Cooperative requirement of the Gli proteins in neurogenesis

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Summary

The Gli proteins are critical components of multiple processes in development, homeostasis and disease, including neurogenesis and tumorigenesis. However, it is unclear how the Gli code, the sum of their combinatorial positive and negative functions, dictates cell fate and behavior. Using an antisense approach to knockdown gene function in vivo, we find that each of the three Gli proteins is required for the induction of all primary neurons in the amphibian neural plate and regulates the bHLH/Notch neurogenic cascade. Analyses of endogenous Gli function in Gli-mediated neurogenesis and tumorigenesis, and in animal cap assays, reveal specific requirements that are context specific. Nuclear colocalization and binding studies suggest the formation of complexes, with the first two zinc fingers of the Gli five zinc-finger domain acting as a protein-protein interaction site. The Gli proteins therefore appear to form a dynamic physical network that underlies cooperative function, greatly extending the combinatorial possibilities of the Gli code, which may be further fine-tuned in cell fate specification by co-factor function.

Key words: Gli, Neurogenesis, Tumor, Morpholino, Neural plate, *Xenopus*, Antisense

Introduction

Neurogenesis from primary precursors or stem cells in vertebrates involves the specification of neuronal fates. One of the simplest models to study vertebrate neuronal specification in vivo is the development of the first neuronal circuits in the early frog embryo. These 'primary' neurons arise soon after gastrulation and the formation of the neural plate, and form a basic network that includes the relay of sensory input to spinal motoneurons to insure an early escape response. Primary neurogenesis in the frog embryo results in the formation of three rows of neurons within the early neural plate: motoneurons close to the midline, sensory neurons close to the lateral edges of the neural plate and interneurons in between.

Several intercellular signaling pathways have been implicated in the control of neurogenesis in vertebrates, including the bHLH/Notch and retinoic acid pathways (e.g. Ruiz i Altaba and Jessell, 1991; Chitnis et al., 1995; Franco et al., 1999; Pierani et al., 1999; Sharp and Goldstone, 2000), as well as the Sonic hedgehog-Gli (Shh-Gli) pathway (Lee et al., 1997; Hynes et al., 1997; Brewster et al., 1998; Ruiz i Altaba et al., 1998; Franco et al., 1999; Lai et al., 2003; Machold et al., 2003; Palma and Ruiz i Altaba, 2004; Palma et al., 2005). The Gli proteins are obligatory mediators of Hh signals, but it is not clear how they act. It has been proposed that the crucial step is the overall read out of positive and negative Gli functions: the Gli code (Ruiz i Altaba, 1997; Ruiz i Altaba, 1998) (reviewed by Ruiz i Altaba et al., 2003).

Gain- and loss-of-function analyses in different species show: that the Gli proteins function in a context-dependent manner; that Gli2 and Gli3, unlike Gli1, harbor strong dominant-negative function in C-terminally truncated forms; and that there is partial redundancy (reviewed by Ruiz i Altaba et al., 2003). Dominant-negative assays in the frog neural plate and the chick neural tube indicate the requirement of overall positive Gli function for normal patterning (Brewster et al., 1998; Persson et al., 2002; Meyer and Roelink, 2003). Similarly, in mice, the phenotype of the double Gli2/Gli3 null, which does not seem to express Gli1, indicates a requirement of Gli proteins for proper neurogenesis and patterning (Bai et al., 2004), consistent with the absolute requirement in flies for Ci, the Gli homologue, in Hh-mediated patterning (Methot and Basler, 2001).

A problem in understanding Gli function resides in part on the difficulty of examining similar alterations for the three Gli proteins in any given system, as their functions are context dependent. The following summarizes current knowledge.

(1) Gli1 behaves as a positive activator. In frog embryos, *Gli1* gain of function mimics Shh signaling (Lee et al., 1997; Ruiz i Altaba, 1998; Ruiz i Altaba, 1999). This result contrasts with the reported normality of *Gli1*$^{-/-}$mice (Park et al., 2000; Bai et al., 2002), although minor embryonic defects are seen when compounded with mutation in Gli2 (Park et al., 2000). In zebrafish, however, *Gli1* mutants have a phenotype (Karlstrom et al., 2003). Gli1 has been also reported to be superfluous for mouse tumorigenesis (Weiner et al., 2002), but it is required for the proliferation of human tumor cells (Sanchez et al., 2004).

(2) Gli2 can act as a positive or negative element in Hh signaling. In frogs, it can induce motoneurons while inhibiting floor plate and neural crest differentiation (Ruiz i Altaba, 1998;

Brewster et al., 1998). In mice, it is mostly a weak activator of floor plate differentiation in the early CNS (Matise et al., 1998; Ding et al., 1998). Here, its function can be replaced by that of Gli1 (Bai and Joyner, 2001) but in zebrafish it harbors mostly negative function (Karlstrom et al., 1999; Karlstrom et al., 2003), although there is also evidence for limited positive effects (Tyurina et al., 2005). At late CNS stages, mouse Gli2 is required for normal brain growth and stem cell maintenance (Palma and Ruiz i Altaba, 2004). In humans, GLI2 is required for proper midline development with its loss of function leading to holoprosencephaly and pituitary deficiencies (Roessler et al., 2003), a phenotype with similarities to that of loss of *Shh* in mice and humans (Chiang et al., 1996; Belloni et al., 1996; Roessler et al., 1996).

(3) Gli3 can induce neurogenesis and repress neural crest and floor plate differentiation in the frog neural plate (Brewster et al., 1998; Ruiz i Altaba, 1998). In zebrafish, it can act as both an activator and a repressor in vivo, and can cooperate with or inhibit Gli1 functions in vitro (Tyurina et al., 2005). Mouse mutants for Gli3 display neural tube closure defects and polydactyly (Johnson, 1967; Schimmang et al., 1992; Hui and Joyner, 1993; Theil et al., 1999; Tole et al., 2000), the latter much like humans with a defective *GLI3* gene (Vortkamp et al., 1991; Kang et al., 1997; Radhakrishna et al., 1997). Gli3 harbors strong repressive function, possibly in C′Δ forms (such as Gli2 and Ci, but not Gli1) detected in vivo (Aza-Blanc et al., 1997; Ruiz i Altaba, 1999; Shin et al., 1999; von Mering and Basler, 1999; Aza-Blanc et al., 2000; Wang et al., 2000). Gli3 plays a critical role in Shh signaling in the early neural tube as Shh inhibits *Gli3* transcription (Ruiz i Altaba 1998) and, importantly, loss of *Gli3* can partially rescue the phenotype of *Shh* mouse mutants (Litingtung and Chiang, 2000). In the brain, Gli3 is also required for growth and the regulation of stem cell behavior (Palma and Ruiz i Altaba, 2004).

Together, these data on patterning and cell type specification by the three Gli genes in different species demonstrate the importance of Gli function. The Gli code is thus thought to regulate the CNS homeodomain code, the latter being required for specification of different neuronal subtypes (Briscoe et al., 2000). However, the variable results make a unifying interpretation difficult. Similarly, their function in primary neurogenesis is not completely clear. Pan-Gli dominant-negative assays and gain-of-function analyses with individual Gli genes indicate that the Gli proteins are key components of neurogenesis (Brewster et al., 1998), but their individual requirement in the neurogenic program has not been determined. Here, we have used a knockdown antisense approach to determine the requirement of each Gli protein in cell fate specification, using primary neurogenesis as a model system.

Materials and methods

Animals, explants and microinjection

Xenopus laevis were used and reared by standard techniques. Morpholino oligos (MO-C, CCTCTTACCTCAGTTACAATTTATA; MO-1, CGGGCGGACACTGGCGGGACGC; MO-2, GCACAGA-ACGCAGGTAATGCTCCAT; and MO-3, TAGTGCTACGG-GACTGGGCTTCCAT) were microinjected at 20 ng/10 nl/embryo and capped synthetic RNAs were injected at 2 ng/10 nl/embryo unless otherwise noted, into the animal pole of one cell at the two-cell stage. *lacZ* RNA used as a lineage tracer was co-injected at <0.5ng/embryo. Animal caps were dissected from stage 8 embryos with a hair knife. Frog MOs do not recognize the human injected RNAs.

In vitro translation

Gli1, *Gli2*, *Gli3* and *GLI1* cDNAs in pCS2 vectors (0.5 to 1.0 μg) and 1 μM of MO-C, MO-1, MO-2 and MO-3 were used to test for protein production using the TNT Coupled Reticulocyte Lysate Systems Kit (Promega) in the presence of ^{35}S-methionine. The levels of specific Gli proteins were measured by autoradiography after 7% SDS-PAGE, immersion in Enhance (Amersham) and drying.

