Differential requirement for Gli2 and Gli3 in ventral neural cell fate specification

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Abstract

Sonic hedgehog (Shh) directs the development of ventral cell fates, including floor plate and V3 interneurons, in the mouse neural tube. Here, we show that the transcription factors Gli2 and Gli3, mediators of Shh signaling, are required for the development of the ventral cell fates but make distinct contributions to controlling cell fates at different locations along the rostral–caudal axis. Mutants lacking Patched1 (Ptc1), the putative receptor of Shh, were used to analyze Gli functions. Ptc1−/− mutants develop floor plate, motor neuron, and V3 interneuron progenitors in lateral and dorsal regions, suggesting that the normal role of Ptc1 is to suppress ventral cell development in dorsal neural tube. The Ptc1−/− phenotype is rescued, with restoration of dorsal cell types, by the lack of Gli2, but only in the caudal neural tube. In triple mutants of Gli2, Gli3, and Ptc1, dorsal and lateral cell fates are restored in the entire neural tube. These observations suggest that Gli2 is essential for ventral specification in the caudal neural tube, and that in more rostral regions, only Gli3 can promote development of ventral cells if Gli2 is absent. Thus, Shh signaling is mediated by overlapping but distinct functions of Gli2 and Gli3, and their relative contributions vary along the rostral–caudal axis.

Keywords: Mouse embryo; Mutant; Neural tube; Ventral cell fates; Shh; Ptc1; Gli2; Gli3; Rostral–caudal axis

Introduction

The Hedgehog (Hh) signaling plays major roles in the development of diverse organisms from Drosophila to human (Ingham and McMahon, 2001). In Drosophila, two transmembrane proteins, Patched (Ptc) and Smoothened (Smo), are involved in the reception of Hh signals (Chen and Struhl, 1996; Quirk et al., 1997), and the transcription factor Cubitus interruptus (Ci) acts as the ultimate transcriptional transducer (Methot and Basler, 2001). The prevailing view is that Ptc inhibits Smo function in the absence of Hh, which permits the proteolytic cleavage of Ci into a transcriptional repressor form (Aza-Blanc et al., 1997). The binding of Hh to Ptc alleviates the repressive action of Ptc on Smo and, when Smo is derepressed, Ci proteolysis is inhibited and Ci is converted into a transcriptional activator. Several features of Hh signal transduction are evolutionarily conserved in vertebrates. While Drosophila Ci is the only transcription factor that mediates Hh signaling, vertebrates have at least three Ci homologues (Gli1, Gli2, and Gli3). Although all three Gli proteins have been implicated in vertebrate Hh signal transduction, it remains unclear how they operate together in mediating Hh signaling in vivo (Ruiz i Altaba, 1999).

Mutant studies indicate that Gli2 and Gli3 are involved in neural tube development, whereas Gli1 is dispensable (Ding et al., 1998; Theil et al., 1999; Park et al., 2000; Tole et al., 2000; Bai et al., 2002). Shh is essential for directing the formation of...
floor plate, motor neurons, and V2 and V3 interneurons, since these cells are absent in Shh−/− embryos (Chiang et al., 1996). Gli2 appears to be a major mediator of the Shh signaling, since Gli2−/− embryos lack floor plate and have reduced number of V3 interneurons (Ding et al., 1998; Matise et al., 1998). In contrast, Gli3 seems to be essential for the development of dorsal cell types in the brain, since dorsal forebrain is reduced in Gli3−/− embryos (Theil et al., 1999; Tole et al., 2000; Aoto et al., 2002). The repressor function of Gli3 in ventral neural tube development has been demonstrated by a restoration of motor neuron and V2 interneuron in the spinal cord of Gli3−/−;Shh−/− mice (Litingtung and Chiang, 2000). However, several observations suggest that Gli3 is also involved in activating ventral neural tube development. First, in Gli1−/−;Gli2−/−;Gli3−/− neural tube, the ventral patterning defects are much milder than those in Shh−/− mutants, suggesting that either Gli3 or a non-Gli activity induces/promotes the development of ventral cell fates (Park et al., 2000). Second, Gli3 activates transcription from the promoters of Gli1 and Ptc1, which are known Shh target genes, in cultured cells (Dai et al., 1999; Shin et al., 1999). It remains unclear whether Gli3 is partially redundant with Gli2 and promotes ventral cell differentiation.

