raised in rabbits immunized with a bacterially derived fusion protein containing the zinc finger DNA-binding domain (aa 255-408). Specific antibodies were purified by affinity chromatography through an affigel (Bio Rad)-Gli fusion protein column and used at 1/100. This antisera does not recognize frog Gli proteins. Rabbit polyclonal antibodies against frog HNF-3β protein (Ruiz i Altaba et al., 1995a) were used at 1/8000. Affinity-purified rabbit polyclonal antibodies against serotonin (purchased from Dr Hadassa, Columbia University) were used at 1/1500. Rabbit polyclonal antibodies against Nkx2.1 (TTF-1, Lazzaro et al., 1991; kindly provided by Dr Di Lauro) were used at 1/5000. Mouse 9E10 anti-myc monoclonal antibody was purchased from Santa Cruz, Inc. and used at 1/500. Monoclonal antibodies against the SC35 protein (Fu and Maniatis, 1990; kindly provided by T. Maniatis) were used as hybridoma supernatant at 1/5.

Embryos were fixed in MEMFA (Patel et al., 1989) for 20 minutes at room temperature. Preincubations, secondary antibodies, washes and peroxidase reactions were as described (Ruiz i Altaba et al., 1995a). Embryos were then dehydrated, cleared, sectioned and photographed as those used for whole-mount in situ hybridization. COS-7 cells were fixed in MEMFA for 5 minutes. Fluorochrome-coupled secondary antibodies (Boehringer Mannheim; Tago) were used at 1/200. Fluorescent images were photographed with an Axiophot microscope or collected with a Princeton Instruments cooled CCD (KAF 1400 chip) camera.

RESULTS

Cloning and expression patterns of the *Gli* genes in frog embryos

We have isolated cDNAs for the three *Gli* genes from frog embryos and analysed their embryonic expression patterns as compared to those of *Shh*, *Pintallavis*/*HNF-3β* and *N-tubulin*.

Among the *Gli* genes, homology in the zinc finger region was very high (between 82% and 96% identity) although the first zinc finger showed less conservation, as only fingers 3-5 bind DNA (Pavletich and Pabo, 1993), and was used to classify the frog cDNAs (Fig. 1A) together with sequences outside of the DNA-binding domain (not shown). The deduced frog and human Gli1 proteins showed 47% identity in the N-terminal region, 88% identity in the zinc finger region and 24% identity in the C-terminal region (Fig. 1B). The frog Gli1 and human Gli3 proteins showed 22% identity in the entire N-terminal region, 93% identity in the zinc finger region and 24% identity in the C-terminal region. In contrast, partial N-terminal sequences of fGli3 (not shown) and hGli3 showed 73% identity whereas their zinc finger regions showed 92% identity. Similarly, partial N-terminal sequences of fGli2 (not shown) and mouse Gli2 showed 71% identity and their zinc finger DNA-binding domains were 94% identical. Together, this shows that the identified frog *Gli1* gene is the homolog of the human and mouse *Gli1* genes.

Expression of *Gli1* and *Gli3* was first detected at low levels in the animal cap region of early gastrula-stage (stage ~10.5) embryos, a time when *Shh* expression was undetectable and that of *Pintallavis* was evident in the organizer region (Fig. 2A-E). By stage ~11.5, *Gli1* RNA was detected in deep midline neural plate cells overlying the notochord (Fig. 2G,Q) in a similar manner to that of *Pintallavis* and *Shh* at later stages (Fig. 2P; Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1995a). At stage 11.5 *Pintallavis* and *Shh* RNAs were expressed in the underlying notochord (Fig. 2F,H). In contrast, *Gli2* expression was detected widely in the rest of the neural plate with higher levels in the future hindbrain region (Fig. 2I). Similarly, *Gli3* expression was absent from the midline and highest in the anterior and lateral edges of the neural plate (Fig. 2J). *Gli3* was also expressed in the posterior-ventral mesoderm (Fig. 2J and not shown).

By midgastrula stages (stage ~12.5), expression of *Gli2* and *Gli3* was similar but expression of *Gli1* was no longer detected in midline cells but rather in cells immediately adjacent to the midline (Fig. 2V). The position of *Gli1*$^+$ cells adjacent to the midline appeared to be transiently coincident with the earliest differentiating *N-tubulin*$^+$ primary medial neurons (Fig. 2W,X; Chitnis et al., 1995). The lateral-to-midline expression of *Gli1* and its previous midline expression were dependent on the persistent presence of an underlying notochord as *Gli1* expression was not detected in the neural ectoderm of complete exogastrula (from stages ~11.5-13; Fig. 2U).

At midneurula stages (stage ~14), *Shh* and *Pintallavis* were expressed in midline neural plate cells, which will form the floor plate of the neural tube (Fig. 2K,M). *Gli* gene expression was maintained (Fig. 2L,N,O) with that of *Gli1* highest in cells lateral to the midline (Fig. 2L,R) and that of *Gli2* homogeneously in the neural plate outside of the midline (Fig. 2S), although the posterior neural plate showed lower levels of *Gli2* expression (Fig. 2N). *Gli1*$^+$ cells flanking the midline appeared to overlap with the medial boundary of *Gli2* expression (Fig. 2L,N). *Gli3* showed highest expression in the anterior neural ridge region (Fig. 2O). More posteriorly, there was a graded expression of *Gli3* from the lateral edges to the midline (Fig. 2O,T). Expression of the *Gli* genes in somitic mesoderm occurred in a manner similar to that in the neural plate: *Gli1* closest to the notochord, *Gli2* throughout and *Gli3* in a graded manner with highest levels distal from the midline (Fig. 2R-T and not shown).

In tadpoles (stages ~32-36), the *Gli* genes showed partly overlapping patterns of expression (Fig. 2Y-Za). In the brain, expression of *Gli1* was detected prominently in a diencephalic stripe similar to that of *Shh* (Figs 2Y, 6C). *Gli2* was prominently expressed in the telencephalon, dorsal and ventral anterior diencephalon and in cells close to the dorsal midbrain/hindbrain junction and dorsal hindbrain (Fig. 2Z). High *Gli3* expression was found throughout the dorsal aspect

of the neural tube especially at the diencephalic/midbrain and midbrain/hindbrain junctions (Fig. 2Za).

Along the D-V axis of the posterior neural tube, expression of *Gli1* was confined to the ventricular zone and there was no expression in the floor plate (Fig. 2Zd), where HNF-3β and *Shh* are detected (Fig. 2Zb, Zc). Expression of *Gli2* and *Gli3* was found in the dorsal ventricular zone (Fig. 2Ze, Zf) with low levels in the roof plate. *Gli* gene expression was reciprocal to that of *N-tubulin* in differentiated neurons distal from the ventricle (Fig. 2Zg).

Expression patterns of the *Gli* genes in the early mouse neural plate and neural tube

Expression of three *Gli* genes has been previously examined in mouse embryos (Walterhouse et al., 1993; Hui et al., 1994). However, these studies did not resolve their early neural expression.

In the posterior neural tube of E8.5-8.75 mouse embryos, the three *Gli* genes were found to be expressed throughout the neural plate (Fig. 3A-C) except that *Gli3* expression was very low or absent at the ventral midline. At this time, posterior midline neural plate cells are beginning to express *HNF-3β* (Fig. 3D) and *Shh* expression is still confined to the notochord (Fig. 3E). In more anterior (and thus older) regions of the neural plate, the expression of all three *Gli* genes was maintained throughout the neural plate with the exception of the floor plate (Fig. 3F-H).

In anterior regions of the closed neural tube of E9.5 embryos, *Gli* gene expression was further restricted (Fig. 3I-K). *Gli1* transcripts were confined to ventricular cells adjacent to the floor plate (Fig. 3I) whereas *Gli2* and *Gli3* mRNAs were detected in the dorsal ventricular zone and immediately adjacent cells (Fig. 3J,K). At this time, the expression of both *HNF-3β* and *Shh* is detected in the floor plate and *Shh* transcripts are also present in the notochord (Fig. 3L,M). In the spinal cord of E11.5 embryos, *Gli1* expression was confined to two patches in the ventral ventricular zone (Fig. 3N) and that of *Shh* to the floor plate and at much lower levels to the motor neuron pools (Fig. 3O). Thus, in both frog and mouse embryos, *Gli* gene expression is preferentially detected in precursor cells that are first found throughout the neural plate and later in the ventricular zone. Moreover, expression in midline cells ceases when these cells begin to express floor plate markers such as HNF-3β. However, all three *Gli* genes are coexpressed in the early mouse neural plate with clear coexpression of *Gli1* and *Gli2* at the ventral midline whereas only *Gli1* is expressed in the prospective floor plate in frog embryos.

A

Zinc Finger Region

I **II**

```
          TNCHW..C.. EFDTQEQLVH HINN.HIHGE KKE---FVCRW. DCSREQKPFK AQYMLVVHMR RHTGEKP
hGli 1    .D.R.DG.SQ ...S..... ...SE..... R..---...H.G G....LR... ......... .......
mGli 1    .D.R.DG.SQ ...S..... ...SE..... R..---...H.G G....LR... ......... .......
fGli 1    .....ES.TK ......H... ....E..... ...---...H.Q .....LR... G......... .......
hTHP      .....ED.TK .Y........ ....E..... ...---.....Q A.T..... ......... ...R...
mGli 2    ...R.AD.TK .Y........ ....E..... ...---.....Q A.T..... ......... .......
fGli 2    .....DG.SK .....D.... ....D..... ...---.....Q ......... ......... .......
hGli 3    .....EG.AR .......... ....D..... ...---.....L ......... ......... .......
mGli 3    ...R.ED.TR .....D.... ....D..... ...---.....L ......... ......... .......
fGli 3    .....EG.SR .......... ....D..... ...---.....L ......... ......A... .......
ci        .....RR.RI ..I..DE..K ....D..QTN ..A---.....E ..T.GE.... ......... .......
tra-1     LT.R.KS.NS S.Q.LKA..D .VQES.VQST EQ.HHAWR.E.E G.D.NET-.. .L...IV.VR .......
```

III **IV** **V**

```
          HKC TFEGC.KAYSRLENLKTHLR SHTGEKPYVC EHEGCNKAFS NASDRAKHQN RTHSNEKPYV CKIPGCTKRY TDPSSLRKHV KTVHGPDAHV
hGli 1    ... .....R.S.......... ........M. .....S.... ......... ......... ..L....... ......... .........
mGli 1    ... .....R.S.......... ........M. .Q...S.... ......... ......... ..L....... ......... .........
fGli 1    ... .....N.......... ......... ......... ......... ......... ......... ......... ......... .........
hTHP      ... .....S.......... ......... ......... ......... ......... .....I ....V..... ......... .........
mGli 2    ... .....S.......... ......... ......... ......... ......... .....I ......... ......... .........
fGli 2    ... .....F.......... ......... A........ ......... ......... .....I ..V....... ......... .........
hGli 3    ... .....T.......... ......... ......... ......... ......... ......... ......... ......... ......E...
mGli 3    ... .....T.......... ......... ......... ......... ......... ......... ......... ......... ......E...
fGli 3    ... .....S.......... ......... ......... ......... ......... ......... ......... ......... .........
ci        ... .....F.......... ........T. .YP..S.... ......... ......... .....I ..A....... ......... .........
tra-1     N.. EYP..G.E.........R. T.......K. .FAD.E.... ......... .....L...S .Q..Q...S. .........I .A...D.EYE
```

Fig. 1. Comparison of deduced amino acid sequences of Gli1 proteins. (A) Comparison of the amino acid sequences of the zinc finger DNA-binding domains of Gli family members. The five zinc fingers are denoted by roman numerals and the consensus sequence is shown on top. Dots refer to conserved residues. Spaces introduced to maximize alignment are denoted by dashes. The cysteines and histidines of the finger structure predicted to establish contacts with the zinc ion are shown in bold. Note the high degree of conservation in the last three zinc fingers, which are the only ones to contact DNA as determined in a protein-DNA co-crystal structure (Pavletich and Pabo, 1993). The frog fGli1, fGli2 and fGli3 sequences derive from this work. The human hGli1 and hGli3 sequences derive from Kinzler et al. (1988) and Ruppert et al. (1990). The human tax-helper-protein (THP) derives from Tanimura et al.(1993). The mouse mGli1, mGli2 and mGli3 sequences derive from Hui et al. (1994). The fly cubitus interruptus (ci) sequence derives from Orenic et al. (1990). The nematode tra-1 sequence derives from Zarkower and Hodgkin (1992). (B) The deduced amino acid sequences of the full-length frog and human Gli1 proteins are shown aligned to highlight conserved regions. The five zinc finger DNA-binding domain is shown boxed and other conserved regions underlined. The human Gli1 (hGli1) sequence is from Kinzler et al. (1988). The frog Gli1 (fGli1) sequence has been submitted to GenBank under accession number U57454.

