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Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling

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SUMMARY

Gli family zinc finger proteins are mediators of Sonic hedgehog (Shh) signaling in vertebrates. The question remains unanswered, however, as to how these Gli proteins participate in the Shh signaling pathway. In this study, regulatory activities associated with the Gli2 protein were investigated in relation to the Shh signaling. Although Gli2 acts as a weak transcriptional activator, it is in fact a composite of positive and negative regulatory domains. In cultured cells, truncation of the activation domain in the C-terminal half results in a protein with repressor activity, while removal of the repression domain at the N terminus converts Gli2 into a strong activator. In transgenic mouse embryos, N-terminally truncated Gli2, unlike the full length protein, activates a Shh target gene, *HNF3β*, in the dorsal neural tube, thus mimicking the effect of Shh signal. This suggests that unmasking of the strong activation potential of Gli2 through modulation of the N-terminal repression domain is one of the key mechanisms of the Shh signaling. A similar regulatory mechanism involving the N-terminal region was also found for Gli3, but not for Gli1. When the Shh signal derived from the notochord is received by the neural plate, the widely expressed Gli2 and Gli3 proteins are presumably converted to their active forms in the ventral cells, leading to activation of transcription of their target genes, including *Gli1*.

Key words: *Gli1, Gli2, Gli3,* Sonic hedgehog, neural tube, *HNF3β,* Mouse

INTRODUCTION

The Hedgehog (Hh) family of signaling molecules is involved in variety of inductive processes in invertebrate and vertebrate development (see review by Hammerschmidt et al., 1997). One member of this family, Sonic hedgehog (Shh), is a major regulator of dorsoventral patterning of the central nervous system and somites, anteroposterior patterning of limb buds, and left-right asymmetry in vertebrates (reviewed by Goodrich and Scott, 1998).

The roles of Shh have been studied most extensively in the development of the central nervous system. Shh signaling organizes the ventral pattern of the neural tube. In the most ventral part, the floor plate, expression of *HNF3β*, is induced by the high concentration of Shh emanating from the underlying notochord (Echelard et al., 1993; Marti et al., 1995b; Roelink et al., 1995). The differentiated floor plate cells also express *Shh*, and Shh proteins derived from both notochord and floor plate in combination presumably form a gradient of activity, and this Shh gradient regulates the differentiation of a variety of cells which are arranged in a ventrodorsal array in the lateral walls of the ventral neural tube (Ericson et al., 1995; Hynes et al., 1995; Marti et al., 1995a; Roelink et al., 1995; Chiang et al., 1996).

On binding of the Shh protein to the transmembrane receptor Patched (Ptc), the signal-receiving cells express a set of target genes (Marigo et al., 1996a; Stone et al., 1996). *Ptc* and *Gli1* are induced in all types of cells, while *HNF3β* is induced only in a limited number of tissues, including the central nervous system (Marigo et al., 1996b,c; Lee et al., 1997; Sasaki et al., 1997). Several lines of evidence indicate that Gli proteins function as downstream transcription factors of Shh signaling. In vertebrates, three *Gli* genes, *Gli1* (originally designated as *Gli*), *Gli2* and *Gli3* have been identified, which encode proteins with closely related zinc finger motifs as DNA binding domains (Ruppert et al., 1988; Hui et al., 1994; Marigo et al., 1996b; Ruiz i Altaba, 1998). Evidence for direct involvement of Gli in gene transcription comes from its regulation of the floor plate-specific enhancer of the *HNF3β* gene. This enhancer contains a binding site for Gli proteins which mediates the Shh response and is essential for the enhancer activity (Sasaki et al., 1997). There are indications that Gli1 and Gli2 are activators of Shh target genes. For example, Gli1 is highly expressed in cells adjacent to the source of Shh signal,

and human GLI1 activates the Shh-responsive element in a cell transfection assay (Platt et al., 1997; Sasaki et al., 1997). Overexpression of Gli1 induces *HNF3β* expression and floor plate development at ectopic sites in transgenic mouse embryos and in frog embryos (Hynes et al., 1997; Lee et al., 1997). Mouse embryos homozygous for *Gli2* mutations display diminished expression of Shh target genes, *Ptc* and *Gli1*, and lack a floor plate in the neural tube (Ding et al., 1998; Matise et al., 1998).

Regulation of Hh target genes by Gli proteins is better understood in *Drosophila* than in vertebrates. In the fly, the Gli protein Cubitus interruptus (Ci) acts downstream of Hh (Forbes et al., 1993; Alexandre et al., 1996; Von Ohlen et al., 1997). There is evidence suggesting that Ci is a bipotential transcriptional regulator whose activity is controlled by the Hh signal. Ci is expressed widely in populations of cells responsive to Hh signaling (Domínguez et al., 1996). In the absence of a Hh signal, Ci protein is processed into a repressor form by proteolysis, and the Hh signal stimulates maturation of Ci into a labile transcriptional activator (Aza-Blanc et al., 1997; Chen et al., 1998; Ohlmeyer and Kalderon, 1998). The conservation of some protein domains between Gli and Ci suggests that analogous regulation may control the activities of the Gli proteins.

Given that Gli proteins are likely involved in the Shh signaling pathway, an important question is whether post-translational regulation of Gli protein activity is also a mechanism essential for the Shh signaling in vertebrates. This question has been complicated partly by the fact that transcription of *Gli* genes is also affected by the Shh signal, which is not the case for *Ci* and *Hh* in *Drosophila* (Marigo et al., 1996b; Hynes et al., 1997; Lee et al., 1997; Sasaki et al., 1997; Ruiz i Altaba, 1998). In this work, we first focused on Gli2. *Gli2* is expressed widely in populations of cells responsive to Shh signaling, and its transcriptional regulation by the Shh signal is minimal. In contrast, the Shh signal strongly induces expression of *Gli1*, while it represses *Gli3*. We showed that Gli2 is a complex transcriptional factor carrying both activation and repression domains. Full-length Gli2 carrying both domains acts as a weak activator. Deletion of the N-terminal repression domain of Gli2 converted the protein into a strong activator in cultured cells, and its expression in transgenic mice mimicked Shh signaling. These results suggest that, in the absence of the Shh signal, the biological activity of Gli2 is suppressed by the N-terminal domain. The existence of such a regulatory mechanism is consistent with the hypothesis that Gli2 is a primary mediator of Shh signaling. We also found that Gli3, but not Gli1, has a similar mechanism whereby the N-terminal region modulates the activator function. Therefore, Gli2 and Gli3 proteins appear to be primary mediators of Shh signaling, and Gli1 is presumably secondarily activated at the transcriptional level.

