Development 128, 5201-5212 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV14502

DEVELOPMENT AND DISEASE

The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis

Nadia Dahmane[1,*], Pilar Sánchez[1], Yorick Gitton[1], Verónica Palma[1], Tao Sun[1], Mercedes Beyna[1], Howard Weiner[2] and Ariel Ruiz i Altaba[1,†]

[1]Skirball Institute of Biomolecular Medicine, Developmental Genetics Program and Department of Cell Biology, NYU School of Medicine, 540 First Avenue, New York, NY 10016, USA
[2]Department of Neurosurgery, NYU School of Medicine, 540 First Avenue, New York, NY 10016, USA
*Present address: Institute for Developmental Biology, CNRS, Marseille, France
†Author for correspondence (e-mail: ria@saturn.med.nyu.edu)

Accepted 24 September 2001

SUMMARY

The mechanisms that regulate the growth of the brain remain unclear. We show that *Sonic hedgehog* (*Shh*) is expressed in a layer-specific manner in the perinatal mouse neocortex and tectum, whereas the *Gli* genes, which are targets and mediators of SHH signaling, are expressed in proliferative zones. In vitro and in vivo assays show that SHH is a mitogen for neocortical and tectal precursors and that it modulates cell proliferation in the dorsal brain. Together with its role in the cerebellum, our findings indicate that SHH signaling unexpectedly controls the development of the three major dorsal brain structures. We also show that a variety of primary human brain tumors and tumor lines consistently express the *GLI* genes and that cyclopamine, a SHH signaling inhibitor, inhibits the proliferation of tumor cells. Using the in vivo tadpole assay system, we further show that misexpression of GLI1 induces CNS hyperproliferation that depends on the activation of endogenous Gli1 function. SHH-GLI signaling thus modulates normal dorsal brain growth by controlling precursor proliferation, an evolutionarily important and plastic process that is deregulated in brain tumors.

Key words: Mouse, *Xenopus*, GLI, SHH, Brain, Tumor, Neocortex, Tectum, Growth, CNS

INTRODUCTION

The regulation of normal brain growth, on one hand, and of tumor formation, on the other, are two tightly related processes that remain poorly understood. Several secreted signals have been previously shown to participate in brain development (Kilpatrick et al., 1995; Li et al., 1998; Mabie et al., 1999). The secreted protein Sonic hedgehog (SHH) is involved in different aspects of the development of the early CNS, controlling cell differentiation as well as cell proliferation. For example, SHH is required for the differentiation of floor plate cells and ventral neurons in the early neural tube (Echelard et al., 1993; Roelink et al., 1994; Ruiz i Altaba et al., 1995), and it later regulates granule cell precursor proliferation in the cerebellum (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999).

Gli zinc-finger transcription factors participate in Hedgehog (HH) signaling and *Gli1* is consistently induced in cells that receive a HH signal [reviewed by Ruiz i Altaba (Ruiz i Altaba, 1999)]. In vivo, we have shown that Gli1 is sufficient to induce basal cell carcinoma (BCC)-like skin tumors in the epidermis of frog embryos (Dahmane et al., 1997), a result later reproduced in mice (Grachtchouk et al., 2000; Nilsson et al., 2000) that further validates our approach (Wallingford et al., 1997). The expression of *GLI1* in proliferating cells of BCCs, medulloblastomas (MBs) and rhabdomyosarcomas (Dahmane et al., 1997; Goodrich et al., 1997; Hahn et al., 1998; Xie et al., 1997; Pietsch et al., 1997; Vorechovsky et al., 1997; Reifenberger et al., 1998), suggests a wider role of the SHH-GLI pathway in tumorigenesis than previously suspected. However, even though *GLI1* was originally identified in a glioma line (Kinzler et al., 1987; Ruppert, 1991), its proposed role in glial brain tumors has not been supported (Salgaller et al., 1991; Xiao et al., 1994).

We have investigated a role for the SHH-Gli pathway in normal and abnormal precursor proliferation in the dorsal brain. Specifically, we have investigated whether the cerebral cortex and the tectum (colliculi), two major, dorsal, layered and late developing structures of the brain, share common mechanisms for growth regulation with the cerebellum. We show that the SHH-Gli pathway modulates the growth of the dorsal brain and that it is deregulated in brain tumors, possibly being a cause for their initiation from precursor cells, as Gli1 is sufficient to induce CNS hyperproliferation. The ability of cyclopamine to inhibit the proliferation of brain tumor cells suggests that the SHH pathway is also be required for tumor maintenance.

MATERIALS AND METHODS

Animals, human tissue samples and cell lines

Swiss-Webster mouse embryos were staged counting the morning after conception as embryonic day (E) 0.5. *Shh* homozygote mutant mice were a kind gift from C. Chiang (Chiang et al., 1996). Human tumor samples were derived from the operating room or from the NYU tumor banks. Brain tumor cell lines were obtained from ATCC and grown according to its specifications. GL261was a kind gift of Dr D. Zagzag. Frog (*Xenopus laevis*) embryos were obtained, reared and staged by standard methods. Tadpoles were ~2 days old. All statistical analyses were carried out using the Student's t test and deviations are shown as s.e.m.

Explants, dissociated cells, cell treatments and chemicals

Neocortical or tectal explants from embryonic day (E) 17.5 to postnatal-day (P) 3 mice were taken from the parietal region, or adjacent to the dorsal midline in the prospective superior and inferior colliculi, respectively. After removal of the meninges, the explants were grown on floating filters in serum-free media (Nothias et al., 1998; Dahmane and Ruiz i Altaba 1999). After 12 hours in culture, SHH was added and incubation continued for a further 48 hours. Explants for RNA preparation were directly collected in Trizol (Gibco-BRL). For dissociated cells, parietal cortical pieces of P3 brains were pooled (~10 explants per experiment) and treated with trypsin for 10 minutes (0.25 mg/ml at room temperature). Tissue was triturated manually in DNase (0.5 mg/ml), and cells were centrifuged, resuspended in supplemented serum-free media and plated at a density of ~400 cells/mm^2 in poly-L-lysine-coated 16- or 8-well slides. After 12 hours the media were replaced and SHH protein added if required. Cells were cultured for a further 48 hours and processed for immunocytochemistry after fixation in 4% paraformaldehyde for 1 minute. Primary gliomas from the

