

Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex

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Summary

Stem cells are crucial for normal development and homeostasis, and their misbehavior may be related to the origin of cancer. Progress in these areas has been difficult because the mechanisms regulating stem cell lineages are not well understood. Here, we have investigated the role of the SHH-GLI pathway in the developing mouse neocortex. The results show that SHH signaling endogenously regulates the number of embryonic and postnatal mouse

neocortical cells with stem cell properties, and controls precursor proliferation in a concentration-dependent manner in cooperation with EGF signaling. These findings identify a crucial mechanism for the regulation of the number of cells with stem cell properties that is unexpectedly conserved in different stem cell niches.

Key words: Hedgehog, GLI, Neocortical stem cells, Mouse

Introduction

The regulation of the numbers of stem and precursor cells during development is of crucial importance for the control of brain size (Rakic, 1995; Caviness et al., 1995). Of the different growth factors reported to affect cell proliferation in the CNS (see Vaccarino et al., 1999; Coffin et al., 1999; Lillien and Raphael, 2000), Sonic hedgehog (SHH) has been recently implicated in cell proliferation and growth of the late embryonic and postnatal dorsal brain (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999; Dahmane et al., 2001; Lai et al., 2003). SHH is expressed in a layer-specific manner by differentiated cells in these structures, weakly in precursor populations, and is proposed to affect *Gli*⁺ cell proliferation in germinal zones (Dahmane et al., 2001). In mice, genetic analyses of the only known mediators of HH signals, the GLI transcription factors, have shown that GLI1 function is redundant (Park et al., 2000), whereas GLI2 and GLI3 show specific defects but also have overlapping functions (Franz, 1994; Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Theil et al., 1999). The GLI proteins thus act through a partly redundant combinatorial code that interprets HH signals in a context-dependent manner (reviewed by Ruiz i Altaba et al., 2002a). Even though SHH-GLI signaling affects precursor proliferation, it remains unclear whether SHH-GLI signaling affects stem cells in the vertebrate brain.

To address this question, we have investigated the role of SHH-GLI signaling in the developing mouse neocortex, analyzing the mid- and late-gestation brain phenotypes of *Shh*, *Gli2* and *Gli3* mutants. The results of these experiments, together with those testing for the function and requirement of SHH signaling in vivo as well as in vitro, carried out under conditions in which self-renewal and multipotentiality can be tested, indicate that the SHH-GLI pathway controls the number

of cells with stem cell properties in the developing neocortex. In addition, we find synergism between SHH and EGF signaling. We discuss the implications of these findings for brain development, for the ability to manipulate stem cell lineages and for tumorigenesis.

Materials and methods

Animals and treatments

Tissue from outbred Swiss-Webster mice were used for all in vitro assays unless otherwise specified. The *Shh* (Chiang et al., 1996), *Gli1* (Park et al., 2000) and *Gli2* (Mo et al., 1997) mutants from our colony were in this background. *Gli3^{X^{TJ}}* (Johnson, 1967; Hui and Joyner, 1993) mice in a C57 background were obtained from the Jackson laboratory (Bar Harbor, Maine). Octyl-modified recombinant SHH-N proteins were a kind gift of Ontogeny/Curis. Cyclopamine (cyc; Toronto Research Biochemicals) was used at 2.5–10 µg/ml dissolved in ethanol for in vitro experiments. Ethanol alone, at the same doses, was used in untreated, control in vitro samples. For all experiments that involved the use of cyc in vivo, five- to ten-week-old inbred C57/B16/J mice were used. Here, cyc was used at 1 mg/ml, conjugated with 2-Hydropropyl-β-Cyclodextrin (HBC; Sigma; prepared as a 45% solution in PBS) (van den Brink et al., 2001). These mice were injected daily, intraperitoneally (IP), for one week, with HBC alone as a control or with cyc-HBC at 10 mg/kg/day. For the in vivo treatment followed by the preparation of neocortical neurospheres (nsps), pregnant mothers were injected for 5 days with HBC alone or with cyc-HBC at 10 mg/kg/day.

BrdU incorporation, histology, immunofluorescence and in situ hybridization

BrdU treatment (20 mg/kg, IP injection), microtome (12 µm) or cryostat (10–18 µm) sections, immunofluorescence, in situ hybridization, and Hematoxylin and Eosin staining was performed as described (Dahmane et al., 2001). BrdU was added to cortical nsp

cultures, at 3 μ M, 7 hours prior to culture fixation. The following antibodies were used: beta III tubulin TuJ1 antibodies (1/300; Babco), Nestin (1/200, PharmingenBecton Dickinson), activated caspase 3 (1/50, R&D Systems), GFAP (1/500, Sigma), O4 (1/40; Roche). Pyknotic cells were identified by intense DAPI labeling. Cells undergoing apoptosis were identified by TUNEL reactivity and/or activated caspase 3 expression. Clone 53 derives from a screen for neocortical genes with patterned expression, and was used as a marker of vz/svz cells. Sense probes confirmed the specificity of in situ hybridization reactions. All probability values were obtained using the Student's *t*-test. Cells were quantified by counting the number of marker-positive cells as a percentage of DAPI-positive cells per ocular grid area at $\times 40$ magnification (5-12 random areas per experiment, from at least three independent samples).

RT-PCR and genotyping

Conditions and sequences were as described previously (Dahmane et al., 2001). Other primers were: mGLI2-1 (forward), gca gct ggt gca tca ta; and mGLI2-2 (reverse), cgg tgc tca tgt gtt tg. Reactions conditions were $T_m=55^\circ\text{C}$, producing an 828 bp band for the wild-type allele and a 913 bp band for the *Gli2* mutant allele. Primers for *Ihh* and *Dhh* were used at $T_m=58^\circ\text{C}$, and gave expected band sizes of 267 bp for *Ihh* and 311 bp for *Dhh* (Tekki-Kessaris et al., 2001). *Egfr* and *Emx1/2* primers were used at $T_m=55^\circ\text{C}$ and gave the expected band sizes of 459 bp for *Egfr*, 183 bp for *Emx1* and 247 bp for *Emx2* (Represa et al., 2001; Yoshida et al., 1997).

Neurospheres

Mid and late embryonic cortical nsps were obtained by standard procedures (see Doetsch et al., 2002). Cells were incubated in neurosphere medium [Neurobasal Medium (GIBCO), containing N2 (GIBCO), 2 mM glutamine, 0.6% (w/v) glucose, 0.02 mg/ml insulin, antibiotics and 15 mM HEPES] with 10 ng/ml of EGF (human recombinant, GIBCO) and 10ng/ml of bFGF (Upstate Biotech) unless otherwise noted. For proliferation assays, nsps were plated at 3000 cells/well onto polyornithine/laminin coated Lab-Tek chamber slides (Nunc) in the presence of EGF and FGF, and grown for 1 week. For differentiation assays, growing nsps were plated at 20,000 cells/well onto polyornithine/laminin coated (10 mg/ml) Lab-Tek chamber slides without growth factors and incubated for 5-7 days. For cloning assays, cells were plated either by dilution at 1 cell/well in 96-well plates (Nunclon), with 50% conditioned media [50% nsp-defined media containing EGF (10 ng/ml) and bFGF (10 ng/ml)], or by assessing clonal sphere colony formation at low cell densities in 1/3 conditioned media documented to yield clonal cultures (Reynolds and Weiss, 1996; Tropepe et al., 2000; Seaberg et al., 2002). The number and size of cloned nsps was counted after one week in culture. For cloning assays with *Gli2*^{-/-} and wild-type littermate neurospheres at E18.5, healthy cells from the first or second passage were visually chosen for their round morphology and homogeneous appearance under the microscope, and manually transferred to single wells in 96-well plates (Nunclon). This was to avoid the potential problem of damaged *Gli2*^{-/-} cells, which are fragile, after dissociation of pelleted neurospheres.

