The amino-terminal region of Gli3 antagonizes the Shh response and acts in dorsoventral fate specification in the developing spinal cord

Néva P. Meyer and Henk Roelink*

*Molecular and Cellular Biology Program, Department of Biological Structure, Center for Developmental Biology,
University of Washington, Box 357420, Seattle, WA 98195, USA*

Abstract

A concentration gradient of Shh is thought to pattern the ventral neural tube, and these ventral cell types are absent in $shh^{-/-}$ mice. Based on in vitro and genetic studies, the zinc finger-containing transcription factors Gli 1, 2, and 3 are mediators of the Shh intracellular response. The floorplate and adjacent cell types are absent in $gli1^{-/-};gli2^{-/-}$ mice, but part of the $Shh^{-/-}$ phenotype in the neural tube is alleviated in the $Shh^{-/-};gli3^{-/-}$ double mutant. This is consistent with the predicted role of Gli3 as a repressor of the Shh response. Gli3 repressor activity is blocked by Shh. In order to test the role of the repressor form of Gli3 in the neural tube, a truncated version of Gli3 (Gli3R*) was designed to mimic a Pallister Hall allele. Gli3R* acts as a constitutive repressor independent of Shh signaling. Misexpression of Gli3R* in the chick neural tube caused a ventral expansion of class-I, dorsal progenitor proteins and a loss of class-II, ventral progenitor proteins consistent with expected activity as a repressor of the Shh response. Activation of the BMP response is sufficient to maintain $gli3$ expression in neural plate explants, which might be a mechanism by which BMPs antagonize the Shh response.
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Introduction

Dorsoventral patterning of neuronal fate in the developing spinal cord occurs as a result of opposing gradients of the morphogens Sonic Hedgehog (Shh) and Bone Morphogenetic Proteins (BMPs). Shh and BMPs are long-range, secreted signaling factors that act in a concentration-dependent manner and can antagonize each other during dorsoventral fate specification (Liem et al., 1995, 2000). Shh and BMPs are distributed in opposing gradients in the neural tube with Shh emanating from the floorplate and notochord (Briscoe et al., 2001; Echelard et al., 1993; Krauss et al., 1993; Marti et al., 1995; Roelink et al., 1994; Tanabe et al., 1995) and BMP family members being released from the roof plate and ectoderm (Lee et al., 1998; Liem et al., 1995, 1997). The differential activation of the Shh and BMP responses within a cell ultimately determines the cellular

phenotype by modulating the expression of homeodomain and bHLH transcription factors inside each, single cell (Barth et al., 1999; Briscoe et al., 2001; Briscoe and Ericson, 1999; Ericson et al., 1997; Lee et al., 1998; Liem et al., 1995, 1997; Roelink et al., 1995; Timmer et al., 2002). Because the expression of these transcription factors ultimately determines neuronal cell fate, they are referred to as progenitor domain transcription factors (Briscoe and Ericson, 1999; Briscoe et al., 2000; Ericson et al., 1997; Gowan et al., 2001; Sander et al., 2000; Vallstedt et al., 2001).

Depending on the distance from the floorplate in the ventral neural tube, Shh signaling activates expression of homeodomain progenitor proteins, such as Nkx2.2 and Nkx6.1, which have been labeled class II progenitor proteins (Briscoe et al., 2000), while repressing expression of other progenitor domain proteins, such as Pax7, Pax6, Dbx1, Dbx2, and Irx3, which have been termed class-I progenitor proteins. The mutually repressive interactions between class-I and class-II proteins result in defined boundaries of protein expression. The ensuing progenitor

* Corresponding author. Fax: +1-206-543-1524.
E-mail address: roelink@u.washington.edu (H. Roelink).

domain code determines subsequent differentiation of neuronal subtypes in discrete regions along the dorsoventral axis of the neural tube (see Fig. 1B, Briscoe et al., 2000, 2001; Ericson et al., 1997). In the spinal cords of mice lacking *shh*, class II progenitor proteins are not properly induced, and expression of class I proteins becomes expanded ventrally. This results in an absence of almost all ventral cell types, including floorplate, V3 interneurons, motor neurons, and V0, V1, and V2 interneurons (Chiang et al., 1996; Litingtung and Chiang, 2000b).

BMPs are a second family of morphogens important for the dorsoventral fate specification in the neural tube. BMPs are initially expressed in the ectoderm overlying the neural tube and later in the dorsal part of the neural tube, including the roof plate (Lee et al., 1998; Liem et al., 1995, 1997). Although it has been difficult to demonstrate an absolute requirement of BMPs for dorsal cell fate patterning (Barth et al., 1999; Dudley and Robertson, 1997; Lee et al., 1998; Nguyen et al., 2000), ectopic activation of the BMP response results in a shift of the dorsal progenitor domain boundaries. Specifically, activation of the BMP response causes a ventral shift in the boundary of the dorsally expressed homeodomain transcription factors Pax6, Pax7, Msx1, and Msx2 and represses expression of intermediate and ventral homeodomain proteins, such as Dbx1 and Dbx2 (Timmer et al., 2002). Taken together, these results demonstrate that progenitor proteins can be both Shh- and BMP-responsive.

A critical question that arises is how a graded response to Shh results in the appropriately localized expression of progenitor proteins. Downstream mediators of the Shh response are the Gli1, Gli2, and Gli3 zinc finger transcription factors. In the developing neural tube, Gli2 mediates Shh-induced induction of floor-plate and V3-specific gene expression, since these cell types are lost in $gli2^{-/-}$ mice (Bai et al., 2002; Ding et al., 1998; Matise et al., 1998). The role of Gli3 in mediating the Shh response is less obvious; $gli3^{-/-}$ mice have no apparent defect in their developing spinal cord (Ding et al., 1998; Litingtung and Chiang, 2000b). However, loss of gli3 partially restores the $shh^{-/-}$ phenotype, as evidenced by the restoration of most ventral cell types, including motor neurons, V0, V1, and V2 interneurons, which are absent in $shh^{-/-}$ mice (Litingtung and Chiang, 2000b). The simplest explanation of this observation is that Gli3 acts as a repressor of the Shh response. Work by several labs has demonstrated that, in the absence of Shh, Gli3 is processed into amino- and carboxy-terminal fragments (Aza-Blanc et al., 2000; Dai et al., 1999; Ruiz i Altaba, 1999; Wang et al., 2000). The amino-terminal fragment, which harbors the zinc-finger domain, is a repressor of Shh-response genes. Activation of the Shh response prevents formation of this repressor form, allowing the transcription of Shh-response genes (Aza-Blanc et al., 2000; Dai et al., 1999; Marine et al., 1997; Sasaki et al., 1999; Shin et al., 1999; Wang et al., 2000).

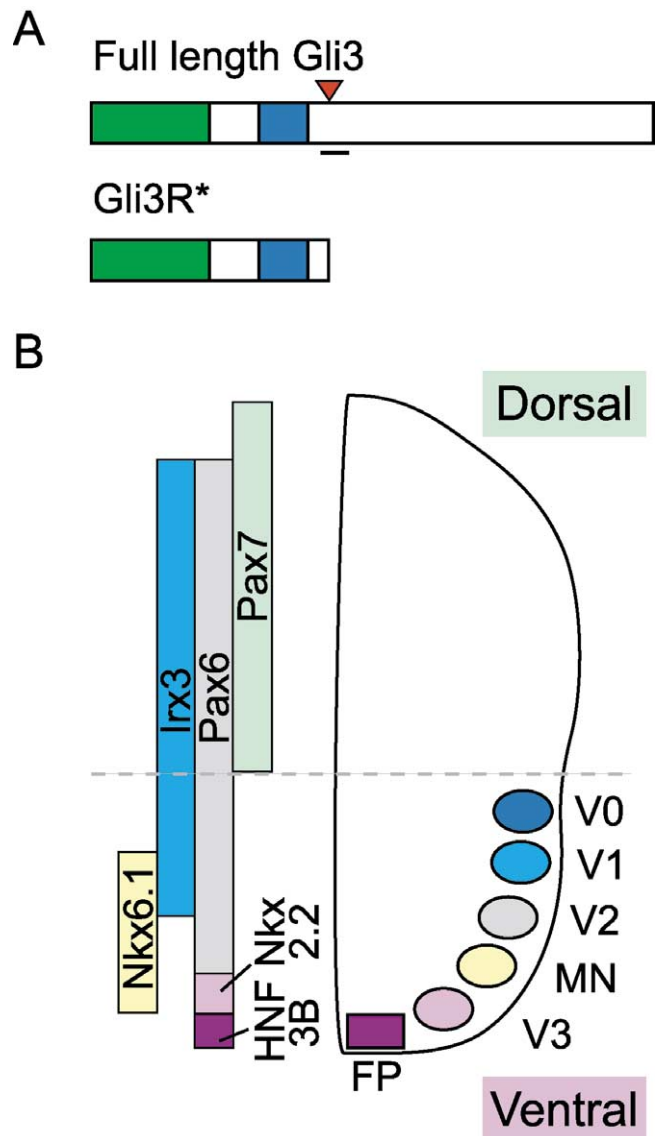In order to determine the role of Gli3R during neuronal