Gli1 induces ectopic HNF-3β expression

Given the distinct expression of the *Gli1* gene in midline neural plate cells at the time of floor plate induction in frog embryos, we tested whether gain of function of *Gli1* could mimic gain of function of *Shh*. We assayed for ventral neural tube differentiation by injecting plasmids driving the expression of cDNAs into the animal pole of one cell in 2-cell frog embryos, which results in the unilateral mosaic expression of injected genes in the ectoderm including the neural tube. Injected embryos at the tadpole stage were then labeled with specific antibodies to HNF-3β, a ventral marker (Fig. 6A) that is ectopically expressed after Shh injection (Ruiz i Altaba et al., 1995a).

Embryos injected with plasmids driving the expression of the full-length frog Gli1 open reading frame displayed ectopic HNF-3β expression within the neural tube as well as in the epidermal ectoderm (Table 1; Figs 4, 6D and not shown). To test for protein production, this clone contained myc tags fused in frame to the N terminus of Gli1. Myc-tagged Gli1 protein (myc-fGli1) was localized primarily in the cytoplasm of transfected COS cells with low nuclear levels although injected frog blastomeres showed both cytoplasmic and nuclear labeling (Fig. 5A,B).

To test whether the myc tags were altering the activity of the Gli1 protein, a deletion clone was made leaving only the intact Gli1 open reading frame. Tadpoles injected with this construct also showed ectopic expression of HNF-3β (Table 1; Fig. 4) demonstrating the ability of the normal endogenous Gli1 protein to induce HNF-3β expression. As controls, injection of a scrambled fGli1 cDNA (fGli1-inv) and uninjected controls did not show ectopic HNF-3β (Fig. 6A; Table 1). Because cells at the ventral midline of the neural tube do not inherit plasmids injected into the animal-most region of 2-cell embryos (Dale and Slack, 1987; Ruiz i Altaba et al., 1995a), aberrant migration or proliferation of endogenous floor plate cells cannot underlie the appearance of HNF-3β$^+$ cells at ectopic locations.

Gli1 functions in the nucleus and requires N-terminal sequences that can be replaced by the transactivating domain of VP16

The analysis of the subcellular localization of injected and transfected Gli1 protein showed it was localized to both the nucleus and cytoplasm (Fig. 5A,B). We tested for the requirement of cytoplasmic Gli1 for HNF-3β - inducing activity by targeting Gli1 to the nucleus. To this end, we created a myc-tagged fGli1 protein carrying an SV40 nuclear localization signal in its N terminus. Injection into frog embryos and transfection of this construct into COS cells showed that the NLS-myc-fGli1 protein was nuclear (Fig. 5C,D). Injected tadpoles showed a very high incidence of ectopic HNF-3β expression (Table 1; Fig. 4). The ability of nuclear Gli1 to mimic the effects of Gli1 suggests that it functions in the nucleus

B

```
fGli1     MASRQCPPAAVFNSMNP-PVNSYVEHCYLR------SPNVMAEGMNEMPYCHQTNLMTSH 53
hGli1               MFNSMTPPPISSYGEPCCLRPLPSQGAPSVGTEGLSGPPFCHQANLMSGP 50

fGli1     HGFGLAQGSDHLAGTDGSRFSTPRSTMKLSKKRAMSISPLSDASIDLQTMIRTSPNSLVA 113
hGli1     HSYGPARETN--SCTEGPLFSSPRSAVKLTKKRALSISPLSDASLDLQTVIRTSPSSLVA 108

fGli1     FINSRCSSASGSYGHLSIGTISPSLGYQNCLNHQRPQAGPYGSNPLMPYNSHEHLSSRGM 173
hGli1     FINSRCTSPGGSYGHLSIGTMSPSLGFPAQMNHQKGPSPSFGVQPCGP-----HDSARGG 163

fGli1     SMLQP--RSSVKHCQLKSEPLSITGLDTIGSKRLEDGSEGDISSPASVGTQDPLLGLLDG 231
hGli1     MIPHPQSRGPFPTCQLKSE------LDMLVGKCREEPLEGDMSSPNSTGIQDPLLGMLDG 217

fGli1     RDDLEKDDGKHEPETVYETNCHWESCTKEFDTQEHLVHHINNEHIHGEKKEFVCHWQDCS 291
hGli1     REDLEREE-KREPESVYETDCRWDGCSQEFDSQEQLVHHINSEHIHGERKEFVCHWGGCS 276

fGli1     RELRPFKAQYMLVVHMRRHTGEKPHKCTFEGCNKAYSRLENLKTHLRSHTGEKPYVCEHE 351
hGli1     RELRPFKAQYMLVVHMRRHTGEKPHKCTFEGCRKSYSRLENLKTHLRSHTGEKPYMCEHE 336

fGli1     GCNKAFSNASDRAKHQNRTHSNEKPYVCKIPGCTKRYTDPSSLRKHVKTVHGPEAHITKK 411
hGli1     GCSKAFSNASDRAKHQNRTHSNEKPYVCKLPGCTKRYTDPSSLRKHVKTVHGPDAHVTKR 396

fGli1     HRGDGMLRAQPGHEGPGNQNVKGENQLDMEASSACKEDGRLAVPDITLKSQPSPGGQSSC 471
hGli1     HRGDG-----PLPRAPSISTVEPKRERE---GGPIREESRLTVPEGAMKPQPSPGAQSSC 448

fGli1     SSERSPLGSTNNNDSGVEMNANTGGSFEDLTNLDDIPSVDSMGTAGASALRKLENLRIDK 531
hGli1     SSDHSPAGSAANTDSGVEMTGNAGGSTEDLSSLDEGPCI---AGTGLSTLRRLENLRLDQ 505

fGli1     LNQLRKTPSSGKMVKLPSIHNSGPQGDMSVVCGPLGMSHNQHGIELPASSHVNHLNDRRN 591
hGli1     LHQLR—PIGTRGLKLPSLSHTGTTVSRRV--GP------------PVS------LERRS 543

fGli1     STTSTMSSAYTVSRRSSVVSPYLPNQRAGDSGNMVDSYDISTDPSGHSNEAVCASGLPGL 651
hGli1     SSSSSISSAYTVSRRSSLASPFPPGSPPENG----------------------ASSLPGL 581

fGli1     TPAQQYRLKAKYAAAT-GGPPPTPLPNMERMNTNNRMAFASSDYRGSAISSLQRRHSSNE 710
hGli1     MPAQHYLLRARYASARGGGTSPTAASSLDRIG-----GLPMPPWRSRA------------ 624

fGli1     YHNYGTGIIHPAQAPGAGI-RRASDPARTGGDIQAVPKVQRFKSMTNMNVSMMGRQGTSI 769
hGli1     --------EYPGYNPNAGVTRRASDPAQ-AADRPAPARVQRFKS--------LGCVHTPP 667

fGli1     QQAYGGS--DANLQRHMFSPRPPSITENVFMETAGPDEVCHTKEQGFIQSNEMQHYMNYQ 827
hGli1     TVAGGGQNFDPYLPTSVYSPQPPSITENAAMDARGLQE------------EPEVGTSMV 714

fGli1     GQGSQLTAPNDHMNFNPQIHGLDGQSQNVYSHSQRAISNMHLNAENYSGQSNVSNFNQCQ 887
hGli1     GSG-----LNPYMDFPPTDTLGYGGPEGAAAEPYGARGPGSLPL----GPGPPTNYGPNP 765

fGli1     MTAHNQHFQNTRQAYNCANLPVQWNEVSSGTMDNPVQRPNHQIMHQNMSPGNHCQSQLSN 947
hGli1     CPQQASYPDPTQET---------WGEFPSHSGLYPGPKALGGTYSQCPRLEHYGQVQV-- 814

fGli1     CTVPESTKQGCPVNRNSCQQGMYMNNQKYNHGGQVQVKPEQQFHHSAPAMMSCQNMKHPS 1007
hGli1     -----KPEQGCPVGSDSTGLAPCLN----AHPSEGPPHPQPLFSH----------YPQPS 855

fGli1     RQEHHFTKTNTMP----LSSEATNC-DYQGQQDSTQNSCFNVGLNLNLLSPPGRRSQTPI 1062
hGli1     PPQYLQSGPYTQPPPDYLPSEPRPCLDFDSPTHST------------------------ 890

fGli1     MQVKEIMVRNYVQSQQALMWEQHPKSMAMMTNSGDDVDTRQNQHKNTLNAAVYMGPKYMN 1122
hGli1     GQLKAQLVCNYVQSQQELLWEGGGREDA-------------PAQEPSYQSPKFLGGSQVS 937

fGli1     YQGKPSPNNLMSPSSQDSQSSHTKAMGSPSSQCYNFDMMPHPPCGPKPLSRQHSVSSQST 1182
hGli1     PSRAKAPVNTYGPGFGPNLPNHKSGSYPTPSPCHENFVV--------------GANRASH 983

fGli1     YMGSPNQLSPSYQSSESSPRRMACLPPIQPQSEVTNNTSMMYYTGQMEMHQSKPGVHKLT 1242
hGli1     RAAAPPRLLPPLPT---------CYGPLK------------------------------ 1003

fGli1     TPLNLNQTSCDGHQHGQYNASHSFLKTVPYTSSCPAANTLDSLDLENTQIDFTAIIDDAD 1302
hGli1     --VGGTNPSC-GHPEVGRLGGGPALYPPPEGQVC---NPLDSLDLDNTQLDFVAILDE-- 1055

fGli1     NALMPGNISPNVLAGSSQASSHLTTLRNTGAVVPNMVVGDLNSMLSSLAGENKYLNTMS  1361
hGli1     ----PQGLSPPPSHDQRGSSGHTPPPSGP----PNMAVGNMSVLLRSLPGETEFLNSSA  1106
```

even though stable protein is found predominantly in the cytoplasm.

To test for a possible role of Gli1 as a transcriptional regulator, we first identified sequences outside the DNA-binding domain responsible for function. An N-terminal truncated myc-tagged protein (myc-fGli1N′Δ) was stable (not shown) but was unable to induce ectopic HNF-3β expression (Table 1; Fig. 4). This deletion was then used to make a chimera in which the strong transactivating domain of VP16 (Campbell et al., 1984; Triezenberg et al., 1988) was fused in frame to fGli1N′Δ. Injection of this chimeric protein (VP16-fGli1) also resulted in the ectopic expression of HNF-3β (Table 1; Fig. 4). The ability of VP16-fGli1 to mimic myc-fGli1 strongly suggests that Gli1 normally acts as a positive transcriptional transactivator.

Widespread expression of human Gli1 but not Gli3 or fly cubitus interruptus induces ectopic HNF-3β expression

To extend our findings with fGli1, we tested the activities of other members of the *Gli* gene family in our in vivo assay. Injection of the human Gli1, but not Gli3, myc-Gli3 or ci, cDNAs led to the ectopic expression of HNF-3β (Figs 4, 6B,E; Table 1). Ectopic HNF-3β expression in hGli1-injected embryos was detected in the neural tube as well as in the non-neural ectoderm (Fig. 6H,I).