Results

SHH and EGF signaling synergize to regulate neocortical precursor proliferation

In order to test for a direct effect of SHH signaling on neural stem cells, but lacking the means for the prospective identification of such cells in vivo, we have used neurosphere (nsp) culture to test for stem cell behavior. EGF can sustain nsp growth at late embryonic stages, whereas at earlier stages FGF is required (Reynolds and Weiss, 1996; Tropepe et al., 1999; Martens et al., 2000). Addition of recombinant human SHH (5

nM) to nsp media containing (10 ng/ml) EGF, or EGF plus (10 ng/ml) FGF2, induced higher levels of *Gli1* expression but did not increase cell proliferation of neocortical nsps from mid [embryonic day (E) 15.5] and late (E18.5) gestation stages (not shown). SHH (5nM) alone was also unable to initiate or sustain nsp growth (not shown). The induction of *Gli1*, however, indicated the ability of nsps to respond to SHH signaling, but proliferation parameters may already have been at a maximum with saturated growth factor levels. E15.5 and E18.5 wild-type cortical nsps were then selected in media containing 10 ng/ml EGF without FGF, which is only required at earlier stages, and were passed and grown at different concentrations of EGF. SHH (5 nM) synergized with EGF at concentrations of between 2.5 and 0.05 ng/ml to promote nsp growth, as measured by BrdU incorporation (Fig. 1A). Similarly, growth of nsps at 1 ng/ml EGF showed a concentration-dependent increase in proliferation by SHH between 1 and 5 nM (Fig. 1B). SHH treatment increased the levels of *Gli1* (Fig. 1D). In addition, nsp cultures expressed *Shh*, as well as *Ihh*, *Dhh* and *Gli1-3*, the RNAs of which were also detected in fresh cortical tissue (Fig. 1D; Fig. 2L; and not shown).

SHH signaling is required for normal proliferation and self-renewal of neocortical neurospheres

To address the role of SHH signaling on stem cell self-renewal, we assayed the number of E15.5 wild-type nsp cells able to give rise to secondary nsps in cloning assays. SHH treatment, in the presence of 2.5 ng/ml of EGF, increased the number of derived nsps over that of control by ~3-fold (Fig. 1C). Conversely, treatment with 5 μ M cyc, a specific inhibitor of HH signaling (Incardona et al., 1998; Cooper et al., 1998), reduced the number of secondary cloned nsps by 50% (Fig. 1C), and also decreased the level of *Gli1* (Fig. 1D).

SHH often acts in a concentration-dependent manner (reviewed by Ingham and McMahon, 2001). Therefore, to test for a concentration-dependent effect of inhibition of HH signaling by cyc, wild-type E15.5 and postnatal day 2 (P2) neocortical EGF-responsive nsps were selected and treated with varying concentrations of cyc. Treated and control nsps were assayed for BrdU incorporation and cloned. Increasing concentrations of cyc lead to a progressive decrease in the level of BrdU incorporation and in the number of secondary clones, as compared with non-treated nsps (Fig. 1E-H). SHH pathway activity thus regulates the size of the pool of nsp cells with self-renewal properties in a concentration-dependent manner.

Pharmacological inhibition of HH signaling in vivo

To test whether an in vivo effect of cyc could be discerned, cyclodextrin-conjugated cyc, or just cyclodextrin (HBC) as control, was injected intraperitoneally, daily, into pregnant mothers (van den Brink et al., 2001) carrying E10.5-E12.5 embryos, and these were collected five days later. By E10.5, the ventral forebrain has already received HH signaling and the eyes have formed. Consequently, cyc treatment did not cause cyclopia and the treated E15.5-E17.5 embryos appeared normal. Analyses of fresh neocortical tissue dissected ~12 hours after the last cyc injection showed that in vivo cyc treatment decreased the expression of *Gli1*, a loyal marker of SHH signaling (Lee et al., 1997), and abolished that of *Egfr* (Fig. 1I), whereas expression of *Gli2* and *Gli3* was unchanged