Fig. 1. Diagrams of the Gli3R* and of class-I/class-II expression domains in the spinal cord. The Gli3R* construct was made by truncating full-length Gli3 after the amino-terminal repressor domain (green) and zinc finger domain (blue). The site of truncation (red arrowhead) was made within the putative cleavage domain, which is marked by a bar (A). Shh signaling activates expression of class-II progenitor proteins, such as Nkx2.2 and Nkx6.1, while repressing expression of class-I progenitor proteins, such as Pax7, Pax6, Dbx1, Dbx2, and Irx3. The mutually repressive interactions between class-I and class-II proteins result in defined boundaries of protein expression, and the ensuing progenitor domain code determines subsequent differentiation of neuronal subtypes in discrete regions along the dorsoventral axis of the neural tube (B). V0, V1, V2, V3, interneurons; MN, motorneurons; FP, floorplate.

lineage restriction in developing spinal cords, we created a truncated Gli3 construct (Fig. 1B; Gli3R*) modeled after an allele found in Pallister Hall Syndrome (Shin et al., 1999). Gli3$_{PHS}$ is insensitive to Shh regulation and appears to act as a constitutive repressor. We observed that expression of Gli3R* caused an expansion of class-I progenitor domain proteins, such as Pax7, Pax6, and Irx3, while repressing
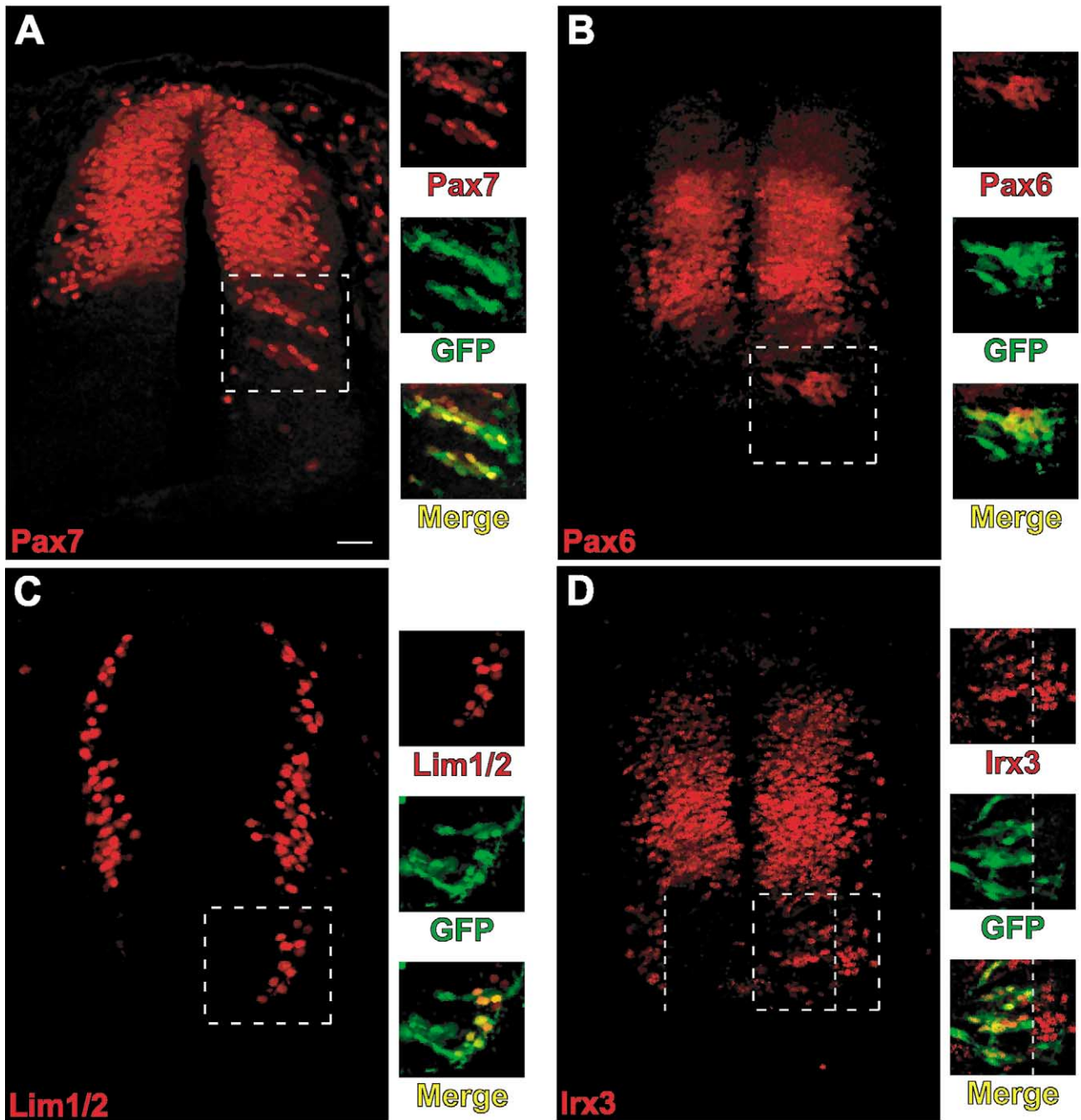
Fig. 2. Gli3R* expression allows the expansion of class-I progenitor transcription factors. Expression of the class-I homeodomain proteins Pax7 (A), Pax6 (B), and Irx3 (D) was cell-autonomously upregulated by Gli3R* misexpression on the right side of the spinal cord at HH stage 20-21. In addition to the dorsal-intermediate domain of Irx3 expression, there also is a population of ventrolateral cells that express Irx3, lateral to the dashed line (D). These cells were not included in analysis used for Table 1, since this is a normal domain of Irx3 expression. Ectopic expression of Irx3 induced by Gli3R* was scored medial to the dashed line (D). The interneuron marker Lim1/2 was also expanded upon Gli3R* misexpression (C). The box outlined by the white, dashed line in each panel is displayed to the right. Expression of GFP, which marks Gli3R*-positive cells, antibody marker, and the merge between the two is shown. Scale bar is 20 μm. Dorsal is up in all spinal cord cross-sections shown.

class-II progenitor proteins, such as Nkx2.2 and Nkx6.1, resulting in a cell-autonomous loss of ventral neurons. Moreover, BMP4 was able to maintain *gli3* expression in neural plate explants. These results demonstrate a critical role for Gli3 in dorsoventral patterning and suggest that the establishment of a gradient of Gli3 repressor is achieved by combined morphogenetic signaling of Shh and BMPs and that this gradient primarily determines establishment of progenitor domains and subsequent differentiation of neural cells in the spinal cord.

Materials and methods

Construction of Gli3R*

Quail *gli3* was a gift from Charles Emerson (Borycki et al., 2000). A total of 315 base pairs, which contained a 65-bp insertion including a premature stop codon, were replaced with cDNA cloned by reverse transcription from quail embryo RNA. The primers used were: CACA-GAGAGAGAAGGAACGCAATC (5′) and GGTGAG-GAGTGCTGAAGGGAGAC (3′). A shortened form of *gli3* encoding amino acids 1-677, which is truncated after the zinc finger domain, was generated by PCR using the primers: AAAGCGGCCGCTTGGCGACGGCAGGTATT (5′) and AAATCATGACCTCCCCAGTCGCACCCTG (3′). Full-length *gli3* or the truncated form of *gli3* (Gli3R*) was expressed from a CMV promoter in pMES-IRES-eGFP, which was kindly provided by Catherine Krull.

Chick electroporation

Fertilized White Leghorn eggs (SPAFAS, CT) were incubated at 37°C until Hamilton–Hamburger stage 10 (Hamburger and Hamilton, 1992). For electroporations, DNA at a concentration of 5–10 μg/μL was injected into the neural tube at the level of the second to last forming somite, and embryos were electroporated by using a BTX ElectroSquare Porator T820. Two types of electroporation were performed. For sideways electroporation, tungsten electrodes were placed on either side of the embryo at 4 mm apart, and embryos were pulsed twice at 25 V for 25 ms. For ventral electroporation, tungsten electrodes were placed on either side of the embryo, but one was inserted into the yolk slightly ventral to the embryo, while the other electrode was placed 1 mm away and slightly dorsal to the embryo. Embryos were pulsed twice at 7 V for 25 ms.