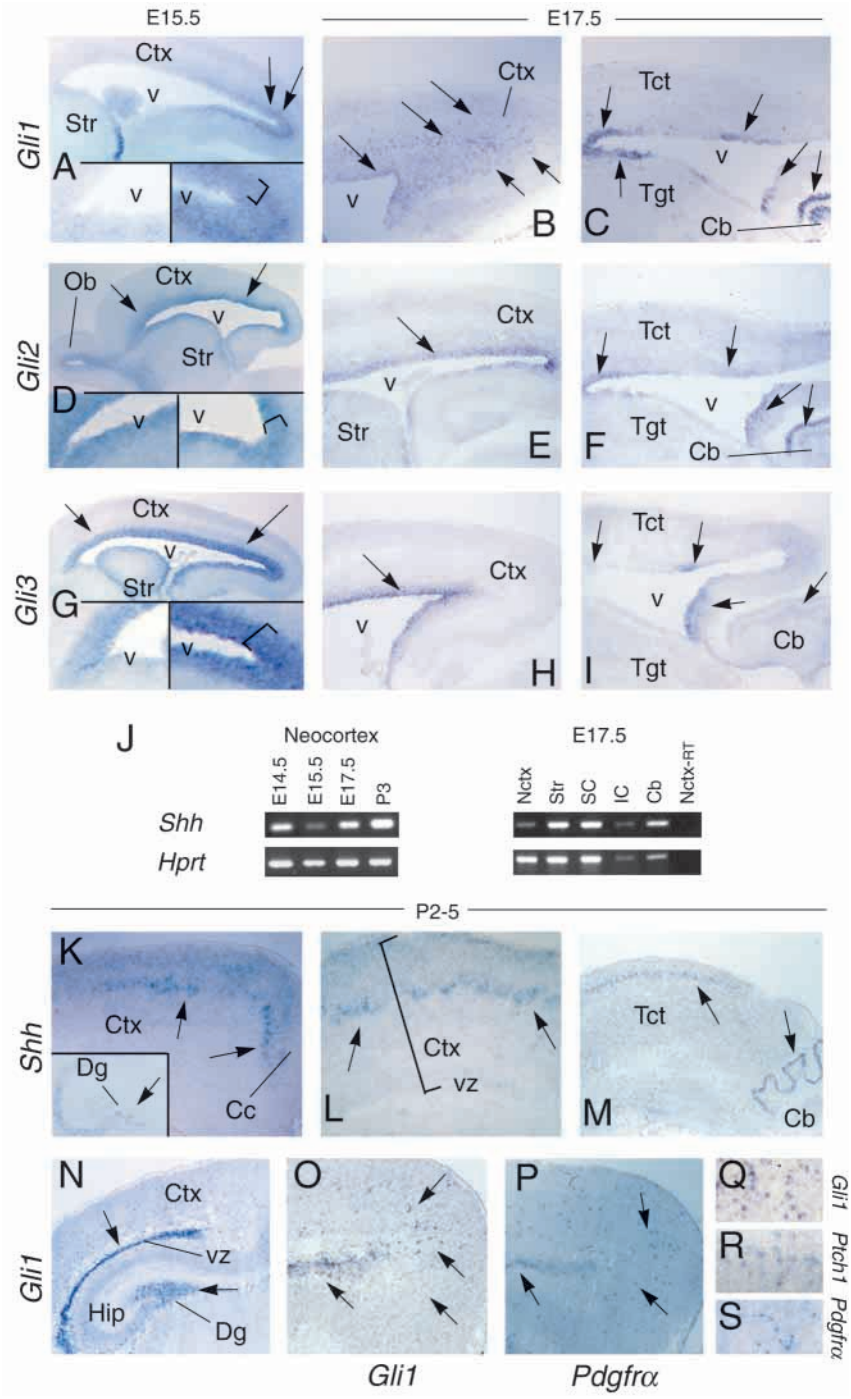

Fig. 1. Perinatal expression of *Gli* and *Shh* in the mouse dorsal brain. (A,D,G) Expression of *Gli1* (A), *Gli2* (D) and *Gli3* (G) in the vz/svz of the cerebral neocortex (Ctx) and striatum (Str) at embryonic day (E) 15.5. The three *Gli* genes are also co-expressed in the vz of the striatum and olfactory bulb (D and not shown). (B,E,H) Expression of *Gli1* (B), *Gli2* (E) and *Gli3* (H) in the cerebral cortex (Ctx) at E17.5. *Gli* gene expression is detected near the ventricle (v) with *Gli1* also expressed in scattered cells within the cortical plate. (C,F,I) Expression of *Gli1* (C), *Gli2* (F) and *Gli3* (I) in the midbrain at E17.5. Expression of the *Gli* genes is regionalized in the vz of the tectum (Tct) and tegmentum (Tgt). Expression is also detected in the EGL and Purkinje layer of the cerebellum (Cb). *Shh* is also detected in the tegmentum as well as in the amygdala (not shown). (J) RT-PCR analyses of *Shh* expression in the parietal neocortex of E14.5-postnatal day (P)3 mice (right) and of its expression at E17.5 in the neocortex (Nctx), striatum (Str), superior colliculus (SC), inferior colliculus (IC) and cerebellum (Cb). A no RT control is also added (Nctx-RT). *Hprt* is used to control for RNA levels. (K-M) *Shh* is expressed in a layer-specific manner in the P2 neocortex (Ctx; K,L) and cingulate cortex (Cc; K), as well as in the tectum (Tct) and cerebellum (Cb), where it is found in the Purkinje layer. *Shh* is also expressed in the hippocampal dentate gyrus (Dg, also detected by RT-PCR, not shown). Similar expression was detected at P1 and P5. (N,O) *Gli1* expression is found in the vz/svz of the cortex and in scattering cells (O) in a P5 mouse brain. *Gli1* expression is also prominent in the dentate gyrus (Dg) of the hippocampus (Hip). Similar expression was detected at P1 and P5. (P) Expression of the oligodendrocyte precursor marker *Pdgfrα* in scattered cells and in a subset of cells near the ventricle. (Q-S) High magnification of the similar expression patterns of *Gli1* (Q), *Ptch1* (R) and *Pdgfrα* (S) in scattering cells in the P5 cortex. Arrows point to sites of expression. v, ventricle. All panels show sagittal sections except N and the inset in K, which display coronal sections.

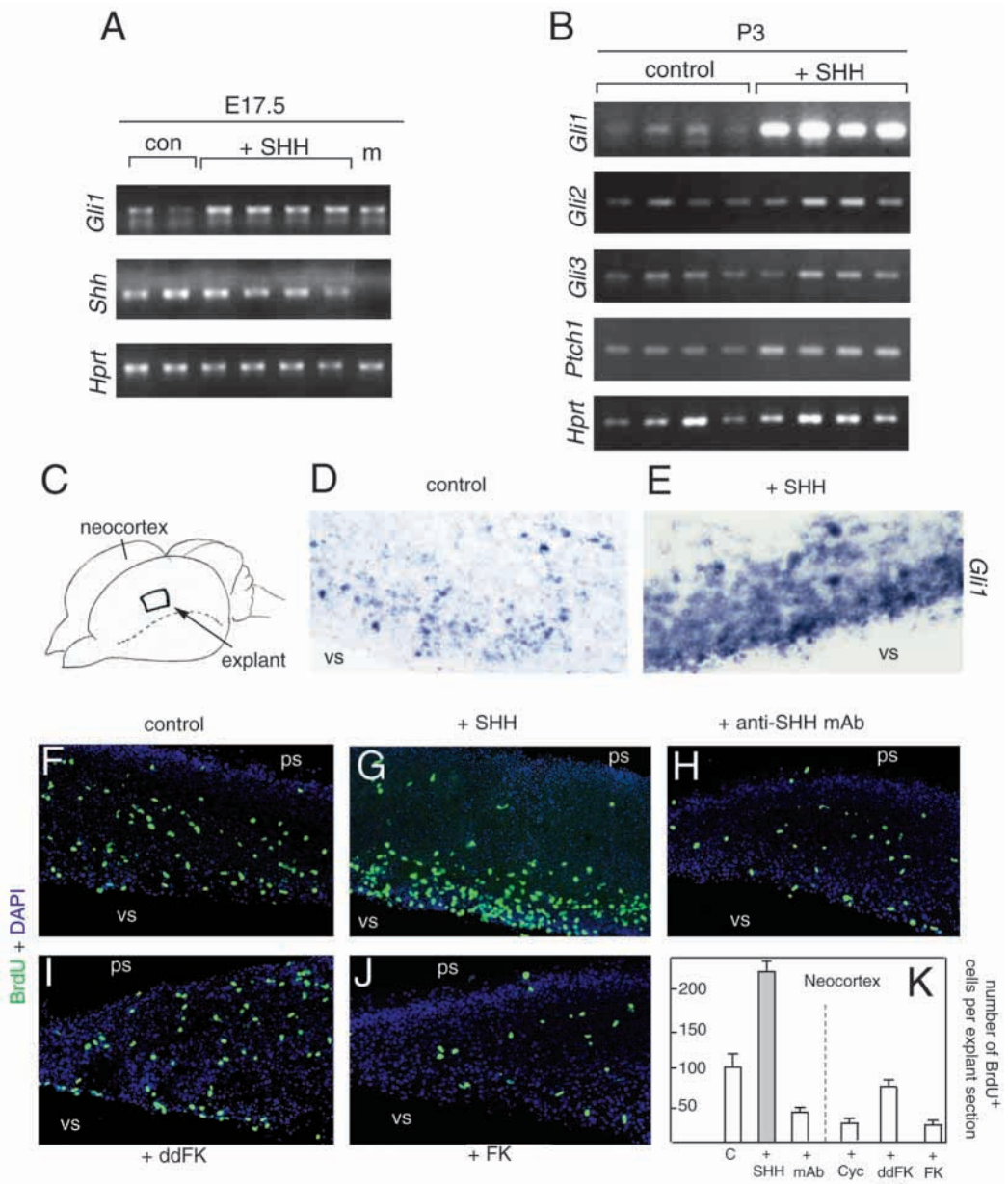

Fig. 2. SHH upregulates *Gli1* transcription and promotes proliferation of vz cells in perinatal mouse neocortical explants. (A) RT-PCR analyses of gene expression in untreated control (con) or SHH-treated (+SHH) mouse E17.5 cerebral cortical explants and meninges (m). One-fifth the concentration of SHH (1 nM) induced a smaller increase in *Gli1* expression (not shown). The expression of the housekeeping gene *Hprt* is used as internal quantitative control. Dorsal brain-derived SHH could affect adjacent cells of the meninges, which express *Gli1*. (B) RT-PCR analyses of gene expression in untreated control or SHH-treated mouse P3 cerebral cortical explants. At this time, the choroid plexus expresses low levels of both *Gli1* and *Shh* (not shown). (C) Sketch of a lateral view of a ~P3 mouse brain showing the position of explanted tissue within the parietal neocortex. (D,E) In situ hybridization analyses on cryostat sections of P3 mouse cerebral cortex explants left untreated (D) or grown in the presence of SHH (E). Each panel shows a representative section. vs, ventricular surface. (F-J) Localization of BrdU-positive cells in the vz of a P3 mouse cortical explants left untreated (F), or treated with SHH (G), anti-SHH blocking monoclonal antibody (H), dideoxyforskolin (I) or forskolin (J). ps, pial surface; vs, ventricular surface. (K) Quantification of cell proliferation by SHH treatment or inhibition of SHH signaling in P3 mouse cortical explants. Numbers of cells±s.e.m. are given. $n>10$ sections of six independent explants counted for each condition. Ten independent explants were used for RT-PCR in two separate experiments, and four or five independent explants for in situ analyses.

operating room were dissociated with papain and plated in U118 media containing 10% fetal calf serum (FCS) or in DMEM:F12 serum-free media supplemented with BIT-9500 (Stem Cell Technologies) and 20 ng/ml of each of FGF2, EGF and PDGF. After two to three passages, the cells had a homogenous appearance and were then tested. Recombinant N-SHH was a kind gift from Ontogeny and was used at 5 nM. For blocking experiments, anti-SHH mAb 5E1 (Ericson et al., 1996) was used at 20 μg/ml (obtained from the University of Iowa Hybridoma Bank). Cyclopamine (a kind gift from the Poisonous Plant Laboratory or purchased from Toronto Research Chemicals) was used at 0.5-5 μM for 48 hours before assaying. Cell lines were plated at 60% confluency the night before cyclopamine treatment. FK and ddFK (Sigma) were used at 50 μM.

Microinjection, RNAs and antisense oligonucleotides

Injection of synthetic RNAs into frog embryos was performed into one cell at the two-cell stage, targeting the future CNS and epidermis. Frog or human *Gli1* RNAs (Lee et al., 1997) were injected at 2 ng/10 nl/embryo. The N-terminal Myc-epitope tag in the frog Gli1 and Gli2 proteins (Lee et al., 1997) was used to monitor protein distribution. *lacZ* RNA was co-injected at 0.2 ng/10 nl/embryo and was used as a lineage tracer through X-gal staining, yielding an insoluble blue precipitate. Morpholino antisense oligonucleotides were purchased from Gene Tools and used at 0.5 mM. These were frog *Gli1*, 5′CGGGCGGACAC-TGGCGGGACGC3′; frog *Gli2*, 5′GCACAGAACGCAGGTAATGC-TCCAT3′; and frog *Shh*, 5′GAGATTCGAGTTCGCAACCAGCATC3′. In all cases, the oligonucleotides were designed to be complementary to regions near the initiation ATG codon and are predicted to inhibit translation (Heasman et al., 2000).

RT-PCR and in situ hybridization

In situ hybridization on serial ~20 μm cryostat sections with

digoxigenin-labeled antisense RNA probes and full-length frog, mouse or human *Gli1* cDNA clones and histology were as previously described (Dahmane et al., 1997; Lee et al., 1997; Park et al., 2000). Visualization of the low levels of *GLI1* and *PTCH1* expression in CNS tumors and mouse brains older than E17 required long (2 days at room temperature) chromogenic development of the in situ hybridization reactions. Specificity was confirmed using sense RNA probes. RT-PCR of human tumors or cell lines was performed for 27, 32 and 37 cycles to determine the linear amplification range. PCR primers and specific reaction conditions are available upon request. A probe for *DDR1* was made as described (Weiner et al., 2000). A 0.6 kb RT-PCR clone was used as a template for probe production for mouse *Pdgfrα* (platelet-derived growth factor receptor α).