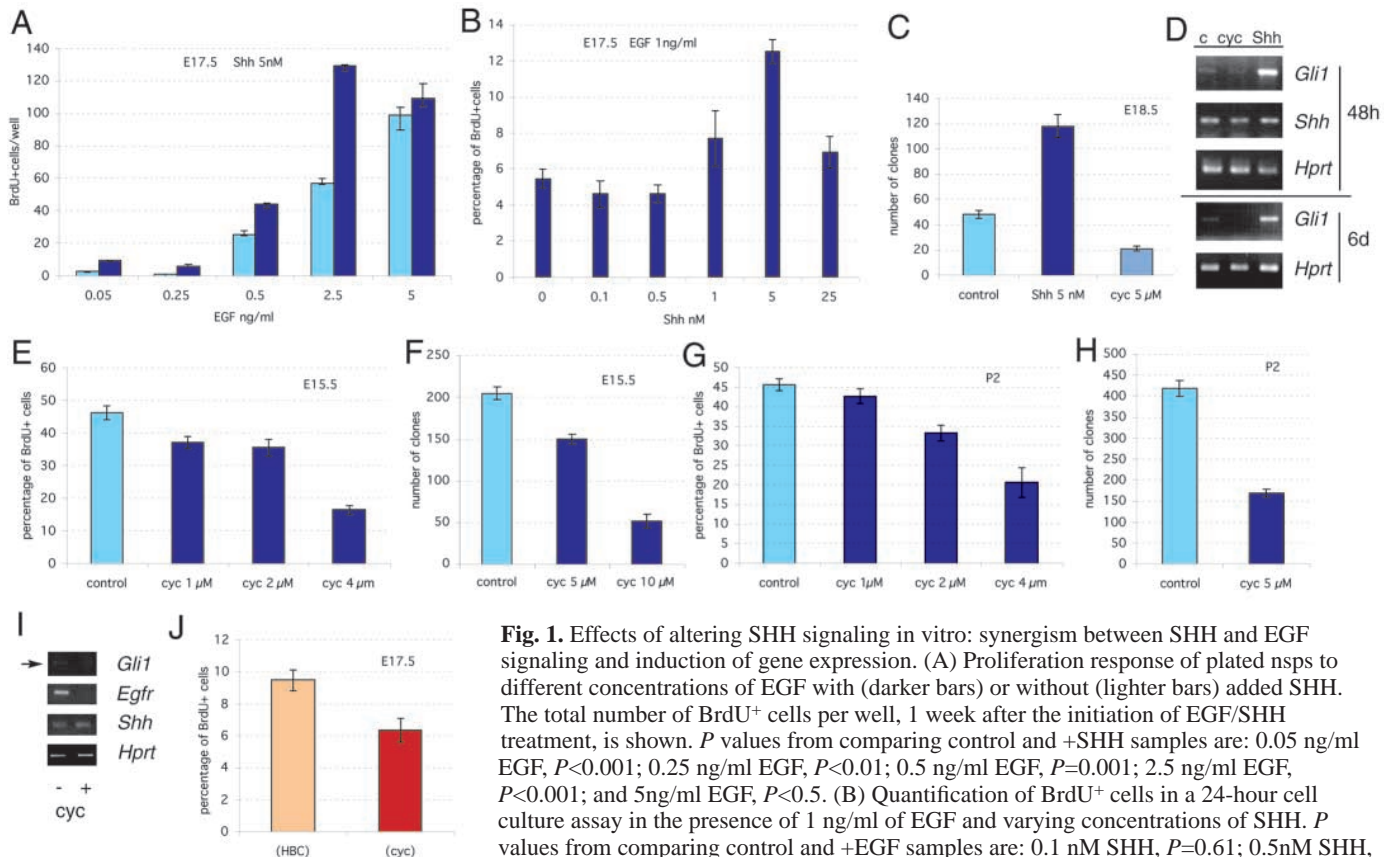


Fig. 1. Effects of altering SHH signaling in vitro: synergism between SHH and EGF signaling and induction of gene expression. (A) Proliferation response of plated nsps to different concentrations of EGF with (darker bars) or without (lighter bars) added SHH. The total number of BrdU⁺ cells per well, 1 week after the initiation of EGF/SHH treatment, is shown. *P* values from comparing control and +SHH samples are: 0.05 ng/ml EGF, *P*<0.001; 0.25 ng/ml EGF, *P*<0.001; 0.5 ng/ml EGF, *P*=0.001; 2.5 ng/ml EGF, *P*<0.001; and 5 ng/ml EGF, *P*<0.5. (B) Quantification of BrdU⁺ cells in a 24-hour cell culture assay in the presence of 1 ng/ml of EGF and varying concentrations of SHH. *P* values from comparing control and +EGF samples are: 0.1 nM SHH, *P*=0.61; 0.5 nM SHH, *P*=0.58; 1 nM SHH, *P*<0.5; 5 nM SHH, *P*=0.013; and 25 nM SHH, *P*=0.596. Similar

results were obtained with 48-hour cultures. (C) Cloning dilution assay (at 1 cell/μl, 2000 cells per well in 1/3 conditioned media and 10 ng/ml of EGF in uncoated 6-well plates) after a 1 week treatment of nsps in 2.5 ng/ml EGF, in the presence of either 5 nM SHH or 5 μM cyc. The total number of clones per well were counted in triplicate (*P*<0.0001). (D) RT-PCR analyses of E15.5 nsps, treated for 48 hours or 6 days in the presence of 5 nM SHH or 10 μM cyc. (E,G) Comparison of the percentage of BrdU⁺ cells at different concentrations of cyc, in the presence of 10 ng/ml EGF, at E15.5 (E) and P2 (G) after 24 hours. (F,H) 48-hour cloning assays in presence of 10 ng/ml of EGF and cyc as indicated. (F) 12.5 E15.5 cells/μl plated in 1 ml media in uncoated 12-well plates (*n*=3) and (H) 25 P2 cells/μl plated in 2 ml media in uncoated 6-well plates (*n*=6). The experiments in A-H were performed with cells selected from the beginning in EGF only. Similar differences were obtained counting clones after one week in culture. (I) RT-PCR analyses of fresh dissected cortex of control and cyc-treated embryos. Regulation of *Egfr* expression by SHH was also seen at E17.5 and P3 (not shown). All RT-PCR assays were repeated 2-3 times. (J) Quantification of BrdU incorporation in acutely dissociated cultures plated in the absence of growth factors from HBC- (control) or cyc-treated neocortexes in vivo. Cells were grown in the presence of one-third conditioned media without additional cyc. Histograms in all figures show mean±s.e.m.

(not shown). Acute dissociation of neocortical tissue at this time, and its subsequent culture without growth factors, confirmed that in vivo cyc treatment inhibits cell proliferation (Fig. 1J). To expand this finding, we analyzed neocortical cell behavior from mice mutant for *Shh*, *Gli2* and *Gli3*, as *Gli1* null mice appear normal (Park et al., 2000).

***Shh* mutant mice show deficits in cells able to form neurospheres**

Shh^{-/-} mice die at birth showing overt signs of cyclopia and lacking all ventral CNS cell types (Chiang et al., 1996). Their dorsal-only CNS comprises an *Emx1*⁺, *Tbr1*⁺ forebrain cortex (Chiang et al., 1996; Dahmane et al., 2001) (Fig. 2A). The *Shh*^{-/-} cortex produced nsp cultures in full media, but these were fewer and smaller than those from wild-type cortices, and contained fewer BrdU⁺ cells (Fig. 2B-G,M-O). Analyses of gene expression confirmed the loss of *Shh* transcripts in the few *Shh*^{-/-} nsps that formed (representing a small pool of viable cells). A decrease in *Ptch1* and *Dhh* expression was detected,

whereas the expression of *Ihh*, *Gli1* and *Gli2* were unchanged, and the expression of *Gli3* expression was slightly higher (Fig. 2L). These *Shh*^{-/-} nsps expressed nestin (Fig. 2H) and were tripotential, as judged by the ability to differentiate as Tuj1⁺ neurons, GFAP⁺ astrocytes or O4⁺ oligodendrocytes (Fig. 2I-K and not shown). Cloning assays showed that *Shh*^{-/-} nsps contain approximately one quarter of the number of nsp-forming stem cells of wild-type nsps at E15.5 (Fig. 2O). At E18.5, there were very few, if any, mutant nsps (Fig. 2O).

***Gli2* mutant mice display a mid and late embryonic dorsal brain phenotype**

Gli2^{-/-} mice also die at birth, displaying defects in multiple organs (Mo et al., 1997; Ding et al., 1998; Matise et al., 1998). We have found novel dorsal brain phenotypes of *Gli2*^{-/-} mice at mid and late gestation stages in an outbred background. *Gli2* null embryos present a variably penetrant severe phenotype, displaying exencephaly by E13.5 (also seen at E17-E18.5; not shown), and a consistent milder phenotype

characterized by expanded but thinner telencephalic vesicles, most clearly seen posteriorly, and an overtly reduced tectum and cerebellum (Fig. 3A-C). We have focused here on the non-exencephalic *Gli2*^{-/-} mice. Histological analyses showed that E18.5 *Gli2*^{-/-} telencephalic vesicles have a thinner proliferative zone (an ~30-50% reduction of the vz/svz; Fig.

3D). *Gli2*^{-/-} mice have fewer BrdU⁺ precursors in the cortex at mid and late gestation periods (Fig. 1E-G), suggesting defects in neuronal as well as glial cell populations. The decrease is most notable in the deeper proliferative area (the svz). Local variations without a clear pattern in the density of BrdU⁺ nuclei were also observed (not shown), indicating an additional degree of neocortical disorder in these mutant mice. TUNEL and activated caspase 3 analyses did not show an increase in apoptosis (not shown).

In the *Gli2*^{-/-} neocortex, *Gli1* transcripts were absent, there was a consistent decrease in the expression of mutant *Gli2* transcripts, and the expression of *Gli3* was diminished (Fig. 3J-O). The expression of *Neurod1*, which marks neuroblasts, and that of one vz/svz-specific cDNA, *clone 53*, showed a reduction of neuroblasts and vz/svz cells, respectively, by ~50% in *Gli2*^{-/-} cortex compared with that of wild-type littermates (Fig. 3P-U). Decreases in the number of *Neurod1*⁺ cells were detected in the *Gli2*^{-/-} cerebellum, which showed abnormal early foliation and more pronounced posterior defects (Fig. 3H,I), and in the hippocampus (Fig. 3P,Q; arrow).