Immunocytochemistry

After electroporation, embryos were allowed to develop for 2 days (approximately HH stage 20-21) and fixed in phosphate-buffered 4% paraformaldehyde, washed extensively, sunk in phosphate-buffered 30% sucrose, and embedded in OCT (Sakura). Cryosections (18 μm) were taken between the forelimbs and the region just anterior to the hindlimbs. Sections were incubated in primary antibodies for either 1.5 h at room temperature or overnight at 4°C. Sections were then incubated with the appropriate secondary antibody for 45 min at room temperature, washed, and mounted using Biomeda Crystal Mount. Fluorescence was visualized by using a Zeiss Axioskop 2 MOT microscope, and confocal images were captured by using LSM5 Pascal software. These images were processed by using Adobe Photoshop.

The following antibodies were kindly provided by Tom Jessell: rabbit anti-Chx10 (Liu et al., 1994), 81-5C10 (mouse anti-HB9) (Tanabe et al., 1995), M2 (guinea pig anti-lsl1/2), and rabbit anti-Irx3. The rabbit anti-Mash1 (Horton et al., 1999) and rabbit anti-Math1 (Helms and Johnson, 1998) antibodies were a gift from Jane Johnson. Additional antibodies used were GN13 (mouse anti-Pax6), mouse anti-Pax7 (Kawakami et al., 1997), 4G1 (mouse anti-Msx1/2) (Liem et al., 1995), 4F2 (mouse anti-Lim1/2) (Tsuchida et al., 1994), 4G11 (mouse anti-En1/2) (Ericson et al., 1997), 74-5A5 (mouse anti-Nkx2.2) (Ericson et al., 1997), 64-4C7 (mouse anti-HNF3β) (Ruiz i Altaba et al., 1995), and 5E1 (mouse anti-Shh) (Ericson et al., 1996). Secondary antibodies used were species-specific and conjugated to Cy3, Texas Red (Jackson ImmunoResearch Labs), or Alexa 568 (Molecular Probes).

In situ hybridization

In situ hybridizations were performed on whole mounts and sections as previously described (Jasoni et al., 1999; Schaeren-Wiemers and Gerfin-Moser, 1993). For neural plate explant assays, pieces of neural plate tissue were removed from HH stage 10 embryos and grown on top of Vitrogen collagen gels buffered with DMEM (GibcoBRL) and NaOH according to the manufacturer's instructions (Cohesion). Wnt3a-conditioned medium at a concentration of 1:1 with complete neurobasal medium (Gibco BRL), appropriate control medium, or BMP4 in complete neurobasal medium was added immediately upon pillow solidification. Wnt3a supernatant was made by growing mouse fibroblast L cells, which were stably transfected with a Wnt3a expression construct (Shibamoto et al., 1998), in Optimem (GibcoBRL) for 4 days. Control supernatant was obtained from mock-transfected L cells. Recombinant human BMP4 (R&D Systems) was resuspended according to the manufacturer's instructions and used at a concentration of 10 ng/mL. After 24 h, the explants were fixed in phosphate-buffered, 4% paraformaldehyde for 4 h at 4°C, and then processed for in situ hybridization (Jasoni et al., 1999; Schaeren-Wiemers and Gerfin-Moser, 1993). Antisense *gli3* riboprobe was generated from the pBK-qGli3 plasmid (Borycki et al., 2000), labeled with digoxigenin (Roche), and purified using Centri-Sep columns (Princeton Separations). Images were visualized by using a Nikon Microphot-SA microscope and then captured with SPOT 3.02 software and processed with Adobe Photoshop. All explant results were reproduced with explants grown in collagen pillows (Placzek et al., 1991; (Yamada et al., 1993), however, because anti-*gli3* probe penetration was much more efficient when the explants were grown on top of collagen gels, this was the preferred method for explant in situ hybridization.

Results

Gli3R* activates class-I progenitor proteins

Gli3R is required to mediate dorsalization of the spinal cord in the absence of Shh, and thus is expected to act as a repressor of the Shh response. We tested this putative ac-

Table 1
Cell-autonomous affect of Gli3R* misexpression

1	2	3	4	5	6	7	8	9
Marker	% Ectopic cells also GFP$^+$	Ectopic cells counted	Marker	% GFP colabeled cells	GFP$^+$ cells counted	Marker	% Chx10 and GFP$^+$ cells	# Cells counted
Pax7	100 ± 0.0	204	Nkx6.1	5.3 ± 3.4	178	Chx10	0.0 ± 0.0	48
Pax6	91 ± 5.0	34	Isl1/2	0.4 ± 1.3	161			
Irx3	97 ± 4.9	105	HB9	0.3 ± 0.8	110			
Lim1/2	89 ± 11.4	65	Nkx2.2	0.4 ± 1.7	142			
En1/2	47 ± 22.4	85						

Note. Cells ectopically expressing indicated markers in the ventral spinal cord (column 1) were scored for GFP expression (column 2), to assess cell autonomy of Gli3R* activity in expanding the expression domain of these markers. To assess the efficacy of Gli3R* in inhibiting the expression of proteins present in the ventral aspect of the neural tube (column 4), the number of GFP$^+$/marker$^+$ cells was determined (column 5). The percentages displayed in Table 1 are an average of percentages calculated from each spinal cord section examined (columns 2, 5, and 8). The standard deviation of each set of percentages also was calculated along with the total number of cells counted (columns 3, 6, and 9). Because the number of Chx10$^+$ cells on the electroporated side of each spinal cord section was so small (on average 2), all of these cells were scored for the presence of GFP (column 8).

tivity via misexpression of a truncated Gli3 construct (Gli3R*) that mimics Gli3R but is insensitive to Shh signals. Gli3R* was misexpressed in chick spinal cords by electroporation, and localized Gli3R* expression was monitored in a cell-autonomous manner by expression of GFP from an internal ribosome entry site (IRES) within the Gli3R* construct.

One of the earliest effects of Shh in the neural plate is the ventral exclusion of Pax7, which is then only expressed in dorsal cells (Ericson et al., 1996; Kawakami et al., 1997). Repression in the ventral region of the neural tube is one of the hallmarks of dorsal class-I transcription factors. High levels of Shh signaling also repress Pax6, although low levels of Shh are required for the induction of Pax6 (Ericson et al., 1997). Additionally, BMP signaling is involved in maintenance of low levels of Pax6 expression in the dorsal spinal cord (Timmer et al., 2002). Misexpression of Gli3R* in the ventral cells of the neural tube resulted in an expansion of the dorsal progenitor transcription factors Pax7 and Pax6 (Fig. 2A and B). Expression of Gli3R* in the ventral aspect of the spinal cord was correlated with a significant change in Pax6 expression, which could be seen by high levels of Pax6 in these ventral cells (Fig. 2B). Gli3R* expression in the dorsal neural tube did not change normal levels of Pax6 in this region (data not shown). All ectopic Pax7$^+$ cells examined and almost all ectopic Pax6$^+$ cells coexpressed GFP (Table 1), demonstrating that Gli3R can very efficiently deregulate repression of both Pax7 and Pax6. In addition, expansion of the interneuron marker Lim 1/2 (Ericson et al., 1997) upon Gli3R* misexpression (Fig. 2C) is consistent with an expansion of this dorsal-intermediate domain of high Pax6 and Pax7 expression.

Another class-I homeodomain progenitor protein is Irx3, whose expression is repressed by Shh signaling and contributes to the specification of cell fate in the spinal cord. Irx3 represses motor neuron fate specification while inducing V2 interneuron fate specification (Briscoe et al., 2000). Irx3 is normally expressed in the dorsal and ventrolateral

region of the spinal cord. Ventral misexpression of Gli3R* was correlated with ectopic Irx3 expression in the ventromedial spinal cord (Fig. 2D). About 97% of ectopic Irx3$^+$ cells examined also expressed GFP (Table 1). The Gli3R*-induced activation of these class-I progenitor proteins is consistent with their normal repression by Shh and demonstrates the ability of Gli3R to antagonize Shh response. These experiments show that the presence of Gli3R in a cell is sufficient to allow the expression of class-I markers, even in the presence of a Shh signal.

Gli3R* represses class II progenitor proteins

Gli3R* is sufficient to allow expression of class-I transcription factors, which are known to repress expression of class-II transcription factors. Based on the expansion of class-I progenitor proteins, we expected Gli3R* misexpression to cause a cell-autonomous inhibition of class-II protein expression. The class-II homeodomain progenitor protein Nkx6.1 is expressed just ventral to the V1 interneuron domain and is necessary at many axial levels for fate specification of motor neurons and V2 interneurons. In addition, misexpression of Nkx6.1 prevents specification of V1 interneuron fate (Briscoe et al., 2000; Sander et al., 2000). Our results showed that GFP$^+$ cells rarely expressed Nkx6.1, even within the normal domain of Nkx6.1 expression, indicating that Gli3R* acts as a repressor of Nkx6.1 expression (Fig. 3A, and Table 1). The presence or absence of the progenitor proteins Nkx6.1, Pax6, and Irx3 are important for fate specification of many different ventral neuron classes. Therefore, the pattern of Pax6 and Irx3 expansion accompanied by loss of Nkx6.1 predicts a loss of ventral neuron classes.