Immunocytochemistry

BrdU incorporation in explants and dissociated cells was for 2 hours at 6 μg/ml. Primary tumor cultures were labelled with BrdU for 14 hours. Pregnant mice were injected intraperitoneally with a single dose of 50 μl of 10 mg/ml BrdU and embryos dissected 2 hours afterwards. For tadpoles, one 20 nl injection of 10 mg/ml BrdU into the lumen of the CNS and one into the endoderm were performed 1 hour before fixation. Sections of embryos or explants (14-20 μm) were prepared using a cryostat. Immunocytochemistry with monoclonal anti-BrdU antibody (Becton-Dickinson), monoclonal anti-vimentin antibody (Santa Cruz), monoclonal anti-neuronal tubulin Tuj1 antibody (Babco), rat monoclonal anti-Nestin antibody (University of Iowa Hybridoma Bank) or O4 monoclonal antibody (Chemicon, also a kind gift of Bob Miller) was performed on frozen sections and cells using fluorescein-conjugated secondary antibodies (Boehringer Mannheim).

RESULTS

The Gli genes are expressed in proliferative zones of the brain

First, we sought to clarify the patterns of expression of the Shh and *Gli* genes (Echelard et al., 1993; Roelink et al., 1994) in the late embryonic and postnatal mouse brain. By E15.5, the three *Gli* genes are expressed in the ventricular zone (vz) of the future cerebral cortex and tectum (Fig. 1A,D,G and not shown), where they are detected as early as E13.5 (Hui et al., 1994). However, *Gli1* was expressed at higher levels in the deeper cortical vz/subventricular zone (svz), *Gli2* mostly in vz cells and *Gli3* in these two populations (Fig. 1A,D,G). *Gli1* and *Gli3*, but not *Gli2*, also show a transient graded distribution along the anteroposterior axis (Fig. 1A,D,G). At E17.5, they remain co-expressed in the vz of the cerebral cortex (Fig. 1B,E,H), tectum and tegmentum (Fig. 1C,F,I). *Gli* expression in the tectum is localized within the vz, suggesting heterogeneity in precursor cells. At this stage, *Shh* is expressed in the tegmentum, but is not detectable by in situ hybridization in the tectum or cerebral cortex. However, more sensitive RT-PCR analyses indicated that *Shh* is indeed found in these regions from E14.5 to P3 (Fig. IJ). The neocortical meninges expressed *Gli1* but not *Shh* (Fig. 2A).

Perinatally, the *Gli* and *Shh* genes display largely mutually exclusive expression domains. *Shh* RNA is found in the Purkinje layer in the cerebellum, and in the superficial layers of the tectum (Fig. 1M) and cerebral cortex (probably layer V; Fig. 1K,L) (Traiffort et al., 1999). Lack of *Shh* expression in dorsal progenitors would be consistent with its reported absence from late embryonic and postnatal neurospheres (Zhu et al., 1999). *Shh* and *Gli1* were also detected in the dentate gyrus of the hippocampus (Fig. 1K,N).

Gli1, but not *Gli2* or *Gli3*, is also expressed at low levels by single cells scattered throughout the brain (Fig. 1B,O,Q). Given that there is massive gliogenesis in the perinatal cortex, if *Gli1*$^+$ scattering cells are glial progenitors that respond or have responded to SHH, the pattern of *Gli1* expression should be similar to that of its target *Ptch1* and possibly to that of *Pdgfrα*, a marker of oligodendrocyte progenitors (Goodrich et al., 1996; Pringle and Richardson, 1993). The expression of *Gli1* was found in a scattered pattern similar to that of *Ptch1* and *Pdgfrα* in serial sections (Fig. 1Q-S), although the low levels of their expression, together with the lack of specific antibodies, precluded double-labeling analyses.

SHH regulates *Gli1* expression in neocortical explants

SHH secreted perinatally from differentiated cells in the cortical plate could affect precursor cells in the vz/svz. To test this possibility, we cultured isolated explants of late embryonic (E17.5) and postnatal (P3) mouse cerebral cortex in vitro (Fig. 2C). SHH treatment dramatically increased *Gli1* expression over that seen in untreated sibling explants (Fig. 2A,B). Upregulation was higher at P3 (Fig. 2B). In situ hybridization analyses confirmed this effect (Fig. 2D,E) and localized *Gli1* expression mostly to vz cells (Fig. 2E). In response to SHH, the expression of *Ptch1* showed a small but consistent upregulation, that of *Gli2* and *Gli3* showed a little variation, and that of endogenous *Shh* remained unchanged, whereas the expression of *Pdgfrα* in control and SHH-treated samples was uninformative, as its expression was found at high levels in both cases (Fig. 2A,B and not shown).

SHH is a required mitogen for neocortical and tectal vz cells

To test whether SHH could act as a mitogen for precursor cells, we cultured P3 neocortical explants for 48 hours and added BrdU to the media 2 hours before fixation. SHH treatment led to an approx. twofold increase in the number of BrdU-positive cells when compared with control untreated explants (Fig. 2F,G,K: control, 105±20 cells/explant section; SHH treated, 226±14 cells/explant section; $P<0.005$). This increase is similar to that seen in cerebellar precursors after SHH treatment (Dahmane and Ruiz i Altaba, 1999). As with *Gli1* expression, the great majority of BrdU-positive cells in SHH-treated explants are in the vz (Fig. 2G).

Treatment of neocortical explants with a blocking anti-SHH antibody, extensively used previously to block SHH function specifically (mAb 5E1) (Ericson et al., 1996; Dahmane and Ruiz i Altaba, 1999; Weschler-Reya and Scott, 1999) produced a twofold decrease in BrdU incorporation after 48 hours in culture when compared with untreated controls (Fig. 2H,F,K: control, 105±20 cells/explant section; mAb 5E1-treated, 45±8 cells/explant section; $P<0.005$). To corroborate this result, we also attempted to block SHH signaling with cyclopamine and forskolin (FK) and with the inactive derivative 1,9-dideoxyforskolin (ddFK) as control. Cyclopamine treatment inhibits the response to SHH signaling (Incardona et al., 1998; Cooper et al., 1998), by acting on the Patched-Smoothened membrane receptor complex (Taipale et al., 2000). Cyclopamine treatment resulted in a twofold decrease in cell proliferation as measured by BrdU incorporation (Fig. 2K: untreated, 72±5.6 cells/explant section; cyclopamine treated, 38±3.4 cells/explant

section, $P<0.001$). FK treatment leads to an upregulation of PKA activity and this inhibits the SHH pathway intracellularly (Fan et al., 1995; Dahmane and Ruiz i Altaba, 1999). FK-treated explants showed a twofold reduction in BrdU incorporation after 48 hours in culture when compared with the inactive derivative 1,9-dideoxyforskolin (ddFK)-treated sibling explants (Fig. 2I-K: ddFK-treated, 73±10 cells/explant section; FK treated, 35±5 cells/explant section; $P<0.005$).

To test if the results in the cortex could be extended to the tectum, we cultured explants of prospective superior and inferior colliculi. Both responded identically and we do not differentiate between them here. SHH upregulated expression of *Gli1* and *Ptch1* and increased the proliferation of cells (Fig. 3A-E). Expression of *Gli2* or *Gli3* was not upregulated (Fig. 3B). SHH treatment of tectal explants induced an approx. twofold increase in the number of BrdU-positive cells (Fig. 3C,E: control, 20.1± 3.6 cells/explant section; SHH treated, 40± 5 cells/explant section; $P=0.005$). As in the cortex, the increase in the number of BrdU-positive cells was seen near the vz (Fig. 3C,D), and FK, but not ddFK, was also able to inhibit BrdU incorporation by approx. two-fold (Fig. 3E: ddFK treated, 12.8±5.9 cells/explant section; FK treated, 7.3±2.5 cells/explant section; $P<0.01$).

SHH is a mitogen for nestin-positive neocortical precursors

To investigate the identity of BrdU-positive cells seen after SHH treatment, we tested for the mitogenic effects of SHH on dissociated P3 cortical cells. Addition of BrdU for 2 hours after 48 hours in culture showed an approx. twofold increase in the percentage of BrdU-positive cells over total cell number per field in SHH-treated versus control untreated samples (Fig. 3E, first panel: control, 7.1±0.8%; SHH treated, 14.8±0.4%; $P<0.005$). Double-labeling analyses showed that BrdU-positive cells with nuclear labeling were also nestin-positive cells with cytoplasmic labeling (Fig. 4A,B,E; 88±2.5% BrdU-positive cells per field were also nestin positive in control samples, and 93±3.2% in SHH-treated samples). Because the intermediate filament nestin identities precursors (Lendahl et al., 1990), including radial glia (Hockfield and McKay, 1985), this result indicates that SHH acts on precursor cells to increase their proliferation, a result consistent with the proliferation of E13.5 neurospheres after SHH treatment (Kalyani et al., 1998). By contrast, only very rarely did BrdU-positive cells express the astrocyte marker GFAP or the oligodendrocyte marker O4 (Fig. 4C-E and not shown). The identity of BrdU-positive cells as Nestin-positive precursors is in accordance with the localization of BrdU-positive cells in the vz/svz of explants (Fig. 2F,G).

The brain of *Shh* mutant late embryos show decreased proliferation in the vz/svz

Our experimental in vitro data predicts that lack of SHH in vivo will result in a downregulation of proliferation in the vz/svz of the neocortex. *Shh*-null mice die at birth and display severe holoprosencephaly (Fig. 5A), resulting in part from the complete lack of ventral CNS structures and the malformation of the face (Chiang et al., 1996). However, dorsal CNS structures are present, as demonstrated by the maintained expression of the *Emx1*, *Otx2* and *En2* in the mutant neocortex, diencephalon and tectum, respectively (Chiang et al., 1996). It is thus feasible to ask whether the dorsal-only brain of *Shh* null mice (Fig. 5L,M) displays normal proliferation prenatally.

Both RT-PCR analyses and in situ hybridization of E18.5 *Shh* homozygote null embryos revealed that the presumptive cortex expresses the neocortical marker *Tbr1* and is present adjacent to the proboscis, which does not express *Tbr1* (not shown). To analyze cell proliferation, a pulse of excess BrdU was given to pregnant mothers 2 hours before embryo collection. Within the cortex, cell proliferation was decreased nearly fourfold in mutant versus control embryos (Fig. 5B,C,F,G; 11.2±0.6% BrdU-positive cells in the vz/svz of wild-type embryos versus 3±0.7% BrdU-positive cells in mutant embryos, $P<0.001$; counting the vz/svz as a width of 10 cell diameters from the ventricle). Nevertheless, nestin-positive vz/svz cells (Fig. 5D,E), Tuj1-positive neurons (Fig. 5F,G) and GFAP-positive astrocytes (not shown) could be observed in both wild-type and mutant cortices. More posterior brain regions also showed a marked decrease in proliferation. For example, in the midbrain areas, the malformed tectum showed one-fifth to a half the number of BrdU-positive cells, and E18 mutant mice lacked a recognizable cerebellum, even though the adjacent choroid plexus was clearly present (Fig. 5H,I). Indeed, by E15.5, the cerebellum was already drastically reduced morphologically (Fig. 5L arrow), in line with previous work on the indispensable role of SHH in cerebellar development (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999).

