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Gli2, but not *Gli1*, is required for initial Shh signaling and ectopic activation of the Shh pathway

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SUMMARY

The Shh signaling pathway is required in many mammalian tissues for embryonic patterning, cell proliferation and differentiation. In addition, inappropriate activation of the pathway has been implicated in many human tumors. Based on transfection assays and gain-of-function studies in frog and mouse, the transcription factor Gli1 has been proposed to be a major mediator of Shh signaling. To address whether this is the case in mouse, we generated a *Gli1* null allele expressing *lacZ*. Strikingly, *Gli1* is not required for mouse development or viability. Of relevance, we show that all transcription of *Gli1* in the nervous system and limbs is dependent on *Shh* and, consequently, Gli1 protein is normally not present to transduce initial Shh signaling. To determine whether *Gli1* contributes to the defects seen when the Shh pathway is inappropriately activated and *Gli1* transcription is induced, *Gli1;Ptc* double mutants were generated. We show that *Gli1* is not required for the ectopic activation of the Shh signaling pathway or to the early embryonic lethal phenotype in *Ptc* null mutants. Of significance, we found instead that *Gli2* is required for mediating some of the inappropriate Shh signaling in *Ptc* mutants. Our studies demonstrate that, in mammals, *Gli1* is not required for Shh signaling and that Gli2 mediates inappropriate activation of the pathway due to loss of the negative regulator *Ptc*.

Key words: *Gli3*, *Ptc*, Neural patterning, Mouse, Mutants

INTRODUCTION

GLI1, which encodes a member of the Gli-Kruppel family of transcription factors, was initially identified as an amplified gene and potential oncogene in a human glioblastoma (Kinzler et al., 1987). A family of Gli genes that includes *Gli1*, *Gli2* and *Gli3* was cloned from human and mouse, and found to be expressed in many organs during mouse development (Ruppert et al., 1988; Hui et al., 1994). The fly homolog of Gli, Cubitus interruptus (Ci), is involved in mediating all Hedgehog (Hh) signaling (Methot and Basler, 2001). Given that Hh proteins are critical for many developmental processes in vertebrates, considerable effort has been made in the past decade to elucidate the function of each of the Gli genes in the Hh pathway (for reviews, see Matise and Joyner, 1999; Ingham and McMahon, 2001). In particular, the roles of the Gli proteins downstream of Sonic Hedgehog (Shh) signaling have been studied in ventral patterning of the nervous system.

In fly, Hh regulates Ci function by inhibiting processing of Ci into a repressor protein, and at the same time potentiating the full-length activator protein (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998; Methot and Basler, 1999). Gli2 and Gli3, but not Gli1, have been similarly found to have an N-terminal repressor domain and be cleaved into a repressor form (Dai et al., 1999; Sasaki et al., 1999; Aza-Blanc et al., 2000; Wang et al., 2000). *Gli2* and *Gli3* also have been found to be required for development and Shh signaling. Loss of *Gli2* function results in defective Shh signaling in the floorplate of the neural tube and other tissues (Mo et al., 1997; Ding et al., 1998; Matise et al., 1998), indicating Gli2 is an activator in the Hh pathway. By contrast, loss of mouse *Gli3* results in dorsal brain defects and limb polydactyly that are associated with ectopic activation of the Shh pathway (Hui and Joyner, 1993; Masuya et al., 1995; Buscher et al., 1997). The *Gli3* mutant phenotype suggests that Gli3 functions primarily as a repressor in the Shh pathway. Indeed, biochemical studies have shown that Shh functions to inhibit the formation of the repressor form of Gli3 (Wang et al., 2000), and removal of *Gli3* function in *Shh* mutants largely rescues the *Shh* mutant defects, showing that part of the *Shh* mutant phenotype is due to an excess of Gli3 repressor (Litingtung and Chiang, 2000).