A major group of neurons that differentiate in the ventral spinal cord are the motor neurons, which arise from cells that are Pax6$^+$ (low levels), Nkx6.1$^+$, and Irx3$^-$ (Briscoe et al., 2000; Ericson et al., 1997). Based on the expansion of high-level Pax6, an expansion of Irx3, and the loss of
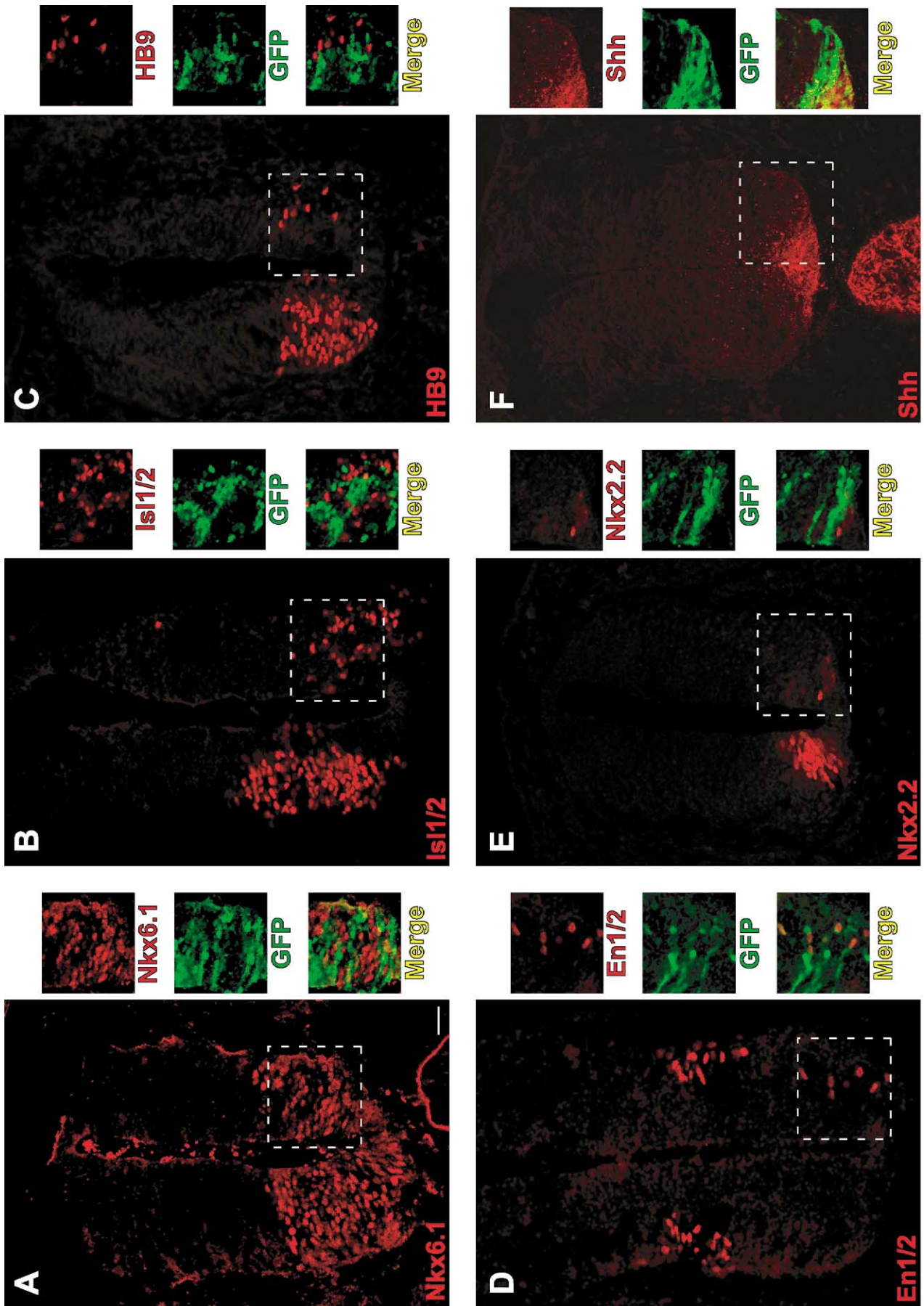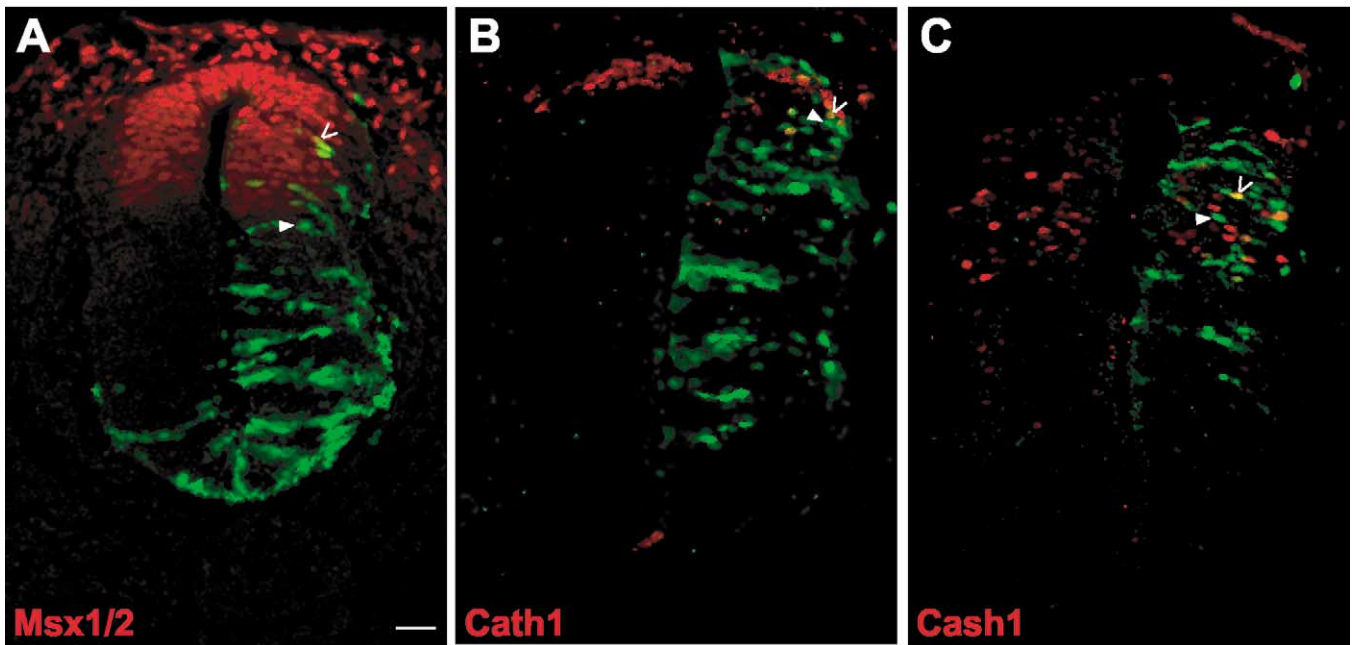
Fig. 4. Gli3R* expression does not affect dorsal-most markers in the developing spinal cord. Expression of the dorsal homeodomain protein Msx1/2 (A) was unaffected by coexpression of Gli3R* in the dorsal region of the spinal cord. Gli3R* did not alter expression of the dorsal bHLH proteins Cath1 (B) or Cash1 (C) either. In (A–C), a representative colabeled cell, positive for both antibody staining and GFP is marked with an open arrowhead, and a representative green cell positive for GFP alone is indicated by a closed arrowhead. Scale bar is 20 μm.

Nkx6.1 expression, we expected the motor neuron pool to be reduced in Gli3R* electroporated embryos. Motor neuron fate specification was monitored by expression of the markers lsl1/2 and HB9, both of which are important for motor neuron formation (Arber et al., 1999; Pfaff and Kintner, 1998; Pfaff et al., 1996; Tanabe et al., 1998; Thaler et al., 1999). Gli3R* misexpression repressed lsl1/2 and HB9 expression in a cell-autonomous manner (Fig. 3B and C). The loss of lsl1/2 and HB9 expression occurred even in GFP^{+} cells that were completely surrounded by lsl1/2[+] or HB9^{+} cells. This cell-autonomous repression of motor neuron markers occurred in 100% of cells examined (Table 1).