To investigate whether Gli3 can induce ventral cell development in the neural tube and to examine how Gli3 functions differently from Gli2, we have performed an epistasis analysis using Ptc1, Gli2, and Gli3 mutant mice. In Ptc1−/− mutants, ventral cells develop independently of Shh; the signaling cascade is constitutively activated (Goodrich et al., 1997). If Gli2 and/or Gli3 were essential for inducing ventral cells, ventral cell development in Ptc1−/− embryos would be blocked by the inactivation of Gli2 and/or Gli3 function. Therefore, analysis of Ptc1 mutant mice that also lack Gli2, Gli3, or both, would allow us to examine the contributions of Gli2 and/or Gli3 to the development of ventral cells.

Here, we show that when Gli2 function is eliminated in the Ptc1−/− background, the ventralized phenotype is suppressed in the caudal neural tube but remains unaffected in the brain and rostral neural tube. Simultaneous inactivation of both Gli2 and Gli3 in Ptc1−/− embryos completely suppresses the Ptc1−/− phenotype along the entire neural axis, demonstrating that Gli2 and Gli3 are essential for the development of ventral cell fates. On the other hand, lack of Gli3 alone has no effects on the Ptc1−/− phenotype. Thus, Gli3 can activate the ventral specification in the brain and rostral neural tube, but the effect can be observed only when Gli2 is absent. Our observations suggest that overlapping but distinct functions of Gli2 and Gli3 induce the development of ventral cell fates.

Materials and methods

Mice

Gli2 mutant mice contain a targeted deletion of the DNA-binding zinc finger motifs of the gene (Mo et al., 1997). Gli3 mutant mice are spontaneous null mutants with a large 3′ deletion of the gene (Hui and Joyner 1993; Buscher et al., 1998; Maynard et al., 2002). Ptc1 mutant mice are targeted mutants carrying an exon 1 deletion (Goodrich et al., 1997). Since the exon 1 is replaced by the lacZ gene, we can monitor the Ptc1 expression by the activity of β-galactosidase. All mutant mice were maintained in a mixed 129/Sv and CD1 background. Both Gli3 and Ptc1 are located on chromosome 13. We performed the following genetic crosses to obtain Gli3+/−;Ptc1+/− mice. First, we mated Gli3+/− mice with Ptc1+/− mice to obtain Gli3+/−;Ptc1+/− mice. In these doubly heterozygous mutants, the Gli3 and Ptc1 mutations are located on different chromosome 13. We next crossed the Gli3+/−;Ptc1+/− males with wild-type female to select for meiotic recombinants that carry both mutations on the same chromosome 13. To generate triply heterozygous mutant mice of Ptc1, Gli2, and Gli3, Gli3+/−;Ptc1+/− male mice are mated with Gli2+/− female mice. The genotyping of mutant mice and embryos was performed as described previously (Goodrich et al., 1997; Mo et al., 1997; Maynard et al., 2002).

Histology and in situ hybridization

Section immunohistochemistry using mouse Foxa2, Isl1/2, Nkx2.2, Pax7, and Shh-specific antibodies (University of Iowa Hybridoma Bank) was performed as described (Ding et al., 1998). Shh, Isl1/2, and Foxa2 were localized in sections by using confocal microscopy as described (Ericson et al., 1997). Secondary antibodies coupled to fluorescent dyes were from Jackson Laboratories. Whole-mount immunohistochemistry using rabbit Foxa2 antibody was also performed as described (Ding et al., 1998). Whole-mount in situ hybridization was performed according to a standard protocol (Hui et al., 1994). The Shh and Gli1 cDNA were used as templates to make digoxigenin-labeled RNA probe (Echelard et al., 1993).

Detection of β-galactosidase activity

X-gal staining to detect Ptc1-lacZ expression was carried out as reported (Ding et al., 1998).