Because the human cDNAs were obtained from glioma cells (Kinzler et al., 1987), the different activities of the *Gli1* and *Gli3* cDNAs could be due to differential expression from the injected plasmids as amplification has been proposed to be the mechanism of action of Gli1 in oncogenesis (Kinzler et al., 1987). Indeed, the induction of ectopic HNF-3β expression by Gli1 proteins was concentration dependent (Table 1 and not shown). However, quantitative RT-PCR revealed a similar expression on average of all tested genes from injected plasmids (not shown), indicating that their differential action is not due to differential expression.

To ascertain that the inactivity of Gli3 was not due to absence of protein, we analysed the expression of hGli1 and hGli3 proteins from the injected cDNAs. The human *Gli1* and *Gli3* cDNAs (Kinzler et al., 1987; Ruppert et al., 1990) directed the expression of stable protein as determined by labeling with specific antibodies against hGli1 and the production of myc-tagged Gli3 protein (Fig. 5 and not shown). hGli1 accumulated preferentially in the nucleus (Fig. 5E,F), like HNF-3β (Fig. 5I), although some cells showed also cytoplasmic labeling (Fig. 5F,K). Myc-tagged hGli3 protein was primarily cytoplasmic with a low level of nuclear labeling (Fig. 5G,H,K). The local-

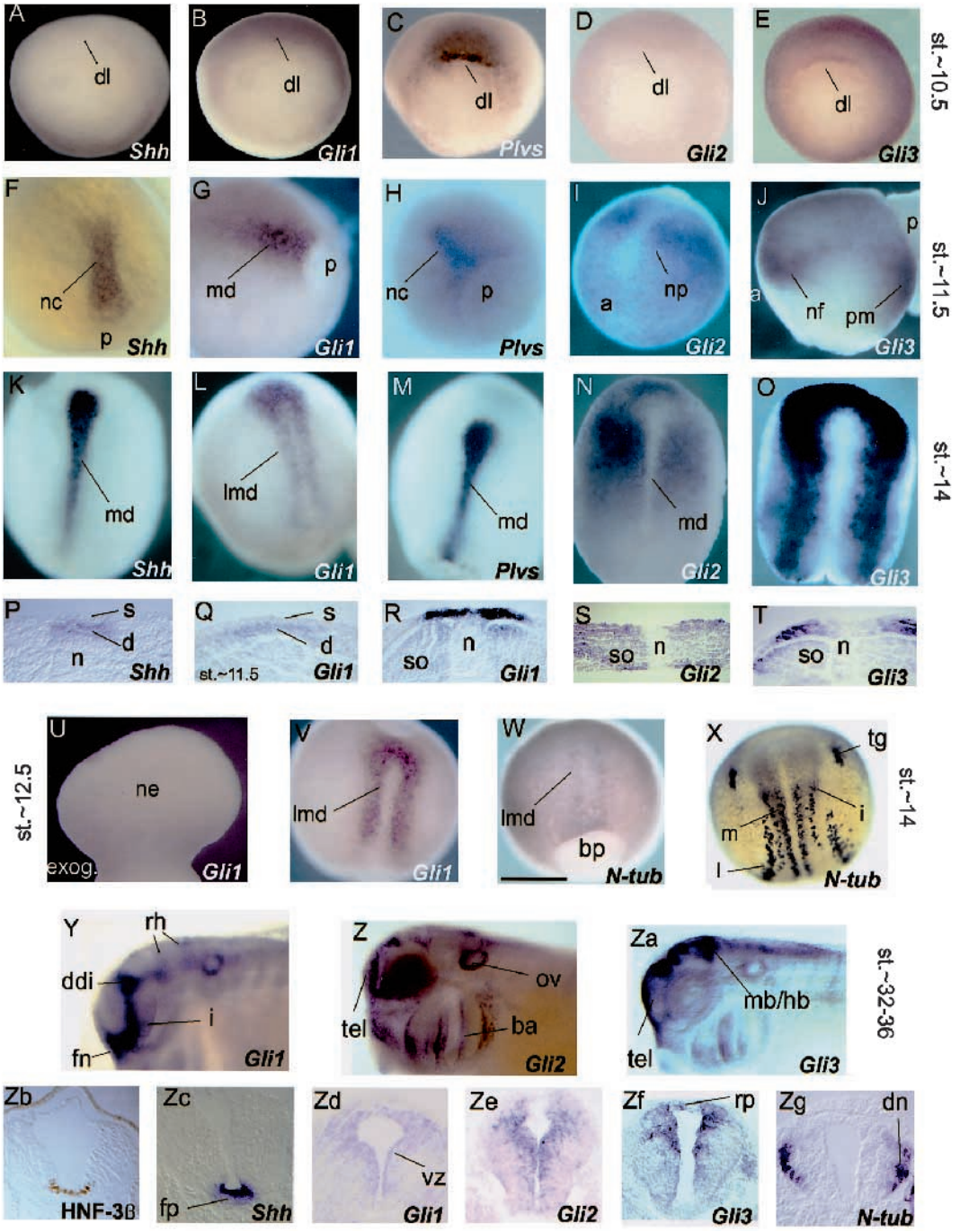

Fig. 2. *Gli* gene expression in frog embryos; expression of *Sonic hedgehog* (*Shh*), *Gli1*, *Pintallavis* (*Plvs*), *Gli2*, *Gli3*, *neural-tubulin* (*N-tub*) and HNF-3β at different stages as indicated on the side. (A-E) At early gastrula stages (~stage 10.5) *Shh* RNA (A) is not yet detected and that of *Pintallavis* is clearly seen in the organizer region (C). Expression of *Gli1* (B) and *Gli3* (E) but not *Gli2* (D) is detected in the animal cap, mostly on the dorsal side. All panels show a vegetal view with dorsal side up. (F-J) At midgastrula stages (stage ~11.5), expression of *Shh* (F) and *Pintallavis* (H) is detected in the nascent notochord (nc) as seen in cleared embryos. Non-cleared embryos show the expression of *Gli1* in midline (md) neural plate cells (G), the expression of *Gli2* in non-midline neural plate (np) cells (I) and that of *Gli3* (J) in the neural plate folds (nf) and posterior mesoderm (pm). All panels show dorsal views from different angles and the direction of the A-P axis is indicated (a, anterior; p, posterior). (K-O) At neurula stages (stage ~14), expression of *Shh* and *Pintallavis* is detected in midline (md) neural plate cells (K, M) whereas that of *Gli1* is found in cells lateral to the midline (lmd; L). *Gli2* (N) is found in neural plate cells with the exception of the midline (md) and anterior area overlying the prechordal plate whereas *Gli3* (O) is detected in a graded fashion with highest levels laterally and absent from the midline. All panels show dorsal views with anterior end up. (P-T) Cross sections show expression of *Shh* (P) in deep (d) and not superficial (s) midline neural plate cells overlying the notochord (n) at stage ~14. *Gli1* expression is also detected at stage ~11.5 in deep midline cells (Q) but, by stage 14, expression is predominantly found in cells lateral to the midline (an anterior section is shown here, R; compare to panel V). Expression in the somites (so) is found also adjacent to the notochord (n). *Gli2* (S) is detected throughout the neural plate and somites but not in midline neural plate cells or notochord and *Gli3* (T) is detected in a graded fashion with highest levels laterally both within the neural plate and somites. (U-X) *Gli1* expression at stage 12.5 is normally found in cells lateral to the midline (lmd; V) but it is not detected in the neural ectoderm (ne) of complete exogastrulae (exog; U) at this time or at earlier stages (not shown). This lateral to the midline expression coincides with the position of the first *N-tubulin* expressing cells (W), most likely primary motor neurons, which precede the appearance of the well-characterized three stripes of primary neurons in the midneurula embryo located in a medial (m), intermediate (I) and lateral (l) position (stage ~14, X). Expression is also detected in trigeminal ganglion neurons (tg). All panels show dorsal views with anterior end up. bp: blastopore. (Y-Za) Expression of *Gli1* (Y), *Gli2* (Z) and *Gli3* (Za) in the anterior area of a tadpole (stages ~32-36) embryo. Note the similarity in the expression of *Gli1* with that of *Shh* (Fig. 6C). *Gli1* is expressed in the lateral and dorsal diencephalon, medial hindbrain, dorsal rhombomeres 1 and 3, otic vesicle and eye. It is also expressed in the frontonasal (fn) process and branchial arches (not shown) but not in the infundibulum (i). *Gli2* (Z) and *Gli3* (Za) are mostly coexpressed in the dorsal neural tube including the telencephalon (tel). High Gli3 expression is detected at the midbrain/hindbrain boundary (mb/hb) and diencephalon/midbrain boundary. Coexpression of the three *Gli* genes is detected in the eye, otic vesicle, frontonasal process and branchial arches. (Zb-Zg) Cross sections of the hindbrain-cervical spinal cord of tadpoles (stages ~34-36). (Zb,Zc) The expression of HNF-3β protein and *Shh* mRNA is detected in floor plate (fp) cells. Note the basal position of nuclei expressing HNF-3β and the ventricular position of the *Shh* mRNA-containing cytoplasm. *Gli1* (Zd) is expressed in the ventricular zone with the exception of the floor plate. *Gli2* (Ze) and *Gli3* (Zf) are coexpressed in the dorsal ventricular zone with low levels or no expression in the roof plate (rp). Differentiated neurons (dn) expressing *N-tubulin* are found distal form the ventricle (Zg). Scale bar is 0.5 mm in A-E,H-J; 0.4 mm in F, G, K-O, U-Za; 120 μm in P-T, 60 μm in Zb-Zg.

Table 1. Quantitation of the HNF-3β-inducing effects of *Gli* genes and chimeras

Injected Gene	Normal	Ectopic	n
fGli1	4	96	71
myc-fGli1	34	66	107
myc-fGli1 N'Δ	99	1	178
VP16- fGli 1	40	60	76
NLS-myc-fGli1	2	98	216
hGli1 (100)	72	28	50
hGli1	20	80	97
hGli3	94	6	113
Ci	96	4	93
fGli1-inv	99	1	160
Uninjected	95	5	184

All numbers refer to percentages except the total number (n) of injected embryos. All injections delivered 200 pg of plasmid DNA to the animal pole of 2-cell embryos unless in hGli1(100) where 100 pg were delivered. Each sample represent the results of several independent injection experiments to minimize variability due to the different genetic backgrounds of the frogs. Embryos were scored as having the normal expression of the markers only or as having normal and ectopic expression independent of the number of ectopic sites.

ization of cytoplasmic Gli proteins in COS cells was partly coincident with that of tubulin and suggestive of an association with the cytoskeleton (Fig. 5L and not shown). Within the nucleus, hGli1 was often detected in subdomains that were always distinct from those containing the splicing factor SC35 (Fig. 5J; Fu and Maniatis, 1990), unlike other zinc finger proteins having dual transcriptional and splicing functions (Larsson et al., 1995).

These results together with the identical abilities of the endogenous frog and human glioma Gli1 proteins in terms of HNF-3β induction indicate that Gli1 proteins are normally active and functionally equivalent. In the following assays, we have therefore used different Gli1 proteins interchangeably and hGli3 as a negative control.

Gli1 induces floor plate development

Ectopic expression of HNF-3β in embryos injected with Gli1 proteins suggested the ectopic differentiation of floor plate cells. To further test this, we assayed injected embryos for the expression of *Shh* mRNA as a second floor plate marker of the posterior neural tube (Fig. 6C). Widespread expression of Gli1 proteins, but not hGli3, resulted in the ectopic transcription of *Shh* within the tadpole neural tube (Fig. 6F,G and not shown; Table 2). Similarly, the floor-plate-specific marker *F-spondin* (Fig. 6J, Ruiz i Altaba et al., 1993b) was induced within the tadpole neural tube by injection of Gli1 but not Gli3 proteins (Fig. 6K,L and not shown; Table 2). These results demonstrate that widespread expression of Gli1 leads to the ectopic differentiation of floor plate cells.