The expression of Gli3R* in the ventral spinal cord leads to the appearance of Nkx6.1[−], lrx3[+], and Pax6^{+} cells. We wanted to address what fate these cells adopt. Since expression of these three proteins is characteristic of cells in the intermediate region of the spinal cord, we examined markers for cell fate in this region. The Chx10 protein is a marker for V2 interneurons, which develop just dorsally to the motor neuron domain (Ericson et al., 1997). We expected Gli3R* to repress Chx10 expression, since V2 interneurons normally express Nkx6.1, in addition to Irx3 and Pax6

(Briscoe et al., 2000). However, we did not find a significant loss of Chx10^{+} cells in the electroporated side of the spinal cord (Table 1), suggesting that Gli3R* expression cannot inhibit the specification of V2 interneurons. Still, given the small number of Chx10^{+} cells, it is difficult to make a conclusive, negative statement, but the complete absence of GFP[+], Chx10^{+} cells suggests that cells expressing Gli3R* do not become V2 interneurons.

Since Gli3R*-expressing cells do not seem to become V2 interneurons, we next examined these cells for expression of the V1 interneuron fate marker, En1/2. V1 interneurons develop in the region just dorsal to the V2 interneuron domain and are Nkx6.1[−], lrx3[+], and Pax6^{+}. In addition, ectopic Nkx6.1 has been shown to repress the V1 interneuron fate (Briscoe et al., 2000). The presence of ectopic Irx3^{+}, Pax6^{+}, Nkx6.1^{−} cells in the ventral spinal cord predicates the presence of ectopic En1/2^{+} cells. Widespread misexpression of Gli3R* was accompanied by a ventral expansion of En1/2^{+} cells (Fig. 3D). This indicates that many Nkx6.1[−], Irx3^{+}, Pax6^{+} cells acquire a V1 fate. However, many cells that misexpressed Gli3R* did not express En1/2 (Table 1, and Fig 3D). The fate of these cells remains

Fig. 3. Gli3R* expression prevents expression of class-II progenitor proteins. Expression of the class-II, homeodomain proteins Nkx6.1 (A) and Nkx2.2 (E) was repressed by misexpression of Gli3R*, as was the expression of the motor neuron markers Isl1/2 (B) and HB9 (C). Although Gli3R* and Isl1/2 were not expressed in the same cells within the motor neuron domain, Isl1/2+ dorsal interneurons were found to express Gli3R*. Misexpression of Gli3R* in the ventral spinal cord also was associated with the misexpression of En1/2 (D), a marker for V1 interneurons. The ectopic expression of En1/2 occurred non-cell-autonomously in about 50% of the cells examined (Table 1). Expression of Shh (F) in the floorplate was not affected by Gli3R* misexpression in this region. The area outlined by the dashed box in each panel is used to show expression of GFP, which marks Gli3R*-positive cells, antibody marker, and the merge between the two on the right. Scale bar is 20 μm.

unclear. In agreement with the ectopic expression of Pax7, Pax6, and Irx3 in ventral neural tube cells, Gli3R*-expressing cells appear to adopt a dorsal-intermediate fate.

Gli3R* represses fate specification of V3 interneurons

The class-II progenitor protein Nkx2.2 and the class I progenitor Pax6 are mutually exclusive, and loss of *pax6* in mice leads to a dorsal expansion of the Nkx2.2 domain (Briscoe and Ericson, 1999; Briscoe et al., 2000; Ericson et al., 1997). Therefore, based on the Gli3R*-mediated ectopic activation in Pax6 expression ventrally, we expected Nkx2.2 expression to be decreased. Misexpression of Gli3R* precluded Nkx2.2 expression in all cells examined within the V3 progenitor domain (Fig. 3E). Because Shh has been shown to induce expression of class-II proteins, it is possible that Gli3R* could interfere with Shh expression and thus repress ventral cell fate specification. This does not seem to be the case as Gli3R* did not significantly change the expression pattern of Shh in the floorplate (Fig. 3F), although expression of the floorplate marker HNF3β was decreased (data not shown). In addition to evidence for a role of the amino-terminal or repressor region of Gli3 in specifying fate in the dorsal neural tube, we also examined the activity of full-length Gli3. In contrast to Gli3R*, misexpression of full-length Gli3 did not alter Pax7, Nkx2.2, or lsl1/2 expression (data not shown).

Gli3R* does not affect the dorsal-most progenitor domains

Since Gli3R* causes a ventral expansion of dorsally expressed proteins such as Pax7, we also examined whether or not Gli3R* is able to cause an expansion of dorsal fate markers that are not directly Shh-responsive. The Msx1 and Msx2 proteins are expressed in the dorsal neural tube and can be induced by BMP signaling (Liem et al., 1995; Takahashi and Le Douarin, 1990; Timmer et al., 2002). In addition, Msx2 seems to be important for patterning of dorsal progenitor domains (Timmer et al., 2002). Electroporation of Gli3R* did not alter the domain of Msx1 or Msx2 expression (Fig. 4A). The bHLH proteins Cath1 (chicken atonal homologue 1) and Cash1 (chicken achaete scute homologue 1) are also expressed in the dorsal neural tube (Ben-Arie et al., 1996; Gowan et al., 2001; Lee et al., 1998). Cath1, and to some extent Cash1, may define specific dorsal progenitor domains (Gowan et al., 2001), much like the homeodomain proteins in the ventral neural tube. Neither Cath1 nor Cash1 expression domains was altered by Gli3R* misexpression, although there was overlap and co-expression with Gli3R* (Fig. 4B and C). The inhibition of the Shh response causes an expansion in Pax6 and Pax7 expression, but not in Msx1/2 expression (Incardona et al., 1998). This suggests that the presence of Gli3R* has a differential effect on dorsal cell types. This appears to be reflected by Gli3R*, which does not affect the expression of Cath1, Cash1, or Msx1/2. More likely, the expression domain of these proteins is determined solely by BMP-mediated signaling.

BMPs maintain gli3 expression

As Gli3 is able to mediate the Shh response in the spinal cord, it is important to understand how high levels of *gli3* expression become restricted to the dorsal neural tube. *Gli3* is initially expressed throughout primitive streak (Fig. 5B) and neural plate (Fig. 5C and D) of HH stage 10 chick embryos (Fig. 5A). However, as the neural tube begins to close, detectable levels of *gli3* become laterally restricted in the neural plate (Fig. 5D) and thus subsequently restricted to the dorsal neural tube (Fig. 5E) of HH stage 10 chick embryos and remain in the dorsal neural tube at least through HH stage 21 (data not shown; Hui et al., 1994; Sasaki et al., 1997).

Gli3 expression in the neural tube is consistent with induction by either Wnt or BMP signaling from the dorsal neural tube and ectoderm. In order to test this, explant tissue from the intermediate region of the neural plate was exposed to Wnt3a and BMP4. Wnt proteins can induce *gli3* expression in somites (Borycki et al., 2000); however, Wnt3a was not able to induce or maintain *gli3* expression in intermediate neural plate explants (Fig. 5G), even though this preparation of Wnt3a was active in other assays (Michelle Braun, personal communication). Consistent with its dorsalizing activity, BMP4 was able to maintain *gli3* expression in intermediate neural plate explants (Fig. 5I). This indicates that BMPs are involved the dorsal restriction of *gli3* expression. The BMP-mediated upregulation of this Shh repressor may be a potential mechanism by which BMPs antagonize the Shh response.

Discussion

Shh-induced neural tube pattern and Gli3

Gli3 plays a central role in mediating the Shh response. In the absence of Shh signaling, Gli3 is processed into a repressor (Gli3R), which in turn blocks transcription of Shh-response genes (Fig. 6). This role is not immediately apparent in *gli3*$^{-/-}$ mice, whose spinal cords are phenotypically almost the same as those of wild-type mice (Ding et al., 1998; Litingtung and Chiang, 2000b). However, most ventral cell types, except for those in or next to the floorplate, develop normally in *gli1*$^{-/-}$;*gli2*$^{-/-}$ mice (Ding et al., 1998; Matise et al., 1998), indicating that a significant fraction of the Shh response is mediated independently of Gli1 and Gli2. An important role of Gli3 is revealed by its ability to mediate the ventral to dorsal fate transformation observed in *shh*$^+$ embryos. The morphogenetic activity of Shh, acting via the inhibition of Gli3 processing suggests a model in which high levels of Gli3R activity allow dorsal

fate specification, while low levels allow ventral fate specification.