In situ hybridization and β-gal staining

Whole-mount in situ hybridization was performed with digoxigenin-labeled single stranded RNA probes followed by nitroblue tetrazolium (NBT) plus 5-bromo-Y-chloro-3-indolyl phosphate (BCIP) (purple) substrates. *Gli1*, *Gli2* and *Gli3* (Lee et al., 1997; Ruiz i Altaba, 1998); *Pintallavis* (Ruiz i Altaba and Jessell, 1992); *Sonic hedgehog* (Ruiz i Altaba et al., 1995); *Zic2*, *Slug*, *Snail* and *N-tubulin* (Brewster et al., 1998) probes were as described. Other probes were made with the following enzymes: *Xash3* (Ferreiro et al., 1994), *Not*I and T3; *Xmyt* (Bellefroid et al., 1996), *Bam*H1 and T7; *Xaml* (Tracey et al., 1998), *Sal*I and T7; and *Sox3* (Penzel et al., 1997), *Eco*RI and T3. For β-gal staining, fixed embryos were incubated with X-gal substrates (purple red/pink precipitates) before dehydration with methanol.

BrdU incorporation

5-bromo-deoxyuridine (BrdU 10 nl of 3 mg/ml; Sigma) were injected into three areas of stage 13 embryos previously injected with MO-3. Incorporated BrdU was detected immunocytochemically with diaminobenzidine (Hardcastle and Papalopulu, 2000; Dahmane et al., 2001). Mouse anti-BrdU antibodies (mouse from Becton Dickinson) were used at 1:100. Horseradish peroxidase-coupled secondary anti-IgG antibodies (Amersham) were used at 1:200.

Quantitative real-time PCR

Total RNA extracted from animal caps was denatured for 10 minutes at 65°C in the presence of random hexamer primers, immediately cooled in ice water, then reverse transcribed using SuperScript II reverse-transcriptase. Real-time PCR was performed with an Opticon™ machine (MJR). Reactions were carried out in 30 μl containing 1 μl of template, 200 nM of each forward and reverse primer, and 1× IQ™ SYBR Green Supermix (BioRad). To quantify transcripts, dilutions of cDNA controls (from sibling embryos non injected) were run in parallel. All experiments were performed in triplicate.

Cell transfection and immunocytochemistry

Gli1, *Gli2*, *GLI3* and *Zic2* cDNAs were subcloned into Flag-tagged or myc-tagged vectors either as full-length clones or deletion mutants (Lee et al., 1997; Brewster et al., 1998; Ruiz i Altaba et al., 1999; Liu et al., 1998). Deletion mutations were carried out using restriction sites within the gene, resulting in the following constructs: GLI3N′ΔStuI (amino acids 1-390 deleted), GLI3C′ΔClaI (amino acids 745-1596 deleted), GLI3C′ΔXhoI (amino acids 558-1596 deleted), Gli2C′Δ (amino acids 559-1468 deleted), Gli1C′ΔPstI (amino acids 551-1397 deleted). Other deletion mutations were carried out using PCR: Zic2N′Δ (amino acids 1-260 deleted), Zic2C′Δ (amino acids 451-501 deleted), VP16Zic2ZF (Brewster et al., 1998), GLI3ZF1,2 (amino acids 473-547) and Zic2ZF1,2 (amino acids 270-351). Immunocytochemistry of transfected COS-7 cells was as described (Ruiz i Altaba, 1999).

Co-immunoprecipitation and western analyses

COS-7 cells were transfected with indicated plasmids at 60% confluency with lipofectamine. Forty-eight hours post-transfection,

cells were washed with PBS, lysed with RIPA buffer [150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 7.5, protease inhibitor cocktail (Sigma P-2714) plus 100 mM PMSF] and incubated with either 2 μl Flag Ab (Sigma F3165) or 10 μl Myc antibody (Santa Cruz, sc-789) for 1 hour at 4°C. ProteinA/G beads (20 μl; Santa Cruz) were added and incubated for 1 hour at 4°C. Beads were then washed four times with ice-cold RIPA buffer, sample buffer added, the mixture boiled and subjected to 10% SDS-PAGE, transferred onto membranes and probed with opposing antibody to immunoprecipitation. For immunoblotting, anti-Myc and anti-Flag antibodies were used at concentration of 1/250 and 0.5 μg/ml, respectively. Binding was visualized using ECL reagents (Amersham). Total protein from each experiment was run separately on SDS-PAGE gels for confirmation of protein expression and lack of degradation.

Results

Gli1, Gli2 and Gli3 are required for the development of primary neurons

Several morpholino-modified antisense oligonucleotides (MO) were designed overlapping or adjacent to the initiator ATG of each Gli mRNA. Initial tests for efficacy of their ability to inhibit target mRNA translation used in vitro translation assays. 0.5 to 1 μg of pDNA encoding full-length *Gli1*, *Gli2* or *Gli3* frog (*Xenopus laevis*) cDNAs under the control of a CMV promoter (Lee et al., 1997; Brewster et al., 1998; Ruiz i Altaba, 1998) were mixed with 1 μM of each MO and subjected to in vitro transcription/translation in the presence of ^{35}S-labelled methionine. An unrelated, control MO (MO-C) was used for all assays. Presence of the specific MO for each target sequence around the initiation ATG resulted in a significant reduction of radiolabeled Gli species (Fig. 1A). The MOs were specific for each target, although efficiencies appeared to vary slightly. The best three MOs were then chosen for further use.

In vivo tests were carried out by injecting 20 ng (~60 nM assuming a volume of 4 μl for the embryo) of each MO into one cell of two-cell embryos. This allowed the uninjected cell, which gives rise to one side of the bilateral embryo, to always act as the internal control. All microinjections contained a trace of *lacZ* RNA to allow the unequivocal recognition of the injected half after X-Gal staining (e.g. red in Fig. 1D,E,G). Injections were performed into the animal cap region to favor targeting to the neural plate and ectoderm, bypassing effects on the role of Gli2 and Gli3 in mesoderm (Brewster et al., 2000). Diffusion of the injected MOs (25 bp) was different than that of the injected *lacZ* RNA (~3000 bp; not shown), making the precise localization of high β-Gal expression in the injected half unimportant.

Injection of MO-C had minor effects, comparable with injection of tracer alone, on the development of primary neurons assayed at stage 14 through the expression of the neuronal-specific marker *N-tubulin* by whole-mount in situ hybridization (MO-C: 1% embryos with reduction of *N-tubulin*, *n*=16; Fig. 1F). By contrast, injection of morpholinos specific for Gli1 (MO-1), Gli2 (MO-2) or Gli3 (MO-3) inhibited primary neuron differentiation [MO-1: 66.7%, *n*=12 (Fig. 1C); MO-2: 50%, *n*=19 (Fig. 1D); MO-3: 83.9%, *n*=26 (Fig. 1E) embryos with fewer primary neurons than MO-C injected embryos] in all three populations. Quantification of the number of *N-tubulin*$^+$ neurons per embryo in the injected half versus the uninjected half in each population is given in Fig. 1B.

The specificity of MO action was further proven by rescue experiments. Co-injection of 10, 5 or 2.5 ng of MO plus 1 ng of the corresponding Gli RNA resulted in an increasing number of rescued embryos with normal neuronal pattern: 16, 46 and 50% rescue for *Gli1*; 44 and 56% for the two lower amounts of MO for *Gli2*; and 25, 42 and 67% rescue for *Gli3* (Fig. 1G,H; data not shown). Only one embryo injected with 1 ng *Gli3* RNA