Results

Multiple ventral cell fates can develop in Ptc1−/− neural tube

The transcription of Ptc1 appears to be directly activated by Hh signaling in both Drosophila and mouse and therefore serves as a useful indicator of the activation of Hh target gene expression (Goodrich et al., 1997). Ptc1−/− mice show maximal activation of Hh target gene expression as revealed by the ubiquitous expression of Shh, Foxa2, and Ptc1-lacZ and develop an entirely ventralized neural tube...
Gli1 is only transiently derepressed in Ptc1\(^{-/-}\) mutants

During neural tube development, Gli1 is initially induced at the ventral midline. At later stages, its expression is extinguished in the floor plate region (Sasaki et al., 1997). In cultured cells and transgenic embryos, Gli1 overexpression activates Ptc1 transcription (Hynes et al., 1997; Dai et al., 1999; Sasaki et al., 1999). Like Ptc1, Gli1 is a transcriptional target of Hh signaling and its expression is derepressed in Ptc1 mutants at 8.5 dpc (Goodrich et al., 1997). At 9.5 dpc, however, no Gli1 transcript was detected at the thoracic level of the mutant neural tube, where Shh expression is ubiquitous (Fig. 2C, D, G, and H). In the caudal neural tube, where Shh is restricted to the ventral half, high levels of Gli1 RNA are present in the dorsal half (Fig. 2G and H). Importantly, the elevated level of Gli1 expression in Ptc1\(^{-/-}\) embryos does not correlate with the increased expression of Shh, Foxa2, Ptc1-lacZ, Nkx2.2, and Isl1/2, suggesting that Gli1 is unlikely to be the major mediator in specifying ventral cell fates. Instead, Gli2 and/or Gli3 may play more critical roles in the augmented Hh target gene expression in Ptc1 mutants.
Gli2 activates not all of ventral cells development in Ptc1−/− neural tube

Loss of floor plate and V3 progenitors in the caudal part of Gli2−/− neural tube suggests that Gli2 is the major mediator of Hh signaling in the caudal region (see Fig. 4C, C1, C2, D, D1, and D2) (Ding et al., 1998). Based on this observation, we would expect that the absence of Gli2 function would block transcription of Shh targets in the caudal neural tube of Ptc1−/− embryos. To test this hypothesis, we examined the phenotype of Gli2−/−; Ptc1−/− embryos. Although Gli2−/−; Ptc1−/− embryos appeared to be less growth-retarded than Ptc1−/− embryos, they still exhibited severe exencephaly in the brain and rostral neural tube and died around 10–11 dpc (Fig. 3B and D).

Ptc1-lacZ expression was used to examine the effect of Gli2 inactivation. An obvious reduction of Ptc1-lacZ expression in Gli2−/− embryos suggested that Gli2 is the major activator of Ptc1 expression (Fig. 3A, C, E, G, I, and K). In Ptc1−/− embryos, X-gal staining was found in most of the cells except for the caudal endoderm (Fig. 3B, F, and J, and data not shown). However, in Gli2−/−; Ptc1−/− embryos, only very weak staining could be detected in the mesodermal cells, and a drastic reduction of staining was found in the neural tube, indicating that Gli2 is required for the Ptc1 expression in both neural and mesodermal cells (Fig. 3F, H, J, and L). In the double mutants, Ptc1-lacZ expression is still derepressed in the brain and rostral neural tube (Fig. 3H). This difference in Ptc1-lacZ expression between the rostral and caudal regions suggests that regulators other than Gli2 activate Ptc1-lacZ expression in the brain and rostral neural tube.

Consistent with the expression of Ptc1-lacZ, the development of floor plate cells in Gli2−/−; Ptc1−/− embryos is prevented in the caudal neural tube but not in the brain and rostral neural tube. In Gli2−/−; Ptc1−/− embryos, the floor plate marker Shh was not detectable from the trunk to tail level, but it was found in the brain and the thoracic level of the neural tube (Fig. 4E, E1, and E2). In Gli2−/−; Ptc1−/− neural tube, Foxa2 protein was produced ubiquitously from the brain to the thoracic level, but was not detected in the rostral neural tube (Fig. 4F, F1, and F2). The floor plate phenotype in the caudal neural tube of Gli2−/−; Ptc1−/− embryos was quite similar to that in Gli2−/− neural tube (Fig. 4C2, D2, E2, and F2), whereas the phenotype in the rostral neural tube resembled that of Ptc1−/− neural tube (Fig. 4F1 and H1). These results suggest that Gli2 function is indispensable for floor plate specification in the caudal neural tube, but not in the rostral neural tube. Transcription factors other than Gli2 must be responsible for Hh target gene activation in the brain and rostral neural tube of Ptc1−/− mutants. As discussed above, a clear candidate is Gli3.