Interestingly, gain-of-function studies in mouse and frog embryos have shown that *Gli1*, but not *Gli2* or *Gli3*, can mimic Shh function by inducing proliferation and activating Shh

target genes, including *Hnf3b* in the dorsal CNS (Hynes et al., 1997; Lee et al., 1997; Park et al., 2000). In addition, *Gli1* is always expressed near *Shh* and can be transcriptionally activated by Shh (Grindley et al., 1997; Hynes et al., 1997; Lee et al., 1997). Based on these findings, it was proposed that Gli1 is the key transcription factor acting in the Shh pathway (Hynes et al., 1997; Ruiz i Altaba, 1997). In support of this assertion, *GLI1* is expressed in human tumors thought to be caused by elevated Shh signaling, such as basal cell carcinomas (Dahmane et al., 1997; Reifenberger et al., 1998). Furthermore, endogenous *Gli1* in frog was recently shown to be required for a transiently supplied exogenous human GLI1 protein to induce hyperproliferation in the nervous system (Dahmane et al., 2001).

To investigate the function of *Gli1* in mouse development, we previously generated a *Gli1* mutant allele lacking the exons that encode zinc fingers 2-5 (zinc-finger deletion or $Gli1^{zfd}$) (Park et al., 2000). In contrast to the predictions based on gain-of-function assays, mice homozygous for the $Gli1^{zfd}$ mutation are viable and do not have obvious defects. Further analysis, however, revealed an alternatively spliced transcript produced by the mutant allele that could encode a Gli1 protein lacking only the zinc fingers. It is therefore possible that the $Gli1^{zfd}$ allele is hypomorphic, and that *Gli1* may be required for mouse development.

To address definitively the requirement for *Gli1* in Shh signaling, as well as in mouse development, we generated a new *Gli1*-null allele in which the coding sequences of *Gli1* are replaced with *lacZ* ($Gli1^{lz}$). Using this allele, we show that Shh is required for the initial transcriptional activation of *Gli1*, and thus Gli1 cannot transduce the earliest Shh signaling in tissues. Consistent with this, *Gli1*-null mutants develop properly and adults appear normal. We also tested the requirement for *Gli1* and *Gli2* in patched (*Ptc*; *Ptch* – Mouse Genome Informatics) mutants in which the Shh pathway is ectopically activated. Our results show that ectopic activation of the Shh pathway via loss of the negative regulator Ptc is not dependent on *Gli1*, but significantly is dependent on *Gli2*.

MATERIALS AND METHODS

Generation of *Gli1* null mutant knock-in mice

W4 ES cells (Auerbach et al., 2000) were electroporated with a $Gli1^{lz}$ targeting construct followed by gancyclovir and G418 double selections according to Matise et al. (Matise et al., 2000). Targeted ES cell clones were identified by restriction enzyme digestion and Southern blot analysis of ES cell DNA using 5′ and 3′ external probes (see Fig. 1). The 5′ probe identified a 9 kb fragment in the targeted allele and a >20 kb fragment in the wild-type allele following *Eco*RV digestion. The 3′ probe identified a 4.5 kb fragment in the targeted allele and a 9 kb fragment in the wild-type allele with *Xba*I digestion. The targeting frequency was one in four ES cells surviving double selections. Three targeted cell lines were then injected into C57BL/6 blastocysts to generate chimeras (Papaioannou and Johnson, 2000). Chimeras were then bred with 129SvEv and Black Swiss mice (Taconic) to establish F_1 heterozygotes and three independent germline transmitting mouse lines were established. The floxed *neo* gene was removed by breeding with *TK-Cre* transgenic mice (W. A., unpublished). PCR analysis was used for routine genotyping with the following primers (see Fig. 1):

P1, CCAGTTTCTGAGATGAGGGTTAGAGGC;
P2, TTGAATGGGGAATACAGGGGCTTAC;
P3, GCATCGAGCTGGGTAATAAGCGTTGGCAAT;
P4, GACACCAGACCAACTGGTAATGGTAGCGAC.

P1 and P2 identify the wild-type allele, whereas P3 and P4 (against *lacZ*) identify the targeted allele.