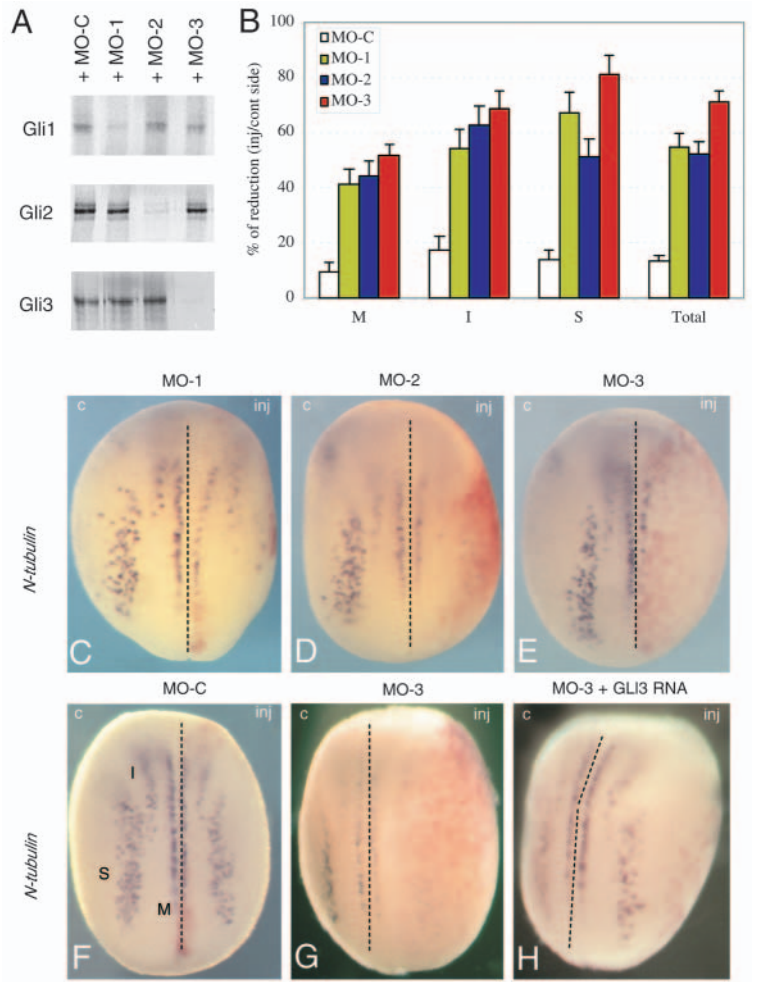

Fig. 1. Primary neurogenesis requires Gli1, Gli2 and Gli3. (A) PAGE analyses of in vitro transcription/translation of *Gli1*, *Gli2* and *Gli3* cDNAs mixed with 1 μM of each MO. (B) Percentage of reduction of *N-tubulin*$^+$ primary neurons: motoneurons (M), interneurons (I), sensory neurons (S) and total neurons (T) in stage 14 embryos injected with each MO. Neurons were counted in the embryos with reduced *N-tubulin* expression. The percentage of each cell type was calculated by dividing the number of neurons on the injected side by the number of neurons on the control side. The statistical analysis was performed using Student's *t*-test. Error bars are s.e.m. (C-H) Examples of *N-tubulin* expression on stage 14 embryos injected (inj.) with MO-1 (C), MO-2 (D), MO-3 (E,G), MO-C (F) and MO-3 plus GLI3 RNA (H), the latter showing phenotypic rescue. The β-gal tracer is red.

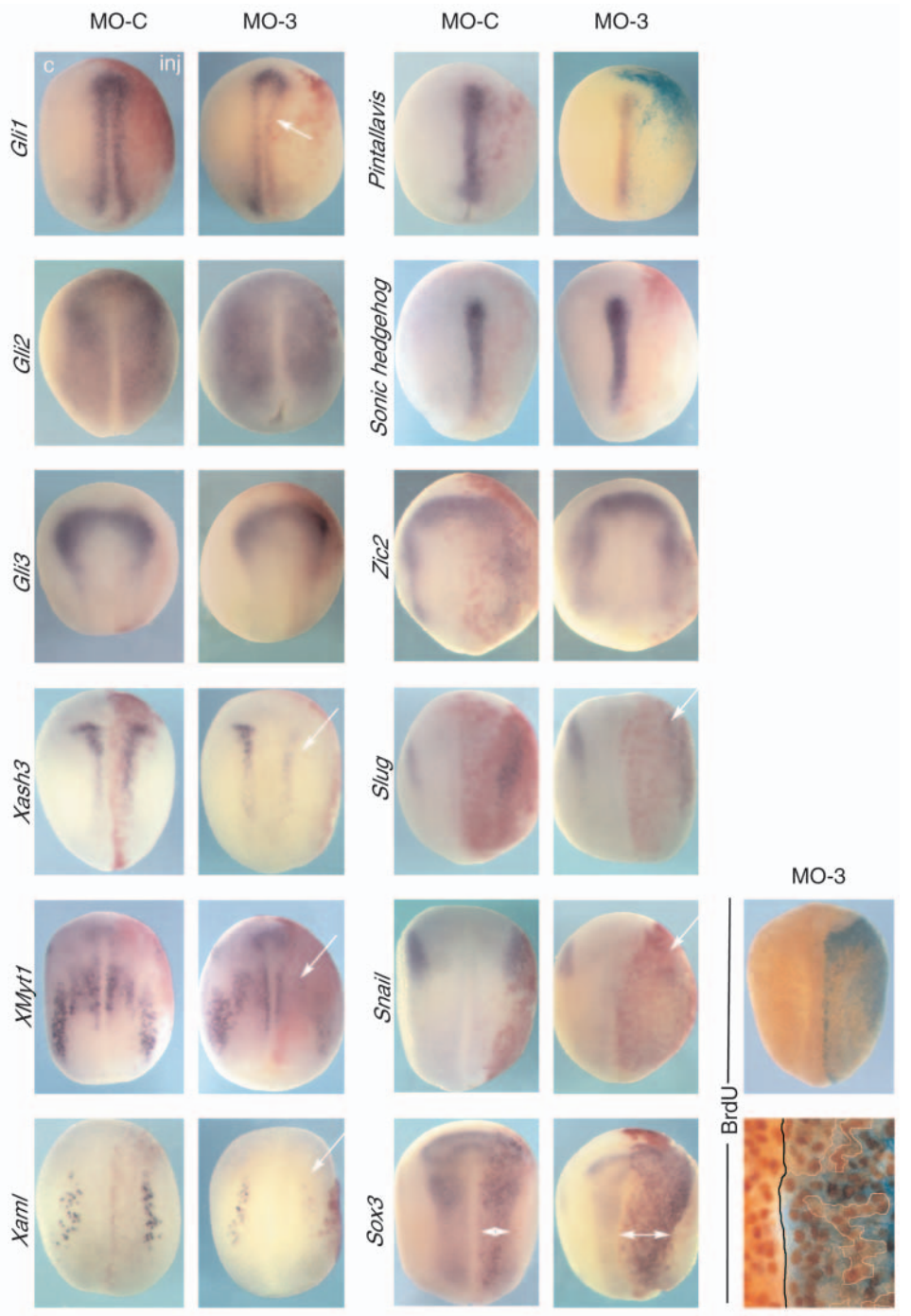

Fig. 2. Effects of Gli3 in neural plate patterning. Whole-mount in situ hybridizations on stage 14 embryos injected (inj.) with MO-C or MO-3 with probes as indicated on the left side of each panel. The β-gal tracer is red except in *Pintallavis*-MO-3 and the BrdU panels, in which it is blue. Arrows indicate the inhibition of *Gli1*, *Xash3*, *XMyt1*, *Xaml*, *Slug* and *Snail* expressions. Double-headed arrows indicate the size of the neural plate assessed by *Sox3* expression. BrdU incorporation in stage 14 embryos injected with MO-3 is shown in brown. A high magnification panel (bottom right) shows BrdU incorporation in the neural plate of a unlilaterally injected MO-3+*lacZ* embryo.

plus 2.5 ng MO-3 showed ectopic neurogenesis, indicating that the MO rescues ectopic neurogenesis by the appropriate injected Gli RNA (and not by the other two Gli genes, see below) and Gli RNA rescues the suppression of endogenous neurogenesis by the appropriate MO.

Gli3 is required for early neurogenic and neural crest gene expression

Given the crucial role that the repression of the negative function of Gli3 plays in Shh signaling in the amniote neural tube (Litingtung and Chiang, 2000), we sought to test further the role of Gli3 in neural plate patterning.

Knockdown of Gli3 did not alter the expression of *Gli2* (n=33) or of the midline markers *Pintallavis* (n=19) and *Sonic hedgehog* (n=20) at stage 14 (Fig. 2). It inhibited *Gli1* expression adjacent to the midline (58%, n=19) and minimally its own transcription (6%, n=26), but not that of *Zic2* (n=29) near the edges of the neural plate (Fig. 2). However it impaired the expression of the neural crest markers *Slug* (51%, n=29) and *Snail* (48%, n=33; Fig. 2). In addition, MO-3 inhibited the expression of the early bHLH neurogenic cascade genes *Xash3* (43%, n=25) and *Xmyt1* (26%, n=38; Fig. 2). The inhibition of neuronal populations was confirmed by the loss of *Xaml* (60%, n=28) expression, a marker of primary sensory neurons (Fig. 2). MO-3 slightly increased the size of the neural plate as judged by $Sox3^+$ staining (65%, n=43), but did not appreciably change $BrdU^+$ incorporation (n=35 embryos tested; Fig. 2 and not shown). This was evident comparing the injected and control halves of the neural plate and also in cases where there was cell mixing in the neural plate between expressing and non-expressing cells (Fig. 2).