MATERIALS AND METHODS

Cloning of Gli cDNAs

To obtain full coding sequences for mouse *Gli1* and *Gli2*, cDNA libraries of E10.5 and E11.5 mouse embryos were constructed and screened with partial mouse *Gli1* (Sasaki et al., 1997) and *Gli2* (Hui et al., 1994) cDNAs as probes. The longest cDNAs were subcloned into Bluescript (Stratagene) and the coding regions were sequenced. The missing 5′ end of *Gli1* was cloned by 5′ RACE. The resulting cDNAs carrying the complete regions were designated pGli1-full/BS (#40) (4.0 kb) (DDBJ/EMBL/GenBank accession number AB025922) and pGli2-full/BS (#3) (7.5 kb), respectively. Their cDNA sequences were identical to those previously reported (Hughes et al., 1997; Liu et al., 1998).

Transfection assay

Expression plasmids for mouse *Gli1* and *Gli2*, and human *GLI1* (Kinzler et al., 1988) (accession number X07384) and *GLI3* (Ruppert et al., 1990) were constructed by isolating the coding regions of the cDNAs by eight cycles of PCR using Pfu DNA polymerase (Stratagene) and then cloning them into the pcDNA3.1-His plasmid (Invitrogen). Gli2 deletion constructs except for ΔN2 were prepared utilizing the following unique restriction sites: *Hinc*II (ΔC1), *Xba*I (ΔC2), *Pvu*I (ΔC3), *Apa*I (ΔC4), *Nsp*V (ΔN3) and *Eco*RV (ΔN1). Gli2-ΔN2, Gli1-ΔN and Gli3-ΔN sequences were prepared by PCR. Expression plasmids of a fusion protein of the GAL4 DNA-binding domain and various portions of Gli2 were constructed using a pCMV/SV2-GAL4-DBD plasmid (Kamachi et al., 1999). The following restriction sites were used to generate the Gli2 fragments: *Sma*I (C), *Sma*I and *Xba*I (A1), *Xba*I (A2) and *Hinc*II (C1), except that the N fragment was prepared by PCR. Primer sequences are available upon request.

Transfection of the rat neural stem cell line MNS70 (Nakagawa et al., 1996) was performed by the standard $CaPO_4$ method as described by Sasaki et al. (1997) with the following modifications. Cells were plated into PRIMARIA 6-well plates (Becton Dickinson) at a density of 1×10^6/well. Each $CaPO_4$/DNA mixture contained effector (1.5 μg), reporter (1.5 μg) and reference (SV-β-gal, 0.5 μg) plasmids. As a reporter, either 8×3′Gli-BS-δ51LucII (Sasaki et al., 1997), or pG4-TK-Luc (Sekido et al., 1997) was used, depending on the effector plasmid. Cells were harvested 1 day after DNA addition. Luciferase activities were normalized with respect to the β-galactosidase activity. The experiments shown in Fig. 4B were performed with 10T1/2 cells since the basal expression level of the reporter in 10T1/2 is much higher than in MNS70. Consistent results were obtained with MNS70 cells. In the case of transfection of 10T1/2 cells, cells were plated into standard 6-well plates at a density of 5×10^4 cells/well. All transfections were repeated in at least two independent experiments, which gave reproducible results.

Production of transgenic mouse embryos

The plasmid pEn2-sp/NotI/pA was constructed by cloning a 1.2 kb DNA fragment of a rabbit *β-globin* gene containing an intron and a poly(A) signal at downstream of the 7.9 kb *En2* promoter fragment (Logan et al., 1993). In addition, a unique *Not*I site was introduced between the intron and poly(A) signal. cDNA fragments isolated from pcDNA3.1-His-Gli plasmids were cloned into the *Not*I site of the pEn2-sp/NotI/pA. The resulting plasmids were used for transgene preparations.

Transgene DNAs were purified by a gelase (Epicentre Technologies) method (Hogan et al., 1986). Transgene DNA (2-3 ng/μl) was injected into the pronuclei of (C57BL/6 × C3H)F2 fertilized mouse eggs as described by Hogan et al. (1986). Genotypes of the embryos were determined by PCR of genomic DNA from extra-embryonic tissues or embryonic tails. The primers used were as follows:

Common 5′: 5′-TGTACACCATGGACCCTCATG-3′
Gli1 3′: 5′-TTTGACTGAACTCCGAGGAGGAG-3′
Gli2 3′: 5′-TCGAGGGGTCACACGTGGACTAGAG-3′
Gli2-ΔN2 3′: 5’-GATGACCACCTCGGCCTCCTG-3′
hGli3 3′: 5′-TCTGTGGCTGCATAGTGATTGC-3′
hGli3-ΔN 3′: 5′-TTGGACCTCTTGTTGTGCATCG-3′

The number of transgenic embryos obtained for each construct

were: three for Gli1-full, seven for Gli2-full, four for Gli2-ΔN2, two for Gli3-full and two for Gli3-ΔN.

Immunohistochemistry

Dissected embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C overnight, dehydrated with ethanol and stored at −20°C. Dehydrated embryos were transferred into butanol and embedded in paraffin. Sections (7 μm) were processed for immunohistochemistry with rabbit antiserum against mouse HNF3β (Yasui et al., 1997) or mouse monoclonal antibody for Lim(1+2) (4F2) (Tsuchida et al., 1994) in combination with anti-rabbit or anti-mouse IgG conjugated with alkaline phosphatase (Jackson Laboratories), respectively. Procedures were as described by Wall et al. (1993).

RESULTS

Three Gli proteins have different activities in cell culture

Previously, we established an assay system for regulation of gene expression by Shh signaling using a rat neural stem cell line, MNS70. In this system, Shh or human GLI1 (hGLI1) activate a reporter gene carrying eight copies of a Gli-binding site derived from the *HNF3β* floor plate enhancer (Sasaki et al., 1997) (Fig. 1A). In this study, we characterized the individual activities of the three different Gli proteins using newly cloned murine *Gli1* and *Gli2* cDNAs (Fig. 1B). Mouse Gli1 acted as a strong transcriptional activator, comparable to hGLI1. Mouse Gli2 activated the reporter, but to a level significantly lower than that induced by Gli1. On the other hand, human GLI3 failed to activate and instead repressed the reporter expression, as noted previously (Sasaki et al., 1997). Therefore, the three Gli proteins have distinguishable activities: Gli1, strong activation; Gli2, weak activation; and Gli3, repression. In the following experiments, mouse *Gli1* and *Gli2*, and human *GLI3* are designated simply as *Gli1*, *Gli2* and *Gli3*.