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![Diagram of Gli2 expression](image-url)
Fig. 3. Gli2 activates Ptc1-lacZ expression in Ptc1−/− embryos. Lateral views of the embryos after whole-mount X-gal staining in (A–D). The position of the transverse sections in (E–L) is indicated by red line in (A–D). Ptc1-lacZ expression detected by X-gal staining is restricted to the ventral region of the wild-type neural tube at 9.5 dpc (A, E, I), while ubiquitous Ptc1-lacZ expression is detected in Ptc1−/− mutant embryos (B, F, J). Arrows indicate the dorsal border of the Ptc1-lacZ-expressing domain (E, H, I). In Gli2−/− embryos, the Ptc1-lacZ expression is significantly reduced (C, G, K). Only weak signal is detected in the ventral midline (G, arrowheads), while no signal is in the tail (K). Gli2−/−;Ptc1−/− embryos are normal in size, and their external morphology is similar to those of the wild-type except for the opened neural tube from the brain to the thoracic level (D). Ptc1-lacZ expression in the double mutants is less than that in Ptc1−/− mutants (F, H, J, L). Whereas the expanded ventral expression is observed in the rostral neural tube (H), the expression has almost disappeared in the caudal neural tube (L). Scale bar, 1 mm (A–D), 50 μm (E–L). Scale bar in (D) is for (A, C, D).

No apparent effect of Gli3 inactivation on Ptc1−/− phenotype

To examine whether Gli3 is required for the induction of ventral cell fates in Ptc1−/− embryos, we generated Gli3−/−;Ptc1−/− embryos (see Materials and methods). Gli3−/−;Ptc1−/− embryos die at about 9–10 dpc and exhibit morphology to that of Ptc1−/− embryos (data not shown). Like Ptc1−/− embryos, Gli3−/−;Ptc1−/− embryos have ubiquitous Ptc1-lacZ expression (data not shown). These results indicate that inactivation of Gli3 cannot suppress the Ptc1−/− phenotype, suggesting that Gli3 and Gli2 might be redundant. We therefore examined triple homozygous mutants of Gli2, Gli3, and Ptc1.

Simultaneous inactivation of Gli2 and Gli3 largely nullifies the Ptc1−/− phenotype

Gli2+/−;Gli3+/−;Ptc1+/− mice were generated, and intercrosses were used to generate triply homozygous embryos. The morphology of the triply homozygous mutants was very similar to that of Gli2−/−;Gli3−/− double mutants (Fig. 5B, C, G, H, L, M, Q, and R). The expression level of Ptc1-lacZ was much lower in Gli2−/−;Gli3−/−;Ptc1−/− embryos than in Gli2−/−;Ptc1−/− embryos (Fig. 5C and E). The expression level of Ptc1-lacZ in Gli2−/−;Gli3−/−;Ptc1−/− embryos was only slightly higher than in Gli2−/−;Gli3−/−;Ptc1+/− embryos (Fig. 5B and C). This is likely due to the two copies of lacZ gene in Gli2−/−;Gli3−/−;Ptc1−/− embryos versus the single copy of the reporter in Gli2−/−;Gli3−/−;Ptc1+/− embryos. In Gli2−/−;Gli3+/−;Ptc1−/− embryos, there was a slight but significant reduction of Ptc1-lacZ expression compared with that in Gli2−/−;Ptc1−/− embryos (Figs. 5D and E, and 6S and T). Thus, the expression level of Ptc1-lacZ in Gli2−/−;Ptc1−/− embryos is evidently dependent on the dosage of Gli3. These results clearly indicate that Gli3 activates Ptc1-lacZ reporter gene expression in the brain and rostral neural tube.