The three Gli proteins are required for neuronal differentiation induced by the ectopic activation of neurogenic pathways

Neurogenesis can be triggered by the activation of the bHLH neurogenic pathways, which specify neuronal fate, and by activated Notch signaling, which is involved in lateral inhibition (reviewed by Chitnis and Kintner, 1995; Kintner, 2002). Injection of neurogenin 1 (*Ngn1a*) RNA resulted in massive *N-tubulin*$^+$ neuronal differentiation (Ma et al., 1996). Similarly, expression of *NeuroD* (Lee et al., 1995), another bHLH gene that acts downstream of *Ngn1a* also resulted in a strong neurogenic phenotype (stage 14-15; Fig. 3;

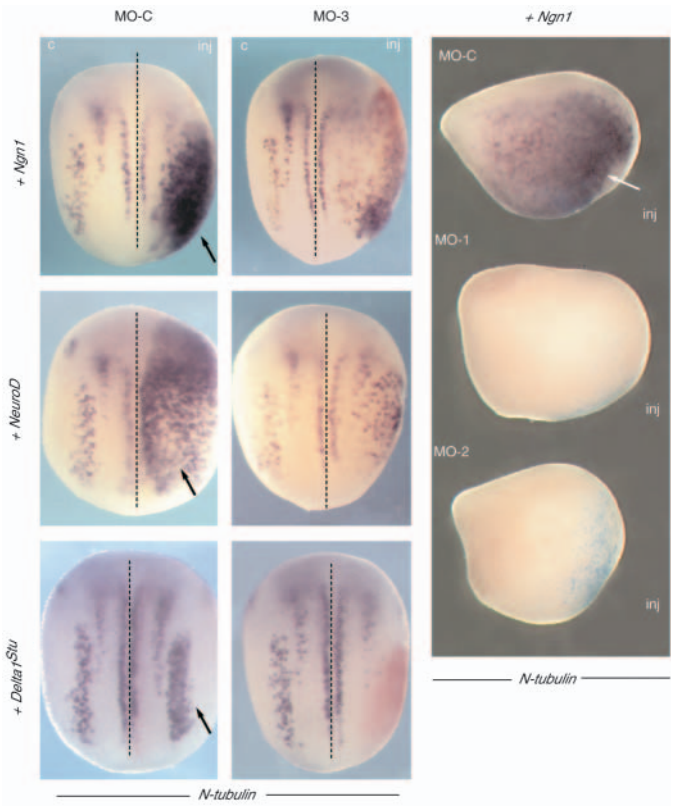

Fig. 3. Gli1, Gli2 and Gli3 are required for ectopic neurogenesis by activated neurogenic pathways. (Left panels) *N-tubulin* expression (purple) on stage 14-15 embryos co-injected (inj.) with *Ngn1*, *NeuroD* or *Delta1*^{Stu} and MO-C or MO-3, as indicated. Arrows indicate ectopic neuronal differentiation caused by *Ngn1*, *NeuroD* or *Delta1*^{Stu}. Panels show dorsal views and the β-gal injection tracer is in red/pink for *Delta1*^{Stu}+MO-3. (Right panel) Lateral views of injected embryos at stage 13 showing the β-gal injection tracer in light blue. Ectopic neurogenesis as determined by *N-tubulin* expression (purple, arrow) is only detected after injection of 1ng *Ngn1a* RNA plus MO-C, but not after co-injection of *Ngn1a* plus MO-1 or MO-2. Dorsal side is upwards, anterior towards the right.

Ngn1a+MO-C, 94% ectopic induction, *n*=34; *NeuroD*+MO-C, 79% ectopic induction, *n*=33). These phenotypes were reversed by MO-3 (Fig. 3; *Ngn1a*+MO-3, 89% reduction, *n*=28; *NeuroD*+MO-3, 86% reduction, *n*=29). In a second set of experiments, each Gli MO inhibited neurogenesis by co-injected *Ngn1a* RNA (stage 13; Fig. 3, right panels; *Ngn1a*+MO-C, 86% ectopic *N-tubulin*, *n*=70; *Ngn1a*+MO-1, 100% reduction, *n*=21; *Ngn1a*+MO-2, 100% reduction, *n*=43; *Ngn1a*+MO-3, 100% reduction, *n*=22, not shown). In addition, neurogenesis induced by a mutant form of *Delta* (*Delta1*^{Stu}), which cell-autonomously inhibits Notch signaling and thus bypassing normal lateral inhibition (Chitnis et al., 1995; Chitnis and Kintner, 1996) (Fig. 3; *Delta1*^{Stu}+MO-C, 75% ectopic induction, *n*=29), was reversed by MO-3 (Fig. 3; *Delta1*^{Stu}+MO-3, 76% reduction, *n*=34).

Gli-induced neurogenesis requires endogenous Gli function in a species-specific manner

Misexpression of either frog Gli or human GLI proteins in the frog neural plate results in a neurogenic phenotype (Lee et al., 1997; Ruiz i Altaba, 1998; Brewster et al., 1998). We thus tested whether Gli1/GLI1-driven neurogenesis requires endogenous Gli function. Expression of frog full-length *Gli1* and MO-C resulted in the ectopic differentiation of *N-tubulin*$^{+}$ primary neurons (*Gli1*+MO-C: 79% embryos with ectopic neurogenesis, *n*=23; Fig. 4A,B), a phenotype that was diminished or abolished in all three neuronal populations by knockdown of each Gli protein (*Gli1*+MO-1: 15% of embryos with ectopic neurons, *n*=22; *Gli1*+MO-2: 27%, *n*=21; *Gli1*+MO-3: 0%, *n*=28; Fig. 4A). However, human GLI1-induced neurogenesis (*GLI1*+MO-C: 87%, *n*=22; Fig. 4C) did not require Gli2 (*GLI1*+MO-1: 50%, *n*=20; *GLI1*+MO-2: 79%, *n*=27; *GLI1*+MO-3: 14%, *n*=25; Fig. 4C,D). Quantification of the change in the number of neurons is given in Fig. 4B,D.

GLI1-induced tumorigenesis requires endogenous Gli1 and Gli3 function

As a second test for the requirement of endogenous Gli function in Gli-induced processes we have used the induction of tadpole skin tumors by GLI1 (Dahmane et al., 1997). We have previously shown that expression of human GLI1 induces epidermal hyperplasias or tumors which are marked by the expression of β-gal from the co-injected *lacZ* tracer (Dahmane et al., 1997), and that co-injection with MO-1 inhibited tumor formation (Dahmane et al., 2001). As expected, injection of *GLI1* and MO-C resulted in massive tumor induction and co-injection with MO-1 decreased the tumorigenic phenotype (Fig. 5A,B: *GLI1*, 88% tumor formation, *n*=59; *GLI1*+MO-1, 23% tumor formation, *n*=56). Paralleling the neurogenic results, co-injection of MO-3, but not MO-2, also reduced tumor formation (Fig. 5A,B: *GLI1*+MO-3, 19% tumor induction, *n*=51; *GLI1*+MO-2, 90% tumor induction, *n*=50).

Gli proteins cooperate in a dynamic fashion to regulate gene expression in a target-and context-specific manner

To extend the results with whole embryos, we have used the animal cap assay to further test and quantify the specific requirements of each Gli gene in target gene induction. Gene expression was tested in pooled animal cap samples from sibling embryos after injection of each Gli RNA along with MO-C or MOs for the other Gli genes. The analyses were carried out in triplicate and quantified with real-time PCR. Animal caps expressing frog Gli RNAs plus MOs were collected at stage 12 and stage 14 to allow for a temporal comparison, while those expressing full-length synthetic human GLI RNAs plus MOs were collected at stage 14 to allow for a species of origin comparison. Tabulation of the results is shown for Gli plus MO-C as the ratio of the value of Gli plus MO-C over that in uninjected animal caps (asterisks in Table 1). The value of these numbers is not important, as it depends on the kinetics of the exact primer pairs used. However, a positive value means induction and a negative one repression. The values given for Gli plus MO-1, MO-2 or MO-3 is the ratio of the number for the specific combination of Gli plus MO over that of the same Gli plus MO-C, i.e. 1 means no change and therefore no effect after gene knockdown. As an arbitrary threshold, we have chosen to highlight changes of at least 40% with green representing activation or activator function and red representing repression or repressor function (Table 1). It is not