Gli2 has a bipartite activation domain in the carboxy-terminal region

Recent analyses of mutant embryos of mouse and zebrafish suggest that Gli2 is involved in the Shh pathway (Ding et al., 1998; Matise et al., 1998; Karlstrom et al., 1999). To understand the action of Gli2 at the molecular level, we analyzed the regulatory activities associated with the Gli2 protein. Since it is known that hGLI1 has an activation domain at its C terminus (Yoon et al., 1998), we made a series of C-terminal deletion mutants of Gli2 and tested their activities in MNS70 cells, resulting in the finding that a short deletion from the C-terminal end (ΔC1) totally abrogated the activation potential (Fig. 2A). Mutant proteins with larger deletions of Gli2 (ΔC2-ΔC4) acted as repressors and reduced the reporter expression to below the basal level. These results indicate that the activator function of Gli2 requires the C-terminal portion of the protein, and that the small region at the C terminus (residues 1452-1544) is especially critical.

Because of the importance of the C-terminal portion of Gli2, the potential of this region as an activation domain was tested by tethering it to the GAL4 DNA-binding domain (GAL4-BD) and measuring its activity on a GAL4-binding site-containing reporter (Fig. 2B). Fig. 2C shows the strong transactivation potential of the region of the Gli2 protein more C-proximal than the zinc fingers (BD-C; 642-1544). This activation domain can be divided into two parts, which individually display transactivation potential (BD-A1: 642-1183 and BD-A2: 1184-1544). This result may suggest that at least two different activation mechanisms are associated with Gli2. However, the integrity of the entire C-terminal region (domain C) may be required for activation by Gli2, since a deletion of the small C-terminal end region C1 (1452-1544), which by itself did not show a significant activation potential (Fig. 2C), abolished the activator function of Gli2 (Fig. 2A).

Gli2 contains a repression domain at the N-terminal side, and has the potential to be a repressor or a strong activator

In *Drosophila*, the absence of the Hh signal results in proteolysis of Ci into a C-terminally truncated form which acts as a repressor (Aza-Blanc et al., 1997). Since the C-terminal deletion of Gli2 resulted in the loss of activator function, we hypothesized that Gli2 may be subject to similar processing and therefore we tested the repressive activities associated with the C-terminally truncated proteins whose coding regions are shown in Fig. 3A. Expression of Gli2-ΔC2 or Gli2-ΔC4 in MNS70 cells clearly repressed the reporter

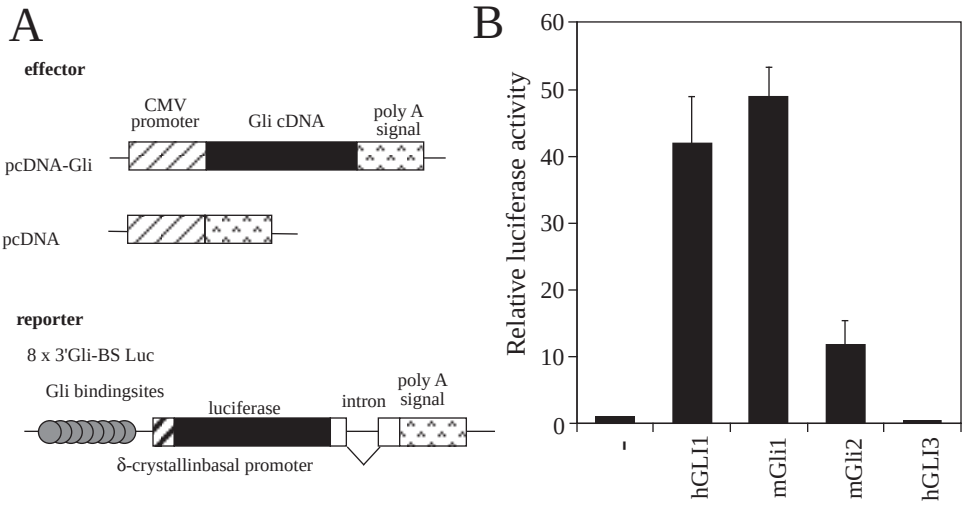

Fig. 1. Full length Gli proteins have different activities in MNS70 cells. (A) Schematic representation of effector genes and the reporter gene in the co-transfection assay. Effectors express Gli proteins under the control of the CMV promoter. The reporter consists of an 8-mer of a Gli protein-binding site sequence (3′Gli-BS) of the mouse *HNF3β* floor plate enhancer, the basal promoter of the chicken *δ-crystallin* gene and the *luciferase* gene. (B) Effect of exogenous Gli proteins in MNS70 cells. Mouse Gli1 and human GLI1 strongly activated the reporter. Mouse Gli2 weakly activated the reporter while human GLI3 repressed it. The results represent the average of two samples with standard errors.

expression to below the basal level, indicating that they act as repressors (Fig. 3B). When these Gli2 mutants were co-expressed with full length Gli1 or Gli2, Gli2-ΔC2/4 strongly inhibited the transactivation by Gli1 or Gli2, confirming the strong repressive activity of Gli2-ΔC2/4.

Since Gli2-ΔC2/4 exerted a strongly repressive effect, we asked whether Gli2 had a repression domain at its N terminus. Fig. 4A shows that removal of residues 1 to 279 (Gli2-ΔN2; 280-1544) converted Gli2 into a strong activator, indicating the existence of a repressive regulatory domain in this N-terminal region. Consistent with this, an N-terminal end fragment (BD-N; 1-308) of Gli2 showed a strong transrepression activity (Fig. 4B). In summary, Gli2 is a complex transcriptional regulator consisting of both repression and activation domains. Consequently, Gli2 can act as either a repressor or a strong activator if one of these regulatory domains is inactivated.

Gli2-ΔN2 induces HNF3β and ventral cells at ectopic dorsal sites in vivo

The N-terminal deletion mutant Gli2-ΔN2 strongly activated transcription, in contrast to the full length Gli2, which had a moderate activation potential (Fig. 4A). Therefore, we speculated that the activity of Gli2 is modulated by Shh signaling through abrogation of the repressive activity of the N-terminal domain. We took advantage of the fact that Gli2-ΔN2 may mimic the activity of the Shh-activated form of Gli2 in order to test the above speculation by examining the activity of Gli2 and Gli2-ΔN2 in transgenic mice using the *En2* promoter/enhancer. This promoter drives transgene expression in cells straddling the boundary of the midbrain/hindbrain region of the neural tube. In the anterior hindbrain of E12.5 embryos, transgene expression is restricted to the dorsal side (Fig. 5A) (Logan et al., 1993). Expression of *HNF3β*, *Shh* and *hGLI1* under the control of this enhancer promotes differentiation of ventral cells in the dorsal neural tube (Sasaki and Hogan, 1994; Hynes et al., 1997).