In contrast to the decrease in the proliferation of the vz/svz of the brain, other parts of the mutant embryo appeared to proliferate normally or even to overproliferate. For example, basal epidermal cells appeared to have the same amount of BrdU-positive cells in mutants and wild-type littermates (Fig. 5J,K; 34.3±1.3 BrdU-positive cells per field in the basal layer of the epidermis in mutant embryos versus 36.2±3.1 BrdU-positive cells per field in the basal layer of the interfollicular epidermis of wild-type littermates, $P>0.5$), even though the mutant skin lacked hair follicles (Fig. 5K) (St-Jacques et al., 1998). The liver, by contrast, appeared to overproliferate, as judged by the number of BrdU- and HNF-3β-labelled cells (not shown).

GLI gene expression in primary brain tumors and brain tumor cell lines

Our findings in normal development raise the possibility that inappropriate activation or maintenance of the SHH-GLI pathway could lead to hyperproliferation, the basis of tumorigenesis. To test this idea, we first analyzed sporadic human brain tumors for the consistent expression of the *GLI* genes. We tested by RT-PCR seven glial tumors and primitive neuroectodermal tumors (PNETs), including those from the cerebellum (medulloblastomas), because the latter have been shown previously to harbor *PTCH1* mutations, suggesting the activation of the SHH-GLI pathway (Wolter et al., 1997; Raffel et al., 1997). We found that all the tumors tested expressed *GLI1*, although at different levels (Fig. 6A). Additional analyses for a total of 22 tumors (Fig. 6B) showed that all samples contained the three *GLI* transcripts. Expression of *PTCH1* followed the expression of *GLI1/2*, while that of *SHH* was not consistently detected (Fig. 6B).

In situ hybridization analyses of 22 independent brain tumors showed that *GLI1* was expressed in neuronal and in glial tumors, including glioblastoma multiforme and low grade glioma (Fig. 6C,E). *GLI1* and *PTCH1* mRNAs were detected in the regions containing tumor cells and not in surrounding normal tissues (Fig. 6E,G,F,H).

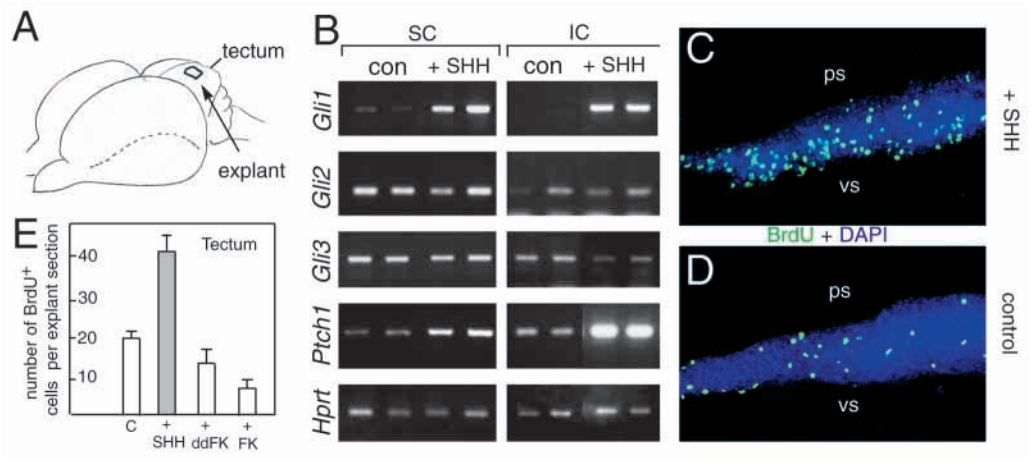

Fig. 3. SHH induces gene expression and proliferation in tectal explants. (A) Sketch of a lateral view of a ~P2 mouse brain showing the position of explanted tissue within the tectum. (B) RT-PCR analyses of gene expression in untreated control (con) or SHH-treated (+SHH) mouse P2 superior (SC) or inferior (IC) colliculi explants. Expression of *Gli1* and *Ptch1* is clearly upregulated. The low expression of *Gli1* is barely detectable in the IC but is clearly seen after additional cycles. (C,D) Increase in the number of BrdU-positive cells in IC explants treated with SHH (C) when compared with an untreated IC explant (D). ps, pial surface; vs, ventricular surface. Seven independent explants were used for RT-PCR from each the IC and SC. (E) Quantification of cell proliferation by SHH treatment or inhibition of SHH signaling on P2 mouse IC explants. Numbers of cells±s.e.m. are given. *n*>10 sections of five independent explants counted for each condition.

Analyses of human brain tumor cell lines, including seven glioblastoma (U87MG, U118MG, U138MG, A172, T98G, M059K, M059J), two glioma (Hs683, mouse GL261), one neuroglioma (H4), three astrocytoma (CCF-STTG1, SW1088, SW1783), three medulloblastoma (Daoy, D283, D341) and two neuroblastoma (SK-N-AS, IMR32) lines, showed that all brain tumor cell lines co-expressed *GLI1*, *GLI2* and *PTCH1* (Fig. 6I). *GLI3* was expressed by all but one (D341) and only a subset (U87MG, U138MG, Daoy, M059K, SW1783) expressed *SHH* (not shown).

As a control, we also tested a panel of unrelated sporadic human tumors by RT-PCR and found that *GLI1* was expressed consistently in prostate carcinomas (9/11 cases) but not in those from the breast (1/7), suggesting that prostate cancer may also result from deregulated SHH-GLI signaling.

Cyclopamine modulates the proliferation of a subset of brain tumor cells

Expression of the *GLI* and *PTCH1* by glioma cells raised the possibility that these harbor mutations that activate the pathway at different levels. Indeed, we expected that only a fraction of these possible mutations would affect the PTCH-SMO receptor complex. To address this possibility we have tested the effects of cyclopamine, a drug that inhibits the function of oncogenic Smoothened forms (Taipale et al., 2000). The glioblastoma/glioma lines U87, U118, U138, M059K, Hs683, C6, GL261, astrocytoma lines, SW1088 and SW1783, and the medulloblastoma line Daoy were tested and four responded to cyclopamine treatment by decreasing BrdU incorporation by ~25-50% (Fig. 6J). These are the glioma lines U87, U118 and U138, and the medulloblastoma line Daoy: untreated U87, 22.5±1.4% BrdU-positive cells/field; 0.5 μM cyclopamine treated, 16.5±0.9% BrdU-positive cells/field, $P<0.005$; and 5 μM cyclopamine-treated, 13.2±1.2% BrdU-positive cells/field, $P<0.001$; untreated U118, 12.9±1.3% BrdU-positive cells/field; 0.5 μM cyclopamine treated, 6.7±0.8% BrdU-positive cells/field, $P=0.001$; and 5 μM cyclopamine treated, 7.1±1.1% BrdU-positive cells/field, $P<0.005$; untreated U138, 13.3±0.7%

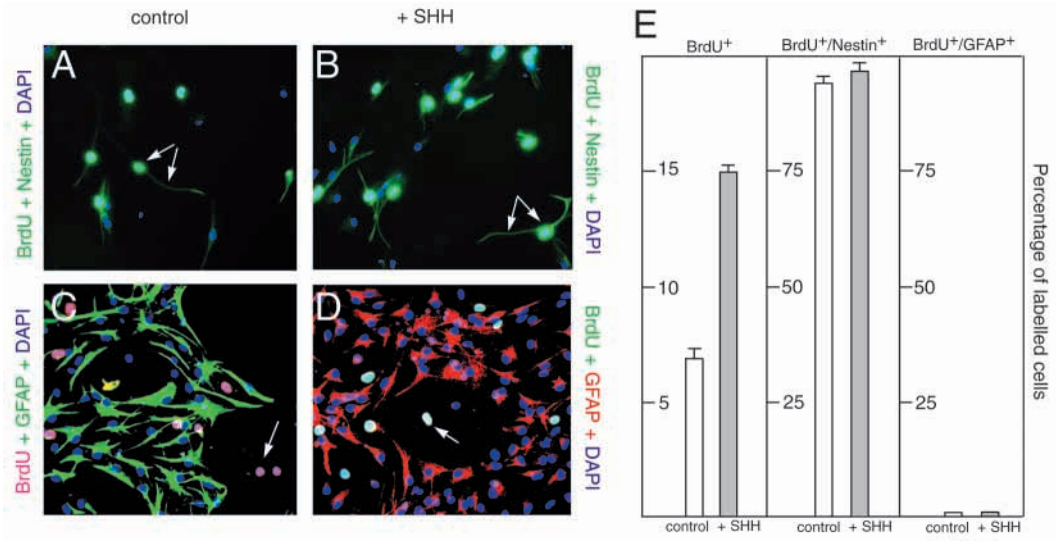

Fig. 4. SHH induces proliferation of nestin-positive cells in the postnatal mouse cerebral neocortex. (A,B) Co-labeling of P3 mouse cortical dissociated cells with anti-nestin and anti-BrdU antibodies. In both control (A) and SHH-treated (B) samples, dividing cells express cytoplasmic nestin (arrows). Nuclei are counterstained with DAPI. Response to SHH induction in dissociated cells included upregulation of *Ptch1* (not shown). (C,D) Labeling of dissociated cells with anti-GFAP and anti-BrdU antibodies. BrdU-labeled cells (arrows) do not express GFAP in control (C) or SHH-treated (D) samples. Nuclei are counterstained with DAPI. (E) Quantification of labeling results with dissociated cells. Numbers are percentages of single- or double-labeled cells±s.e.m. over the total number of cells as seen by DAPI staining. *n*>1000 cells counted for each sample.