BMP-mediated dorsoventral patterning

The formation of dorsal cells does not require Gli3, even though Gli3 is required for the ventral to dorsal transformation seen in the absence of Shh. We propose that there are several independent ways to get correct dorsoventral patterning. The first path can occur in a Shh–Gli3-independent manner and presumably relies on a gradient of BMP activity (Fig. 6). This activity gradient is set up by BMP expression in the ectoderm and roof plate of the neural tube and the expression of BMP antagonists in the notochord and somites (Lee et al., 1998; Liem et al., 1995, 1997, 2000). Results from zebrafish mutants that carry mutations in the BMP pathway (*swirl* or *bmp2b*$^{-/-}$ and *somitabun* or *smad5*) suggest that appropriate BMP levels are required for normal fate specification throughout the neural tube; and that loss of varying amounts of activation of the BMP response affects fate specification at all dorsoventral levels (Barth et al., 1999). In addition, activation of the BMP response through misexpression of constitutively active BMP receptors in the chick neural tube results in ectopic expression of some class-I proteins and repression of some class-II protein expression (Timmer et al., 2002).

Loss of the BMP antagonist *noggin,* which is normally expressed in the notochord and roof plate of mice, results in a loss of Shh-dependent ventral cell types as evidenced by the downregulation of lsl1, Nkx2.2, and En1. This is despite an apparently normal Shh signal, which was monitored by the upregulation of *patched* expression (McMahon et al., 1998). Taken together, these results suggest that a BMP concentration gradient spans the entire neural tube and can pattern dorsoventral fate in the spinal cord in the absence of *gli3* and *shh*. Apparently, a gradient of BMP response is sufficient to induce most cell types along the dorsoventral axis of the neural tube. This might indicate a relatively minor role for Shh signaling in dorsoventral patterning. However, not only is there a requirement for Shh signaling in the induction of the most ventral cell types, other cell types appear to arise as a consequence of Shh-mediated regulation of Gli3R activity, working in conjunction with the BMP response (Fig. 6).

Shh/Gli3-mediated dorsoventral patterning

The second path contributing to dorsoventral fate patterning occurs in a Shh–Gli3-dependent manner and acts through a gradient of Gli3R activity to pattern the ventral-intermediate region of the spinal cord. This Shh/Gli3-mediated initiation of dorsoventral fate specification is expected to occur independently of BMP signaling, at least in the ventral and intermediate regions of the spinal cord. A common element between the BMP-mediated and Shh-mediated dorsoventral fate specification paths is the regulation

of class-I and class-II protein expression. Activation of the BMP response acts upon class-I proteins, while activation of the Shh response acts upon class-II proteins. The subsequent antagonism between class-I and class-II proteins results in correct dorsoventral patterning (Fig. 6).

Our data support the Shh/Gli3 loop of our model by demonstrating the ability of Gli3R to prevent ventral fate specification when Shh regulation is absent. Because Gli3R is a repressor, we expect that Gli3R represses class-II protein expression, which gives rise to ectopic class-I protein expression (Fig. 6). In our assays, Gli3R prevented expression of class-II progenitor proteins, while mediating the ectopic expression of class-I proteins in the ventral neural tube. The existence of a Shh gradient in the ventral neural tube likely acts to directly set up the gradient of Gli3R activity by preventing Gli3 processing. In addition to regulation of Gli3R activity, Shh signaling is also important for midline cell specification through Gli1 and Gli2 activation.

Gli3-mediated interference with Gli1/2 induced transcription

In order to understand how Gli3R antagonizes the Shh response in the spinal cord, it is useful to understand how the *Drosophila* homolog of the Gli proteins, Cubitus interruptus (Ci), controls transcription. Ci has both an activator form and a repressor form. In the wing imaginal disc, the cleaved form of Ci represses transcription of hedgehog (*hh*), *decapentaplegic* (*dpp*), and *patched* (*ptc*). At the same time, activated Ci increases transcription of *ptc* and *dpp*. In this way, the activator and repressor forms of Ci act on an overlapping but distinct set of genes (Aza-Blanc and Kornberg, 1999; Ingham, 1998; Litingtung and Chiang, 2000a).

In much the same way, Gli3R most likely blocks transcription of some genes that can be activated by Gli1 and Gli2 and some genes that cannot. In support of this, the combination of Gli1 and Gli3 can substitute for Ci function in the developing wing disc (von Mering and Basler, 1999) Along these lines, the Gli1 and Gli3 core DNA binding sites are the same, although the flanking sequences differ and both Gli1 and Gli3 are able to bind to the same DNA sequence (Kinzler and Vogelstein, 1990; Ruppert et al., 1990; Vortkamp et al., 1995). In addition, Gli3 has been shown to block Gli1- and Gli2-mediated transcriptional activation from the Gli DNA binding site (Ruiz i Altaba, 1998, 1999; Sasaki et al., 1997; Wang et al., 2000). The competition for identical sites between these proteins could explain in part why Gli3 levels are so important for antagonism of the Shh signal. This mechanism could be important for regulation of genes like *nkx2.2* because removal of *gli1* and *gli2* results in an almost complete loss of Nkx2.2. In contrast, progenitor genes important for motor neuron specification are expressed normally in *gli1*$^{-/-}$;*gli2*$^{-/-}$ mice (Ding et al., 1998; Matise et al., 1998), indicating that the expansion of these class-II progenitor proteins occurs independently of Gli1/2 activity. Our data indicate that Gli3 represses these proteins and that this is likely to occur
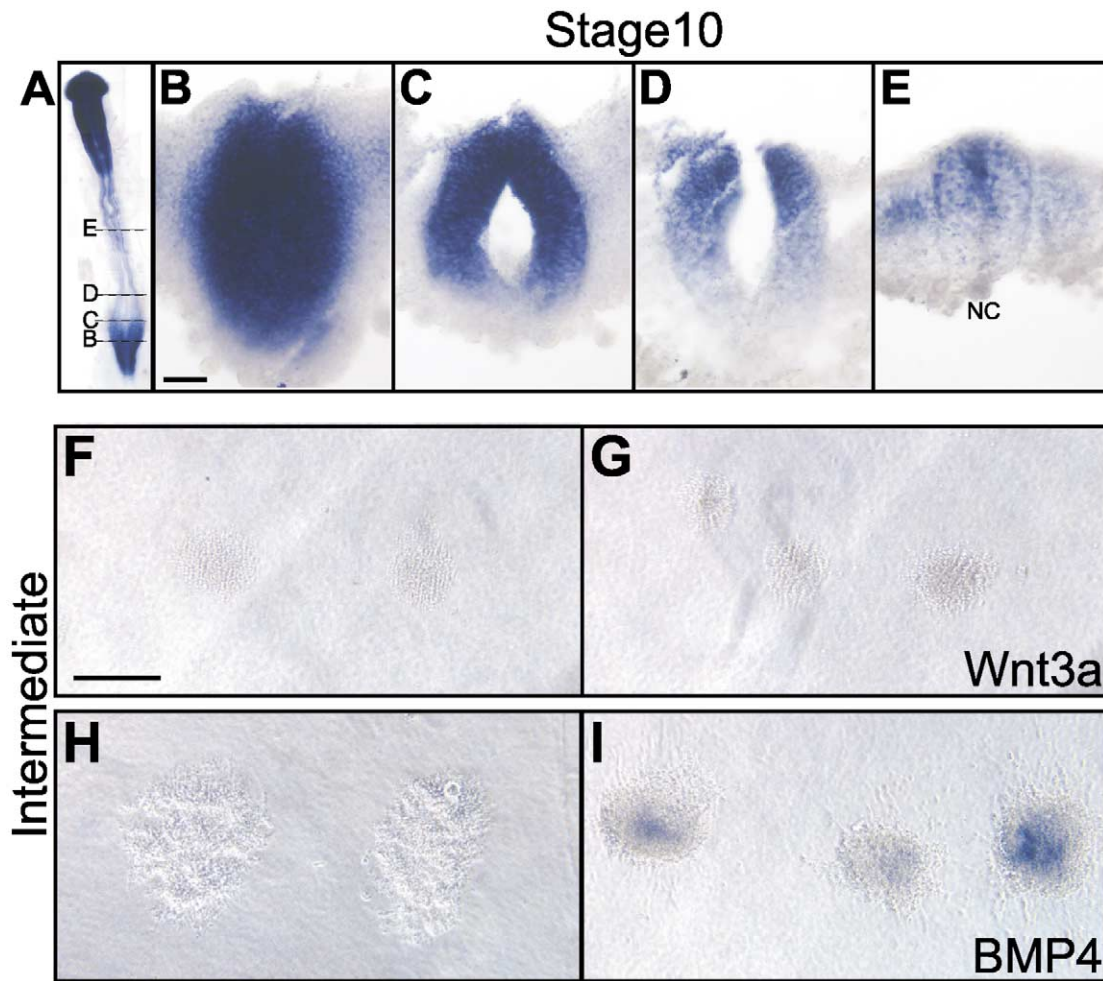
Stage10