Transgenic embryos with *Gli2-ΔN2* displayed expansion of the dorsal mid/hindbrain at E14.5, but transgenes with full length *Gli2* did not cause any morphological change (data not shown). Such embryos were first analyzed for the expression of a floor plate-specific gene, *HNF3β*, which is a direct target of Shh signaling (Sasaki and Hogan, 1994; Marti et al., 1995a; Roelink et al., 1995; Ruiz i Altaba et al., 1995). The *Gli2-ΔN2* transgenic embryos expressed HNF3β in small patches at ectopic dorsal sites of the neural tube (Fig. 5D). As expected from the function of *HNF3β* as a floor plate regulator (Sasaki and Hogan, 1994), the morphology of the cell clusters in the dorsal neural tube which ectopically expressed HNF3β resembled that of the floor plate (compare insets of Fig. 5B,D). Thus, Gli2-ΔN2 mimicked the effect of the Shh signal.

Ectopic HNF3β was always found in small patches, although the transgene is thought to be widely expressed within the *En2* expression domain. One possible explanation for this is low expression of the transgene around the level of the threshold for *HNF3β* induction. The level of transgene expression

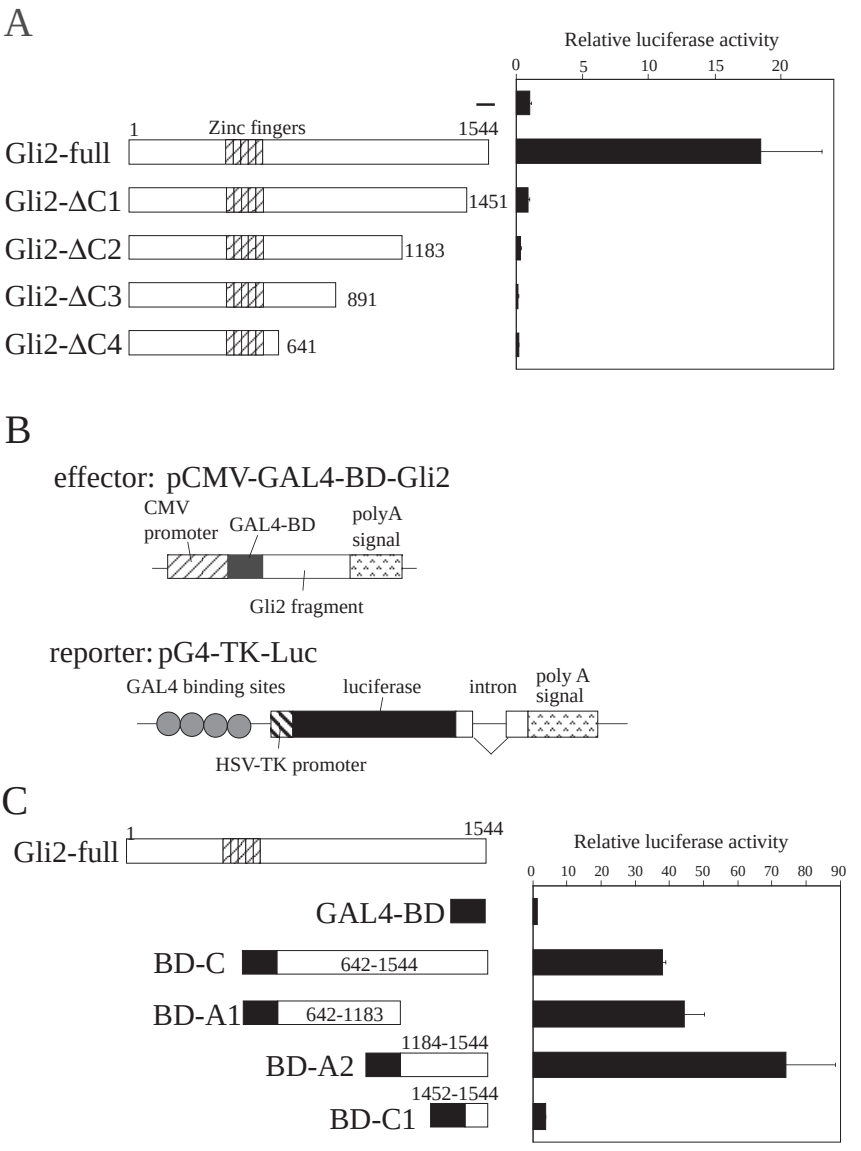

Fig. 2. Gli2 has a bipartite activation domain in the carboxy-terminal portion. (A) C-terminally truncated Gli2 proteins are schematically shown on the left. Clustered zinc fingers are indicated by hatched boxes. Numbers refer to amino acids in the protein sequences. Relative luciferase activities are shown on the right. Only full length Gli2 activated the reporter, while all other C-terminally truncated forms did not.
(B) Schematic representation of effectors and the reporter used in the domain analysis. Effectors code for fusion proteins of the GAL4 DNA-binding domain and a portion of Gli2. The reporter consisted of a 4-mer of the GAL4 binding site, the *HSV-TK* promoter (–197 – +56) and the *luciferase* gene. (C) Activities of isolated C-terminal portions of Gli2. Solid boxes represent GAL4-BD. The full length C-terminal portion induced transcriptional activation, and this portion could be divided into two subdomains, A1 and A2, which possessed individual activation potentials.