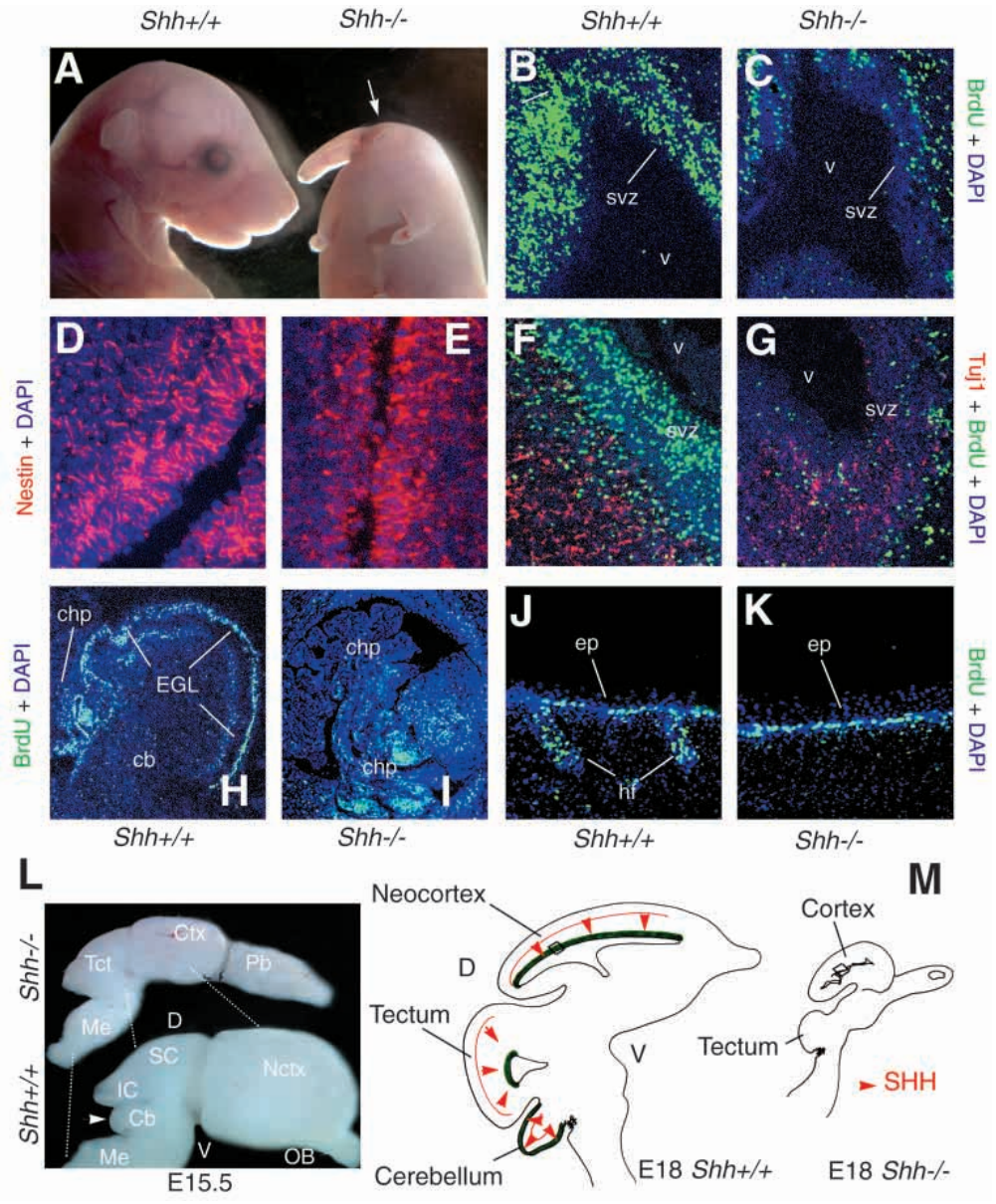

Fig. 5. Decreased precursor proliferation in the brain of *Shh* null embryos. (A) Morphological appearance of an E18.5 *Shh* null (right) and a wild-type littermate (left). Arrow points to the position of the forebrain, immediately posterior to the proboscis. (B,C) Details of the ventricular areas of the neocortex of wild-type (B) and in the forebrain of E18.5 *Shh* null embryos seen in sagittal sections (C) showing the marked decrease in proliferation in the vz/ svz in the mutant. (D,E) Expression of nestin in the vz/svz of both wild-type (D) and mutant (E) cortices. (F,G) Expression of the neuronal tubulin marker Tuj1 (red) in the neocortical plate of a wild-type embryo (F) and in the cortex region of a *Shh*-null sibling (G). (H,I) The characteristic proliferation of EGL cells in the E18.5 wild-type cerebellum (H) is missing in a sibling mutant brain (I), where no structure is morphologically recognizable as a cerebellum adjacent to the posterior choroid plexus (chp). (J,K) Proliferation of interfollicular cells appears normal in the wild-type (J) and mutant (K) epidermis (ep). Hair follicles (hf) are absent in the mutants. (L) Lateral views of dissected E15.5 wild-type (bottom) and *Shh* mutant (top) brains showing the existence of a morphologically defined cortex (Ctx), tectum (Tct) and medulla (Me) areas, as well as an elongated proboscis (Pb) anteriorly. The corresponding parts of the mutant brain and the wild-type littermate are indicated by white lines. Note the apparent presence of superior (SC) and inferior (IC) colliculi in both brains, but the apparent absence of the cerebellum (Cb, arrowhead) in the mutant. The olfactory bulb (OB) is also indicated in the normal brain. D, dorsal; Nctx, neocortex; V, ventral. (M) Camera lucida drawings of the outlines of sagittal sections of the brains of an E18 wild-type embryo (left) and a *Shh*-null sibling (right) at scale. The red lines and arrowheads indicate the source and deduced action of SHH protein in the neocortex, tectum and cerebellum.

BrdU-positive cells/field; 0.5 μM cyclopamine treated, 9.5±0.6% BrdU-positive cells/field, $P<0.005$; and 5 μM cyclopamine-treated, 7.5±1.2% BrdU-positive cells/field, $P=0.001$; untreated Daoy, 37.9±2% BrdU-positive cells/field; 0.5 μM cyclopamine treated, 27.7±1.5% BrdU-positive cells/field, $P=0.001$; and 5 μM cyclopamine-treated, 27.1±1.7% BrdU-positive cells/field, $P=0.001$. While it is unclear why these four lines respond differently, these results show that their proliferation is modulated by cyclopamine-sensitive targets. Non-responsive cells could have mutations that affect the activation of the pathway downstream of the receptor complex.

Cyclopamine was also tested in three primary cortical gliomas that were dissociated and cultured in vitro. Dividing cells from all three tumors expressed vimentin (not shown), which marks neural precursors in culture (Palmer et al., 1999) among other cell types. These cells also expressed *GLI1* and *GLI2* but not *Shh*. Treatment with 5 μM cyclopamine resulted in the inhibition of BrdU incorporation in one of them by ~60% (untreated tumor 3, 11.4±1.5% BrdU-positive cells/field; cyclopamine treated, 3.6±0.5% BrdU-positive cells/field, $P<0.001$), while the other two were unresponsive (untreated tumor 4, 4.5±0.5% BrdU-positive cells/field; treated, 4.6±0.5% BrdU-positive cells/field, $P>0.9$; and untreated tumor 5, 2.4±0.7% BrdU-positive cells/field; treated, 3.2 ±0.3% BrdU-positive cells/field, $P=0.3$).

Deregulated GLI1 function is sufficient to induce hyperproliferation of CNS cells with precursor character

The results with brain tumors and cell lines suggest that the deregulated SHH-GLI pathway may be involved in abnormal

proliferation. To directly test this idea, we have misexpressed Gli1 in the CNS of the developing frog embryo. Tadpoles expressing Gli1 after unilateral injections developed ipsilateral neural tube hyperplasias (Fig. 7A; 24/36 embryos), first detected at tailbud stages, that expressed the β-gal lineage tracer (Fig. 7C; 15/15 embryos). Most hyperplasias appeared in the hindbrain and spinal cord, consistent with the more frequent distribution of the injected materials in these areas, and showed an increase in the number of BrdU-positive cells (more than fivefold: 19±1 BrdU-positive cells on average per control neural tube side in three sections counted of independent control embryos and 91±5.6 BrdU-positive cells per injected neural tube side in three sections of independent Gli1-injected tadpoles; $P<0.005$) when compared with the normal, uninjected contralateral side where BrdU-positive cells were confined to the vz zone (Fig. 7D). As expected, abnormal tissue contained HNF-3β-positive floor plate cells and neurons (Fig. 7B and not shown) (Lee et al., 1997; Ruiz i Altaba, 1998) but a large proportion of the hyperplastic masses had an undifferentiated appearance. In cases where the injected *Gli1* RNA localized to the epidermis, the resulting skin hyperplasias or BCC-like tumors (Dahmane et al., 1997) also showed a marked increase in the number of BrdU-positive cells (not shown) over that seen in the normal, contralateral epidermis. Gli2 or Gli3 did not have these effects because they induced ectopic mesoderm earlier (Brewster et al., 2000).

The expression of *Gli1* in glial tumors, together with the involvement of the SHH-GLI pathway in the development of glial lineages (Pringle et al., 1996; Poncet et al., 1996; Orentas et al., 1999; Nery et al., 2001), raised the possibility that Gli1-induced hyperplasias could contain glial precursors. *Pdgfrα* labels oligodendrocyte and other precursors (Pringle and Richardson, 1993) and is a marker of gliomas (Smits and Funa, 1998). In tadpoles, *Pdgfrα* is first expressed within the CNS in a small group of bilateral cells of the central vz of the diencephalon (Fig. 7E), overlapping *Gli1* expression (not shown). *Gli1*-injected tadpoles showed ectopic expression of *Pdgfrα* within the hyperplasias (Fig. 7F; 20/45). Control uninjected tadpoles (0/60) failed to show ectopic expression of *Pdgfrα*.

To test if these hyperplasias include precursor cells with vz character, we have used the expression of endogenous *Gli1* as a marker, as this gene is only transiently expressed in vz cells in normal development (Lee et al., 1997). Sectioning human *GLI1* RNA-injected embryos after in situ hybridization with a frog *Gli1* probe (which does not cross-hybridize with the injected, exogenous human RNA under the conditions used) (Dahmane et al., 1997) showed that the majority (12/16 embryos) had unilateral hyperplasias ectopically expressing endogenous *Gli1* (Fig. 7G,H).

Activation of endogenous Gli1 function is required for hyperproliferation

The expression of endogenous *GLI1* in tumors and in induced tadpole hyperplasias (Fig. 6, Fig. 7G,H) raised the possibility that its function is required in tumor formation. This could be consistent with the observation that abnormal growths in the CNS or epidermis were first detected towards the late neurula-early tailbud stages, when most of the injected material had already been degraded. To test if endogenous Gli1 is required for tumor development, we injected human *GLI1* RNA along with a morpholino antisense oligonucleotide specific for the endogenous frog *Gli1* mRNA that does not recognize the injected human *GLI1* RNA. Injection of human *GLI1* and *lacZ* RNAs resulted in the development of both CNS and skin hyperplasias (Fig. 7I; 35/36). By contrast, co-injection of these same RNAs plus morpholino anti-frog *Gli1* resulted in normal development without tumor formation (Fig. 7J; $n=1/83$). This effect is specific, as morpholino anti-frog *Gli2* ($n=47/52$), morpholino anti-frog *Shh* ($n=48/50$) or a control unrelated morpholino ($n=49/49$) did not prevent tumor development by co-injected human *GLI1* (not shown).