Fig. 5. BMPs maintain *gli3* expression in neural plate explants in culture. *gli3* expression was analyzed in a HH stage 10 embryo (A). In the intact embryo shown in (A), anterior is up. *gli3* expression in cross-sections of stage 10 primitive streak (B), neural plate (C, D), and neural tube (E), is shown through the four different levels indicated on the intact embryo. *gli3* is initially expressed throughout the neural plate and subsequently becomes restricted to the dorsal neural tube (D), where it remains expressed at least until stage 21 (not shown). Dorsal is up in all spinal cord cross-sections shown. *gli3* expression in neural plate explants was monitored by in situ hybridation. Growth in medium containing recombinant BMP4 maintained *gli3* expression in 12/13 intermediate explants (I). Culturing explant in Wnt-conditioned (G), control-conditioned (F), or medium alone (H) did not result in the induction or maintenance of *gli3* (0/17, 0/24, and 2/14, respectively). *gli3* expression in dorsal neural plate explants was monitored as positive control. Scale bars are 20 μm.

through a direct transcriptional repression. Similar results were obtained by Persson and coworkers, although they did not address the cell-autonomous character of this Gli3R-mediated repression (Persson et al., 2002).

Gli3 as a potential mediator of BMP-mediated antagonism of the Shh response

Shh and BMPs are known to antagonize the downstream response to each other in the neural tube (Liem et al., 2000; Patten and Placzek, 2002; Watanabe et al., 1998). However, the exact mechanism for this still remains elusive. It is possible that this mutual antagonism occurs through control of class-I and class-II protein expression, which are also mutually repressive. Gli3 is expressed in the dorsal neural tube and can inhibit expression of class-II proteins, acting in opposition to the Shh response. However, in the absence of

Shh, *gli3* expression is maintained in the ventral neural tube (Litingtung and Chiang, 2000b), contributing to the loss of class-II expression. Since a BMP response has been demonstrated in the ventral neural tube (Barth et al., 1999; McMahon et al., 1998; Timmer et al., 2002) and our data show that a BMP signal is capable of sustaining *gli3* expression in neural plate explants, it is likely that BMPs are also responsible for maintaining *gli3* expression in the ventral neural tube in the absence of Shh. Thus, one mechanism of BMP-mediated antagonism of the Shh signal may be maintenance of *gli3* transcription.

Gli3 and disease

The significance of Gli3 expression during development is seen in several human diseases and syndromes. Certainly, mutation or loss of *gli3* significantly affects human devel-
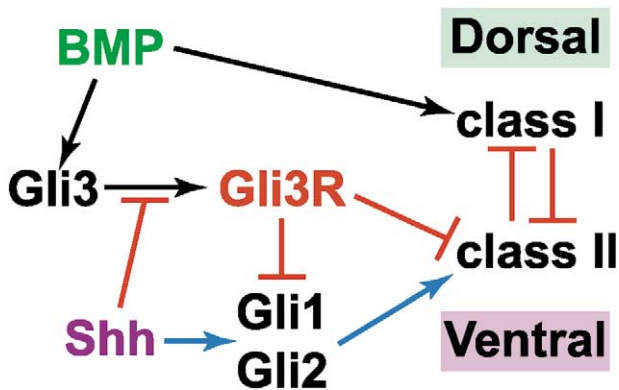
Fig. 6. Gli3R is a key regulator of Shh signaling during dorsoventral fate specification in the developing spinal cord. Correct regulation of class-I and class-II protein expression is critically important for dorsoventral differentiation of the spinal cord. Activation of the BMP response acts on class-I proteins, thought to mediate dorsal differentiation. The activation of the Shh response acts upon class-II proteins involved in the formation of ventral cell types. The subsequent antagonism between class-I and class-II proteins is expected to be responsible for correct dorsoventral patterning. The processing of Gli3 to Gli3R prevents ventral fate specification when Shh signaling is absent. Because Gli3R is a repressor, we postulate that Gli3R represses class-II protein expression. Shh signaling directly activates Gli1 and Gli2, resulting in the induction of Nkx2.2, and class II protein expression, which can be antagonized by Gli3R. We assume that the Shh gradient present in the developing spinal cord is responsible for an opposite Gli3R gradient, which in turns determines the areas in which the class-II proteins are repressed, and consequently the domains where the class-I proteins are expressed.

opment. Loss of one copy of *gli3* results in Greig cephalopolysyndactyly syndrome (GCPS, OMIM#175700), a haploinsufficient disorder that consists of macrocephaly, hypertelomerism, a broadening of the nasal root and forehead, and polysyndactyly (Gollop and Fontes, 1985; Greig, 1926; Kalff-Suske et al., 1999; Vortkamp et al., 1991; Wild et al., 1997). Many of these characteristics can be correlated with enhanced Shh response. For instance, hypertelomerism is caused by an increase in the amount of ventral neural tissue in the forebrain. Mutations in *gli3* that functionally truncate the protein after the DNA binding domain result in Pallister-Hall syndrome (PHS, OMIM#146510), an autosomal dominant disorder that includes hypothalamic hamartomas, a bifid epiglottis, polysydactyly, and abnormal craniofacial features (Kang et al., 1997b; (Ondrey et al., 2000). These mutations in *gli3* truncate the protein such that it resembles the Gli3R cleavage product, and thus further demonstrate the importance of the Gli3 repressor during development (Bose et al., 2002; Kang et al., 1997a, b).

We modeled Gli3R* after a strong PHS allele. The importance of *gli3* gene dosage in both of these disorders once again highlights the necessity of correct Gli3 levels in cells during development. Additionally, amplification of *gli1* is associated with the development of some tumors (Ruppert et al., 1988), implying that Gli-mediated activation of Shh transcriptional targets contributes to these tumors. These observations emphasize a need to better understand the

relationship between Shh signaling response and Gli proteins in activation and/or repression of downstream genes.

In summary, we have shown that Gli3R antagonizes Shh signaling in the ventral neural tube and plays a critical role in cell fate specification of intermediate and ventral neurons. These experiments suggest that Shh and BMP signals are carefully modulated in the developing neural tube. Further work will be necessary to understand the direct targets of the Gli3 repressor and the interplay between the Shh and BMP responses.

References

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T.M., Sockanathan, S., 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 23, 659–674.

Aza-Blanc, P., Kornberg, T.B., 1999. Ci: a complex transducer of the hedgehog signal. Trends Genet. 15, 458–462.

Aza-Blanc, P., Lin, H.Y., Ruiz i Altaba, A., Kornberg, T.B., 2000. Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities. Development 127, 4293–4301.

Bai, C.B., Auerbach, W., Lee, J.S., Stephen, D., Joyner, A.L., 2002. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development 129, 4753–4761.

Barth, K.A., Kishimoto, Y., Rohr, K.B., Seydler, C., Schulte-Merker, S., Wilson, S.W., 1999. Bmp activity establishes a gradient of positional information throughout the entire neural plate. Development 126, 4977–4987.

Ben-Arie, N., McCall, A.E., Berkman, S., Eichele, G., Bellen, H.J., Zoghbi, H.Y., 1996. Evolutionary conservation of sequence and expression of the bHLH protein Atonal suggests a conserved role in neurogenesis. Hum. Mol. Genet. 5, 1207–1216.

Borycki, A., Brown, A.M., Emerson Jr., C.P., 2000. Shh and Wnt signaling pathways converge to control Gli gene activation in avian somites. Development 127, 2075–2087.

Bose, J., Grotewold, L., Ruther, U., 2002. Pallister-Hall syndrome phenotype in mice mutant for Gli3. Hum. Mol. Genet. 11, 1129–1135.

Briscoe, J., Chen, Y., Jessell, T.M., Struhl, G., 2001. A hedgehog- insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. Mol. Cell 7, 1279–1291.

Briscoe, J., Ericson, J., 1999. The specification of neuronal identity by graded Sonic Hedgehog signalling. Semin. Cell Dev. Biol. 10, 353–362.

Briscoe, J., Pierani, A., Jessell, T.M., Ericson, J., 2000. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101, 435–445.

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

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M., Ishii, S., 1999. Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. J. Biol. Chem. 274, 8143–8152.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., Hui, C.C., 1998. Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development 125, 2533–2543.

Dudley, A.T., Robertson, E.J., 1997. Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. Dev. Dyn. 208, 349–362.

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

Ericson, J., Morton, S., Kawakami, A., Roelink, H., Jessell, T.M., 1996. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell 87, 661–673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T.M., Briscoe, J., 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell 90, 169–180.

Gollop, T.R., Fontes, L.R., 1985. The Greig cephalopolysyndactyly syndrome: report of a family and review of the literature. Am. J. Med. Genet. 22, 59–68.