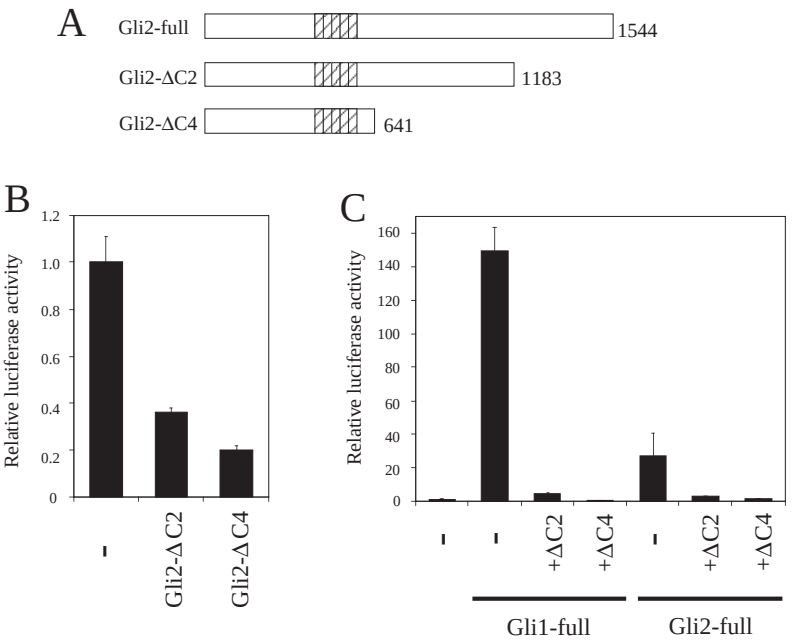

Fig. 3. C-terminally truncated Gli2 proteins are repressors. (A) Schematic representation of C-terminally truncated Gli2 proteins. (B) Gli2-ΔC2/4 functions as a repressor in MNS70. Transfection of Gli2-ΔC2/4 repressed reporter expression to below the basal level. (C) Gli2-ΔC2/4 counteracts activation by full length Gli1 and Gli2. Co-transfection of Gli2-ΔC2/4 and either Gli1 (left) or Gli2 (right) strongly repressed transcriptional activation by Gli1 or Gli2.

appears in fact to be low, because we could not detect the expression of the tagged Gli2-ΔN2 protein encoded by the transgene in these embryos (data not shown). If Gli2-ΔN2 was expressed throughout the *En2* domain at a low level and if it mimicked the effect of the Shh signal, the cells which did not express HNF3β in the *En2* domain should undergo the fate of ventral neurons, which require weaker Shh signaling. To test this possibility, we studied the expression of Lim1/2. In normal embryos, Lim1/2-positive cells were widely distributed in the ventrolateral region except for the floor plate, and formed dense clusters on both sides of the floor plate (arrows in Fig. 5E). In *Gli2-ΔN2* transgenic embryos, dense clusters of Lim1/2-positive cells also existed at the ectopic sites of the dorsal neural tube between or around ectopic HNF3β-expressing cells (Fig. 5F). The ectopic Lim1/2-positive cells were not always adjacent to the HNF3β-expressing cells, suggesting that these cells were produced as an alternative consequence of the transgene expression. Therefore, Gli2-ΔN2 might directly promote differentiation of the ectopic Lim1/2-positive cells rather than as a secondary effect of the HNF3β-positive cells assuming a floor plate function, although the latter possibility was not ruled out. Taken together, these results show that, mimicking the effects of various strength of Shh signaling, Gli2-ΔN2 promotes differentiation of the ventral type of cells. The type of cells induced may be correlated with the expression level of Gli2-ΔN2 in individual cell populations.

In the case of full length *Gli2* transgenics, however, no morphological change of the dorsal neural tube or ectopic expression of HNF3β or Lim1/2 was detected (Fig. 5C, data not shown). Thus, an activated form of Gli2 (Gli2-ΔN2) mimicked the effect of Shh, while full length Gli2 failed to mimic these effects in the absence of the Shh signal. This suggests that the Shh signal activates Gli2 through abrogating the repressive function of the N-terminal region during neural patterning.

Gli3 but not Gli1 is regulated by the N-terminal repression domain

The repression domain at the N terminus is important in controlling Gli2 activity and regulating Shh target genes. We investigated whether the N-terminal domains of the other two

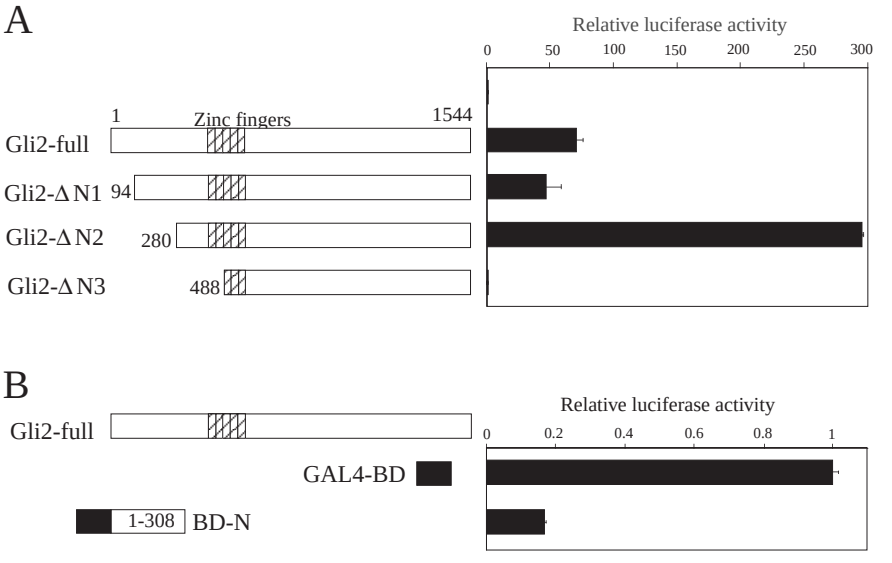

Fig. 4. Gli2 has a repression domain near the N terminus. (A) Activities of N-terminally truncated Gli2 proteins. Gli2-ΔN2 activated transcription much more strongly than full length Gli2. (B) Repressive activity of isolated N-terminal portion of Gli2. The reporter and effector genes were as described in Fig. 2B. The N domain had a strong repressor activity.