DISCUSSION

The results presented here demonstrate that the SHH-GLI pathway has a general role in controlling progenitor cell number in the developing dorsal brain, and contrast with its earlier role in ventral neural tube patterning. The approx. twofold changes in proliferation we detect in neocortical and tectal precursor after manipulating SHH signaling are similar to those we detected in the cerebellum (Dahmane and Ruiz i Altaba, 1999), indicating that SHH signaling modulates the normal growth of the three major dorsal brain regions that are layered and evolutionarily plastic. Additionally, our findings demonstrate that deregulation of SHH-GLI signaling in the CNS leads to hyperproliferation of precursor cells and suggests its involvement in the initiation and maintenance of brain tumorigenesis.

Sources of SHH and effects in the dorsal brain

Shh is the only Hh family member reported to be normally expressed in the mammalian CNS (Echelard et al., 1993; Traiffort et al., 1999), raising the question of the localization of its sources that affect dorsal brain development. SHH is abundantly expressed in ventral brain regions throughout embryogenesis and is required for ventral development (Chiang et al., 1996). By contrast, early dorsal neural tube development requires the absence of SHH as dorsal cells, including those in the prospective cerebral cortex and tectum, can be ventralized by SHH (Roelink et al., 1994; Ericson et al., 1995; Kohtz et al., 1998; Watanabe and Nakamura, 2000; Agarwala et al., 2001). However, forebrain competence for ventralization is lost by ~E11.5 in mice: SHH does not repress the cortical markers *Emx1* or *Tbr1* in E17.5 explants (not shown) as it does in similar ~E10-11.5 explants (Khotz et al., 1998). After this early period, there is a change in the response to SHH. We show that *Shh* is expressed dorsally and that it is an endogenous late embryonic and postnatal mitogen modulating dorsal brain growth. From E14.5 to E17.5, we cannot localize the expression of *Shh* by in situ hydridization in the dorsal brain. As *Shh* may be expressed by layer V cortical neurons (Fig. 1) (Traiffort et al., 1999) and these are born at ~E13-E14, it is possible that their precursors already express *Shh* in the vz, where they may affect neighboring cells. It is also possible, however, that as in the cerebellum (Dahmane and Ruiz i Altaba, 1999), *Shh* is expressed transiently by precursor cells, which later become dependent on SHH secreted from mature neurons at a distance.

SHH could also have survival functions, although TUNEL assays in our cortical explants did not show obvious differences between treated and untreated samples (not shown). Moreover, this effect is first detected at fivefold higher concentrations than those used here (Miao et al., 1997; Oppenheim et al., 1999).

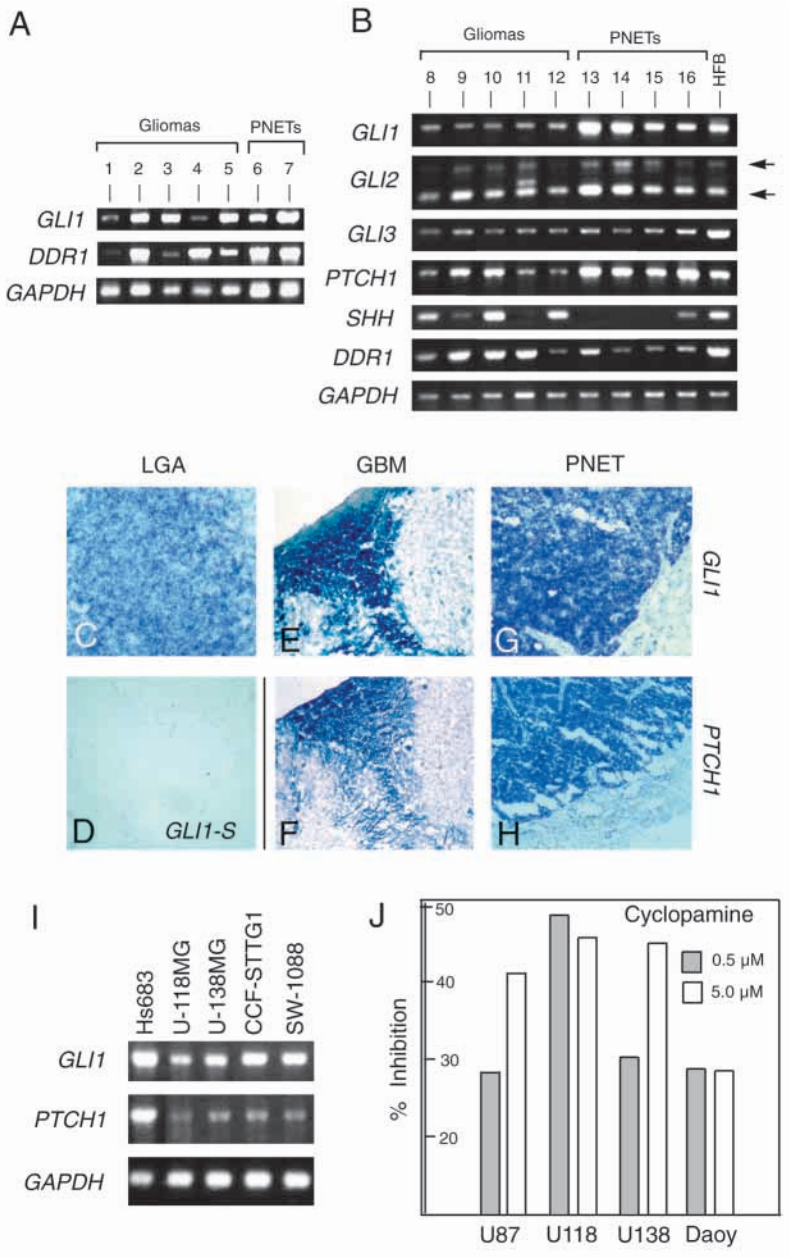

Fig. 6. GLI gene expression in human brain tumors, cell lines and effects of cyclopamine. (A,B) RT-PCR analyses of independent brain tumor samples. Note the varying levels of *GLI1* and *GLI2* expression and its general correlation with the levels of *PTCH1*. HFB, human fetal brain RNA, used here as control; PNETs, primitive neuroectodermal tumors. Varying levels of two *GLI2* bands in B represent previously described differentially spliced forms (arrows) (Tanimura et al., 1998). Levels of expression are interpreted in relation to that of discoidin domain receptor 1 (*DDR1*) mRNA, used to measure the relative amount of tumor in a given sample (Weiner et al., 2000), assuming homogenous expression per tumor cell. Expression of the housekeeping gene *GAPDH* served as positive control to quantify the total amount of mRNA. A total of 22 *DDR1*-positive primary tumors were tested by RT-PCR. Of these, nine glioblastoma multiformes from the frontal, parietal or temporal lobes expressed *GLI1* (9/9), *GLI2* (7/9), *GLI3* (9/9), *PTCH1* (7/9) and *SHH* (7/9). Co-expression of *GLI1* and *SHH* could suggest that the origin of these gliomas is the *GLI1*- and *SHH*-positive SVZ of the lateral ventricle (N. D., D. Lim, A. Álvarez-Buylla, A. R. A, unpublished); one gliosarcoma from the temporal lobe and one anaplastic oligodendroglioma from the parietal lobe expressed all these genes; one low grade glioma from the insula expressed *GLI1*, *GLI3*, *PTCH1* but not *GLI2* or *SHH*; four PNETs from the posterior fossa expressed *GLI1*, *GLI3* and *PTCH1*; one PNET from the thalamus expressed all of these genes; and six PNETs from the cerebellum expressed all genes except *SHH*. Ages of the individuals with gliomas ranged from 20 to 74 years and those with PNETs from 2 to 38 years. There was no correlation of gene expression with gender. (C-H) In situ hybridization of cortical glioma (C-F) and cerebellar PNET (G,H) tumor sections showing the expression of *GLI1* (C,E,G) coincident with that of *PTCH1* (F,H). Sense *GLI1* RNA probes did not show specific hybridization (D; six tumors tested). The localization of regions with tumor was determined by the high levels of *DDR1* expression and the histopathological examination of Hematoxylin- and Eosin-stained sections (not shown). Matched slides of the same tumor are C,D, E,F and G,H. GBM, glioblastoma multiforme; LGA, low grade astrocytoma. A total of 22 *DDR1*-positive tumors were tested by in situ hybridization. Of these, seven GBMs from the temporal and parietal lobes expressed *GLI1* (7/7) and *PTCH1* (6/7); one oligodendroastrocytoma from the temporal lobe and one oligodendroglioma from the frontal lobe expressed both genes; two low grade gliomas from the cerebellum and centrum ovale expressed *GLI1* (2/2) and *PTCH1* (1/2); five juvenile pilocytic astrocytomas from the cerebellum, thalamus and hypothalamus expressed *GLI1* (4/5) and *PTCH1* (2/5); one anaplastic oligodendroglioma from the frontal lobe expressed both genes; two anaplastic astrocytomas from the frontal lobe expressed *GLI1* (2/2) and *PTCH1* (1/2); and three PNETs from the cerebellum, posterior fossa and occipital lobe expressed both genes. The age of individuals with glial tumors ranged from 6 to 60 years and those with PNETs from 3 to 17 years. There was no correlation of gene expression with gender. As controls, one ependymoma from the fourth ventricle and one hemangioma from the cerebellum did not express *GLI1* or *PTCH1*. (I) RT-PCR analyses of human brain tumor cell lines testing for the expression of *GLI1*, *PTCH1* and *GAPDH*. All cells tested express both *GLI1* and *PTCH1*. (J) Percentage inhibition of the proliferation of four human glioma cell lines by cyclopamine at the concentrations indicated as measured by BrdU incorporation.

Our results differ from those obtained in postnatal mice after SHH misexpression in the early forebrain using recombinant viruses, in which infected cells only become oligodendrocytes (Nery et al., 2001). This difference, however, may result from the fact that retroviral infections were performed at ~E9.5, when SHH induces oligodendrocyte differentiation in early forebrain cells (Zhu et al., 1999) but does not yet act as a dorsal mitogen. Later on, SHH continues to induce oligodendrocytes in the ventral forebrain (Tekki-Kessaris et al., 2001). In addition to being a mitogen for precursors, our results therefore raise the possibility that endogenous SHH is involved in late embryonic and postnatal dorsal oligodendrogenesis.