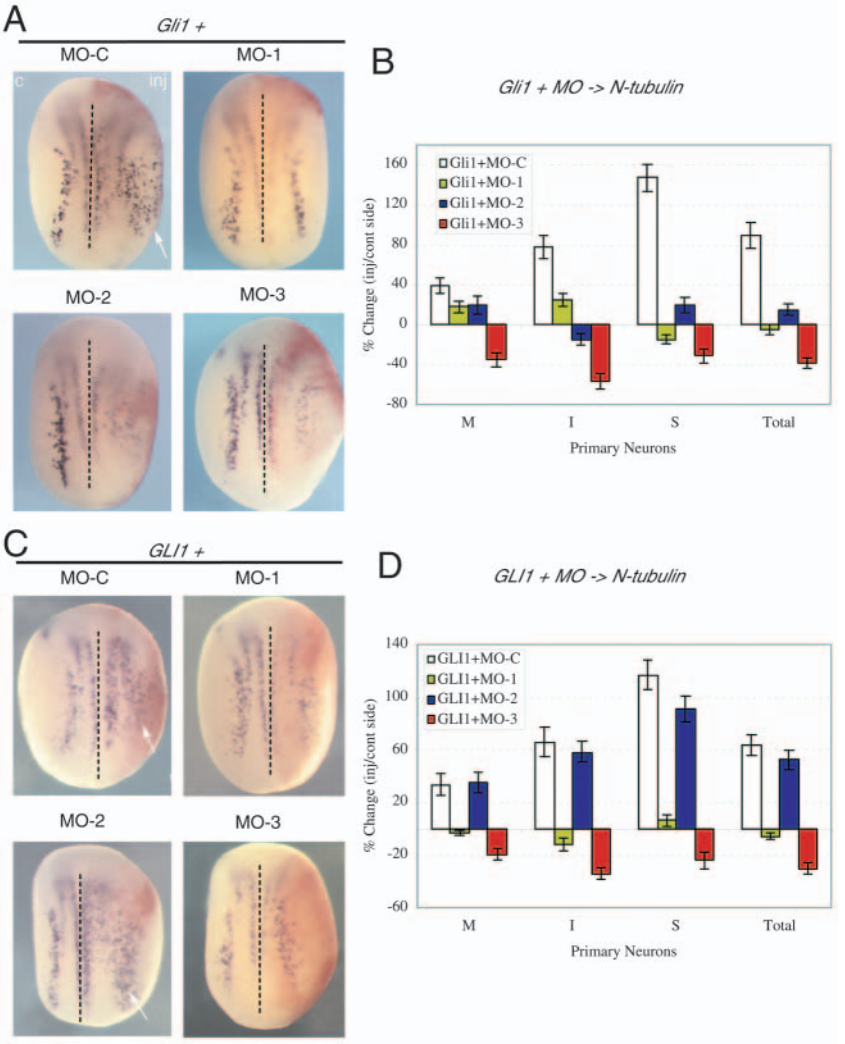

Fig. 4. Gli-induced neurogenesis requires endogenous Gli function. (A,C) *N-tubulin* expression on stage 14 embryos co-injected (inj.) with *Gli1* or human *GLI1* and each MO. Arrows indicate ectopic neuronal differentiation. The β-gal tracer is red. (B,D) Percentage change of primary neurons: motoneurons (M), interneurons (I), sensory neurons (S) and total neurons (T) in stage 14 embryos injected with *Gli1* (B) or human *GLI1* (D) and each MO. Neurons were counted in the embryos that showed a phenotype. The percentage of each cell type was calculated by dividing the number of neurons on the injected side by the number of neurons on the control side. The statistical analysis was performed using Student's *t*-test. Error bars are s.e.m.

known if the majority of these genes are direct targets or whether the Gli proteins act directly as bona fide transcriptional activators or repressors in all cases. The color of the values indicated with asterisks rows refer to the action of the exogenous Gli protein injected, the others refer to the inferred function of the endogenous Gli protein inhibited by the specific MO used. The results are summarized for Gli and neurogenic genes in Fig. 6.

Gli and Ptc

One group of genes tested included the three Gli genes (Lee et al., 1997) as well as *Ptc1* and *Ptc2* (Takabatake et al., 2000; Koebernick et al., 2001), with *Ptc1* being a target of Gli function (Agren et al., 2004) and a measure of induction of the Hh-Gli pathway (Table 1). Gli1 and Gli2 induced each other's expression at both stage 12 and stage 14. Gli3, however, induced *Gli1* only at stage 14, while it induced *Gli2* at both stages. At stage 12, Gli1 and Gli2 repressed *Gli3* expression but at stage 14 Gli1 did not affect *Gli3* expression and Gli2, by contrast, induced *Gli3*. Human GLI proteins induced all the frog Gli genes with the exception that GLI1 did not significantly affect *Gli3* expression (Table 1, Fig. 6). All frog and human Gli proteins induced *Ptc1* and *Ptc2* at both stages (Table 1). Analyses of the requirement of the function of each Gli protein through the co-injection of individual Gli MOs revealed a more complex scenario (Table 1). We describe one case.

Gli3

Gli3 was repressed by injected Gli1 at stage 12. At stage 14, injected Gli1 and GLI1 did not affect its expression. However, endogenous Gli1 acted as a repressor in the presence of GLI1 as here MO-1 induced *Gli3* activation. Injected Gli2 repressed

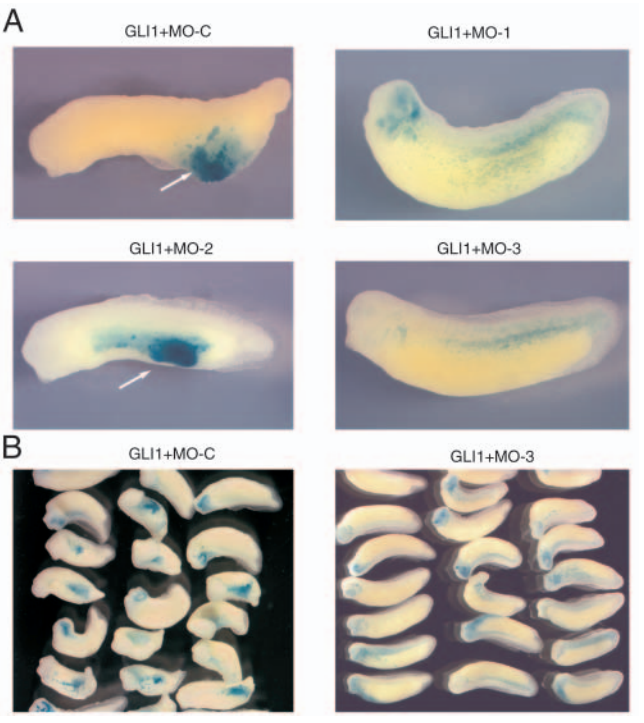

Fig. 5. GLI1-induced tumorigenesis requires endogenous Gli1 and Gli3 function. (A) Tadpoles injected with human *GLI1* and each MO. Arrows show tumors in tadpoles injected with *GLI1* and MO-C or MO-2. (B) Sets of tadpoles injected with *GLI1* and MO-C or MO-3 showing varying overall deformities present in tumor-bearing *GLI1*+MO-C-injected embryos but absent in *GLI1*+MO-3-injected siblings. The β-gal tracer is blue.

Table 1. Quantitative analyses of marker gene expression

	Targets								
Injected	*Gli1*	*Gli2*	*Gli3*	*Ptc1*	*Ptc2*	*Ncam*	*Ngn1a*	*NeuroD*	*Ntub*
Stage 12									
Gli1+MO-C		10.1*	0.5*	36.5*	135.2*	0.6*	1.5*	1.0*	14.0*
Gli1+MO-2			0.7	0.8	0.6	0.8	1.0	1.1	0.9
Gli1+MO-3		1.1		0.5	0.3	1.3	1.1	0.7	0.4
Gli2+MO-C	6.3*		0.5*	12.2*	29.2*	1.0*	1.6*	0.5*	19.8*
Gli2+MO-1			0.7	0.7	0.6	0.6	0.6	0.3	0.5
Gli2+MO-3	0.4			0.5	0.6	2.1	1.4	1.0	0.4
Gli3+MO-C	0.9*	13.2*		10.3*	18.2*	1.4*	4.8*	2.7*	4.6*
Gli3+MO-1		0.5		0.9	0.6	1.0	0.4	0.2	0.7
Gli3+MO-2	0.8			0.5	0.6	0.7	1.2	0.6	0.3
Stage 14									
Gli1+MO-C		21.0*	1.0*	39.5*	84.5*	0.4*	7.5*	7.9*	4.7*
Gli1+MO-2			0.7	1.0	0.7	0.9	0.8	0.7	0.7
Gli1+MO-3		0.9		0.8	0.8	1.3	1.8	0.7	0.5
Gli2+MO-C	20.1*		2.7*	10.4*	23.0*	1.3*	15.1*	1.4*	28.8*
Gli2+MO-1			0.4	0.5	0.7	0.6	0.6	1.5	0.4
Gli2+MO-3	0.3			0.3	0.2	0.6	0.6	2.8	0.1
Gli3+MO-C	6.3*	28.4*		10.6*	12.5*	2.0*	16.3*	3.7*	8.4*
Gli3+MO-1		0.4		0.5	0.6	0.5	0.4	0.8	0.2
Gli3+MO-2	0.4			0.5	1.2	0.7	0.4	0.7	0.1
Stage 14									
GLI1+MO-C	6.9*	5.2*	1.3*	20.9*	64.5*	1.2*	2.6*	0.5*	2.6*
GLI1+MO-1		0.8	3.5	0.1	0.2	2.0	0.5	1.9	0.7
GLI1+MO-2	1.1		1.3	0.9	0.8	5.6	2.5	4.0	1.6
GLI1+MO-3	0.7	0.7		>0	>0	2.4	1.1	3.3	0.2
GLI2+MO-C	19.7*	5.5*	2.0*	18.3*	17.4*	6.3*	4.3*	16.5*	22.8*
GLI2+MO-1		0.9	1.2	0.5	0.8	0.4	1.2	>0	0.3
GLI2+MO-2	1.2		1.9	2.2	1.0	0.4	1.4	>0	0.2
GLI2+MO-3	1.0	1.5		0.8	1.2	0.9	5.2	2.0	0.3
GLI3+MO-C	9.5*	22.2*	3.7*	10.6*	4.9*	4.1*	15.1*	4.6*	34.8*
GLI3+MO-1		0.3	0.3	0.4	1.3	1.2	0.5	0.1	0.2
GLI3+MO-2	0.9		0.3	0.7	1.4	2.4	0.5	0.2	0.7
GLI3+MO-3	1.0	0.8		0.9	1.8	0.4	0.4	0.8	0.7