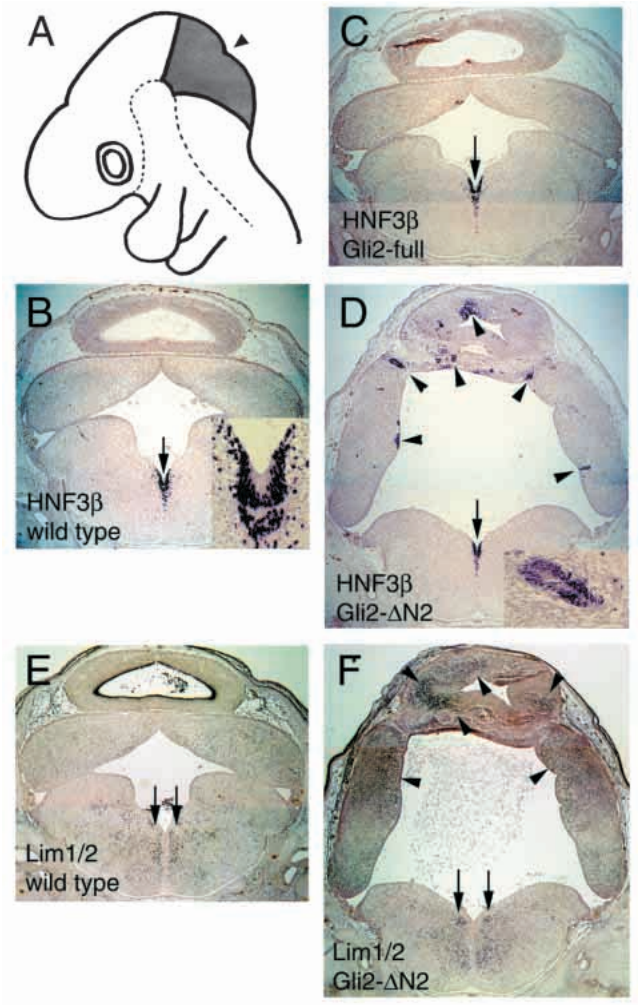

Fig. 5. N-terminally truncated Gli2, but not full length Gli2, can promote ventral cell differentiation in the dorsal neural tube of transgenic mouse embryos. Transgenic embryos which expressed Gli2 or Gli2-ΔN2 under the control of the *En2* enhancer/promoter were produced. (A) A schematic drawing of transgene expression domain at E12.5 (Logan et al., 1993). At this stage, the transgene is expressed in a wide region (shaded) in the dorsal neural tube straddling the boundary of the mid/hindbrain (arrowhead). The transgenes are thought to be expressed widely within the shaded area. (B-F) Cross sections of E14.5 embryos were prepared at the anterior hindbrain level and expression of HNF3β (B-D) or Lim1/2 (E, F) was examined by immunohistochemistry. (B) Expression of HNF3β in the wild type embryo was restricted to the floor plate region (arrow). The inset shows an enlargement of the floor plate. (C) A transgenic embryo expressing Gli2, and showing no ectopic expression of HNF3β or morphological abnormality. (D) A transgenic embryo expressing activated Gli2 (Gli2-ΔN2), and displaying a number of ectopic sites of HNF3β expression (arrowheads). The inset shows an enlargement of one of the ectopic floor plates. The authentic floor plate is indicated by an arrow. (E) Distribution of Lim1/2-positive cells in a normal embryo. They were widely distributed in the ventrolateral region. Arrows indicate the clusters of Lim1/2-positive cells. (F) In a transgenic embryo expressing *Gli2-ΔN2*, dense clusters of Lim1/2-positive cells are also found on the dorsal side of the neural tube (arrowheads).

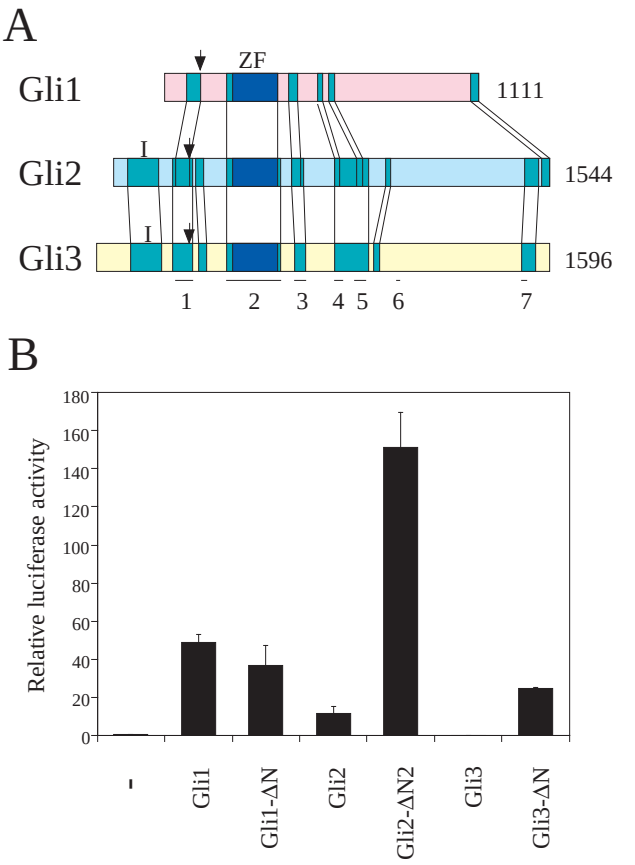

Fig. 6. The N-terminal region of Gli3 has repressive activity similar to that of Gli2, but the N-terminal region of Gli1 lacks such an activity. (A) Schematic drawing showing amino acid sequence similarities between mouse Gli2 and mouse Gli1, and between mouse Gli2 and human Gli3. The dark-blue boxes indicate zinc fingers. The conserved regions, which have greater than 50% identity in a span longer than 20 residues, are indicated in green. Arrows indicate the positions of N-terminal deletions used in B. Horizontal lines with numbers below Gli3 indicate the homology regions reported by Ruppert et al. (1990). (B) The N-terminally truncated Gli1 (Gli1-ΔN) has similar activation potential to the full length Gli1. The N-terminal truncation of Gli3 (Gli3-ΔN) converted Gli3 from a weak repressor into an activator.

Gli proteins have similar properties. Based on the sequence similarities of the Gli proteins, N-terminal deletions analogous to Gli2-ΔN2 were produced for Gli1 and Gli3 (Fig. 6A, arrows indicate the points of deletion). Gli1 and N-terminally truncated Gli1 (Gli1-ΔN; 135-1111) showed similar transcriptional activation in transfected cells, indicating that the N-terminal domain of Gli1 does not have a significant repressive function (Fig. 6B). This result is consistent with the hypothesis that Gli1 is a simple activator and its activity is controlled mainly at the transcriptional level. In the case of Gli3, however, N-terminal truncation (Gli3-ΔN; 345-1596) clearly converted the protein from a non-activator into an activator, although the level of transcriptional activation attained with this form of Gli3 was still lower than the levels obtained with Gli1 and Gli2-ΔN2 (Fig. 6B). This result suggests that Gli3 has a repressive regulatory domain at the N terminus, like Gli2.