Our results also differ from those obtained after misexpression of SHH in the dorsal spinal cord, in which the SHH-induced an approx. twofold increase in the proliferation of early precursors ceases before E18 (Rowitch et al., 1999). Thus, the differential growth of the dorsal regions of the brain and spinal cord could be related to the inability of the latter to proliferate in response to SHH.

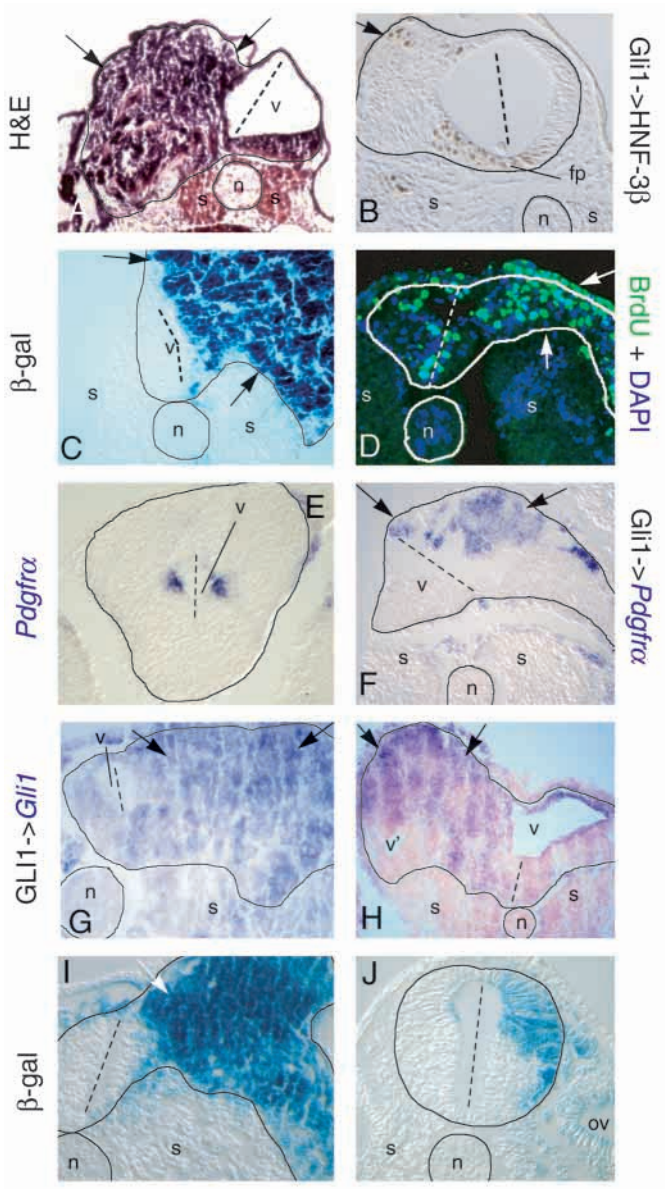

Fig. 7. Gli1 induces hyperplasia in the CNS of frog embryos. (A) Histological appearance of a Gli1-induced hyperplasia at the level of the hindbrain after sectioning and staining with Hematoxylin and Eosin. The unilateral development of hyperplasia is a consequence of the injection of *Gli1* RNA into one cell at the two-cell stage, using the uninjected half of the brain as internal control. (B) Section of a *Gli1*-injected embryo showing the ectopic differentiation of HNF-3β-positive cells within the hyperplastic region at the level of the midbrain. (C) Lineage tracing of a GLI1-induced hyperplasia in the neural tube. The section shows X-gal staining, indicating the development of a tumor from cells inheriting the co-injected *GLI1* and *lacZ* RNAs. (D) Analyses of BrdU incorporation in a GLI1-injected embryo showing a large increase in the number of BrdU-positive cells in the hyperpastic side (arrows) versus the uninjected, control side. (E,F) Endogenous expression of *Pdgfrα* mRNA in the midventricular zone of the diencephalon (E), and its ectopic expression within the CNS hyperplasia of a *Gli1*-injected embryo (F). (G,H) Constitutive expression of endogenous *Gli1* mRNA in hyperplastic regions (G, spinal cord; H, hindbrain) induced by injected human GLI1. (I,J) Hyperplasia resulting from the of injection of human *GLI1* RNA (I) or it complete absence after injection of *GLI1* RNA plus morpholino oligonucleotide anti-frog *Gli1*. X-Gal-stained cross sections are shown. The distribution of X-gal-labeled cells in J is as expected for animal pole injection. n, notochord; ov, otic vesicle; s, somites. All panels show representative cross sections of embryos at stages ~34-38 except A, which show sections of embryos at stage ~40. Arrows indicate induced CNS hyperplasia and/or sites of gene expression within them. Broken lines show the axis of normal bilateral symmetry. The CNS and notochord are independently outlined. The size of the notochord in all sections serves as scale. fp, floor plate; n, notochord; s, somite; v, ventricle; v′, secondary ventricle.

SHH signaling, growth modulation and morphological plasticity

The SHH-GLI pathway may not only modulate growth, and thus size, but also the shape of the brain. This idea derives from the proposal that differential use/localization of SHH would be responsible for the size and foliation patterns of the cerebellar cortex (Dahmane and Ruiz i Altaba, 1999) and from results in the ventral midbrain (Agarwala et al., 2001). Our experimental findings and the differential expression of *Gli* genes in the neocortex and tectum now allow us to extend this proposal to the two other major dorsal brain structures. It remains possible that SHH also regulates hippocampal cell proliferation. Indeed, changes in the spatial and/or temporal regulation of the SHH-GLI pathway within the dorsal brain could underlie the differential growth of the neocortex, tectum and cerebellum during evolution, as the sizes and shapes of these three structures vary enormously in phylogeny. A doubling of cortical cell proliferation could account for the development of the large primate neocortex (Rakic, 2000).

The late layer-specific expression of *Shh* in the dorsal brain (Fig. 1) suggests that it regulates *Gli1*$^{+}$ precursor proliferation in germinative zones. There appears to be, therefore, a common mechanism by which SHH secreted from early differentiated neurons in the neocortex, tectum and cerebellum regulates precursor proliferation and, thus, the number, of later-born cells. This system would allow the independent growth of each dorsal structure during evolution by changing the region-specific action of SHH or its response. In this sense, the action of additional signals, such as BMPs and PACAP (Li et al., 1998; Zhu et al., 1999; Suh et al., 2001), could affect growth by antagonizing the proliferative effects of SHH.

The robust induction of *Gli1* by SHH may pinpoint its primacy in the mediation of SHH signals (Lee et al., 1997; Hynes et al., 1997). However, because *Gli1* null mice appear normal (Park et al., 2000), Gli2/3 could compensate for the loss of Gli1. Consistent with this, all Gli proteins have neurogenic activity (Brewster et al., 1998), and *Gli3* mutant mice have smaller and disorganized cortices (Franz, 1994; Theil et al., 1999; Toole et al., 2000). Nevertheless, in the dorsal brain, as in the early embryonic neural tube (Ruiz i Altaba, 1998; Litingtung and Chiang, 2000), an antagonism between Gli3 and SHH/Gli1 may be crucial for normal development.

Hyperplasia, brain tumorigenesis and deregulation of the SHH-GLI pathway

In addition to sporadic BCCs and PNETs (Dahmane et al., 1997; Raffel, 1997; Xie et al., 1998; Reifenberger et al., 1998), we have found that two new types of sporadic human tumors, glial brain tumors and prostate carcinomas, consistently express the *GLI* genes. This expression may reflect their site of origin or the types

of cells affected. For example, in the *GLI1*-positive follicle or basal layer of the skin, maintained or ectopic GLI function could give rise to a BCC; in the *GLI1*-positive prostate (Podlasek et al., 1999), to carcinoma development; in the *GLI1*-positive cerebellum, to a medulloblastoma; in the *GLI1*-positive svz of the perinatal neocortex or striatum; and in adult regions where *GLI1*-positive precursors reside (such as the adult striatal svz (N. D., D. Lim, A. Álvarez-Buylla and A. R. A., unpublished) (Traiffort et al., 1999; Doetsch et al., 1999)), to a glioma.

Beyond being a marker of the origin of tumors, *GLI* expression is likely to reflect a participation of deregulated SHH-GLI signaling in tumorigenesis: GLI proteins may control precursor proliferation in many organs including the prostate and brain and their deregulation could lead to tumor formation. Support for this idea in brain tumors derives from the ability of cyclopamine to inhibit the proliferation of several human glioma cells, suggesting that these harbor mutations in the SMO-PTCH receptor (Taipale et al., 2000), that provoke constitutive signaling. Further support derives from our finding that the targeted, transient, somatic misexpression of GLI1 is sufficient to initiate a hyperplastic program in tadpoles, which could also occur in transgenic mice (Hynes et al., 1997; Park et al., 2000), that is dependent on endogenous Gli1 function. Moreover, mutations in *PTCH1* and *SMO* have been detected in non-medulloblastoma PNETs (Vorechovsky et al., 1997; Wolter et al., 1997; Reifenberger et al., 1998) but not yet in gliomas. Nevertheless, why individuals with Gorlin syndrome heterozygous for *PTCH1* develop some but not all tumor types associated with SHH-GLI signaling remains unclear. Interestingly, the consistent expression of the *GLI* genes in nearly all human primary brain tumors tested, as well as in all tested tumor cell lines, together with the action of cyclopamine, raises the possibility that SHH-GLI signaling also involved in tumor maintenance. If so, the viability of many human tumors in the brain and other organs could be based on persistent GLI function, thus providing an avenue for rational therapies.

We thank Moses Chao, Arturo Álvarez-Buylla, Sonya Baik, José Mullor and Gord Fishell for comments on the manuscript, the NYU tumor banks and D. Zagzag for specimens and for GL261 cells, M. Mercola for the probe for frog *Pdgfrα*, and B.Miller for O4 monoclonal antibody. A. R. A. and H. W. are members of the Kaplan Cancer Center. P. S. and V. P. were recipients of Fundación Ramón Areces and Pew Latin American Fellowship postdoctoral grants, respectively. H. W. was a recipient of awards from the American Brain Tumor Association/Emily Dorfman Foundation for Children and the Children's Brain Tumor Foundation. This work was supported by grants from the NIH-NINDS, NCI, the March of Dimes and The Pew Scholars Program to A. R. A.

REFERENCES

Agarwala, S., Sanders, T. A. and Ragsdale, C. W. (2001). Sonic hedgehog control of size and shape in midbrain pattern formation. *Science* **291**, 2147-2150.

Brewster, R., Lee, J. and Ruiz i Altaba, A. (1998). Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. *Nature* **393**, 579-583.

Brewster, R., Mullor, J. and Ruiz i Altaba, A. (2000). Gli2 functions in FGF signaling during antero-posterior patterning. *Development* **127**, 4395-4405.