Neocortical cells from *Gli2* mutant mice show compromised neurosphere-forming abilities

Gli2^{-/-} neocortices gave rise to nsps (Fig. 4A-D), containing Nestin⁺ cells (Fig. 4F) that were tripotential (Fig. 4G-I). However, at late embryonic stages mutant nsps progressively became smaller, more delicate, and showed more blebbing than wild-type nsps (Fig. 4A-D,J,K). *Gli2*^{-/-} nsps decreased in numbers during culture and, after a few passes (~6), they were rare, and all died soon after. The *Gli2*^{-/-} nsps surviving at passage ~2-4 lacked *Gli1* expression and showed downregulation of *Ihh* and *Dhh* expression. *Shh* expression was unchanged, whereas *Gli3* and *Ptch1* expression was reduced (Fig. 4E). The expression of *Egfr* was also reduced.

Gli3 mutant mice do not form stable neurosphere cultures

Gli3 mutant (*Xt^l*) mice die at birth. Those that are not exencephalic have a severely reduced cortex (Franz et al., 1994; Theil et al., 1999) (Fig. 5A), and we have used these for our analyses. Primary cultures of E18.5 *Gli3*^{-/-} neocortices in full nsp media yielded transiently forming clumps that

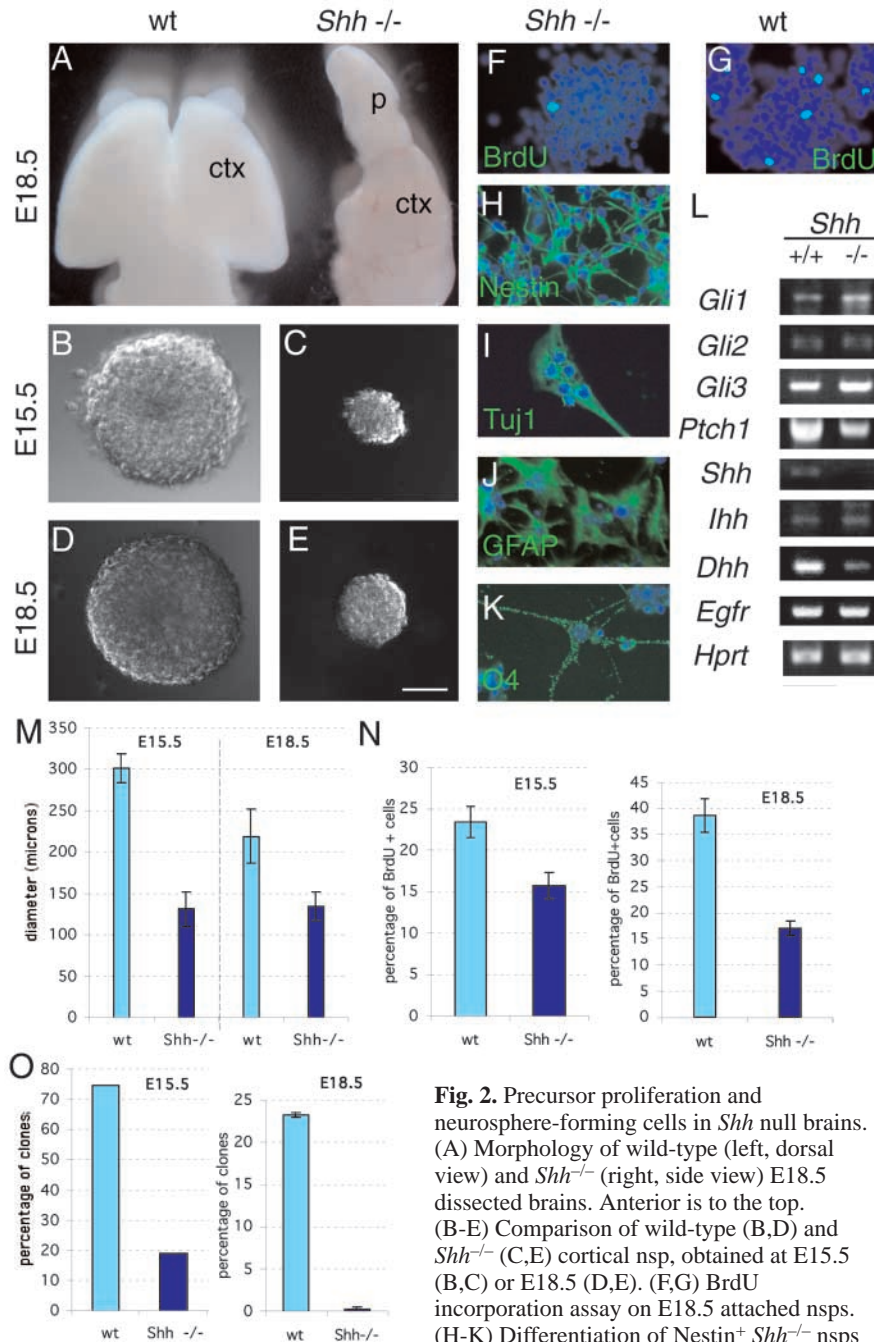


Fig. 2. Precursor proliferation and neurosphere-forming cells in *Shh* null brains. (A) Morphology of wild-type (left, dorsal view) and *Shh*^{-/-} (right, side view) E18.5 dissected brains. Anterior is to the top. (B-E) Comparison of wild-type (B,D) and *Shh*^{-/-} (C,E) cortical nsp, obtained at E15.5 (B,C) or E18.5 (D,E). (F,G) BrdU incorporation assay on E18.5 attached nsps. (H-K) Differentiation of Nestin⁺ *Shh*^{-/-} nsps (H) into Tuj1⁺ neurons (I), GFAP⁺ astrocytes (J) and O4⁺ oligodendrocytes (K). (L) RT-PCR analyses of E18.5 wild-type and *Shh* null nsp cultures. (M) Quantification of wild-type versus *Shh*^{-/-} E15.5 and E18.5 nsp size in clonal dilution assays (E15.5 wild type, *n*=12; *Shh* null, *n*=11; E18.5 wild type, *n*=11; *Shh* null, *n*=14; *P*<0.001 for E15.5; *P*<0.05 for E18.5). (N) Quantification of the number of BrdU⁺ cells (after 1 week in culture for both E15.5 and E18.5) after a 7 hour pulse in wild-type versus *Shh*^{-/-} nsp (E15.5, *P*=0.006; E18.5, *P*<0.001). (O) Quantification of E15.5 and E18.5 wild-type versus *Shh*^{-/-} nsp number (*P*<0.001 for both). Scale bar in E: 620 μm for A; 90 μm for B,C,E; 70 μm for D; 45 μm for F,G; 15 μm for H-K.