With the knowledge of the functional differences of the three Gli proteins in transfected cells, we tested in vivo activities of Gli1, Gli3 and Gli3-ΔN by expressing them in transgenic

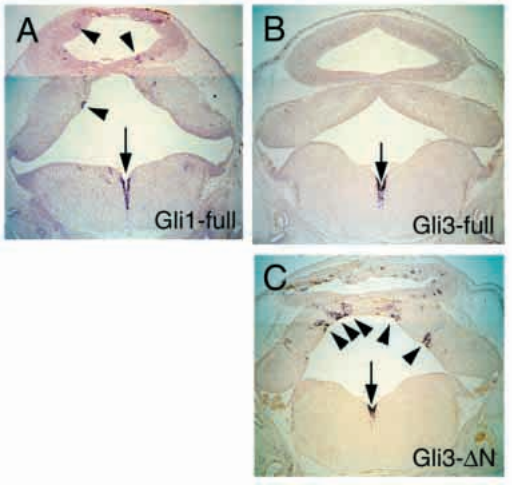

Fig. 7. Full length Gli1 and Gli3-ΔN, but not full length Gli3, induce ectopic HNF3β expression in transgenic embryos. Transgenic mouse embryos which expressed Gli1, Gli3 and Gli3-ΔN were produced and analyzed as described in Fig. 5. (A) Transgenic embryos with full length Gli1 expressed HNF3β at ectopic sites. Positions of the floor plate and ectopic floor plate-like structures are indicated by an arrow and arrowheads, respectively. (B) Transgenic embryos with full length Gli3 did not have any ectopic HNF3β expression or abnormal morphology. (C) Transgenic embryos with Gli3-ΔN displayed ectopic HNF3β expression in the anterior hindbrain together with abnormal morphology.

mouse embryos using the *En2* promoter/enhancer. In the case of Gli1, ectopic expression of full length mouse Gli1 resulted in the expression of HNF3β at ectopic sites (Fig. 7A) as previously reported with human GLI1 (Hynes et al., 1997). This result indicates that overexpression of Gli1 can mimic the effect of Shh signaling. In contrast, the results with Gli3 were similar to those of Gli2: transgenic embryos with full length *Gli3* did not have any abnormal phenotype, but those with *Gli3-ΔN* expressed HNF3β ectopically (Fig. 7B,C). Thus, the function of Gli3 may be regulated in a way similar to that of Gli2 by Shh signaling.

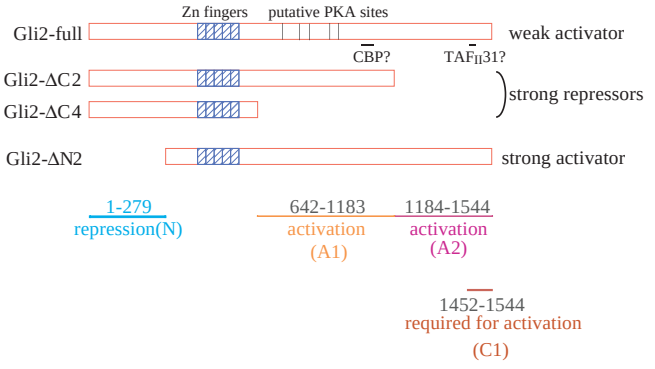

Fig. 8. Summary of the Gli2 domain analysis. A strong repression domain is present close to the N terminus (N). An activation domain which consists of two subdomains, A1 and A2, is present in the C-terminal half of the Gli2 protein. A short region at the C terminus (C1) is essential for the activator function of full length Gli2 protein, but does not have a significant activation potential by itself.

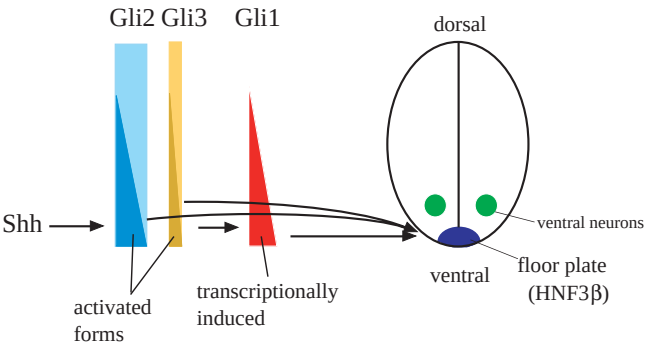

Fig. 9. Role of Gli proteins in mediating the Shh signal and in establishing the ventral pattern of the mouse neural tube. Before and at the time of floor plate induction, high level of Gli2 and low level of Gli3 are expressed throughout the neural plate/neural tube. Shh signal coming from the underlying notochord activates Gli2/3 proteins at the ventral side, and activated forms of Gli2/3 induce expression of the simple activator Gli1. Activated Gli2/3 and induced Gli1 synergistically promote differentiation of the floor plate. Dark colors in the boxes indicate hypothetical distributions of activated proteins.

DISCUSSION

Gli2 is a bipotential transcription factor

The present study demonstrates that Gli2 is a bipotential transcription factor, possessing both activation and repression domains. An activation domain, which can be divided into two subdomains, A1 and A2, is located in the C-terminal half of Gli2, and a repression domain is located in the N-terminal region (N). C-terminally truncated forms of Gli2 proteins function as strong repressors and N-terminally truncated Gli2 functions as a strong activator (Fig. 8).

Two mechanisms of transactivation have been elucidated for Ci and hGLI1. Histone acetyltransferase dCBP binds to the C-terminal region of *Drosophila* Ci as a coactivator, and loss of dCBP impedes Hh signaling (Akimaru et al., 1997). Sequence comparison revealed a motif well conserved between the dCBP interaction domain of Ci and activation domain A1 of Gli2 (Ci: EELILPDEMLQYL; Gli2: DELVLPDDVVQYI), raising the possibility of interaction of CBP/p300 with the A1 domain (Hughes et al., 1997). On the other hand, for human GLI1 it has been suggested that activation by a C-proximal domain may involve direct interaction with $TAF_{II}31$ (Yoon et al., 1998). A sequence bearing a significant similarity to the putative $TAF_{II}31$-interaction motif is also found in domain A2 of Gli2 (hGLI1: DSLDLDNTQLDFVAILDE; Gli2: DSQLLEPPQIDFDAIMDD), and in the C-terminal region of Gli3 and Ci as well (Yoon et al., 1998). It is thus possible that the activator function of Gli2 involves two different mechanisms: interaction with the co-activator CBP/p300, and interaction with a component of the basic transcriptional machinery, $TAF_{II}31$.

Gli2 has a strong repression domain in the N-terminal portion (Fig. 4), and C-terminally truncated Gli2 proteins devoid of the activation domain function as strong repressors (Fig. 3B, C). In *Drosophila*, absence of the Hh signal results in conversion of Ci by proteolysis into a C-terminally truncated form which is a repressor protein (Aza-Blanc et al., 1997). Gli2

has five sites containing the consensus sequence for PKA phosphorylation common to Ci, which are essential for the processing in the case of Ci (Chen et al., 1998), implying that C-terminal processing may also occur for Gli2 in the absence of the Shh signal, although the occurrence of processing remains to be demonstrated.