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.

Cooper, M. K., Porter, J. A., Young, K. E. and Beachy, P. A. (1998). Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603-1607.

Dahmane, N. and Ruiz i Altaba, A. (1999). Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* **126**, 3089-3100.

Dahmane, N., Lee, J., Robins, P., Heller, P. and Ruiz i Altaba, A. (1997). Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature* **389**, 876-881.

Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-716.

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.

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., Morton, 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.

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.

Franz, T. (1994). Extra-toes (Xt) homoxygous mice demonstrate a role for the Gli3 gene in the development of the forebrain. *Acta Anat.* **150**, 38-44.

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 Dev.* **10**, 301-312.

Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.

Grachtchouk, M., Mo, R., Yu, S., Zhang, X., Sasaki, H., Hui, C. C. and Dlugosz, A. A. (2000). Basal cell carcinomas in mice overexpressing gli2 in skin. *Nat. Genet.* **24**, 216-217.

Hahn, H., Wojnowski, L., Zimmer, A. M., Hall, J., Miller, G. and Zimmer, A. (1998). Rhabdomyosarcomas and radiation hypersensitivity in a mouse model of Gorlin syndrome. *Nat. Med.* **4**, 619-622.

Heasman, J., Kofron, M. and Wylie, C. (2000). Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Hockfield, S. and McKay, R. (1985). Identification of major cell classes in the developing mammalian nervous system. *J. Neurosci.* **5**, 3310-3328.

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., Stone, D. M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A. and Rosenthal, A. (1997). Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* **19**, 15-26.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.

Kalyani, A. J., Piper, D., Mujtaba, T., Lucero, M. T. and Rao, M. S. (1998). Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. *J. Neurosci.* **18**, 7856-7868.

Kilpatrick, T. J., Richards, L. J. and Bartlett, P. F. (1995). The regulation of neural precursor cells within the mammalian brain. *Mol. Cell. Neurosci.* **6**, 2-15.

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.

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

Lee, J., Platt, K., Censullo, P. and Ruiz i Altaba, A. (1997). Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. *Development* **124**, 2537-2552.

Lendahl, U., Zimmerman, L. B. and McKay, R. D. G. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585-595.

Li, W., Cogswell, C. A. and LoTurco, J. J. (1998). Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP. *J. Neurosci.* **18**, 8853-8862.

Litingtung, Y. and Chiang, C. (2000). Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat. Neurosci.* **3**, 979-985.

Mabie, P. C., Mehler, M. F. and Kessler, J. A. (1999). Multiple roles of bone morphogenetic protein signaling in the regulation of cortical cell number and phenotype. *J. Neurosci.* **19**, 7077-7088.

Miao, N., Wang, M., Ott, J. A., D'Alessandro, J. S., Woolf, T. M., Bumcrot, D. A., Mahanthappa, N. K. and Pang, K. (1997). Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult in vitro. *J. Neurosci.* **17**, 5891-5899.

Nery, S., Wichterle, H. and Fishell, G. (2001). Sonic hedgehog contributes to oligodendrocyte specification in the mammalian telencephalon. *Development* **128**, 527-540.

Nilsson, M., Unden, A. B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P. G. and Toftgad, R. (2000). Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing Gli1. *Proc. Natl. Acad. Sci. USA* **97**, 3338-3543.

Nothias, F., Fishell, G. and Ruiz i Altaba, A. (1998). Cooperation of intrinsic and extrinsic signals in the elaboration of regional identity in the posterior cerebral cortex. *Curr. Biol.* **8**, 459-462.

Oppenheim, R. W., Homma, S., Marti, E., Prevette, D., Wang, S., Yaginuma, H. and McMahon, A. P. (1999). Modulation of early but not later stages of programmed cell death in embryonic avian spinal cord by sonic hedgehog. *Mol. Cell. Neurosci.* **13**, 348-361.

Orentas, D. M., Hayes, J. E., Dyer, K. L. and Miller, R. H. (1999). Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors. *Development* **126**, 2419-2429.

Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F. and Gage, F. H. (1999). FGF-2 activates a latent neurogenic program on neural stem cells from diverse regions of the adult CNS. *J. Neurosci.* **19**, 8487-8497.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M. and Joyner, A. L. (2000). Mouse Gli mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605.

Pietsch, T., Waha, A., Koch, A., Kraus, J., Albrecht, S., Tonn, J., Sorensen, N., Berthold, F., Henk, B., Schmandt, N., et al. (1997). Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of Drosophila patched. *Cancer Res.* **57**, 2085-2088.

Podlasek, C. A., Barnett, D. H., Clemens, J. Q., Bak, P. M. and Bushman, W. (1999). Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium. *Dev. Biol.* **209**, 28-39.

Poncet, C., Soula, C., Trousse, F., Kan, P., Hirsinger, E., Pourquie, O., Duprat, A.-M. and Cochard, P. (1996). Induction of oligodendrocyte progenitors in the trunk neural tube by ventralizing signals: effects of notochord and floor plate grafts, and of sonic hedgehog. *Mech. Dev.* **60**, 13-32.

Pringle, N. P. and Richardson, W. D. (1993). A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. *Development* **117**, 525-533.

Pringle, N. P., Yu, W.-P., Guthrie, S., Roelink, H., Lumsden, A., Peterson, A. C. and Richardson, W. D. (1996). Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog. *Dev. Biol.* **177**, 30-42.

Rakic, P. (2000). Radial unit hypothesis of neocortical expansion. *Novartis Found. Symp.* **228**, 30-42.

Raffel, C., Jenkins, R. B., Frederick, L., Hebrink, D., Aldrete, B., Fults, D. W. and James, C. D. (1997). Sporadic medulloblastomas contain *PTCH* mutations. *Cancer Res.* **57**, 842-845.

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

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

Rowitch, D. H., St-Jacques, B., Lee, S. M., Flax, J. D., Snyder, E. Y. and McMahon, A. P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J. Neurosci.* **19**, 8954-8965.

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

Ruiz i Altaba, A. (1999). Gli proteins and Hedgehog signaling: development and cancer. *Trends Genet.* **15**, 418-425.

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

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.

Salgaller, M., Pearl, D. and Stephens, R. (1991). In situ hybridization with single-stranded RNA probes to demonstrate infrequently elevated gli mRNA and no increased ras mRNA levels in meningiomas and astrocytomas. *Cancer Lett.* **57**, 243-253.

Smits, A. and Funa, K. (1998). Platelet-derived growth factor (PDGF) in primary brain tumours of neuroglial origin. *Histol. Histopathol.* **13**, 511-520.

St-Jacques, B., Dassule, H. R., Karavanova, I., Botchkarev, V. A., Li, J., Danielian, P. S., McMahon, J. A., Lewis, P. M., Paus, R. and McMahon, A. P. (1998). Sonic hedgehog signaling is essential for hair development. *Curr. Biol.* **8**, 1058-1068.

Suh, J., Lu, N., Nicot, A., Tatsuno, I. and DiCicco-Bloom, E. (2001). PACAP is an anti-mitogenic signal in developing cerebral cortex. *Nat. Neurosci.* **4**, 123-124.

Taipale, J., Chen, J. K., Cooper, M. K., Wang, B., Mann, R. K., Milenkovic, L., Scott, M. P. and Beachy, P. A. (2000). Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* **406**, 1005-1009.

Tanimura, A., Dan, S. and Yoshida, M. (1998). Cloning of novel isoforms of the human Gli2 oncogene and their activities to enhance Tax-dependent transcription of the human T-cell leukemia virus type I genome. *J. Virol.* **72**, 3958-3964.

Tekki-Kessaris, N., Woodruff, R., Hall, A. C., Gaffiels, W., Kimura, S., Stiles, C. D., Rowitch, D. H. and Richardson, W. D. (2001) Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon. *Development* **128**, 2545-2554.

Theil, T., Alvarez-Bolado, G., Walter, A. and Rüther, U. (1999). Gli3 is required for *Emx* gene expression during dorsal telencephalon development. *Development* **126**, 3561-3571.

Toole, S., Ragsdale, C. W. and Grove, E. A. (2000) Dorsoventral patterning of the mouse telencephalon is disrupted in the mouse mutant extra-toes. *Dev. Biol.* **217**, 254-265.

Traiffort, E., Charytoniuk, D., Watroba, L., Faure, H., Sales, N. and Ruat, M. (1999). Discrete localizations of hedgehog signalling components in the developing and adult nervous system. *Eur. J. Neurosci.* **11**, 3199-3214.

Vorechovsky, I., Tingby, O., Hartman, M., Stromberg, B., Noister, M., Collins, V. P. and Toftgard, R. (1997). Somatic mutations on the human homologue of Drosophila patched in primitive neuroectodermal tumors. *Oncogene* **15**, 361-366.

Wallace, V. A. (1999). Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. *Curr. Biol.* **9**, 445-448.

Wallingford, J. B., Seufert, D. W., Virta, V. C. and Vize, P. D. (1997). p53 activity is essential for normal development in Xenopus. *Curr. Biol.* **7**, 747-757.

Watanabe, Y. and Nakamura, H. (2000). Control of chick tectum territory along the dorsoventral axis by Sonic hedgehog. *Development* **127**, 1131-1140.

Wechsler-Reya, R. J. and Scott, M. P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* **22**, 103-114.

Weiner, H. L., Huang, H. Y., Zagzag. D., Boyce, H. M. K., Lichtenbaum, R. and Ziff, E. B. (2000). Consistent and selective expression of the DDR1 tyrosine kinase in human brain tumors. *Neurosurgery* **47**, 1400-1409.

Wolter, M., Reifenberger, J., Sommer, C., Ruzicka, T. and Reifenberger, G. (1997). Mutations in the human homologue of the Drosophila segment polarity gene patched (PTCH) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.* **57**, 2581-2585.

Xiao, H., Goldthwait, D. A. and Mapstone, T. A. (1994). A search for gli expression in tumors of the central nervous system. *Pediatr. Neurosurg.* **20**, 178-182.

Xie, J., Johnson, R. L., Zhang, X., Bare, J. W., Waldman, F. M., Cogen, P. H., Menon, A. G., Warren, R. S., Chen, L. C., Scott, M. P. and Epstein, E. H., Jr (1997). Mutations of the PATCHED gene in several types of sporadic extracutaneous tumors. *Cancer Res.* **57**, 2369-2372.

Zhu, G., Mehler, M. F., Zhao, J., Yung, S. Y. and Kessler, J. A. (1999). Sonic hedgehog and BMP2 exert opposing actions on proliferation and differentiation of embryonic neural progenitor cells. *Dev. Biol.* **215**, 118-129.