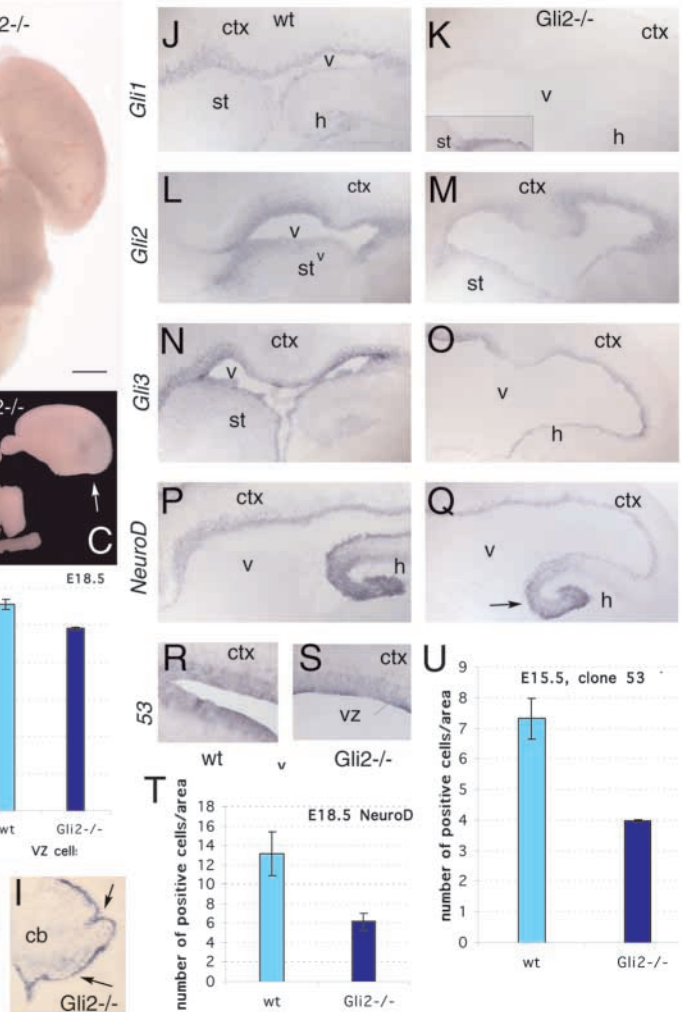
Fig. 3. Morphology and gene expression in *Gli2* null brains.

(A) Dorsal view of the morphology of wild-type (left) and *Gli2*^{-/-} (right) dissected E18.5 brains. Anterior is to the top. (B,C) Comparison of lateral views of dissected cortex, and dorsal views of tectum and cerebellum in wild-type (B) versus *Gli2*^{-/-} (C) brains. Arrows point to the posterior cortex in the two hemispheres, tectum and cerebellum. *Gli2*^{-/-} mutant mice have an apparently normal choroid plexus (not shown). The ballooning of the telencephalic vesicle may be due to the inability of the tissue, which is thinner than normal, to sustain the same degree of intraventricular pressure. (D) Comparison of wild-type and *Gli2*^{-/-} cortices seen in parietal sagittal sections stained for Hematoxylin and Eosin. The intermediate zone and cortical plate layers appear of normal size and cell density.

(E-G) BrdU incorporation in wild-type (F) and mutant (G) cortices, and quantification of cell proliferation (E). The mean number of BrdU⁺ cells per section from wild-type and *Gli2*^{-/-} animals is shown. For simplicity, the vz was

considered as the zone in between the ventricle and ~5-cell diameters away, and the svz as that in between ~5- and 10-cell diameters from the ventricle (svz cells, $P < 0.05$; vz cells, $P < 0.01$). (H,I) Comparison of *Neurod1* expression by in situ hybridization in the cerebellum of wild-type (H) and *Gli2*^{-/-} (I) E18.5 animals. (J-S) Images of in situ hybridization analyses of sagittal sections from E18.5 (J-Q), and coronal sections from E15.5 (R,S), wild type (J,L,N,P,R) and *Gli2*^{-/-} (K,M,O,Q,S) animals probed with *Gli1* (J,K), *Gli2* (L,M), *Gli3* (N,O), *Neurod1* (P,Q) or clone 53 (R,S). Neocortical *Gli1* expression is absent in *Gli2*^{-/-} animals, but a domain of its expression in the striatum was still present (inset, K). Note the small hippocampus in the *Gli2*^{-/-} brain (Q, arrow), compared with in wild type (P). Forebrain pattern appeared largely unchanged as expression of the *Pax6* anteroposterior gradient and of *Dlx2* ventrally were not grossly affected (not shown). (T,U) Quantification of the number of *Neurod1*⁺ (T) and clone 53⁺ (U) cells in the dorsal telencephalon of wild-type versus *Gli2*^{-/-} animals (*Neurod1*⁺, $P < 0.001$; clone 53⁺, $P < 0.001$). cb, cerebellum; cp, cortical plate; ctx, cortex; h, hippocampus; iz, intermediate zone; Med, medulla; st, striatum; svz, subventricular zone; tct, tectum; vz, ventricular zone. Scale bar in A: 800 μ m for A; 1.3 mm for B,C; 75 μ m for D; 50 μ m for F,G,H,I; 130 μ m for J-Q; 300 μ m for R,S.

rapidly degenerated, whereas nsp cultures from wild-type siblings formed stable nsp clusters (Fig. 5E,F). In one out of six cases, we were able to obtain a small number of nsps that survived up to 4 passes. Instead of nsps, E18.5 *Gli3*^{-/-} primary cultures normally yielded isolated attached cells with differentiated morphology that expressed neuronal antigens (Fig. 5E,F). After one week, a time when newly formed nsps are clearly visible in the wild-type control, there were almost no *Gli3* null nsps, and the cells with differentiated morphologies died soon after. At E15.5, the first nsps were detected after one week in both wild-type and mutant cultures, and these were able to be passed but most died in the first pass (Fig. 5B,C). The fraction of nsps that survived were tripotential (not shown). Most of these nsps, however, died after