Gowan, K., Helms, A.W., Hunsaker, T.L., Collisson, T., Ebert, P.J., Odom, R., Johnson, J.E., 2001. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron 31, 219–232.

Greig, D.M., 1926. Oxycephaly. Edinburgh Med. J. 33, 189–218.

Hamburger, V., Hamilton, H.L., 1992. A series of normal stages in the development of the chick embryo. 1951. Dev. Dyn. 195, 231–272.

Helms, A.W., Johnson, J.E., 1998. Progenitors of dorsal commissural interneurons are defined by MATH1 expression. Development 125, 919–928.

Horton, S., Meredith, A., Richardson, J.A., Johnson, J.E., 1999. Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. Mol. Cell. Neurosci. 14, 355–369.

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

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

Ingham, P.W., 1998. Transducing Hedgehog: the story so far. EMBO J. 17, 3505–3511.

Jasoni, C., Hendrickson, A., Roelink, H., 1999. Analysis of chicken Wnt-13 expression demonstrates coincidence with cell division in the developing eye and is consistent with a role in induction. Dev. Dyn. 215, 215–224.

Kalff-Suske, M., Wild, A., Topp, J., Wessling, M., Jacobsen, E.M., Bornholdt, D., Engel, H., Heuer, H., Aalfs, C.M., Ausems, M.G., Barone, R., Herzog, A., Heutink, P., Homfray, T., Gillessen-Kaesbach, G., Konig, R., Kunze, J., Meinecke, P., Muller, D., Rizzo, R., Strenge, S., Superti-Furga, A., Grzeschik, K.H., 1999. Point mutations throughout the GLI3 gene cause Greig cephalopolysyndactyly syndrome. Hum. Mol. Genet. 8, 1769–1777.

Kang, S., Allen, J., Graham Jr., J.M., Grebe, T., Clericuzio, C., Patronas, N., Ondrey, F., Green, E., Schaffer, A., Abbott, M., Biesecker, L.G., 1997a. Linkage mapping and phenotypic analysis of autosomal dominant Pallister-Hall syndrome. J. Med. Genet. 34, 441–446.

Kang, S., Graham Jr., J.M., Olney, A.H., Biesecker, L.G., 1997b. GLI3 frameshift mutations cause autosomal dominant Pallister-Hall syndrome. Nat. Genet. 15, 266–268.

Kawakami, A., Kimura-Kawakami, M., Nomura, T., Fujisawa, H., 1997. Distributions of PAX6 and PAX7 proteins suggest their involvement in both early and late phases of chick brain development. Mech. Dev. 66, 119–130.

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

Krauss, S., Concordet, J.P., Ingham, P.W., 1993. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell 75, 1431–1444.

Lee, K.J., Mendelsohn, M., Jessell, T.M., 1998. Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev. 12, 3394–3407.

Liem Jr., K.F., Jessell, T.M., Briscoe, J., 2000. Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. Development 127, 4855–4866.

Liem Jr., K.F., Tremml, G., Jessell, T.M., 1997. A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell 91, 127–138.

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

Litingtung, Y., Chiang, C., 2000a. Control of Shh activity and signaling in the neural tube. Dev. Dyn. 219, 143–154.

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

Liu, I.S., Chen, J.D., Ploder, L., Vidgen, D., van der Kooy, D., Kalnins, V.I., McInnes, R.R., 1994. Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. Neuron 13, 377–393.

Marine, J.C., Bellefroid, E.J., Pendeville, H., Martial, J.A., Pieler, T., 1997. A role for Xenopus Gli-type zinc finger proteins in the early embryonic patterning of mesoderm and neuroectoderm. Mech. Dev. 63, 211–225.

Marti, E., Bumcrot, D.A., Takada, R., McMahon, A.P., 1995. Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. Nature 375, 322–325.

Matise, M.P., Epstein, D.J., Park, H.L., Platt, K.A., Joyner, A.L., 1998. Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 125, 2759–2770.

McMahon, J.A., Takada, S., Zimmerman, L.B., Fan, C.M., Harland, R.M., McMahon, A.P., 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev. 12, 1438–1452.

Nguyen, V.H., Trout, J., Connors, S.A., Andermann, P., Weinberg, E., Mullins, M.C., 2000. Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway. Development 127, 1209–1220.

Ondrey, F., Griffith, A., Van Waes, C., Rudy, S., Peters, K., McCullagh, L., Biesecker, L.G., 2000. Asymptomatic laryngeal malformations are common in patients with Pallister-Hall syndrome . Am. J. Med. Genet. 94, 64–67.

Patten, I., Placzek, M., 2002. Opponent activities of Shh and BMP signaling during floor plate induction in vivo. Curr. Biol. 12, 47–52.

Persson, M., Stamataki, D., te Welscher, P., Andersson, E., Bose, J., Ruther, U., Ericson, J., Briscoe, J., 2002. Dorsal–ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. Genes Dev. 16, 2865–2878.

Pfaff, S., Kintner, C., 1998. Neuronal diversification: development of motor neuron subtypes. Curr. Opin. Neurobiol. 8, 27–36.

Pfaff, S.L., Mendelsohn, M., Stewart, C.L., Edlund, T., Jessell, T.M., 1996. Requirement for LIM homeobox gene lsl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell 84, 309–320.

Placzek, M., Yamada, T., Tessier-Lavigne, M., Jessell, T., Dodd, J., 1991. Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. Dev. Suppl. 105–122.

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

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

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

Ruiz i Altaba, A., 1999. Gli proteins encode context-dependent positive and negative functions: implications for development and disease. Development 126, 3205–3216.

Ruiz i Altaba, A., Placzek, M., Baldassare, M., Dodd, J., Jessell, T.M., 1995. Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF-3 beta. Dev. Biol. 170, 299–313.

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

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

Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T.M., Rubenstein, J.L., 2000. Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. Genes Dev. 14, 2134–2139.

Sasaki, H., Hui, C., Nakafuku, M., Kondoh, H., 1997. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development 124, 1313–1322.

Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., Kondoh, H., 1999. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 126, 3915–3924.

Schaeren-Wiemers, N., Gerfin-Moser, A., 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry 100, 431–440.

Shibamoto, S., Higano, K., Takada, R., Ito, F., Takeichi, M., Takada, S., 1998. Cytoskeletal reorganization by soluble Wnt-3a protein signalling. Genes Cells 3, 659–670.

Shin, S.H., Kogerman, P., Lindstrom, E., Toftgard, R., Biesecker, L.G., 1999. GLI3 mutations in human disorders mimic Drosophila cubitus interruptus protein functions and localization. Proc. Natl. Acad. Sci. USA 96, 2880–2884.

Takahashi, Y., Le Douarin, N., 1990. cDNA cloning of a quail homeobox gene and its expression in neural crest-derived mesenchyme and lateral plate mesoderm. Proc. Natl. Acad. Sci. USA 87, 7482–7486.

Tanabe, Y., Roelink, H., Jessell, T.M., 1995. Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation. Curr. Biol. 5, 651–658.

Tanabe, Y., William, C., Jessell, T.M., 1998. Specification of motor neuron identity by the MNR2 homeodomain protein. Cell 95, 67–80.

Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J., Pfaff, S.L., 1999. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron 23, 675–687.

Timmer, J.R., Wang, C., Niswander, L., 2002. BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix–loop–helix transcription factors. Development 129, 2459–2472.

Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M., Pfaff, S.L., 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell 79, 957–970.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T.M., Ericson, J., 2001. Different levels of repressor activity assign redundant and specific roles to nkx6 genes in motor neuron and interneuron specification. Neuron 31, 743–755.

von Mering, C., Basler, K., 1999. Distinct and regulated activities of human Gli proteins in Drosophila. Curr. Biol. 9, 1319–1322.

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

Vortkamp, A., Gessler, M., Grzeschik, K.H., 1995. Identification of optimized target sequences for the GLI3 zinc finger protein. DNA Cell Biol. 14, 629–634.

Wang, B., Fallon, J.F., Beachy, P.A., 2000. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 100, 423–434.

Watanabe, Y., Duprez, D., Monsoro-Burq, A.H., Vincent, C., Le Douarin, N.M., 1998. Two domains in vertebral development: antagonistic regulation by SHH and BMP4 proteins. Development 125, 2631–2639.

Wild, A., Kalff-Suske, M., Vortkamp, A., Bornholdt, D., Konig, R., Grzeschik, K.H., 1997. Point mutations in human GLI3 cause Greig syndrome. Hum. Mol. Genet. 6, 1979–1984.

Yamada, T., Pfaff, S.L., Edlund, T., Jessell, T.M., 1993. Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notchcord and floor plate. Cell 73, 673–686.