The development of floor plate was also dependent on Gli3 function in the triple mutants. The expression of Shh and Foxa2 was greatly reduced in Gli2−/−;Gli3−/−;Ptc1−/− embryos (Fig. 5H, M, and R). In the triple mutant embryos,
**Fig. 4.** Gli2 mutation can block the augmented floor plate in \( Ptc1^{-/-} \) embryos. Whole-mount in situ hybridization with \( Shh \) antisense RNA probe and whole-mount immunohistochemistry with anti-Foxa2 antibody in wild-type (A, B), \( Gli2^{-/-} \) (C, D), \( Gli2^{-/-};Ptc1^{-/-} \) (E, F), and \( Ptc1^{-/-} \) (G, H) embryos. Lateral views of the embryos are shown in (A–H). The position of the transverse sections in (A1–H2) is indicated by red line in (A–H). In 9.5-dpc wild-type embryos, \( Shh \) and Foxa2 are expressed in the floor plate (fp) and notochord (nt) (A, A1, A2, B, B1, B2). In \( Gli2^{-/-} \) embryos, no \( Shh \) and Foxa2 are observed in the caudal part of the neural tube (C, C2, D, D2). \( Shh \) and Foxa2 are detected in the fore- and midbrain and neural tube at thoracic level in \( Gli2^{-/-} \) embryos (arrowheads in C, D and fp? in C1, D1). In \( Ptc1^{-/-} \) embryos, \( Shh \) and Foxa2 are up regulated (G, G1, G2, H, H1, H2). In \( Gli2^{-/-};Ptc1^{-/-} \) embryos, expanded \( Shh \) is observed only in the forebrain (E). \( Shh \) is detected in the midline in the thoracic neural tube, while no \( Shh \) in the caudal part (E, E1, E2). The widespread Foxa2 expression in \( Gli2^{-/-};Ptc1^{-/-} \) embryos is much wider than that of \( Shh \), from the forebrain to thoracic levels of the neural tube (F, F1). No Foxa2 is detected in the caudal neural tube (F, F2). Scale bar, 1 mm (A–H), 50 \( \mu \)m (A1–H2). Scale bar in (F) is for (A–F), bar in (H) is for (G, H), bar in (D2) is for (A1–D1, A2–D2), bar in (H1) is for (E1–H1), and bar in (H2) is for (E2–H2).
residual \textit{Shh} expression was observed only in the presumptive zona limitans intrathalamica (Zli) and ventral telencephalon (Fig. 5H and M). The cells in the Zli are essential for setting the boundary between dorsal and ventral thalamus. The expression pattern of \textit{Shh} in \textit{Gli2}^{+/−};\textit{Gli3}^{−/−};\textit{Ptc1}^{−/−} embryos was strikingly similar to its pattern in
Gli2\(^{+/−}\); Gli3\(^{−/−}\) embryos (Fig. 5G, H, L, and M). These observations suggest that Gli2 and Gli3 do not activate the Shh expression in the Zli and ventral telencephalon. The boundary between dorsal and ventral thalamus is established in Shh mutant embryos. Also some part of ventral telencephalon is developed in the mutants, suggesting that Shh signaling is not involved in the induction of Shh itself in these cells (Ishibashi and McMahon, 2002; Ohkubo et al., 2002).

In contrast, Foxa2 protein was completely absent from the neural tubes of Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) and Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos, indicating that Gli2 and Gli3 are essential for Foxa2 expression throughout the neural tube (Fig. 5Q and R). The expanded Shh and Foxa2 expression in Gli2\(^{−/−}\); Ptc1\(^{−/−}\) embryos was significantly reduced in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (Fig. 5N and S). Thus, when Gli2 is absent, the dosage of Gli3 seems to be critical for Shh and Foxa2 expression in the ventral neural tube.

Together, these observations strongly support the notion that Gli2 and Gli3 are required for most, if not all, floor plate cell development in the neural tube of Ptc1 mutant embryos.

**Gli3 activates V3 interneuron and motor neuron progenitors development**

We checked the development of V3 interneuron and motor neuron progenitors in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos to examine whether Gli3 can activate their development. Many Nkx2.2-expressing V3 progenitors were found at the thoracic level of Gli2\(^{−/−}\) and Gli2\(^{−/−}\); Ptc1\(^{−/−}\) neural tube (Fig. 6E) (Ding et al., 1998). Consistent with the Shh and Foxa2 expression, Nkx2.2 expression was absent in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) neural tube, indicating that Gli3 is required for the specification of V3 progenitors in the rostral neural tube if Gli2 was absent (Fig. 6C). We