To determine the requirement of the presence of an endogenous floor plate for the ectopic differentiation of floor plate cells by Gli1, we injected hGli1 into embryos that were then induced to develop as complete exogastrulae, which lack an endogenous floor plate (Ruiz i Altaba, 1992, 1994). Injection of hGli1 led to ectopic HNF-3β expression in patches within the neural ectoderm of complete exogastrulae, in a position distal from the junction with mesoderm (20%, *n*=25; not shown), indicating that floor plate differentiation by Gli1 is independent of the presence of an endogenous floor plate.

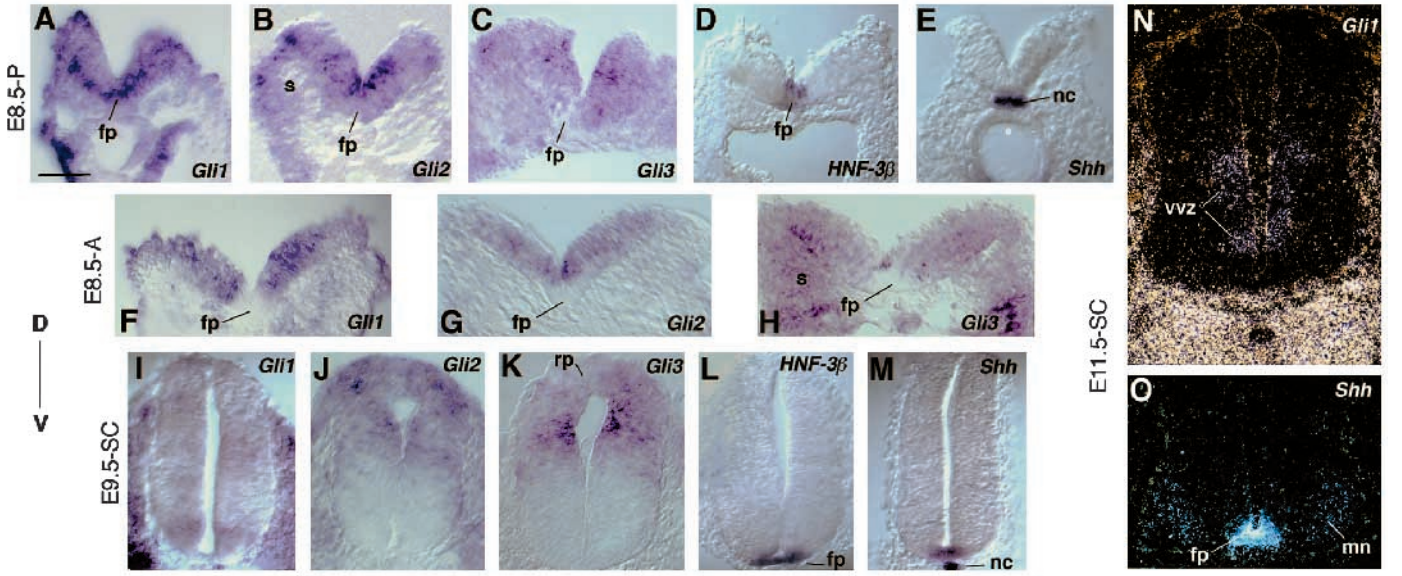

Fig. 3. Neural expression of *Gli* genes in mouse embryos. Expression of transcripts in the posterior neural plate at E8.5-8.75 (E8.5-P; A-E), in the anterior neural plate at E8.5 (E8.5-A; F-H) and in the spinal cord at E9.5 (E9.5-SC; I-M) and E11.5 (E11.5-SC; N, O). (A,F,I,N) Expression of *Gli1* mRNA. Gli1 is expressed throughout the early neural plate (A), is absent from the floor plate in slightly older stages (F) and is then found restricted to the ventral ventricular zone including cells adjacent to the floor plate (I,O). Strong *Gli1* labeling is detected in the condensing sclerotome surrounding the neural tube (O). (B,G,J) Expression of *Gli2* mRNA. *Gli2* is expressed throughout the early neural plate (B) and is absent from the floor plate in slightly older stages (G). In the closed neural tube, *Gli2* is expressed in the dorsal aspect including the ventricular zone (J). (C,H,K) Expression of *Gli3* mRNA. *Gli3* is also expressed throughout the early neural plate (C) and is absent from the floor plate in slightly older stages (H). In the closed neural tube, *Gli3* is expressed in a manner similar to that of *Gli2* (J) with highest expression in the dorsal ventricular zone (K). (D,L) Expression of *HNF-3β* mRNA. *HNF-3β* is first expressed in the neural ectoderm in midline cells of the neural plate shortly after floor plate (fp) induction by the underlying notochord, which also expresses this gene. In the early neural plate (D) midline cells express *HNF-3β* and in the closed neural tube it is found in the floor plate and in a small group of immediately adjacent cells (compare L and M). (E,M,O) Expression of *Shh* mRNA. The early neural plate does not express *Shh* but high expression is detected in the underlying notochord (nc, E). In the closed neural tube (M), *Shh* expression is maintained in the notochord (nc) and new expression is detected in the floor plate. At later stages, weak expression of *Shh* is detected in the motor neuron pools (mn) in limb regions lateral to the floor plate (fp), which maintains high expression (O). Scale bars are 30 μm in (A-H) and 50 μm in (I-O).

Floor plate cells acquire the floor-plate-inducing properties of the notochord (Placzek et al., 1993). We therefore tested for the ability of newly induced floor plate cells in hGli1-injected embryos to induce, in turn, floor plate development in adjacent cells. To distinguish between ectopic floor plate cells derived from injected blastomeres and those that may be induced by homeogenetic induction, we coinjected a lineage tracer (rhodamine-lysine-dextran, RLDx) with hGli1 plasmids. Because RLDx diffuses more rapidly than plasmid DNA (as assessed by the distribution of RLDx and plasmid-derived myc-tagged proteins in coinjection assays; not shown), all cells that inherit DNA are labeled by RLDx but not the opposite. For this analysis, we screened histological sections of injected embryos for regions displaying ectopic HNF-3β expression and a mosaic pattern of RLDx distribution. In these areas, there was

Table 2. Quantitation of the incidence of the ectopic expression of HNF-3β, *Shh*, *F-spondin*, serotonin and Nkx2.1 in *Gli* gene-injected and control embryos

Marker	Injected gene	Normal	Ectopic	n
Shh	hGli 1	78	22	120
	hGli 3	98	2	109
	Uninjected	99	1	94
F-spondin	hGli1	35	65	109
	Uninjected	96	4	53
Serotonin	hGli 1	92	8	304
	hGli 3	100	0	222
	Uninjected	100	0	254
Nkx 2.1	hGli1	64	36	47
	myc-fGli1	61	39	28
	NLS-myc-fGli1	78	22	36
	Uninjected	100	0	35

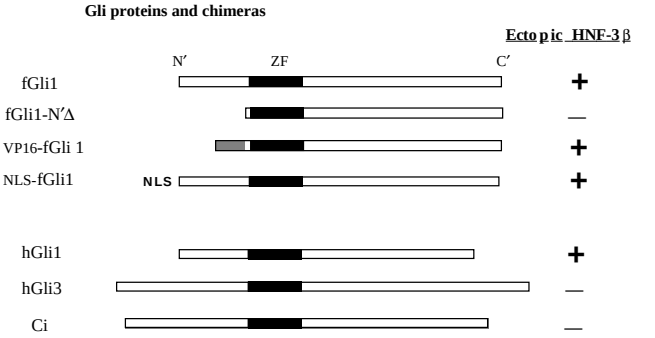

Fig. 4. Diagram showing the ability (+) or inability (–) of different Gli family members and chimeric proteins to induce ectopic HNF-3β expression in injected embryos. The maps are shown to scale centered around the conserved 5 zinc finger DNA-binding domain (black box in fGli1). See text for details.

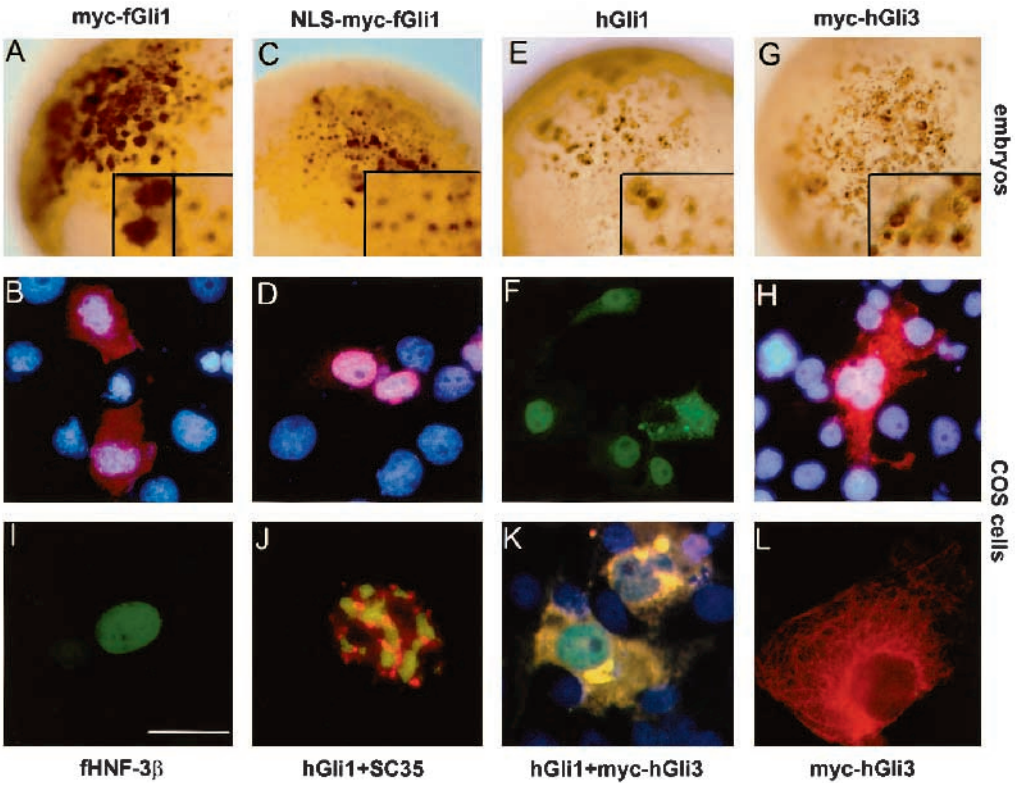

Fig. 5. Subcellular localization of Gli proteins. (A,C,E,G) Localization of Gli proteins in gastrula (stage ~12) injected frog embryos. Insets at lower right show a higher magnification of labeled cells. Labeling was visualized with the peroxidase reaction. All panels show a dorsal view. (B,D,F,H,I-L) Localization in transfected mammalian COS cells. Nuclei in B, D, H and K were counterstained with DAPI, shown in blue. Labeling was visualized by epifluorescence. A,B) Myc-tagged frog Gli1 protein localizes both to the cytoplasm (left inset) and nuclei (right inset). (C,D) A myc-tagged frog Gli1 protein bearing a nuclear localization signal (NLS) in its N terminus localizes primarily to the nucleus although some cytoplasmic labeling is evident. (E,F) Human glioma Gli1 localizes to both the nuclei and cytoplasm with cell-to-cell variability in the amount of cytoplasmic labeling (F). (G,H) Myc-tagged human Gli3 protein localizes to the cytoplasm primarily although a small number of cells showed weak nuclear labeling. (I) Frog HNF-3β protein localizes to the nuclei. (J) Human Gli1 protein (in green) is sometimes detected in nuclear subdomains distinct from those containing the splicing protein SC35 (in red). (K) Cotransfected human Gli1 and Gli3 proteins show a colocalization in the cytoplasm. Gli1 (green) is primarily nuclear whereas Gli3 (red) is mostly cytoplasmic. Part of the cytoplasm is colored yellow due to the normal presence of some Gli1 protein in this compartment (see J). A dark-blue stain depicts weak DAPI labeling to show the position of unlabelled nuclei. (L) Myc-tagged hGli3 protein is detected in a fibre-like pattern in the cytoplasm in apparent association with the cytoskeleton. Scale bar is 160 μm in A,C,E,G; 10 μm in B,D,F,H; 6 μm in I,K,L; 4 μm in J.

a similar incidence of RLDx-labeled and unlabeled HNF-3β$^+$ cells (Fig. 6M,N). Non-RLDx-labeled HNF-3β$^+$ cells were always in close proximity to RLDx-labeled HNF-3β$^+$ cells, indicating that newly induced floor plate cells are functional and can themselves induce adjacent cells towards floor plate differentiation.