Numbers indicate fold change in endogenous gene expression in stage 12 or 14 animal caps with frog Gli or human GLI RNAs and MOs, determined by real-time RT-PCR analyses as calculated by the ΔCT method. Gene expression levels were normalized to EF1α. Changes of at least 40% are in green for activator function and in red for repressor function.

*The action of injected Gli proteins.

MO-C=Gli'x'+MO-C/uninjected AC.

MO-'x'=Gli'x'+MO-'y'/Gli'x'+MO-C.

Gli3 at stage 12, but at stage 14 injected Gli2 and GLI2 induced it. However, injected Gli2 did so in a Gli1-dependent manner, while endogenous Gli2 acted as a repressor with injected GLI2. Finally, induction of *Gli3* by injected GLI3 required Gli1 and Gli2.

Neurogenic cascade

Another group of genes tested comprises *Ncam*, *Ngn1a*, *NeuroD* and *N-tubulin* to reflect neural and neuronal determination and differentiation (Figs 6, 7). *Ncam*, a general neural marker (Balak et al., 1987), was repressed by Gli1 at stages 12 and 14 but induced by Gli3 at both stages. GLI2 and GLI3, but not GLI1, also induced *Ncam*. *Ngn1a*, an early and critical bHLH neurogenic factor (Ma et al., 1996), was induced by all Gli proteins from frog and human origin. By contrast, *NeuroD* (Lee et al., 1995) was repressed by Gli2 and induced by Gli3 at stage 12, whereas it was induced by all three Gli proteins from both species at stage 14, with the exception of GLI1, which acted as a repressor. *N-tubulin*, a marker of differentiated neurons (reviewed by Chitnis and Kintner, 1995), was also induced by all Gli proteins tested at both stages. As with the Gli and Ptch genes, the use of *Gli* MOs uncovered a complex regulatory network. One example is described.

Ngn1a

Ngn1a was induced by all injected Gli proteins. However, at stage 14 but not stage 12, endogenous Gli3 acted as a repressor in the presence of injected Gli1. By contrast, endogenous Gli3 acted as a repressor at stage 12 and an activator at stage 14 in the presence of injected Gli2. Endogenous Gli1 was required for induction of *Ngn1a* by injected Gli2 at stages 12 and 14. Induction of *Ngn1a* by injected Gli3 required endogenous Gli1 at stages 12 and 14 but only endogenous Gli2 at stage 14. Its induction by injected GLI1 required endogenous Gli1 but Gli2 acted as a repressor. By contrast, endogenous Gli2 and Gli3 acted as repressors in the presence of injected GLI2. Finally, all three endogenous Gli proteins were required for induction by injected GLI3.

Cooperative effects of Gli proteins in nuclear localization

The results presented above suggest cooperative functional interactions in target gene regulation. To begin to investigate

the basis of such cooperativity, we first probed the ability of Gli proteins to influence the subcellular localization of each other. Here, we took advantage of the finding that human GLI1 localizes to the nucleus, while frog Gli2 localized to the cytoplasm in transfected COS-7 cells (Fig. 7A,C,D) (Ruiz i Altaba, 1998; Ruiz i Altaba, 1999). Co-transfection of both GLI1 and Gli2 resulted in the preferential localization of Gli2 in the nucleus only when co-expressed with GLI1 (Fig. 7A,C). A similar result was obtained with human GLI3, which normally localized to the cytoplasm (Ruiz i Altaba, 1999) but became nuclear when co-expressed with GLI1 (Fig. 7C,D).

Zic proteins are members of the Gli superfamily that harbor a very similar five zinc-finger DNA-binding domain (Brewster et al., 1998; Nakata et al., 1998). Zic1 has been reported to enhance the nuclear localization of Gli1 and Gli3, and to affect protein function in vitro (Koyabu et al., 2001; Mizugishi et al., 2001). In vivo, Gli proteins and Zic2 interact to establish the pattern of the neural plate where Zic2 functions as an anti-neurogenic factor in primary neurogenesis (Brewster et al., 1998). We therefore tested whether Zic2 could also affect Gli localization in transfected COS-7 cells. Zic2 protein was nuclear (Brewster et al., 1998) (Fig. 7B) and its co-expression with frog Gli1, which is also heavily cytoplasmic (Lee et al., 1997; Ruiz i Altaba, 1999), Gli2 or GLI3 resulted in an increased nuclear localization of each of these Gli proteins (Fig. 7B,D).

Physical interactions through the first two fingers of the five zinc-finger domain in the Gli superfamily

Given the colocalization of co-expressed Gli/Zic2 proteins, we tested whether GLI3 could bind Zic2. Immunoprecipitation and western blot analyses of co-transfected Zic2 and different forms of GLI3 showed that full-length Zic2 can bind N- and C-terminally deleted forms of GLI3 (Ruiz i Altaba, 1999), as well as Zic2 itself (Fig. 7E). This focused our attention on the central zinc-finger domain. Zic1 and Gli proteins bind each other through their last three zinc fingers (Koyabu et al., 2001), a domain that represents the most highly conserved region in these proteins. This interaction may render the proteins unable to bind DNA as the last three fingers also represent the DNA-binding domain (Pavletich and Pabo, 1993). Unlike with Zic1 (Koyabu et al., 2001), we found that the first two fingers of Zic2 or GLI3 are sufficient to bind Zic2 and Gli proteins (Fig. 7F,G). Here, we used C-terminally deleted forms, which are expressed at higher levels than full-length Gli proteins and are therefore easier to detect (Ruiz i Altaba, 1999). The first two zinc fingers of GLI3 specifically recognized Zic2, Gli2 and Gli3, but not Gli1 (Fig. 7F). Similarly, the first two fingers of Zic2 recognized Gli2 and GLI3, but not Gli1 (Fig. 7G). Although other binding sites may exist, this specificity correlated with the ability of Gli2 and Gli3, but not Gli1, to harbor potent dominant-negative function, an activity shared with Zic2 (Ruiz i Altaba, 1999; Brewster et al., 1998).

The first two fingers of Zic2 did not recognize full-length Zic2 (Fig. 7G). This result indicated a high degree of specificity and begged the question of whether Zic2 proteins could form complexes. Analyses of a variety of truncated and chimeric forms of Zic2 (Fig. 7H) showed that Zic2-Zic2 interactions occur through the N-terminal domain, unlike in the case of Gli-Zic2 binding.

Discussion

Using a knockdown approach with specific antisense oligonucleotides we demonstrate here the involvement of each of the three Gli proteins in primary neurogenesis and uncover a network of cooperative interactions that regulates Gli function and target gene expression. Our data provide a framework to understand Gli function in cell fate specification, allowing for fine-tuned outputs and the specification of multiple cell types.