Breeding and genotyping of $Gli2^{lzki}$, $Gli2^{zfd}$ and *Ptc* mutants were as described (Mo et al., 1997; Goodrich et al., 1997; Bai and Joyner, 2001). All mice were kept and analyzed on an outbred Swiss Webster background.

Immunohistochemistry and RNA in situ hybridization

Embryos were fixed in 4% paraformaldehyde for 20 minutes at 4°C before embedding in OCT. Frozen sections were cut at 12 μm. Immunohistochemistry was performed as previously described (Matise et al., 1998) using the following monoclonal antibodies: Shh, Nkx2-2, Isl1/2 and Pax6 (Ericson et al., 1996). Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) was used at a dilution of 1:500. Images were captured via a Hamamatsu cooled CCD camera and colors assigned offline. X-gal staining and whole-mount RNA in situ hybridization were performed as previously described (Bai and Joyner, 2001).

RESULTS

Production of a *Gli1-lacZ* marker allele in mice

In order to determine whether *Gli1* is indeed required during development and to have mice expressing *lacZ* as a read-out of Gli1 activity, we generated a new mutant *Gli1*-null allele by replacing the genomic fragment that encodes the entire Gli1 N-terminal and zinc-finger domains with the *lacZ* gene (Fig. 1). This replacement results in a loss of *Gli1* function, even if alternative splicing occurs from the 5′ untranslated exon to an exon downstream of the *lacZ* insertion. Three independent lines of mice derived from independently targeted ES cells were established from chimeras ($Gli1^{lz}$). Analysis of β-galactosidase activity in all three lines showed identical *lacZ* expression patterns at various embryonic stages (E7.5-E12.5). Furthermore, the *lacZ* expression pattern was indistinguishable from *Gli1* mRNA expression (Hui et al., 1994; Platt et al., 1997), showing that the knock-in allele recapitulates *Gli1* expression (data not shown and see Fig. 3).

Both $Gli1^{lz}$ and $Gli1^{zfd}$ are null alleles not required for development

To determine whether *Gli1* is required for mouse development and Shh signaling, $Gli1^{lz/lz}$ homozygous mutants were generated. Homozygous $Gli1^{lz}$ mutant mice were found to be viable with no obvious phenotypes. Thus, *Gli1* is not required for development or survival of mice.

Although our previous studies showed that homozygous $Gli1^{zfd}$ mutant mice are phenotypically normal, reducing the level of *Gli2* in *Gli1* homozygous mutant mice ($Gli1^{zfd/zfd};Gli2^{zfd/+}$) results in multiple defects including a partial loss of the floor plate, failure of notochord regression, aberrant lung development and lethality (Park et al., 2000). We compared $Gli1^{lz/lz};Gli2^{zfd/+}$ embryos with $Gli1^{zfd/zfd};Gli2^{zfd/+}$ embryos to determine if there are phenotypic differences between the $Gli1^{lz}$ and $Gli1^{zfd}$ alleles. In $Gli1^{lz/lz};Gli2^{zfd/+}$ embryos, as in $Gli1^{zfd/zfd};Gli2^{zfd/+}$ embryos, some floor plate cells were found to be lost at E10.5. In the most severe embryos, HNF3β-expressing cells are not present (Fig. 2A,E), Nkx2.2 interneurons are greatly reduced (Fig. 2B,F), and motor neurons occupy a more