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**Fig. 6.** Gli3 activates Nx2.2 and Isl1/2 and represses Pax7 expression. Section immunohistochemistry with anti-Nx2.2 (A–E), Isl1/2 (F–J), and Pax7 (K–O) antibodies and X-gal staining (P–T) on the transverse section at 9.5 dpc. Brackets ended arrowheads indicate the region with high level signal of immunoreaction and X-gal staining. The position of the sections is at anterior thoracic level. Nx2.2 is expressed in the region dorsolateral to Foxa2 expression in the wild-type (A). Nx2.2 is observed in the ventral half of the opened neural tube of Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) and Gli2\(^{−/−}\); Ptc1\(^{−/−}\) embryos (D, E). No Nx2.2 is detected both in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) and Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (B, C). Isl1/2 is detected in the ventral side in the wild-type (F). In Gli2\(^{−/−}\); Ptc1\(^{−/−}\) embryos, Isl1/2-expressing cells are observed in the ventral half of the opened neural tube (J). The number of Isl1/2-expressing cells is significantly reduced in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (I, arrows). No Isl1/2-expressing cell is detected in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (G, H). Pax7 are detected in the dorsal region of the wild-type (K), whereas very small amounts of the Pax7-expressing cells are detected in the dorsal edge of the opened neural tube in Gli2\(^{−/−}\); Ptc1\(^{−/−}\) and Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (N, O). Restoration of Pax7-expressing cells are detected in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (M). More Pax7-expressing cells are detected in Gli2\(^{−/−}\); Gli3\(^{−/−}\) embryos than those in the wild-type and Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (L). Ptc1-lacZ expression is detected in the ventral cells of the wild-type (P). The expression in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos is restricted to the ventral region (S) compared with the ubiquitous expression in Gli2\(^{−/−}\); Ptc1\(^{−/−}\) embryos (T). Weak Ptc1-lacZ expression is detected in the Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) embryos (R), whereas the expression in Gli2\(^{−/−}\); Gli3\(^{−/−}\); Ptc1\(^{−/−}\) is too weak to observe on the sections (Q). Scale bar, 100 μm.
did not see an obvious difference in the number of Nkx2.2-expressing cells between Gli2\(^{-/-}\):Ptc1\(^{-/-}\) and Gli2\(^{-/-}\); Gli3\(^{+/+}\):Ptc1\(^{-/-}\) neural tube, suggesting that the dosage of Gli3 gene is not critical for the specification of V3 progenitor. Since V3 progenitors were not observed in Gli2\(^{-/-}\); Gli3\(^{-/-}\):Ptc1\(^{+/-}\) neural tube, the specification of V3 progenitors is blocked in the absence of Gli2 and Gli3 function (Fig. 6J) (Ding et al., 1998). We examined Isl1/2 expression using Pax7 expression as a marker. The expression was significantly reduced in Gli2\(^{-/-}\); Isl1/2\(^{-/-}\); Ptc1\(^{-/-}\) neural tube compared with that in Gli2\(^{-/-}\); Isl1/2\(^{-/-}\); Ptc1\(^{+/-}\) embryos (Fig. 6L and J). Thus, when Gli2 is absent, the dosage of the Gli3 gene is critical for Isl1/2 expression in the ventral neural tube. Since motor neurons develop normally in Gli3\(^{-/-}\) embryos, our results suggest that Gli2 and Gli3 have overlapping functions in promoting motor neuron development.

Next, we checked the development of dorsal neural cells using Pax7 expression as a marker. The expression was restored in Gli2\(^{-/-}\); Isl1/2\(^{-/-}\); Ptc1\(^{+/-}\) neural tube, while a small number of Pax7-expressing cells were observed in Gli2\(^{-/-}\); Isl1/2\(^{-/-}\); Ptc1\(^{-/-}\) neural tube (Fig. 6K–O). Pax7-expressing cells were more abundant in Gli2\(^{-/-}\); Isl1/2\(^{-/-}\); Ptc1\(^{-/-}\) mutants than in Gli2\(^{-/-}\); Isl1/2\(^{-/-}\); Ptc1\(^{+/-}\) mutants (Fig. 6L and M). Thus, even without Gli2 and Gli3, the neural tube was ventralized due to the missing Ptc1 function.