We have previously shown that there is a critical involvement of Gli

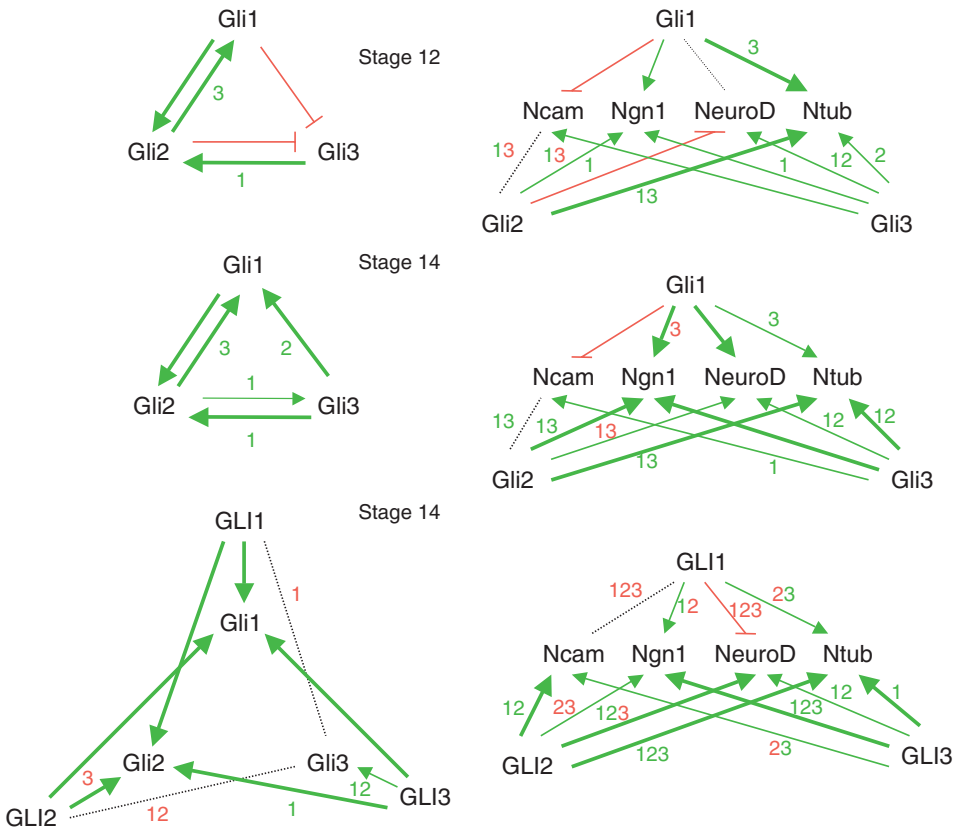

Fig. 6. Diagrammatic summary of Gli function. Frog proteins are in lower case whereas human proteins are in upper case. Green and red indicate activator or repressor activity. Arrows indicate induction and T bars repression. Thick lines refer to changes of fivefold or higher. The numbers next to the arrows or T bars indicate the requirement of the different endogenous Gli genes. The data derive from Table 1.

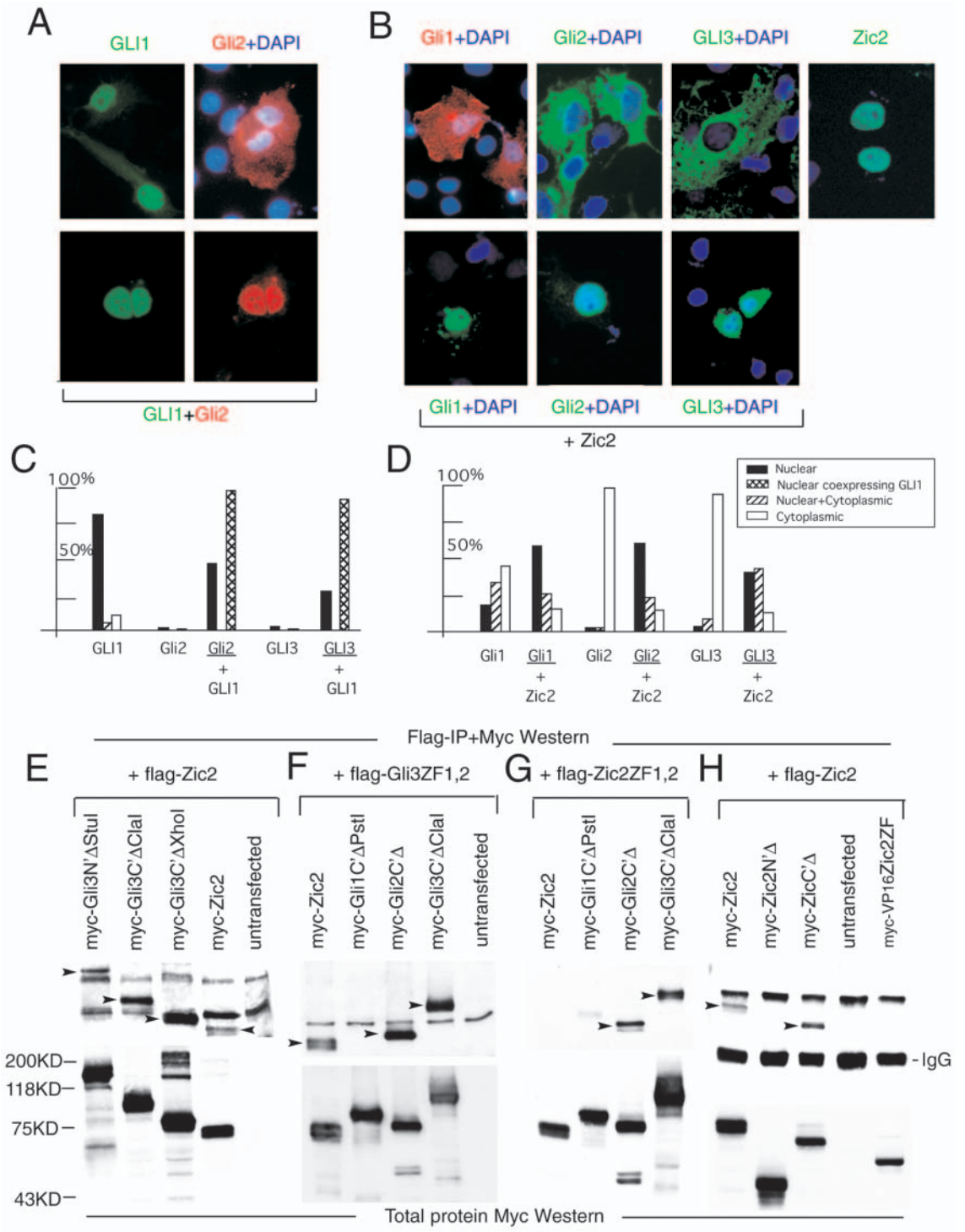

Fig. 7. Cooperative effects and physical interactions within the Gli superfamily. (A) (Top left) Human GLI1 is nuclear in transfected COS cells, here detected with specific polyclonal anti-human GLI antibodies (Lee et al., 1997). (Top right) Myc-tagged Gli2 is cytoplasmic. (Bottom left) Co-expression of GLI1 and Gli2 results in nuclear localization of Gli2 (bottom right). (B) (Top panels) Expression of Myc-tagged frog Gli1, Myc-tagged-Gli2, Myc-tagged GLI3 and FLAG-tagged Zic2 in COS cells. (Bottom panels) Co-expression with Zic2 forces the Gli1, Gli2 and GLI3 cytoplasmic proteins into the nucleus. DAPI staining highlights nuclei (blue). The color of the font indicates the protein visualized by immunostaining. (C,D) Quantification of the subcellular compartmentalization of Gli proteins when transfected alone versus co-transfection with human GLI1 (C), and when transfected alone versus co-transfection with Zic2 (D). (C) Only nuclear values are given for co-transfection of Gli2 and GLI1 and for GLI3 and GLI1. (E-H) (Top panels) Immunoprecipitation of Flag-tagged proteins with anti-FLAG Ab as labeled, followed by western blotting with anti-Myc Ab. Arrowheads indicate the immunoprecipitated species. (Bottom panels) Corresponding western analysis of total Myc-protein input from transfected COS cells as labeled. The horizontal alignment of bottom panels corresponds to the molecular weight markers on the left.

function in the patterning of the neural plate, the CNS primordium (Lee et al., 1997; Brewster et al., 1998; Ruiz i Altaba, 1998; Ruiz i Altaba, 1999). The present data crucially extends this work by showing an unexpected requirement for each Gli protein in the induction of all primary neurons: motor, sensory and interneurons. Our results appear strikingly different from those obtained in mice, as loss of the function of any single or all Gli proteins in mouse embryos does not abolish neural tube neurogenesis (Bai et al., 2004). This difference could be due to the species-and context-dependent function of Gli proteins (e.g. Ruiz i Altaba, 1998; Ruiz i Altaba, 1999; McDermott et al., 2005). Indeed, Gli1 is not required in mice for development or tumorigenesis (Park et al., 2000; Weiner et al., 2002), but it is essential for frog embryo tumors (Dahmane et al., 1997; Dahmane et al., 2001) (this work) and human cancer (Sanchez et al., 2004). Similarly, Gli3 has a major negative function in the mouse neural tube, which must be suppressed by Shh signaling in order to allow ventral neuronal differentiation (Litingtung and Chiang, 2000), but here we demonstrate a sweeping positive role for Gli3 as a required component of primary neurogenesis. Evidence for positive Gli3 activity has been also described in gain-of-function analyses on amphibian primary neurogenesis (Ruiz i Altaba et al., 1998; Brewster et al., 1998) and in a limited fashion in the zebrafish, chick and mouse neural tube (Persson et al., 2002; Meyer and Roelink, 2003; Bai et al., 2004; Tyurina et al., 2005).