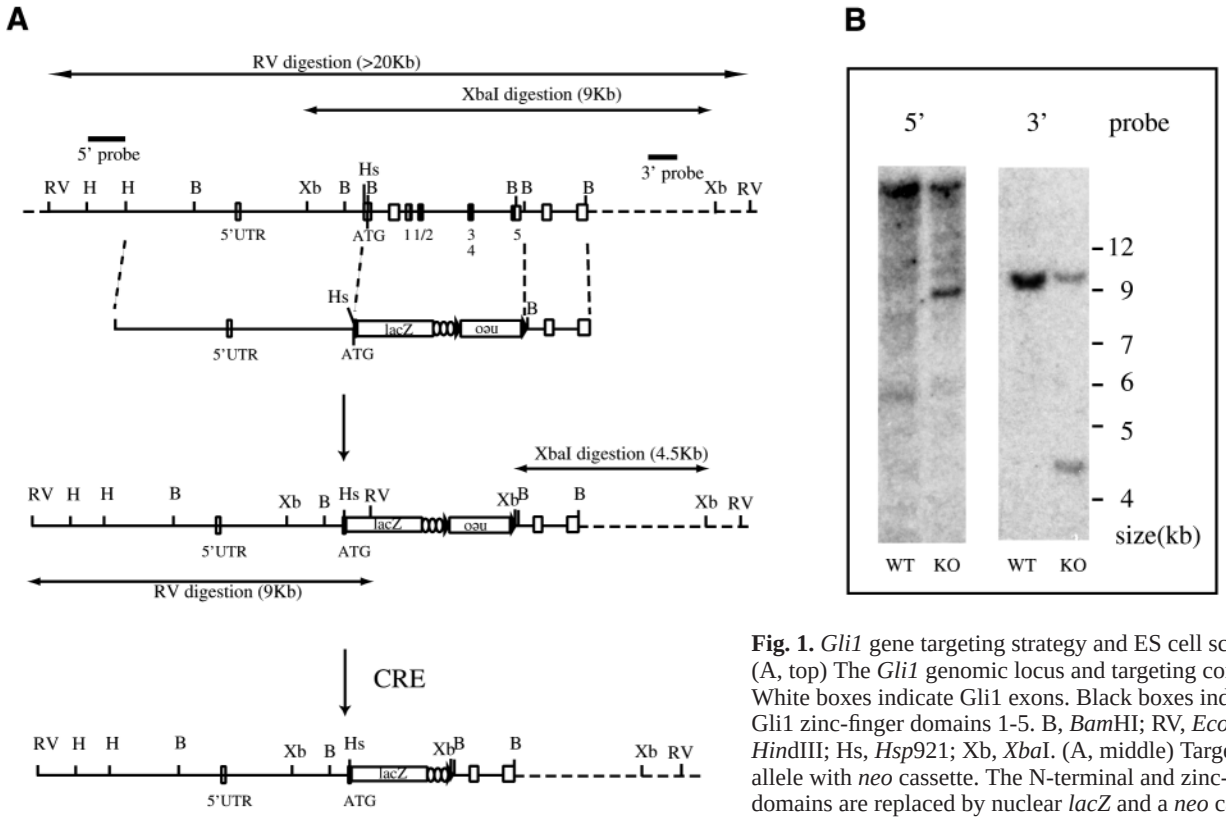

Fig. 1. *Gli1* gene targeting strategy and ES cell screening. (A, top) The *Gli1* genomic locus and targeting construct. White boxes indicate Gli1 exons. Black boxes indicate Gli1 zinc-finger domains 1-5. B, *Bam*HI; RV, *Eco*RV; H, *Hin*dIII; Hs, *Hsp*921; Xb, *Xba*I. (A, middle) Targeted allele with *neo* cassette. The N-terminal and zinc-finger domains are replaced by nuclear *lacZ* and a *neo* cassette. The *neo* cassette is in the opposite orientation relative to *Gli1* transcription. (A, bottom) Targeted allele without *neo* allele. Cre recombinase was used to remove the *loxP-neo-loxP* cassette. (B) ES cell Southern blot hybridization using 5′ and 3′ external probes indicated in A following *Eco*RV and *Xba*I digestion, respectively. The 5′ probe identifies a 9 kb mutant fragment and a >20 kb wild-type fragment, whereas the 3′ probe identifies a 4.5 kb mutant fragment and a 9 kb wild-type fragment.