Ectopic expression of HNF-3β, *Shh* and *F-spondin* were detected throughout the A-P axis with similar frequencies (Fig. 6) taking into consideration the bias towards anterior expression given the site of injection into the animal pole according to the fate map (Dale and Slack, 1987). Expression of Gli1, like Shh (Ruiz i Altaba et al., 1995a), leads to the differentiation of floor plate cells in the forebrain, a region that lacks a normal floor plate suggesting that neural A-P fates are not irreversibly fixed. This alteration of A-P values was striking as D-V identities are normally constrained by the A-P position of precursor cells (Jacobson, 1964; Yamada et al., 1991; Ruiz i Altaba, 1994; Simon et al., 1995, Ericson et al., 1995).

Along the D-V axis, the distribution of ectopic HNF-3β expression sites showed also that there are no restrictions to floor plate differentiation driven by Gli1 proteins (Fig. 6M-P). However, the roof plate showed a high incidence of ectopic sites (Fig. 6N) and similar results were obtained using *Shh* mRNA as a marker in the hindbrain (not shown). These results show that all cells can acquire a floor plate phenotype but that roof plate cells are the most competent to do so in response to Gli1 function.

Gli1 induces ectopic ventral neuronal differentiation

If Gli1 can mimic the actions of Shh, injected embryos should display the ectopic differentiation of ventral neuronal cell types. In the ventral midbrain, HNF-3β is expressed in the floor plate and in cells located away from the ventricular zone showing a rounded nucleus which suggests that they are differentiated neurons (asterisks in Fig. 6P). Embryos injected with hGli1 displayed ectopic cells in the midbrain resembling such putative neurons.

To directly test whether widespread expression of *Gli1* could induce the ectopic differentiation of a defined cell type, we assayed for the expression of the neurotransmitter serotonin (5HT) in injected embryos as a marker of raphespinal neurons. In tadpole (stage ~36) embryos, 5HT$^+$ cells are normally found clustered in the ventral region of rhombomere 1 adjacent to the floor plate (Fig. 7A; van Mier et al., 1986; Ruiz i Altaba and Jessell, 1991). Embryos injected with hGli1 displayed the ectopic differentiation of 5HT$^+$ neurons (Fig. 7B-E; Table 2). These neurons were frequently located near the dorsal midline and displayed axons with aberrant pathways. Ectopic differentiation of 5HT$^+$ neurons outside of the anterior hindbrain was observed in the dorsal diencephalon in only two embryos (not

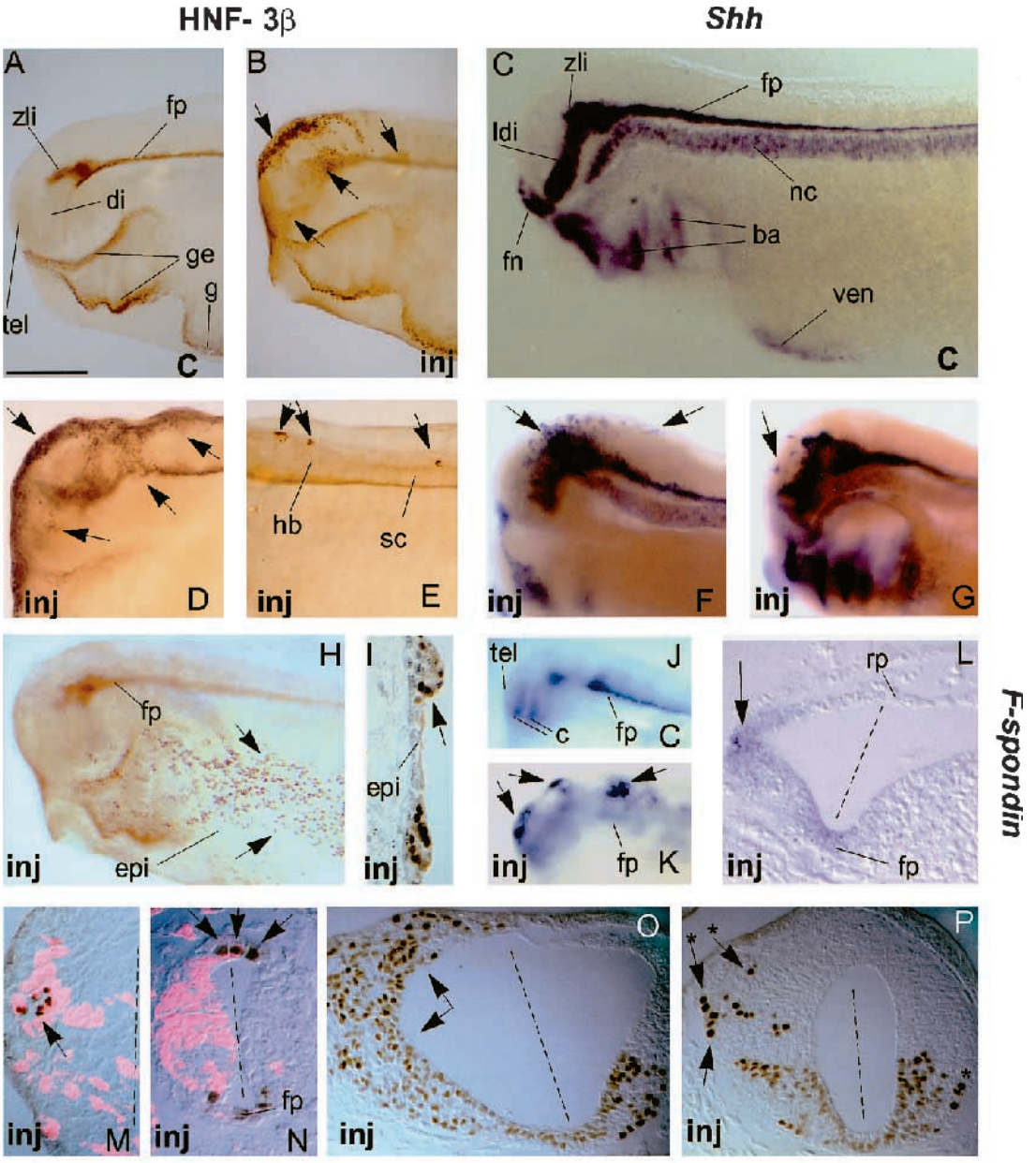

Fig. 6. Ectopic differentiation of floor plate cells driven by injected Gli1. Expression of HNF-3β (A,B,D,E), *Shh* (C,F,G) and *F-spondin* (J,K) in normal controls (c; A,C,J) and hGli1-injected (inj) tadpole (stage ~36) embryos (B,D-G, K) viewed in whole mount. Arrows point to sites of ectopic expression. All panels show side views for whole mounts with anterior end to the left and dorsal side up for cross sections. (A) Normal distribution of HNF-3β protein in the floor plate (fp) as well as in cells, possibly neurons, in the ventral midbrain and near the zona limitans intrathalamica (zli) in the diencephalon (di). HNF-3β protein can also be seen in the forming gut tube (g) and in the endoderm of the gill area (ge). Expression is completely absent from the telencephalon (tel). (B,D,E) Expression of HNF-3β protein after injection of hGli1 (B,E) or myc-fGli1 (D). Note the ectopic expression in the neural tube in the dorsal diencephalon and midbrain as well as in more medial areas. Cells expressing ectopic HNF-3β are detected throughout the brain (B,D), including the diencephalon and telencephalon (B,D), hindbrain (hb) and spinal cord (sc; D,E). (C) Normal distribution of *Shh* mRNA in tadpole embryos. Within the neural tube, *Shh* mRNA is found in a similar pattern to that of HNF-3β including expression in the floor plate (fp), the zone limitans intrathalamica (zli) and in a stripe in the lateral diencephalon (ldi). There is no expression in dorsal areas or in the telencephalon. *Shh* mRNA is also found in the notochord (nc), frontonasal process (fn), branchial arches (ba) and in a small region of the ventral anterior endoderm (ven). See also Ruiz i Altaba et al.(1995a). (F,G) Ectopic expression of *Shh* mRNA in hGli1-injected tadpoles. Ectopic expression is detected in the brain in the telencephalon (G), dorsal diencephalon (F,G), midbrain and hindbrain (F,G). (H,I) Ectopic expression of HNF-3β in the epidermis (epi) of hGli1-injected embryos. The expression in the floor plate (fp) is seen out of focus (H). (I) A cross section showing HNF-3β $^{+}$ cells in the epidermis. (J) Normal expression of *F-spondin* mRNA in the floor plate (fp). Expression is also detected in a small group of cells near the zona limitans intrathalamica and in the forebrain commissures (c). tel, telencephalon. (K,L) Ectopic expression of *F-spondin* in hGli-injected embryos seen in whole-mount (K) or cross section (L). Ectopic expression is detected throughout the dorsal neural tube. Endogenous expression in the floor plate (fp) in K is out of focus. (M-P) Expression of ectopic HNF-3β in cross sections of embryos injected with hGli1 plus RLDx (M,N) or alone (O,P). Since embryos were injected into one cell at the 2-cell stage, the ectopic expression of HNF-3β is usually unilateral. The injected side is marked (inj). (M,N) Localization of ectopic HNF-3β expression in the telencephalon (M) and spinal cord (N) with expressing cells colabeled with coinjected RLDx (in pink; M, N) and not colabeled (right most dorsal cell in N). Of all counted cells (n=27), 48% of HNF-3β^{+} cells were labeled with RLDx and 52% were not. (O,P) Ectopic expression of HNF-3β throughout the D-V extent of the neural tube (midbrain) in the injected half (arrows in O). In addition to cells showing the nuclear size and intensity of labeling of those in the floor plate, there are others with large nuclei normally located in a position suggestive of their neuronal nature (asterisks). These cells are also found at ectopic locations in the injected side (asterisk in P). Along the A-P axis, sites of ectopic expression of HNF-3β, *Shh* and *F-spondin* in the telencephalon constituted 13%, 25% and 18%, in the dorsal diencephalon 13%, 25% and 17%, in the ventral diencephalon 6%, 3% and not determined, in the dorsal midbrain 10%, 17% and 23%, in the dorsal hindbrain 21%, 17% and 32% and in the spinal cord 37%, 13% and 10% of all sites, respectively (with n=106, 40 and 147, respectively). Along the D-V axis, the roof plate displayed a disproportionate incidence of ectopic HNF-3β sites (31% of sites, n=246) given its small size (<1/10th the size of the basal and alar plates), as compared to the basal (25% of sites) or alar (19% of sites) plates. The ventricular zone showed a small number of sites (15%). Scale bar is 1 mm in (A-H,J,K) and 25 μm for (I,L-O).