At the protein level, Gli3 shares many characteristics with Gli2, while Gli1 seems to be different. Gli3 shows significant sequence similarities with Gli2 throughout the protein (Fig. 6A) including the potential CBP/p300 binding site and five PKA sites (Ruppert et al., 1990; Thien et al., 1996). In contrast, Gli1 lacks the potential CBP/p300 site and contains only three PKA sites (Kinzler et al., 1988; Liu et al., 1998). Consistent with this notion, Dai et al. (1999) recently provided biochemical evidence for the existence of a CBP-binding domain and PKA-dependent processing in the case of Gli3, but not in the case of Gli1. In this study, we also demonstrated that Gli3 has a repressive N-terminal domain similar to Gli2, while Gli1 appears not to contain a similar domain (Figs 6 and 7). A conserved motif (homology region 1) reported by Ruppert et al. (1990), may not contribute to the repressive activity of the N-terminal domain since it is also found in Gli1. The repressive activity may be associated with another conserved motif (marked as 'I' in Fig. 6A) which exists in the N-terminal portion of Gli2 and Gli3, but not in Gli1. These observations indicate that Gli2 and Gli3 are bipotential transcriptional regulators, while Gli1 seems to act solely as an activator.

Gli2 acts as the primary mediator of Shh signaling

Mutant mouse embryos have been used to demonstrate the essential role of Gli2 in the Shh signaling pathway. *Gli2*$^{zfd/zfd}$ embryos lack expression of a Shh target gene, *HNF3β*, in the ventral neural tube, and this results in the loss of the floor plate and adjacent cells (Ding et al., 1998; Matise et al., 1998). Gli2 is a bifunctional transcription factor, and removal of the repression domain at the N terminus converts it from a weak activator into a strong activator (Fig. 4A). In transgenic mouse embryos, this active form of Gli2, but not the full length Gli2, could induce expression of *HNF3β* at ectopic sites in the neural tube, mimicking the effect of Shh (Fig. 5). In normal development, *Gli2* is expressed widely throughout all dorsoventral levels of the neural tube at the time of floor plate induction (Lee et al., 1997; Sasaki et al., 1997; Ding et al., 1998), but *HNF3β* is induced only in the prospective floor plate, supporting the idea that Gli2 is active only in the ventral neural tube. All of these observations are consistent with the model that Gli2 protein is activated by Shh signaling as the primary mediator of the Shh signal. In vitro experiments with MNS70 cells also suggest that Gli2, but not Gli1, is a primary mediator of Shh signaling (M. N., unpublished observations). This argument implies that unmasking of the strong activation potential of Gli2 through modulation of the repression domain at the N terminus is an important mechanism of Shh signaling. It is likely that the Shh signal regulates the interaction of a co-repressor protein and the repression domain. Regulation of Gli2 activity may also involve protein processing, regulation of subcellular localization or alteration of protein stability, as described for Ci (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998).

Deletion analysis of Gli3 suggested that, similarly to Gli2, this protein is also regulated by a mechanism involving the N-terminal region (Figs 6 and 7). Therefore, it is possible that Gli3, in addition to Gli2, contributes to the primary response to Shh signaling. There are some observations to support this notion. Firstly, *Gli3* is uniformly expressed in the neural tube at the time of floor plate induction, although its level of expression is low (Lee et al., 1997; Sasaki et al., 1997). Secondly, *Gli1/Gli2* double homozygous mutant embryos still manifest a subset of Shh-dependent ventral patterning in the neural tube, suggesting that the residual Gli3 partly compensates for the activator function of Gli2 (Matise et al., 1998). Thirdly, *Gli2/Gli3* double mutant neural tubes have more severe ventral defects than those of *Gli2* mutants (C.-c. H. unpublished observation).

On the other hand, the analysis using transgenic embryos indicate that Gli1, when expressed, acts as an activator of *HNF3β* even in the absence of Shh signaling (Fig. 7) (Hynes et al., 1997). This characteristic of Gli1 does not fit into the model of it being a primary mediator of Shh signaling. It has been demonstrated that expression of *Gli1* is Shh dependent (Marigo et al., 1996b; Lee et al., 1997; Sasaki et al., 1997; Borycki et al., 1998). It is likely that *Gli1* transcription is activated as a consequence of activation of Gli2/3 proteins, and once synthesized, Gli1 works as a simple activator without dependence on the Shh signal. In this sense, Gli1 is secondarily involved in the Shh signaling pathway.

Roles of Gli proteins in ventral neural tube development

Based on the observations described in this report, we propose that Gli2 and Gli3 function as primary mediators of Shh signaling in the model shown schematically in Fig. 9, which indicates their roles in ventral neural tube development. In mouse embryos, from before the time of floor plate induction, both *Gli2* and *Gli3* are expressed throughout the neural tube, with expression of *Gli2* occurring at a higher level than that of *Gli3* (Lee et al., 1997; Sasaki et al., 1997). The nascent Gli2/3 proteins are presumably not active, and the Shh signal, originating from the underlying notochord, alters Gli2/3 proteins into their active forms in the ventral neural fold/tube. Then, the activated Gli2/3 proteins may induce transcription of the *Gli1* gene, thereby establishing ventrally restricted expression of *Gli1* (Marigo et al., 1996b; Lee et al., 1997; Sasaki et al., 1997). Consistent with this model, Gli3 activates the *Gli1* promoter in a Shh-dependent manner in transfected cells (Dai et al., 1999). Since newly synthesized Gli1 is already in an active form, it can activate target genes without further involvement of the Shh signal. It is thus possible that Gli1 expression is outside of the negative feedback loop in which Shh signaling upregulates Ptc, which inhibits further Shh signal transduction. Activated Gli2/3 and secondarily induced Gli1 synergistically activate Shh target genes, including *HNF3β*, in the ventral neural tube. Apparently, Gli2 plays the most important role in this process, but other unidentified transcriptional factors may also contribute to activation of these target genes (Krishnan et al., 1997). Gli proteins directly activate the *HNF3β* enhancer, and expression of HNF3β subsequently promotes floor plate differentiation (Sasaki and Hogan, 1994; Sasaki et al., 1997). Besides activating target genes, Shh also represses expression of some other genes (*Pax7*, *Gli3* etc.) in the ventral neural tube. The possible roles of Gli proteins in Shh-dependent gene

repression have not yet been elucidated and need to be studied in the future.

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