The demonstration that all three Gli proteins are required for the formation of all primary neurons in the neural plate, together with Gli gain-of-function phenotypes (Lee et al., 1997; Ruiz i Altaba, 1998; Ruiz i Altaba, 1999) and the function of Zic2 as a prepattern anti-neurogenic factor (Brewster et al., 1998), allow us to suggest a further elaboration of our previous model for Gli function in primary neurogenesis: (1) combinatorial Gli/Zic function determines the sites of neuronal differentiation, the neuronal stripes (Brewster et al., 1998); and (2) the different neuronal types induced in different stripes depend on cooperative and quantitatively combinatorial Gli function. The Gli code thus integrates signaling inputs (e.g. Brewster et al., 2000), regulates the bHLH-Notch neurogenic cascade (this work) (Brewster et al., 1998; Franco et al., 1999) and affects neuronal type outcomes (this work) (Ruiz i Altaba, 1998).

Mechanistically, we show that the Gli proteins regulate neurogenic bHLH genes (Brewster et al., 1998) (this work), indicating that they act upstream of neuronal specification, consistent with their expression patterns: the Gli genes are co-expressed at low levels throughout the animal cap, the neural plate primordium, preceding the expression of neurogenic bHLH genes, and are later expressed in partially overlapping domains throughout the mature neural plate (Lee et al., 1997; Mullor et al., 2001). Primary neurogenesis therefore requires Gli function in different neural plate areas that display high and low expression levels, and/or in the neural plate primordium where Gli expression is low and ubiquitous. However, the Gli proteins are also required for neurogenesis directly induced by the ectopic expression of the neurogenic bHLH proteins and the Notch-Delta pathway, which modulates bHLH gene expression (Chitnis et al., 1995; Chitnis and Kintner, 1996), suggesting sustained function of the Gli proteins is required at distinct steps in the neurogenic cascade. This is also consistent with results with the involvement of Zic2 upstream and downstream of the bHLH neurogenic cascade (Brewster et al., 1998). Therefore, although it remains unclear exactly how Gli proteins regulate bHLH protein function – for example, by affecting co-factors or additional components, such as Id (e.g. Liu and Harland, 2003) or homeodomain proteins (Briscoe et al., 2000; Gershon et al., 2000), or by interacting with multiple bHLH proteins – the data highlight a multistep link between bHLH/Notch and Gli activities that is essential for neurogenesis.

In addition to neurogenic function, the Gli proteins have anti-neural crest activity (Brewster et al., 1998). Our previous work has shown that ectopic Gli2 or Gli3 function suppresses neural crest differentiation whereas Zic2 or dominant-negative GLI function induces it (Brewster et al., 1998), consistent with a general positive involvement of Zic proteins in neural crest development (Nakata et al., 1998). Unexpectedly, we demonstrate here that knockdown of Gli3, the Gli gene most prominently expressed in the neural folds, also suppresses neural crest differentiation. An explanation for this apparent discrepancy may reside in the fact that the MOs interfere with the action of both positive (activator) and negative (repressor) forms of Gli2 and Gli3, as the latter form post-transcriptionally. We propose that Gli repressor function at the edges of the neural plate, far from midline sources of Shh, is required to inhibit any positive neurogenic Gli function and thus allow for neural crest differentiation.

The quantification of target gene expression we present indicates that the Gli proteins act in a cooperative fashion that is dynamic, target gene and species dependent. The larger number of functional interactions observed after expression of the human proteins in comparison with those detected after expression of frog Gli proteins, would appear to result from the interplay of four Gli proteins (the three endogenous ones plus the exogenous one injected) versus the interplay of only three players (the injected plus the other two endogenous ones). This cooperative function of Gli proteins and their ability to influence each other's subcellular localization raise the possibility of the existence of protein complexes. In support of this idea, we show that the first two Gli zinc fingers can act as a docking site for other Gli proteins, possibly through the same domain. These findings offer a role for an evolutionarily conserved subdomain that had so far remained functionally orphaned and that shows a higher degree of variability within the Gli superfamily (Lee et al., 1997; Mizugishi et al., 2001) than the last three zinc fingers, which bind DNA (Pavletich and Pabo, 1993). In contrast to Gli-Gli and Gli-Zic interactions, Zic2 forms homocomplexes through the N-terminal region and not through the zinc-finger domain, showing specificity and suggesting the possibility of hetero-oligomers. Together, the data thus suggest the existence of a Gli protein network.

The ability of this protein network to act in a context-dependent manner is most probably reliant upon the availability of interacting co-factors. For example, the binding of Zic2 to C′Δ forms of Gli2 and Gli3, but not of Gli1, demonstrates specificity and suggests functional relevance, as Zic2 and C′Δ forms of Gli2 and Gli3, but not Gli1, have dominant repressive function (Ruiz i Altaba, 1999; Shin et al., 1999; Aza-Blanc et al., 2000). The overall read out of positive and negative Gli function, the combinatorial Gli code, may thus depend on the type of cooperative interactions present – the

network – and the types of factors that dock on or interact with the network. These factors include Zic proteins (Brewster et al., 1998; Koyabu et al., 2001); Ski, which recruits the histone deacetylation complex (Dai et al., 2002); Suppressor of Fused (Kogerman et al., 1999; Ding et al., 1999; Dunaeva et al., 2003); Dyrk1 (Mao et al., 2002); and Hox proteins, some of which can impart activator function to Gli3C′Δ repressors (Chen et al., 2004). Shh signaling, and other signaling inputs (Brewster et al., 2000), may thus act on the Gli code by modifying the combination of Gli proteins that interact in a cooperative network and thus the possible factors that associate.

The cooperative and combinatorial function of Gli proteins may also be critical in cancer (e.g. Ruiz i Altaba et al., 2004) as endogenous Gli1 has been shown to be required for the induction of epidermal and neural tumors by exogenous GLI1 (Dahmane et al., 1997; Dahmane et al., 2001). Here, we extend these data and show that endogenous Gli3 is also required for GLI1-induced tumorigenesis. This finding is consistent with the ability of Gli3, as Gli1 and Gli2, to act as an activator in different contexts (Brewster et al., 1998; Persson et al., 2002; Meyer and Roelink, 2003; Bai et al., 2004; Zuñiga and Zeller, 1999; Zakany et al., 2004). However, the data also reveal a differential requirement for Gli2 and Gli3 as knockdown of Gli2, like that of the other Gli proteins, has drastic effects on target gene expression but it is not required for GLI1-mediated neurogenesis or tumorigenesis. Sustained Gli activity necessary for tumor growth thus appears to require the cooperative function of Gli1 and Gli3. This is interesting as ectopic expression of Gli1, but not Gli3, leads to tumor formation. Instead, ectopic Gli3 leads to supernumerary tail development (Brewster et al., 2000). As ectopic expression occurs at higher than endogenous levels, this result further suggests that the Gli code is both qualitative and quantitative.

We propose that the context-specific cooperative (this work) and combinatorial (Ruiz i Altaba, 1998) action of Gli proteins, forming a Gli network, is a general property and underlies a number of previous unexplained observations in different systems. In frogs, these include: the ability of co-injected Gli2 or Gli3 to inhibit ventral forebrain neuronal and floor plate inductions by exogenous Gli1; and the ability of exogenous Gli3 to inhibit motoneuron induction by co-injected Gli2 (Ruiz i Altaba, 1998). In the mouse neural tube, a Gli network could explain the requirement of Gli1 for positive Gli3 function (Bai et al., 2004). Similarly, in zebrafish and cell culture (Tyurina et al., 2005) a combinatorial and cooperative Gli network could account for the findings that: Gli3 cooperates with Gli1 to induce ventral CNS targets; Gli3 repressor function affects a number of targets differently; Gli3 and Gli2 inhibit Gli1-induced Gli reporter activation; but Gli3 enhances it in the presence of Shh while Gli3 alone is ineffective.

Finally, the finding that amphibian primary neurogenesis and tumorigenesis (Dahmane et al., 1997; Brewster et al., 1998) (this work), on the one hand, and adult mammalian stem cell neurogenesis (Lai et al., 2003; Machold et al., 2003; Palma and Ruiz i Altaba, 2004; Palma et al., 2005) and cancers of different organs (e.g. Dahmane et al., 2001; Thayer et al., 2003; Watkins et al., 2003; Sanchez et al., 2004) (reviewed by Ruiz i Altaba et al., 2004), on the other hand, depend on Hh-Gli signaling, further suggests the extension of a cooperative Gli network to other scenarios. These include stem cells, adult neurogenesis and cancer.

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