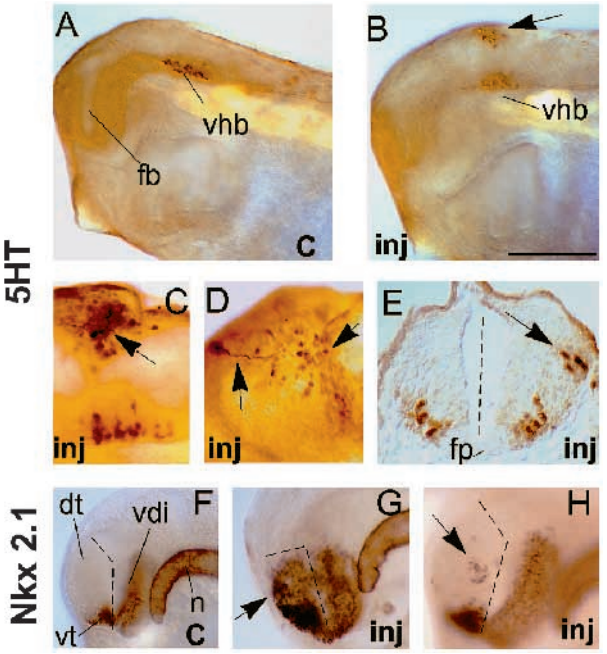

Fig. 7. Ventral neuronal differentiation induced by widespread expression of Gli1. Expression of serotonin (5HT; A-E) and Nkx2.1 (F-H) in control and Gli-1injected embryos. Arrows depict ectopic expression. Panels show lateral (A-D; F-H) views for whole mounts with anterior end to the left; in E dorsal side is up. (A) Normal expression of $5HT^+$ neurons in the ventral anterior hindbrain of stage ~36 tadpoles. Immunoreactivity is detected in the soma and axons growing both cranially and caudally. Control embryos: 19 neurons unilaterally ±0.6 s.e.m.; *n*=26. (B) Normal and ectopic differentiation of $5HT^+$ neurons (arrow) in the hindbrain of an injected tadpole. Note the dorsal position of the ectopic cell bodies. Injected embryos: 8% ectopic sites, *n*=302 with 19 neurons unilaterally ±2.4 s.e.m.; *n*=13. The relatively low percentage of embryos displaying ectopic $5HT^+$ neurons is expected and significant. The incidence of ectopic *Shh* or HNF-3β in the hindbrain was 17% and 21%, respectively. Since the expected incidence of ectopic *Shh* and HNF-3β expression in the anterior hindbrain, where $5HT^+$ neurons differentiate, is less than half of that detected in the entire hindbrain or less than 9% and 11%, these values are very close to the observed 8% incidence of ectopic $5HT^+$ neuronal differentiation. (C,D) Details of the ectopic differentiation of $5HT^+$ neurons in the hindbrain of injected embryos. Note the aberrant pathway of labeled axons (arrows). (E) Cross section through the anterior hindbrain of an injected embryo showing the unilateral ectopic differentiation of $5HT^+$ neurons in the alar plate (arrow) and the bilateral presence of endogenous $5HT^+$ neurons adjacent to the floor plate (fp). (F-H) Expression of Nkx2.1 protein in the forebrain of normal control (F) and embryos injected with NLS-myc-fGli1 (G) or hGli1 (H). Note the normal expression in ventral diencephalic (vdi) and ventral telencephalic (vt) cells. The boundary of the telencephalon is denoted by a broken line in F-H. The notochord (n) sheath shows cross reaction with the antisera. Scale bar is 0.5 mm in A,B, 200 μm in C,D,H, 20 μm in E and 270 μm in F,G.

shown). Ectopic $5HT^+$ neurons were never observed in *Gli3*-injected or uninjected control embryos (Table 2). It is not clear, however, whether the ectopic differentiation of these neurons is induced directly by injected Gli1 or secondarily via an induced ectopic floor plate.

Shh can induce the differentiation in vitro of ventral neurons from different anteroposterior levels of the neural tube (Ericson et al., 1995). We therefore tested for the ability of Gli1 to induce the differentiation of ventral cell types in the forebrain, a region that lacks a floor plate. We chose to examine the expression of Nkx2 homeoproteins expressed by ventral telencephalic and diencephalic neurons (Fig. 7F; Lazzaro et al., 1991; Price et al., 1992; Ericson et al., 1995), the expression of which is responsive to Shh (Barth and Wilson, 1995; Ericson et al., 1995). Ectopic Nkx2.1 protein was detected in dorsal telencephalic regions of embryos injected with hGli1 or fGli1 proteins (Fig. 7G,H; Table 2), demonstrating the ability of Gli1 to induce ventral forebrain development in vivo.

Gli1 induces ectopic midline development at neural plate stages

Floor plate development begins during gastrulation as the notochord underlies and induces midline neural plate cells, but widespread expression of Shh or HNF-3β cannot induce ectopic midline (floor plate) development within the neural plate at these early stages (Ruiz i Altaba et al., 1995a). To investigate whether similar restrictions affect the action of Gli1, we analysed midline marker expression in injected neurulae (stage ~14-16). In these embryos, there was no ectopic expression of HNF-3β in the neural plate of hGli1- or hGli3-injected embryos although a fraction of embryos injected with hGli1 displayed expression in the non-neural ectoderm (10%; *n*=20).

Injected embryos were then analyzed for the expression of two midline neural plate markers: *Pintallavis* and *Shh* (Ruiz i Altaba and Jessell, 1992; Dirksen and Jamrich, 1992; Knöchel et al, 1992; Ruiz i Altaba et al., 1995a; Ekker et al., 1995). hGli1 and VP16-fGli1 proteins, but not hGli3, were able to induce the ectopic expression of these markers at neural plate stages (Table 3). However, their expression occurred in scattered cells mostly but not exclusively in non-neural, epidermal ectoderm (Fig. 8A,B). Similar results were obtained

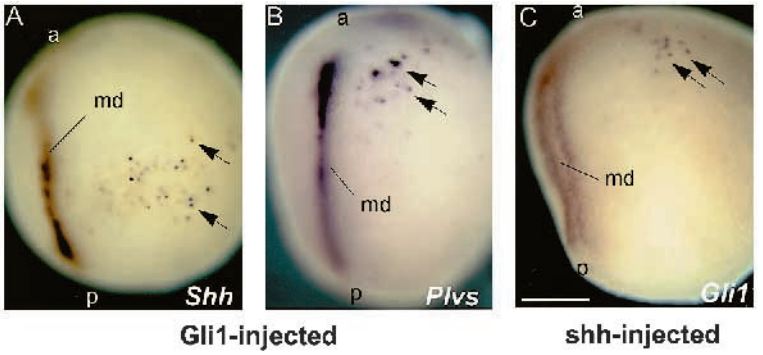

Fig. 8. Ectopic midline gene expression induced by Shh and Gli1 at neural plate stages. (A-C) Ectopic expression of *Shh* (A) and *Pintallavis* (B) after injection of hGli1 (A) or VP16-fGli1 (B). Ectopic gene expression was detected in small patches or single cells, mostly outside of the neural plate. The normal expression of *Shh* and *Plvs* in midline cells (md) is shown out of focus (A,B). (C) Ectopic *Gli1* (D) expression driven by injected Shh. Arrows point to sites of ectopic expression. Panels show dorsolateral views with anterior en up. a; anterior; p: posterior. Scale bar is 200 μm.

Table 3. Quantitation of the incidence of ectopic expression of *Pintallavis*, *Shh*, and *Gli1* at neural plate stages in *Shh*- and *Gli*-injected and control embryos at neural plate stages

Marker	Injected gene	Normal	Ectopic	n
Pintallavis	Shh	58	42	48
	hGli1	82	16	50
	hGli3	97	3	38
	VP16-fGli1	71	29	34
	Uninjected	100	0	24
Shh	hGli1	83	17	24
	hGli3	100	0	39
	VP16-fGli1	85	15	52
	Uninjected	100	0	35
Gli1	Shh	70	30	54
	hGli1	100	0	32
	Uninjected	100	0	34

in Shh-injected embryos (Table 3; Ruiz i Altaba et al., 1995a). Restrictions that operate to prevent ectopic floor plate differentiation by Shh and Pintallavis and at neural plate stages also appear to affect *Gli1* function. Nevertheless, epidermal ectoderm can respond to express midline genes.

We also tested for the expression of the endogenous *Gli1* after injection of the human gene. Injected embryos did not display ectopic *Gli1* (Table 3) suggesting that *Gli1* does not positively autoregulate at these early stages.

Injected Shh induces the ectopic expression of *Gli1*

Because the expression of *Gli1* is detected in midline cells, we tested the possibility that *Gli1* is a target of Shh induction. Embryos injected with Shh displayed the ectopic expression of *Gli1* (Fig. 8C; Table 3). As expected, cells expressing *Gli1* ectopically were found mostly in epidermal ectoderm. *Gli1* induction by injected Shh was detected both at stages when *Gli1* is restricted to the midline proper and later on when its expression is found in cells adjacent to the midline (Fig. 8C and not shown).

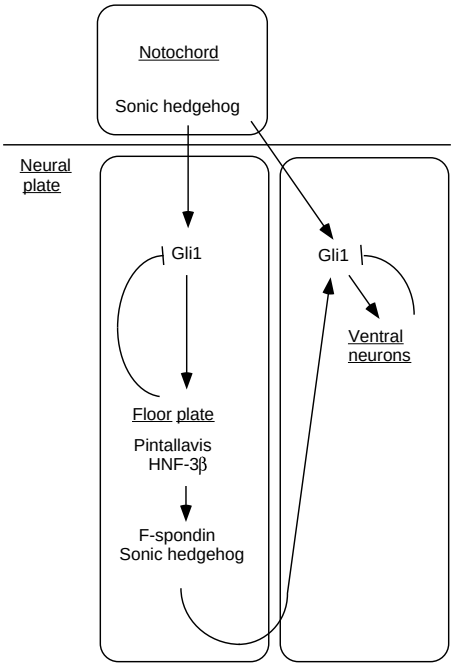

Fig. 9. Schematic diagram of Gli1 function in ventral neural tube patterning by Shh. The diagram depicts Shh signaling from the notochord to the medial neural plate, the future ventral neural tube, with Gli1 functioning in the nuclei of responding cells downstream of the cytoplasmic signal transduction machinery. Gli1 is proposed to induce both floor plate and immediately adjacent ventral neuronal cell types. In a pathway for floor plate differentiation, Gli1 is proposed to act upstream of Pintallavis/HNF-3β which in turn regulate downstream floor plate genes such as *F-spondin* and *Sonic hedgehog*. The lack of *Gli1* expression in floor plate cells and differentiated ventral neurons at late gastrula stages suggests *Gli1* transcription is repressed in these differentiated cells. It is unclear at present how the concentration-dependent effects of Shh on ventral cell type differentiation are interpreted by Gli1 although differential levels of Gli1 activity remain a possibility. See text for details.

DISCUSSION

In this study, we have investigated whether the zinc finger genes of the *Gli* family function in Shh signaling. The coincident or adjacent patterns of expression of *Gli1* and *Shh* in different tissues, including the neural ectoderm and somitic mesoderm, suggest an involvement of Gli1 in the interpretation of the Shh signal. These results therefore indicate a conservation in the response of ci/Gli1 to hh/Shh signals in insects and vertebrates. Within the neural plate, *Gli1* is expressed in midline cells becoming the floor plate when Shh is expressed by the underlying notochord. At later stages, Gli1 is expressed in cells becoming ventral neurons adjacent to the midline when Shh is expressed by the floor plate at the midline. This, together with the induction of *Gli1* by injected Shh suggests that it normally functions downstream of secreted Shh although some late aspects of Gli1 expression could be independent of Shh signaling.

Our results show that widespread expression of the endogenous frog or the human glioma Gli1 proteins induces ectopic ventral pattern in the neural tube in vivo, mimicking our previous results with widespread expression of Shh (Ruiz i Altaba et al., 1995a). The activity of nuclear Gli1 together with the ability of the VP16 transactivating domain to restore function to an N-terminal truncated fGli protein strongly suggest that, like *Drosophila* ci (Alexandre et al., 1996), Gli1 normally acts as a positive transactivator. In the posterior neural tube, Gli1 may therefore function to mediate the induction of floor plate cells and immediately adjacent ventral cell types acting both as a mediator and a target of Shh signaling (Fig. 9). The ectopic expression of Nkx2 proteins in the dorsal telencephalon of Gli1-injected embryos together with the normal expression of Gli1 in the forebrain in a manner similar to that of Shh also implicate Gli function in ventral forebrain patterning.