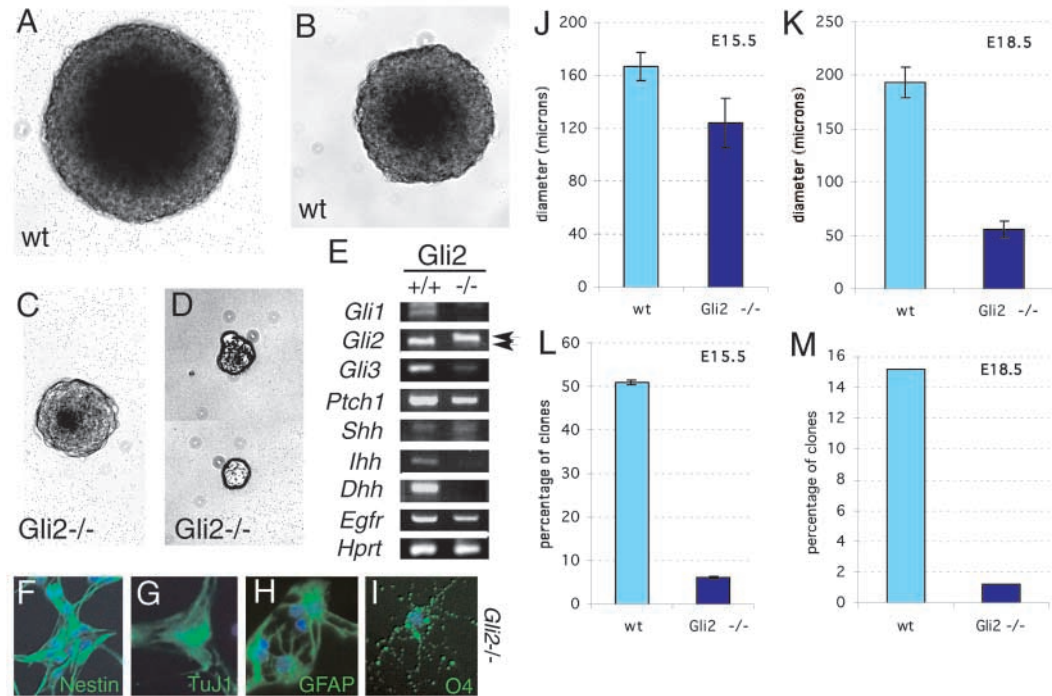


subsequent passes. The context-dependent function of GLI3 was further suggested by the finding that *Gli3*^{-/-} tectal nsps could be easily grown and passed (not shown). Neocortical *Gli3*^{-/-} E15.5 nsps expressed *Emx2* (Fig. 5D), but lacked *Emx1* (Theil et al., 1999) and *Gli3*, confirming their provenance. These cells had unchanged levels of *Gli2*, but expression of *Gli1* was absent, and that of *Shh* and *Ihh* were drastically reduced. By contrast, expression of *Dhh* was enhanced. Expression of *Egfr* and *Ptch1* was slightly reduced.

Discussion

The results we present here indicate that HH signaling represents an endogenous mechanism regulating the behavior

Fig. 4. Neurosphere cultures from *Gli2* null brains. (A-D) Phase contrast images of representative cortical nsps cultured from wild-type (A,B) and *Gli2*^{-/-} (C,D) animals at E18.5. (E) RT-PCR analyses of cortical nsps. Note the loss of *Gli1* expression, the shift in the *Gli2* mutant allele band (arrows), the reduced *Gli3* expression, and the induction of *Ihh* and to a lesser extent *Dhh* in *Gli2*^{-/-} cells. *Hprt* is shown as a control. (F-I) Expression of nestin in precursors of *Gli2*^{-/-} nsps (F), of TuJ1 in neurons (G), of GFAP in astrocytes (H) and of O4 in oligodendrocytes (I) after differentiation. Nuclei were counterstained with DAPI. (J-M) Quantification of nsp size at E15.5 (J) and E18.5 (K). An average of 20 nsps from two independent experiments is shown (E15.5, $P < 0.05$; E18.5, $P < 0.001$). Given their rarity at E18.5, single *Gli2*^{-/-} nsps were measured. (L,M) Quantification of nsps obtained in cloning assays. Three independent experiments are shown for E15.5 ($P < 0.001$) and one for E18.5. Scale bar: 75 μ m for A-D; 10 μ m for F-I.



of cells with stem cell properties in the developing mouse neocortex. The lack of specific, prospective neural stem cell markers, and the finding that nsps can derive from both bona fide stem cells and amplifying precursors that acquire stem cell properties through EGF signaling in vitro (Doetsch et al., 2002), make a strict correlation between in vivo stem cells and cells that can form nsps difficult. However, the effects of manipulating HH-GLI signaling on self-renewal (as revealed in nsp cloning assays) allow the investigation of the role of HH-GLI signaling in regulating stem cell properties.

Our data suggest that cells with stem cell properties increase their numbers in response to higher than normal endogenous HH levels; normal HH levels equate to homeostasis, and reduction of HH signaling below a critical threshold decreases their numbers. In this case, our present and previous data (Dahmane et al., 2001) suggests that HH signaling affects both precursors and neocortical stem cells, although definitive proof requires the prospective identification of stem cells in vivo and their direct analyses in terms of response to SHH-GLI function, which is not yet feasible. The scenario we propose parallels the multiple effects of SHH signaling on adjacent cell types in other parts of the brain (Dahmane and Ruiz i Altaba, 1999), and the separate effects of SHH signaling on the differentiation of a single cell type at different times (Ericson et al., 1996). Our results are also consistent with the finding that *Smoothed*, a crucial component of the HH-GLI pathway, is expressed in populations enriched for SOX2⁺ neural stem cells, as well as in derived nsps from the midgestation mouse telencephalon (D'Amour and Gage, 2003). It remains possible that the smaller size of nsps that we observe after inhibiting SHH-GLI function could also reflect changes in cell identity or cell populations, as wild-type nsps of different sizes can express partially different subsets of genes (Suslov et al., 2002)

and SHH might also affect precursor maturation (Viti et al., 2003). However, the loss of virtually all nsps from E18.5 *Shh* and *Gli3* null mice suggests the general requirement of the HH-GLI pathway.

The phenotype of *Gli2*, *Gli3* and *Shh* mutant mice is likely to include a decrease in the number of neocortical cells with stem cell properties. The existence of severe (exencephalic) and milder *Gli2* and *Gli3* null phenotypes indicates that genetic modifiers affect GLI function. Functional compensation between GLI3 and GLI2 may alleviate the phenotypes, and also explain the maintained response of *Gli2*^{-/-} neocortical explants to SHH (data not shown). Nevertheless, such compensation is likely to be only partial as *Gli2*^{-/-} nsps are severely affected, and GLI2 and GLI3 regulate gene expression differently. For example, loss of either one results in lower *Gli1* levels, but *Dhh* is downregulated in *Gli2*^{-/-} mice whereas it is upregulated in *Gli3* mutants. It remains possible that GLI2 and GLI3 respond to, and/or mediate, non-HH inputs (Brewster et al., 2000).

HH signals that control neocortical expansion may derive from the precursors themselves, as nsps express HH genes, and from differentiated cells located at a distance (Dahmane et al., 2001), possibly paralleling the axonal transport of HH (Huang and Kunes, 1996; Traiffort et al., 2001). A similar situation is likely to occur in the cerebellum, where early external germinal zone precursors transiently express *Shh* (Dahmane and Ruiz i Altaba, 1999) in addition to its strong expression by Purkinje neurons (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999; Dahmane et al., 2001). In the hippocampus, SHH is produced by cells in the hilus of the dentate gyrus (Dahmane et al., 2001), but there is also an effect from *Shh*⁺ septal cells located at a distance (Lai et al., 2003).

The findings that SHH affects the behavior of cells treated

with low doses of EGF, and that high doses of EGF cannot efficiently induce nsp cultures from *Shh*^{-/-} mice, indicates that SHH acts on EGF-responsive cells and that it is unlikely to be only a stem cell survival factor. It is possible that the few nsp observed from *Shh* null mice derive from the complementary action of IHH and DHH. How the EGF and SHH pathways interact is not clear, but it is interesting to note that neocortical precursors express EGFR, and that changes in EGFR expression have been associated with changes in progenitor cell behavior (see Burrows et al., 1997). This raises the possibility that changes in the responsiveness to EGF signaling could modulate or alter the response to SHH signaling or vice versa. Here we provide evidence that SHH signaling may synergize with EGF signaling in the brain through its regulation of *Egfr* expression, paralleling its regulation in the early neural tube and fly brain (Amin et al., 1999; Viti et al., 2003).