**Discussion**

Shh is a ventralizing factor, while its receptor Ptc1 is an antagonist of Shh signaling. Here, we show that Gli2 and Gli3 are required for the development of ventral cell fates that arise in dorsal and lateral regions in Ptc1 mutants. The mutual suppression of the Gli and Ptc1 mutant phenotypes provides evidence that the transcription factors Gli2 and Gli3 specify ventral cell fates, floor plate, V3 interneuron, and motor neuron progenitors. There are striking differences in the functions of Gli2 and Gli3 in activating those targets, both in terms of target specificity and in terms of location in the embryo. While Gli2 is an essential activator in the floor plate and V3 interneurons in the caudal neural tube, in the brain and rostral neural tube, Gli3 can substitute for Gli2 and promote the development of the ventral midline cells. In contrast, both Gli2 and Gli3 equally contribute to the motor neuron development at all axial levels.

Multiple ventral cell fates form even without Ptc1 function

Shh appears to specify multiple cell fates in the ventral neural tube in a dosage-dependent manner (Jessell, 2002). In Shh\(^{-/-}\) mice, the floor plate cells, V3 interneurons, motor neurons, and V2 interneurons do not develop (Chiang et al., 1996; Pierani et al., 1999; Litingtung and Chiang, 2000). The current view is that a morphogen gradient of Shh, ventral-high to dorsal-low, leads to the formation of different cell types at distinct dorsoventral positions in the neural tube (Gritti-Linde et al., 2001). Ptc is a key player in this morphogen gradient model. In Drosophila, Ptc sequesters Hh protein and thus restricts Hh movement through tissue (Chen and Struhl, 1996). Since Ptc itself is increased by the Hh signal, the Hh-induced expression of Ptc may function to accentuate the Hh gradient. Briscoe et al. (2001) have recently demonstrated that Ptc1 acts similarly in the neural tube, restricting the movement of Shh derived from the floor plate.

The simple prediction for Ptc1 mutants is that their cells will be unable to repress Shh target genes and will therefore take on ventral character. If the fates of the different ventral cell fates were due entirely to sensing differences in Shh concentration, the elimination of Ptc1 should mean production of only the ventral cell fate characteristic of the highest Shh concentration. We show here that the actual situation is more complex. Instead, cells take on multiple ventral fates. V3 interneurons and motor neurons, which are normally induced at lower Shh concentrations, develop despite the absence of Ptc1 function (Fig. 1). In contrast to their ordered distributions in the normal neural tube, floor plate cells, V3 interneurons, and motor neurons are interspersed in the Ptc1 mutant neural tube. These observations suggest that, although Ptc1 plays a role in spatial patterning of these cell fates in the neural tube, multiple ventral cell fates form even in the apparent absence of the ability to sense different Shh concentrations.

The blocking of Ptc function by Hh is believed to allow the activity of the Smo transmembrane protein in flies (Chen and Struhl, 1996) and mammals (Stone et al., 1996; Goodrich et al., 1997; Taiaple et al., 2000). An activated form of Smo (Smo-M2) can induce the ectopic differentiation of motor neurons and ventral interneurons (Hynes et al., 2000). These observations argue that the specification of distinct ventral cell fates is not solely mediated by graded Shh signaling. We found that V3 interneuron and motor neuron progenitors are localized in the ventral side, while Pax7-expressing dorsal cells are restored in Gli2\(^{-/-}\); Ptc1\(^{-/-}\) neural tube. Thus, without Ptc1 function, even partially, dorsal and ventral pattern is established. Several molecules expressed in the neural tube, such as Ptc2 (Motoyama et al., 1998), Hip (Chuang and McMahon, 1999) and vitronectin (Martinez-Morales et al., 1997; Pons and Mati, 2000), are induced by Hh signaling and might interact with Hh. These binding proteins may modulate the activity of Smo and/or a...
component downstream of Smo through interactions with Hh.

Gli3 can activate ventral cell fate development

Our experiments demonstrate that Gli3 can induce floor plate and V3 progenitors in the rostral neural tube. Although Gli3 clearly can function as an activator of the floor plate and V3 interneuron development, its function in normal embryos is probably minor. Ventral cell types in Gli3−/− embryos develop normally, and Gli3 inactivation has no effect on the derepressed Shh target genes in Ptc1−/− embryos. Thus, in both normal and Ptc1−/− embryos, Gli2 is the major activator of the ventral cell fate specification. Gli3 can compensate for the loss of Gli2 functions in mediating the Shh signal in the rostral neural tube. In contrast, Gli2 and Gli3 may function equally for the specification of motor neurons. Motor neurons develop normally both in Gli2−/−
and Gli3−/− embryos. Only simultaneous inactivation of Gli2 and Gli3 can abolish motor neuron development. Thus, Gli2 and Gli3 play overlapping roles in specification of motor neurons in the developing neural tube.