Gli1 in a pathway for floor plate and ventral neural tube development

Floor plate development in frogs begins as the nascent notochord induces the overlying neuroectoderm to express floor plate markers such as *Pintallavis* and later *Shh* (Ruiz i Altaba, 1992; Ruiz i Altaba and Jessell, 1992; Dirksen and Jamrich, 1992; Ruiz i Altaba et al., 1993b; 1995a; Ekker et al., 1995). Our data suggest a pathway in which an early consequence of secreted Shh from the notochord is the transcription of *Gli1* and the downstream activation of floor plate and medial (ventral) neuronal determination genes (Fig. 9).

Transcription of *HNF-3β* in neural cells is induced by notochord signals in the absence of protein synthesis (Ruiz i Altaba et al., 1995b). This must be mediated by proteins already present in the neural ectoderm before *Gli1* is transcribed in response to Shh signaling. One possibility to account for this effect is that Shh signaling directly activates early *HNF-3β* transcription. However, the normal expression pattern of *Gli1* predicts that it would be already expressed in explanted neural plate pieces similar to those induced in the presence of cycloheximide (Ruiz i Altaba et al., 1995b). Because *Gli1* transcripts are first detected throughout the prospective neural plate, the dorsal animal cap, preexisting Gli1 protein may be activated in midline cells in response to initial Shh induction. This could be a general mechanism; in mice the three *Gli* genes are coexpressed throughout the early neural plate where initial *Gli* gene transcription would also appear to be independent of Shh signaling.

Gli1 does not appear to positively autoregulate and is only transiently expressed in deep midline cells that later will express *Pintallavis* and *Shh* and are fated to give rise to the floor plate. It is possible that high levels of HNF-3β/Pintallavis or an early floor plate protein repress *Gli1* gene transcription. Autorepression is unlikely as *Gli* transcription is maintained in other cell types in which it may be independent of Shh. Signaling by Shh in floor plate cells and in adjacent ventral neuronal precursors may therefore lead to a regulatory feedback loop in which the activation of *Gli1* is followed by its repression (Fig. 9).

At neural plate stages, embryos injected with Gli1, like those injected with Shh or HNF-3β (Ruiz i Altaba et al., 1995a), failed to show ectopic midline marker expression within the neural plate. Thus, in addition to loss of competence to become floor plate by neural cells (Placzek et al., 1993), there must be a mechanism that normally prevents floor plate differentiation throughout the neural plate (Ruiz i Altaba et al., 1995a). This mechanism may play an important role in neural patterning as the superimposition of the endogenous pattern of expression of *Shh*, *bhh* and *chh* shows that the entire neural plate expresses hh factors (Ekker et al., 1995). In contrast, at neural tube stages, Gli1, unlike HNF-3β/ Pintallavis and Shh, is sufficient to induce floor plate differentiation throughout the neural tube in vivo (Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1993a, 1995a). This, together with their normal patterns of expression, suggest that Pintallavis/ HNF-3β act downstream of Gli1 and that their induction of floor plate cells may require a cofactor. Restrictions to Shh could reflect spatial restrictions on Gli1 expression or function.

Gli2 and Gli3

The pattern of expression of Gli2 in cells close to the neural plate midline suggests that, like Gli1, it could be involved in mediating some of the inductive effects of Shh signaling on non-ventral midline cells. However, later in development, Gli2 would appear to have a Shh-independent function as it is coexpressed with Gli3 in the dorsal ventricular zone, a region that is unaffected by Shh from the notochord or floor plate.

The inability of hGli3 to induce ventral neural tube development is likely to reflect a similar function of fGli3 and is consistent with its predominant expression in dorsal regions. *Gli3* expression is first detected in the animal cap and it is rapidly repressed from midline neural plate cells, possibly representing an early step in the progression of medial cells towards ventral development. Because expression of *Gli3* is coincident or adjacent to sites of BMP4 expression, such as the animal cap, posterior/ventral mesoderm and the dorsal neural tube (Nishimatsu et al., 1992; Dale et al., 1992; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Liem et al., 1995), it is possible that the dorsal-to-ventral graded expression of *Gli3* in the neural plate is related to BMP signaling from epidermal ectoderm. If so, Gli3 could act negatively on Shh signaling.

Gli3 could have a similar function in the developing limb bud and somites, acting in an opposite manner to Gli1. In the somites, the pattern of expression of the three *Gli* genes mimics that in the neural plate. *Gli1* is expressed close to the notochord, which secretes Shh, *Gli3* is expressed close to the lateral plate, which secretes BMP4, and these two signals have opposite effects on somite patterning (Fan and Tessier-Lavigne, 1994; Fan et al., 1995; Pourquié et al., 1995, 1996). In the limb bud, *Gli1* is expressed close to the zone of polarizing activity (ZPA), *Gli3* is expressed more generally but it is absent from the ZPA (Marigo et al., 1996d) and loss of function of *Gli3* leads to the ectopic expression of Shh anteriorly (Masuya et al., 1995).

Within the vertebrate Gli family, there appears to be a diversification of function from that of ci in flies, as Gli1 and Gli3 have distinct patterns of expression and functional properties. Moreover, ci is unable to induce ectopic floor plate differentiation suggesting that, whereas both Gli1 and ci may mediate Shh/Hh signaling, the exact molecular mechanisms involved in these processes are not identical.

Implications for oncogenesis

Gli1 was originally found as an amplified gene in a human glioma cell line (Kinzler et al., 1987) and it can transform fibroblasts in cooperation with E1A (Ruppert et al., 1991). However, it is not known if hGli1 is sufficient to cause tumor formation in vivo. Patched is a Shh receptor (Stone et al., 1996; Marigo et al., 1996c; Chen and Struhl, 1996) that is mutated in patients with the basal cell nevus syndrome, which predisposes them to develop basal cell carcinomas (Hahn et al., 1996; Johnson et al., 1996). Because patched may normally act negatively on Shh signaling (e.g. Forbes et al., 1993), its tumor suppressor activity raises the possibility that deregulated Shh signaling leads to skin cancer. Consistent with this, we note that the Shh signaling pathway can be activated in epidermal ectoderm by ectopic expression of Shh (Ruiz i Altaba et al., 1995a) or Gli1 (this work) as determined by the expression of *HNF-3β*, a Gli1-target gene.

We are grateful to Gord Fishell, Ruth Lehmann, Ed Ziff, Nadia Dahmane, Jessica Treisman, Rachel Brewster, Will Talbot, Alex Joyner and Alex Schier for discussion and comments on the manuscript. We thank E. Ziff, R. Di Lauro, R. Holmgren, A. Joyner, T. Kornberg, T. Maniatis and B. Vogelstein for reagents and G. Fishell for help with computer imaging. K. P. was a recipient of a National Research Service Award with A. Joyner. This work was supported by Skirball Institute start up funds to A. R. A.

REFERENCES

Alexandre, C., Jacinto, A. and Ingham, P. W. (1996). Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the

Cubitus interruptus protein, a member of the Gli family of zinc finger DNA-binding proteins. *Genes Dev*. **10,** 2003-2013.

Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF-3/fork head proteins. *Development* **119**, 1301-1315.

Barth, K. A. and Wilson, S. W. (1995). Zebrafish Nkx2.2 is regulated by sonic hedgehog/vertebrte hedgehog-1 and demarcates a neurogenic zone in the embryonic forebrain. *Development* **121**, 1755-1768.

Belloni, E., Muenke, M., Roessler, E., Traverso, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H. F., Donis-Keller, H., Helms, C., Hing, A. V., Heng, H. H. Q., Koop, B., Martindale, D., Rommens, J. M., Tsui, L.-C. and Scherer, S. W. (1996). Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. *Nature Genetics* **14**, 353-356.

Campbell, M. E. M., Palfreyman, J. W. and Preston, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for the stimulation of immediate early trasncription. *J. Mol. Biol*. **180**, 1-19.

Chang, D. T., Lopez, A., von Kessler, D. P., Chiang, C., Simandl, B. K., Zhao, R., Seldin, M. F., Fallon, J. F., and Beachy, P. A. (1994). Products, genetic linkage, and limb patterning activity of a murine hedgehog gene. *Development* **120**, 3339-3353.

Chen, Y. and Struhl, G. (1996). Dual roles for patched in sequestering and transducing hedgehog. *Cell* **87**, 553-563.

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

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.

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

Conlon, R. A. and Herrmann, B. G. (1993). Detection of messenger RNA by in situ hybridization to postimplantation embryo whole mounts. *Methods in Enzymology* **225**, 373-383.

Dale, L. and Slack, J. M. W. (1987). Fate map for the 32-cell stage of Xenopus laevis. *Development* **99,** 197-210.

Dale, L., Howes, G., Price, B. M. and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development. *Development* **115**, 573-585.

Dirksen, M. L. and Jamrich, M. (1992). A novel, activin-inducible, blastopore lip-specific gene of Xenopus laevis contains a fork head DNA-binding domain. *Genes Dev.* **6,** 599-608.

Domínguez, M., Brunner, M., Hafen, E. and Basler, K. (1996). Sending and receiving the hedgehog signal: control by the Drosophila Gli protein cubitus interruptus. *Science* **272,** 1621-1625.

Eaton, S. and Kornberg, T. B. (1990). Repression of ci-D in posterior compartments of Drosophila by engrailed. *Genes Dev.* **4,** 1068-1077.

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

Ekker, S. C., McGrew, L. L., Lai, C.-J., Lee, J. J., von Kessler, D. P., Moon, R. T. and Beachy, P. A. (1995). Distinct expression and shared activities of members of the hedgehog gene family of Xenopus laevis. *Development* **121,** 2337-2347.

Epstein, D. J., Martí, E., Scott, M. P. and McMahon, A. P. (1996). Antagonizing cAMP dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. *Development* **122,** 2885-2894.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81,** 747-756.

Ericson, J., Mortin, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of sonic hedgehog signaling required for the specification of motor neuron identity. *Cell* **87,** 661-673.

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. *EMBO J*. **13,** 5015-5025.

Fan, C.-M. and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* **79,** 1175-1186.

Fan, C.-M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A. and Tessier-Lavigne, M. (1995). Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81,** 457-465.

Forbes, A.J., Nakano, Y., Taylor, A. M. and Ingham, P. W. (1993). Genetic analysis of hedgehog signalling in the Drosophila embryo. *Development* **1993 Supplement,** 115-124.

Franz, T. (1994). Extra-Toes (Xt) Homozygous Mutant Mice Demonstrate a Role for the Gli-3 Gene in the Development of the Forebrain. *Acta Anat* **150**, 38-44.

Fu, X.-D. and Maniatis, T. (1990). Factor required for mamalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature* **343**, 437-441.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Development* **10,** 301-312.

Goulding, M., Lumsden, A. and Gruss, P. (1993). Signals from the notochord and floor plate regulate the region-specific expression of two pax genes in the developing spinal cord. *Development* **117**, 1001-1016.

Hahn, H., Wicking, C., Zaphiropoulous, P. G., Gailani, M. R., Shanley, S., Chidambaram, A., Vorechovsky, I., Holmberg, E., Unden, A. B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell., D. J., Gerrard, B., Goldstein, A. M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwrught, B. and Bale, A. E. (1996). Mutations of the human homolog of Drosohila patched in the nevoid basal cell carcinoma syndrome. *Cell* **85,** 841-951.

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

Harland, R. M. (1991). In situ hybridization: an improved whole mount method for Xenopus embryos. *Meth. Enzymol*. **36,** 675-685.

Hemmati-Brivanlou, A. and Thomsen, G. (1995). Ventral mesodermal patterning in Xenopus embros: expression patterns and activities of BMP-2 and BMP-4. *Dev. Genetics* **17,** 78-89.

Hui, C.-C. and Joyner, A. L. (1993). A mouse model of Greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nature Genetics* **3,** 241-246.