HH signaling in defined brain domains may be involved in maintaining niches in which stem cells exist and proliferate. Such niches may be defined, in part, by a critical concentration range at which SHH acts, possibly cooperating with EGF. This idea is consistent with the finding that *Shh* and GLI genes are expressed in other stem cell niches, such as the adult forebrain subventricular zone (SVZ) (V.P., N. Dahmane, D. Lim, A. Álvarez-Buylla and A.R.A., unpublished) and the dentate gyrus (Dahmane et al., 2001), and with the requirement of HH signaling in the the subgranular layer of the hippocampus (Lai et al., 2003), in the SVZ (V.P., D. Lim, N. Dahmane, P. Sánchez, Y. Gitton, A. Álvarez-Buylla and A.R.A., unpublished) and for somatic stem cell proliferation in the fly ovary and brain (Zhang and Kalderon, 2001; Park et al., 2003). Together, these findings suggest an unexpected general role of SHH-GLI signaling in the control of the behavior of stem cell lineages throughout the brain and in different species. How some niches, such as the developing neocortex, are extinguished in adulthood, while others persist is unclear but it is possible that alterations in SHH-GLI pathway activity might underlie these changes.

How SHH signaling is integrated with other niche factors is also not known. For example, in the adult SVZ, a niche of persistent stem cell-derived neurogenesis (see Doetsch et al., 1999), BMP signaling inhibits neuronal differentiation and promotes gliogenesis (Lim et al., 2000), whereas endogenous SHH signaling enhances neurogenesis and the number of cells with stem cell properties (V.P., D. Lim, N. Dahmane, P. Sánchez, Y. Gitton, A. Álvarez-Buylla and A.R.A., unpublished). Here, ependymal cells secrete the BMP antagonist noggin, thereby creating a niche in which neurogenesis can occur (Lim et al., 2000). In the developing neocortex, noggin inhibits whereas BMPs enhance differentiation (Li et al., 1998; Li and LoTurco, 2000; Mabie et al., 1999), and BMPs are also proposed to inhibit the

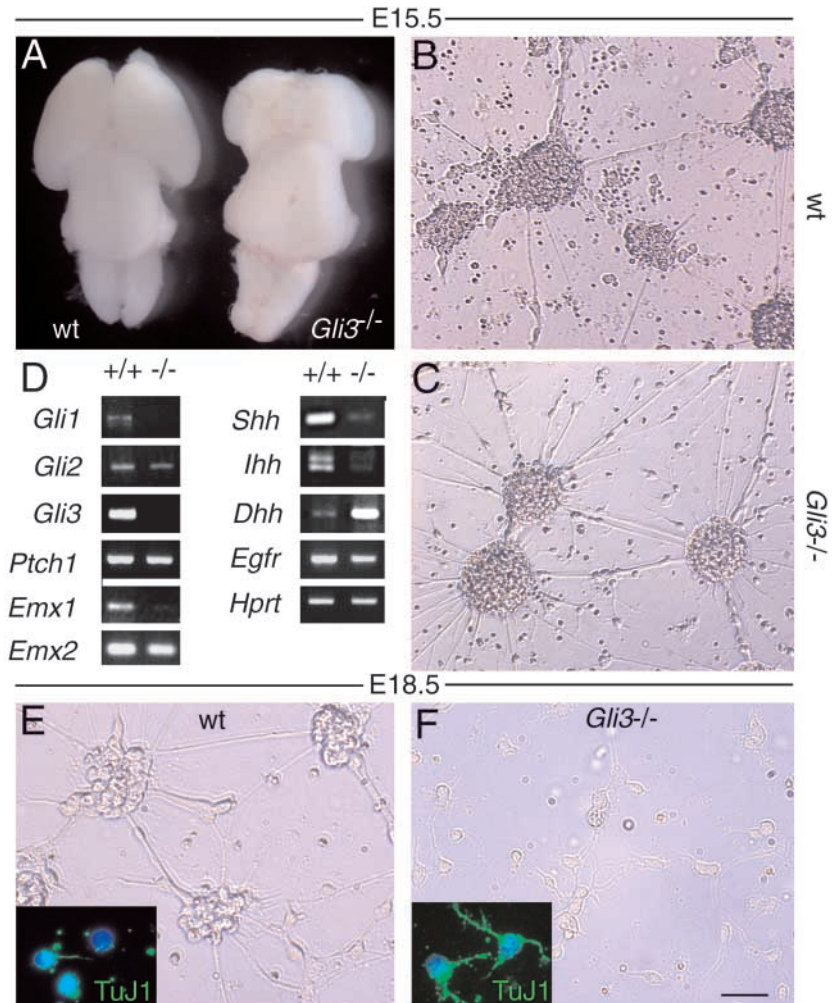


Fig. 5. Morphology and neurosphere cultures from *Gli3* null brains. (A) Dorsal morphology of wild-type (left) and *Gli3*^{-/-} (right) dissected brains at E15.5. Anterior is to the top. (B,C,E,F) Phase contrast images of representative primary cultures of wild-type (B,E) and *Gli3*^{-/-} (C,F) animals at E15.5 (B,C) and E18.5 (E,F). Insets (E,F) correspond to TuJ1⁺ immunostaining of primary cultures. (D) RT-PCR analyses of E15.5 first passage nsp representing the rare survivors. *Hprt* was used as a loading control. RT-PCR analyses at E18.5 were not possible due to the lack of nsp.

transition of stem cells to an EGF-responsive state (Lillien and Raphael, 2000). In the neocortex, as in the SVZ and neural tube (Liem et al., 1995), BMPs and SHH may also therefore act in opposite manners. This raises the possibility that the synergism we report here between EGF and SHH signaling may take place, not only directly through the activation of *Egfr* by SHH, but also indirectly through the inhibition of BMP signaling. This may be supported by the finding that activation of ERKs by EGF leads to the phosphorylation of SMAD1 in the linker region, rendering it unable to respond to activation by activated BMP receptors (Kretzchmar et al., 1997) and thus silencing BMP signaling. The antagonism between SHH and BMP signaling, possibly also integrated at the level of SMAD-GLI interactions (Liu et al., 1998), may thus underlie many aspects of stem cell and neurogenic niches, being a common target for regulators of stem cell properties and neurogenesis.

The present findings support a crucial role of SHH signaling

in building the vertebrate brain, by modulating its size through the regulation of the number of cells with stem cell properties, in addition to controlling precursor proliferation (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999; Dahmane et al., 2001; Lai et al., 2003). Overall or local changes in SHH signaling, or its reception, during evolution may have contributed to the evolving sizes and shapes of the brain, including the expansion of the neocortex in primates, of the tectum in birds and of the cerebellum in electrosensitive fish. The price for such plasticity may be tumorigenesis. Our data, together with previous results on the involvement of the SHH-GLI pathway in tumorigenesis (reviewed by Ruiz i Altaba et al., 2002b), suggest that the many cancers that arise from constitutive HH signaling in various tissues, such as brain, skin, muscle and lung, may derive from cells with stem cell properties that inappropriately maintain an active response to HH signaling, with continued signaling being required for tumor maintenance. In the brain, the synergism between EGF and SHH signaling raises the possibility that enhancement or inappropriate activation of either pathway, such as through EGFR amplification or upregulation of GLI1 function, could give stem cells an advantage to initiate cancer.

In contrast to tumor development resulting from unregulated activity, the controlled modulation of SHH and EGF signaling in vitro and in vivo is likely to lead to the development of protocols to increase the number of cells with stem cell properties in an effort to ameliorate the effects of degenerative diseases.