In the developing neural tube, the function of Gli3 as a repressor of ventral cell fates has been unveiled in Gli3−/−; Shh−/− mutants (Litingtung and Chiang, 2000; Aoto et al., 2002; Rallu et al., 2002). In contrast, the present study allows the detection of the Gli3 activation functions. The seemingly different inferences about Gli3 function can be reconciled if the Shh signal converts Gli3 from a repressor to an activator. In Gli3−/−; Shh−/− mutants, only the repressor form of Gli3 may exist since there is no Shh signal (von Mering and Basler, 1999; Aza-Blanc et al., 2000). Two groups have recently showed that, in the fly, Gli3 can be a Hh-regulated activator and repressor, whereas Gli1 functions only as an activator. They used transgenic overexpression in Drosophila to study the activity of the three mouse Gli transcription factors (Litingtung et al., 2002; te Welscher et al., 2002). These studies show that Gli1 is resistant to cleavage, whereas Gli2 and Gli3 can be cleaved to form repressors, and only Gli3 is cleaved in Hh-dependent manner. Although how much these findings apply to mammals remains to be determined, they are consistent with published observations including the findings reported here.

Gli2 and Gli3 mediate most, if not all, of the Shh response in mice

Many members of the Drosophila Hh pathway are conserved in vertebrates (Ingham and McMahon, 2001). In some cases, when there is only one mammalian homologue, such as Smo, mutant studies have clearly revealed an evolutionarily conserved role in vertebrate Hh signaling (Zhang et al., 2001). In contrast, the role of multiple Gli transcription factors in vertebrate Hh signaling has been a major puzzle. Here, by generating triple mutants of Ptc1, Gli2, and Gli3, we establish the model that Gli2 and Gli3 share overlapping activator functions in induction of ventral cells in developing neural tube. It has been shown that ptc; ci double mutants have a phenotype indistinguishable from that of embryos lacking only ci (Methot and Basler, 2001). Ci therefore appears to mediate all aspects of Hh signaling in the fly. Triple mutants of Ptc1, Gli2 and Gli3 are also phenotypically similar to Gli2−/−; Gli3−/− embryos. We conclude that, similar to Ci in flies, Gli2 and Gli3 function together as activators of Shh target gene transcription in the neural tube of Ptc1−/− embryos. However, the phenotype of Shh mutants is much more severe than that of Gli2−/−; Gli3−/− or Gli2−/−; Gli3−/−; Ptc1−/− embryos. Shh−/− embryos lack midline structure completely, whereas Gli2−/−; Gli3−/− and Gli2−/−; Gli3−/−; Ptc1−/− embryos can develop midline at least in the ventral telencephalon even without many ventral cell fates. We observed residual Ptc1-lacZ expression in the ventral neural tube in Gli2−/−; Gli3−/−; and Gli2−/−; Gli3−/−; Ptc1−/− embryos, while no Ptc1-lacZ expression is detected in Shh−/− embryos (Eggschwiler et al., 2001), suggesting that either Gli1 or an unknown transcription factor, other than Gli2 and Gli3, can mediate Shh signal.

Based on this study, the developing neural tube can be divided into two parts, each with different requirements for Gli2 and Gli3 in the regulation of Shh targets (Fig. 7). In the rostral part, from the forebrain, except for the Zli and ventral telencephalon, to the thoracic level of the neural tube, both Gli2 and Gli3 can activate the specification of floor plate, V3 interneuron, and motor neuron progenitors. In the caudal region, the neural tube at the trunk and tail levels, Gli3 cannot substitute for Gli2 function in the specification of the floor plate and V3 progenitors, but can promote motor neuron development. The expression pattern of Gli2 and Gli3 do not account for the differential contribution, since both Gli2 and Gli3 are expressed in the entire neural tube both in the wild-type and Ptc1 mutant neural tube (data not shown). Factors involved in rostral-caudal patterning of the neural tube must modulate the differential contributions of Gli2 and Gli3 in the activation of the target genes.

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