Hui, C.-C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162,** 402-413.

Hynes, M., Porter, J. A., Chians, C., Chang, D., Tessier-Lavigne, M. and Beachy, P. A. (1995). Induction of midbrain dopaminergic neurons by sonic hedgehog. *Neuron* **15**, 35-44.

Jacobson, C.-O. (1964). Motor nuclei, cranial nerve roots, and fiber pattern in the medulla oblongata after reversal experiments on the neural plate of Axolotl larvae. I. Bilateral operations. *Zool. Bidrag. Uppsala* **26,** 73-160.

Johnson, D. R. (1967). Extra-toes: a new mutant gene causing multiple abnormalities in the mouse. *J. Embryol. Exp. Morph*. **17,** 543-581.

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

Kelsey-Motzny, C. and Holmgren, R. (1995). The Drosophila cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. *Mech. Dev.* **52**, 137-150.

Kintner, C. R. and Melton, D. A. (1987). Expression of the Xenopus N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99,** 311-325.

Kinzler, K. W., Bigner, S. H., Bigner, D. D., Trent, J. M., Law, M. L., O'Brien, S. J., Wong, A. J. and Vogelstein, B. (1987). Identification of an amplified, highly expressed gene in a human glioma. *Science* **236**, 70-73.

Kinzler, K. W., Ruppert, J. M., Bigner, S. H. and Vogelstein, B. (1988). The GLI gene is a member of the Kruppel family of zinc finger proteins. *Nature* **332**, 371-74.

Kinzler, K. W. and Vogelstein, B. (1990). The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. *Mol. Cell Biol*. **10,** 634-642.

Knöchel, S., Lef, J., Clement, J., Klocke, B., Hille, S., Koster, M. and Knöchel, W. (1992). Activin A-induced expression of a fork-head-related gene in posterior chordamesoderm (notochord) of Xenopus laevis embryos. *Mech. Dev*. **38,** 157-165.

Krauss, S., Concordet, J.-P. and Ingham, P. W. (1993). A functionally

conserved homolog of the Drosophila segment polarity gene hedgehog is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75,** 1431-1444.

Larsson, S. H., Charlieu, J.-P., Miyagawa, K., Engelkamo, D., Rassoulzadegan, M., Ross, A., Cuzin, F., van Heyningen, V. and Hastie, N. D. (1995). Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. *Cell* **81**, 391-401.

Lazzaro, D., Price, M., De Felice, M. and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113**, 1093-1104.

Liem, K. F., Jr., Tremmel, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V. and Tabin, C. J. (1996a). Conservation in hedgehog signaling: induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* **122**, 1225-1233.

Marigo, V. and Tabin, C. J. (1996b). Regulation of Patched by Sonic hedgehog in the developing neural tube. *Proc. Natl. Acad. Sci. USA* **93**, 9346-9351.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996c). Biochemical evidence that patched is the hedgehog receptor. *Nature* **384**, 176-179.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996d). Sonic hedgehog differentiatlly regulates expression of Gli and Gli3 during limb development. *Dev. Biol.* **180**, 273-283.

Martí, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995a). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

Martí, E., Takada, R., Bumcrot, D. A., Sasaki, H. and McMahon, A. P. (1995b). Distribution of sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-2547.

Masuya, H., Sagai, T., Wakana, S., Moriwaki, K. and Shiroishi, T. (1995). A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev.* **13**, 1645-1653

Monaghan, A. P., Kaestner, K. H., Grau, E. and Schutz, G. (1993). Postimplanation expression patterns indicate a role for the mouse forkhead/HNF-3α, β and γ genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119**, 567-578.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal table of Xenopus laevis (Daudin)*. Amsterdam: North Holland.

Nishimatsu, S., Suzuki, A., Shoda, A., Murakami, K. and Ueno, N. (1992). Genes for bone morphogenetic proteins are differentiatlly transcribed in early amphibian embryos. *Biochem. Biophys. Res. Com.* **186**, 1487-1495.

Orenic, T. V., Slusarski, D. C., Kroll, K. L. and Holmgren, R. A. (1990). Cloning and characterization of the segment polarity gene cubitus interruptus dominant of Drosophila. *Genes Dev.* **4,** 1053-1067.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of engrailed proteins in arthropods, annelids and chordates. *Cell* **58**, 955-968.

Pavletich, N. P. and Pabo, C. O. (1993). Crystal structure of a five-finger GLI-DNA complex: new perspectives on zinc fingers. *Science* **261**, 1701-1707.

Perrimon, N. (1995). Hedgehog and beyond. *Cell* **80**, 517-520.

Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. M. and Dodd, J. (1990). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250**, 985-988.

Placzek, M., Jessell, T. M. and Dodd, J. (1993). Induction of floor plate differentiation by contact-dependent, homeogenetic signals. *Development* **117**, 205-218.

Pourquié, O., Coltey, M., Breant, C. and Le Douarin, N. M. (1995). Control of somite patterning by signals from the lateral plate. *Proc. Natl. Acad. Sci. USA* **92**, 3219-3223.

Pourquié, O., Fan, C.-M., Coltey, M., Hirsinger, E., Watanabe, Y, Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996). Lateral and axial signals involved in somite patterning: a role for BMP-4. *Cell* **84**, 461-471.

Price, M., Lazzaro, D., Pohl, T., Mattei, M.-G., Ruther, U., Olivo, J.-C., Duboule, D. and Di Lauro, R. (1992). Regional expression of the homeobox gene Nkx2.2 in the developing mammalian forebrain. *Neuron* **8**, 241-255.

Richter, K., Grunz, H. and Dawid, I. B. (1988). Gene expression in the embryonic nervous system of Xenopus laevis. *Proc. Natl. Acad. Sci. USA* **85**, 8086-8090.

Riddle, R., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **751**, 401-1418.

Roberts, W. M., Douglass, E. C., Peiper, S. C., Houghton, P. J. and Look, A. T. (1989). Amplification of the gli gene in childhood sarcomas. *Cancer Research* **49**, 5407-5413.

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

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

Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S. W., Tsui, L.-C. and Muenke, M. (1996). Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. *Nature Genetics* **14**, 357-360.

Ruiz i Altaba, A. (1992). Cooperation of Planar and Vertical Signals in the Induction and Patterning of the Xenopus Nervous System. *Development* **115**, 67-80.

Ruiz i Altaba, A. (1993). In *Essential Developmental Biology – A Practical Approach* (ed. C. Stern and P. W. H. Holland) Oxford: IRL Press.

Ruiz i Altaba, A. (1994). Pattern formation in the vertebrate neural plate. *Trends Neurosci.* **17**, 233-243.

Ruiz i Altaba, A., and Jessell, T. M. (1991). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage Xenopus embryos. *Development* **112**, 945-958.

Ruiz i Altaba, A. and Jessell, T. M. (1992). Pintallavis, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis. *Development* **116**, 81-93.

Ruiz i Altaba, A., Prezioso, V. R., Darnell, J. E. and Jessell, T. M. (1993a). Sequential expression of HNF-3β and HNF-3α by embryonic organizing centers: the dorsal lip/node, notochord and floor plate. *Mech. Dev.* **44,** 91-108.

Ruiz i Altaba, A., Cox, C., Jessell, T. M. and Klar, A. (1993b). Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis. *Proc. Natl. Acad. Sci. USA* **90**, 8268-8272.

Ruiz i Altaba, A., Jessell, T. M. and Roelink, H. (1995a). Restrictions to floor plate induction by hedgehog and winged-helix genes in the neural tube of frog embryos. *Mol. Cell. Neurosci.* **6**, 106-121.

Ruiz i Altaba, A., Placzek, M., Baldassare, M., Dodd, J. and Jessell, T. M. (1995b). Early stages of notochord and floor plate differentiation defined by normal and induced expression of HNF-3β in chick embryos. *Dev. Biol.* **170,** 299-313.

Rupp, R. A. W., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8,** 1311-1323.

Ruppert, J. M., Kinzler, K. W., Wong, A. J., Bigner, S. H., Kao, F. T., Law, M. L., Seuanez, H. N., O'Brien, S. J. and Vogelstein, B. (1988). The GLI-Kruppel family of human genes. *Mol. Cell. Biol.* **8,** 3104-3113.

Ruppert, J. M., Vogelstein, B., Arheden, K. and Kinzler, K.W. (1990). GLI3 encodes a 190-kilodalton protein with multiple regions of GLI similarity. *Mol. Cell Biol.* **10,** 5408-5415.

Ruppert, J.M., Vogelstein, B. and Kinzler, K.W. (1991). The zinc finger protein GLI transforms primary cells in cooperation with adenovirus E1A. *Mol. Cell Biol.* **11**, 1724-1728.

Sánchez-Herrero, E., Couso, J. P., Capdevila, J. and Guerrero, I. (1996). The *fu* gene discriminates between pathways to control *dpp* expression in Drosophila imaginal discs. *Mech. Dev.* **55,** 159-170.

Sasaki, H. and Hogan, B. (1993). Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118**, 47-59.

Sasaki, H. and Hogan, B. (1994). HNF-3β as a regulator of floor plate development. *Cell* **76**, 103-115.

Schimmang, T., Lemaistre, M., Vortkamp, A. and Rüther, U. (1992). Expression of the zinc finger gene Gli3 is affected in the morphogenetic mouse mutant extra-toes (Xt). *Development* **116**, 799-804.

Simon, H., Hornbruch, A. and Lumsden, A. (1995). Independent assignment of antero-posterior and dorso-ventral positional values in the developing chick hindbrain. *Current Biol.* **5,** 205-214.

Slusarski, D.C., Kelsey Motzny, C. and Holmgren, R. (1995). Mutations that alter the timing and pattern of cubitus interruptus gene expression in Drosophila melanogaster. *Genetics* **139**, 229-240.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q, Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F. and Rosenthal, A. (1996). The tumor suppressor gene

patched encodes a candidate receptor for sonic hedgehog. *Nature* **384,** 129-134.

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

Tanabe, Y., Roelink, H. and Jessell, T. M. (1995). Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation. *Current Biology* **5**, 651-658.

Tanimura, A., Teshima, H., Fujisawa, J. and Yoshida, M. (1993). A new regulatory element that augments the Tax-dependent enhancer of human T-cell leukemia virus type 1 and cloning of cDNAs encoding its binding proteins. *J. Virology* **67,** 5375-5382.

Triezenberg, S. J., Kingsbury, R. C. and McKnight, S. L. (1988). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev*. **2,** 718-729.

Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev*. **8,** 1434-1447.

van Mier, P., Joosten, H. W. J., van Rheden, R. and Ten Donkelaar, H. J. (1986). The development of serotonergic raphespinal projections in Xenopus laevis. *Int. J. Develop. Neurosci*. **24**, 465-476.

van Straaten, H. W. M., Hekking, J. W. M., Wiertz-Hoessels, E. L., Thors, F. and Drukker, J. (1988). Effect of the notochord on the differentiation of a floor plate area in the neural tube of the chick embryo. *Anat. Embryol*. **177,** 317-324.

van Straaten, H. W. M. and Hekking, J. W. M. (1991). Development of floor plate, neurons and axonal outgrowth pattern in the early spinal cord of the notochord-deficient chick embryo. *Anat. Embryol*. **184,** 55-63.

Vortkamp, A., Gessler, M. and Grzeschik, K.-H. (1991). GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families. *Nature* **352**, 539-540.

Walterhouse, D., Ahmed, M., Slusarski, D., Kalamaras, J., Boucher, D., Holmgren, R. and Iannaccone, P. (1993). gli, a zinc finger transcription factor and oncogene, is expressed during normal mouse development. *Dev. Dyn.* **196**, 91-102.

Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate notochord. *Cell* **64**, 635-647.

Zarkower, D. and Hodgkin, J. (1992). Molecular Analysis of the C. elegans Sex-Determining Gene tra-1: A Gene Encoding Two Zinc Finger Proteins. *Cell* **70,** 237-249.

(Accepted 18 April 1997)