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References

- Amin, A., Li, Y. and Finkelstein, R. (1999). Hedgehog activates the EGF receptor pathway during *Drosophila* head development. *Development* **126**, 2623-2630.
- Brewster, R., Mullor, J. L. and Ruiz i Altaba, A. (2000). Gli2 functions in FGF signaling during antero-posterior patterning. *Development* **127**, 4395-4405.
- Burrows, R. C., Wancio, D., Levitt, P. and Lillien, L. (1997). Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron* **19**, 251-267.
- Caviness, V. S., Jr, Takahashi, T. and Nowakowski, R. S. (1995). Numbers, time and neocortical neurogenesis: a general developmental and evolutionary model. *Trends Neurosci.* **18**, 379-383.
- 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.
- Coffin, J. D., Wyland, J. J. and Hung, Y. T. (1999). Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. *Nat. Neurosci.* **2**, 246-253.
- 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.
- D'Amour, K. A. and Gage, F. H. (2003). Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **100 Suppl. 1**, 11866-11872.
- Dahmane, N. and Ruiz i Altaba, A. (1999). Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* **126**, 3089-3100.
- Dahmane, N., Sanchez, P., Gitton, Y., Palma, V., Sun, T., Beyna, M., Weiner, H. and Ruiz i Altaba, A. (2001). The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. *Development* **128**, 5201-5212.
- Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui C. C. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. *Development* **125**, 2533-2543.
- Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M. and Álvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-716.
- Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J. M. and Álvarez-Buylla, A. (2002). EGF converts transit amplifying precursors in the adult brain into multipotent stem cells. *Neuron* **36**, 1021-1034.
- 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.
- 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.
- Huang, Z. and Kunes, S. (1996). Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. *Cell* **86**, 411-422.
- 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. *Nat. Genet.* **3**, 241-246.
- Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopropane inhibits sonic hedgehog signal transduction. *Development* **125**, 3553-3562.
- Ingham P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
- Johnson, D. R. (1967). Extra-toes: a new mutant gene causing multiple abnormalities in the mouse. *J. Embryol. Exp. Morphol.* **17**, 543-581.
- Kretzschmar, M., Doody, J. and Massagué, J. (1997) Opposing BMP and EGF signaling pathways converge on the TGF-beta family mediator Smad1. *Nature* **389**, 618-622.
- Lai, K., Kaspar, B. K., Gage, F. H. and Schaffer, D. V. (2003). Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. *Nat. Neurosci.* **6**, 21-27.
- 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.
- Li, W. and LoTurco, J. J. (2000). Noggin is a negative regulator of neuronal differentiation in developing neocortex. *Dev. Neurosci.* **22**, 68-73.
- 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.
- Liem, K. F., Jr, Tremml, 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.
- Lillien, L. and Raphael, H. (2000). BMP and FGF regulate the development of EGF-responsive neural progenitor cells. *Development* **127**, 4993-5005.
- Lim, D. A., Tramontin, A. D., Trevejo, J. M., Herrera, D. G., Garcia-Verdugo, J. M. and Álvarez-Buylla, A. (2000). Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* **28**, 713-726.
- Liu, F., Massagué, J. and Ruiz i Altaba, A. (1998). Carboxy-terminally truncated Gli3 proteins associate with Smads. *Nat. Genet.* **20**, 325-326.
- 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.
- Martens, D. J., Tropepe, V. and van der Kooy, D. (2000). Separate proliferation kinetic of fibroblast growth factor-responsive and epidermal growth factor-responsive neural stem cells within the embryonic forebrain germinal zone. *J. Neurosci.* **20**, 1085-1095.
- Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. A. and Joyner, A. L. (1998). Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2759-2770.
- Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H., Chik, K. W., Shi, X. M., Tsui, L. C., Cheng, S. H. et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123.
- Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M. and Joyner, A. L. (2000). Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605.

- Park, Y., Rangel, C., Reynolds, M. M., Caldwell, M. C., Johns, M., Nayak, M., Welsh, C. J., McDermott, S. and Datt, S.** (2003). Drosophila perlecan modulates FGF and hedgehog signals to activate neural stem cell division. *Dev. Biol.* **253**, 247-257.
- Rakic, P.** (1995). A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. *Trends Neurosci.* **18**, 383-388.
- Represa, A., Shimazaki, T., Simmonds, M. and Weiss, S.** (2001). EGF-responsive neural stem cells are a transient population in the developing mouse spinal cord. *Euro. J. Neurosci.* **14**, 452-462.
- Reynolds, B. A. and Weiss, S.** (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1-13.
- Ruiz i Altaba, A., Palma, V. and Dahmane, N.** (2002a). Hedgehog-Gli signalling and the growth of the brain. *Nat. Rev. Neurosci.* **3**, 24-33.
- Ruiz i Altaba, A., Sánchez, P. and Dahmane, N.** (2002b). Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat. Rev. Cancer* **2**, 361-372.
- Seaberg, R. M. and van der Kooy, D.** (2002). Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J. Neurosci.* **22**, 1784-1793.
- Suslov, O. N., Kukekov, V. G., Ignatova, T. N. and Steindler, D. A.** (2002). Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. *Proc. Natl. Acad. Sci. USA* **99**, 14506-14511.
- Tekki-Kessarlis, N., Woodruff, R., Hall, A. C., Gaffield, 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., Álvarez-Bolado, G., Walter, A. and Rüther, U.** (1999). Gli3 is required for *Emx* gene expression during dorsal telencephalon development. *Development* **126**, 3561-3571.
- Traiffort, E., Moya, K. L., Faure, H., Hässig, R. and Ruat, M.** (2001). High expression and anterograde axonal transport of aminoterminal sonic hedgehog in the adult hamster brain. *Euro. J. Neurosci.* **14**, 839-850.
- Tropepe, V., Sibilio, M., Ciruna, B. G., Rossant, J., Wagner, E. F. and van der Kooy, D.** (1999). Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev. Biol.* **208**, 166-188.
- Tropepe, V., Coles, B. L., Chiasson, B. J., Horsford, D. J., Elia, A. J., McInnes, R. R. and van der Kooy, D.** (2000). Retinal stem cells in the adult mammalian eye. *Science* **287**, 2032-2036.
- Vaccarino, F. M., Schwartz, M. L., Raballo, R., Nilsen, J., Rhee, J., Zhou, M., Doetschman, T., Coffin, J. D., Wyland, J. J. and Hung, Y. T.** (1999). Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. *Nat. Neurosci.* **2**, 246-253.
- van den Brink, G. R., Hardwick, J. C., Tytgat, G. N., Brink, M. A., Ten Kate, F. J., Van Deventer, S. J. and Peppelenbosch, M. P.** (2001). Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. *Gastroenterology* **121**, 317-328.
- Viti, J., Gulacsi, A. and Lillien, L.** (2003). Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. *J. Neurosci.* **23**, 5919-5927.
- 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.
- Wechsler-Reya, R. J. and Scott, M. P.** (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* **22**, 103-114.
- Yoshida, M., Suda, Y., Matsuo, I., Miyamoto, N., Takeda, N., Kuratani, S. and Aizawa, S.** (1997). *Emx1* and *Emx2* functions in development of dorsal telencephalon. *Development* **124**, 101-111.
- Zhang, Y. and Kalderon, D.** (2001). Hedgehog acts as a somatic stem cell factor in the Drosophila ovary. *Nature* **410**, 599-604.