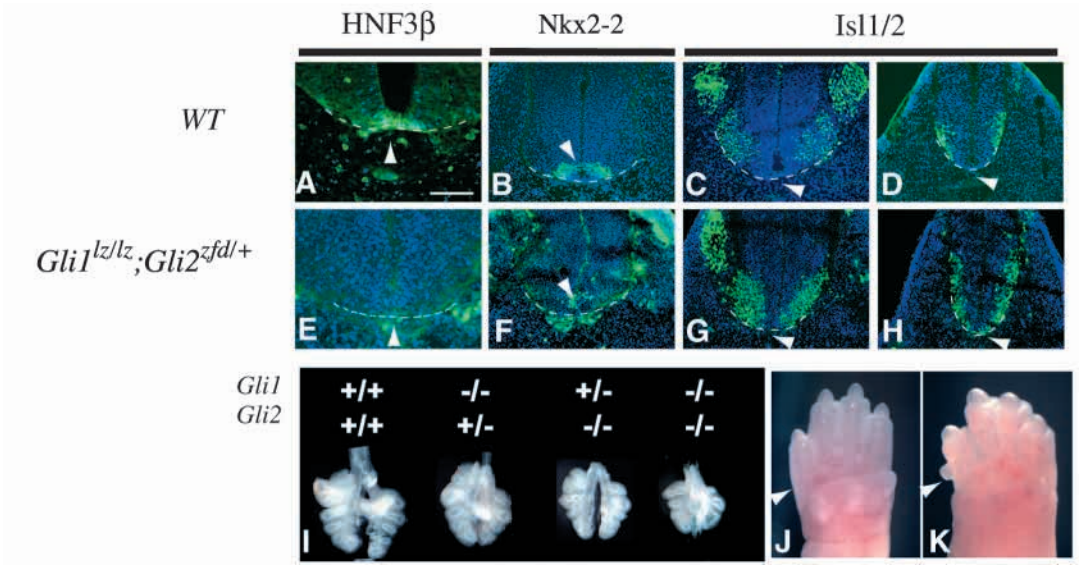

Fig. 2. *Gli1*$^{lz/lz}$;*Gli2*$^{zfd/+}$ embryos have defects similar to *Gli1*$^{zfd/zfd}$;*Gli2*$^{zfd/+}$ embryos. In E10.5 *Gli1*$^{lz/lz}$;*Gli2*$^{zfd/+}$ embryos, there is a variable loss of floor-plate cells. In the most extreme mutant embryos, HNF3β- and Nkx2-2-expressing cells are greatly reduced (compare E and F with A and B), such that Isl1/2-expressing cells occupy the ventral midline of the spinal cord (compare G and H with C and D). (C,G) Sections at forelimb level; (D,H) sections at hindlimb level. Arrowhead indicates ventral midline of spinal cord. (I) At E12.5, *Gli1*$^{lz/lz}$;*Gli2*$^{zfd/+}$ lungs are smaller than wild-type lungs, in addition, *Gli1*$^{lz/lz}$;*Gli2*$^{zfd/zfd}$ lungs have only two lobes and are much smaller than wild-type lungs. At E18.5, a postaxial nubbin was found in the limbs of *Gli1*$^{lz/lz}$;*Gli2*$^{zfd/zfd}$ embryos (K), similar to *Gli1*$^{zfd/zfd}$;*Gli2*$^{zfd/zfd}$ embryos. (J) E18.5 wild-type limb. Scale bar: 0.1 mm in A-H.

ventral position (Fig. 2C,D,G,H). At E12.5, the notochord failed to regress (data not shown). Furthermore, similar to *Gli1*^{zfd};*Gli2*^{zfd} double homozygous mutants, *Gli1*$^{lz/lz}$;*Gli2*$^{zfd/zfd}$ embryos displayed smaller lungs at E12.5 and E18.5 compared with *Gli2*$^{zfd/zfd}$ embryos (Fig. 2I and data not shown), and an extra postaxial nubbin was present in the forelimbs at E18.5 (Fig. 2J,K). Given that the *Gli1*^{lz} and *Gli1*^{zfd} alleles show similar phenotypes as single mutants or combined with *Gli2* mutant alleles, our results show that both *Gli1*^{lz} and *Gli1*^{zfd} are null alleles.

Shh is required for transcription of *Gli1*

One possible reason why *Gli1* is not required during development is that it is not expressed when the Shh pathway is first activated, because *Gli1* transcription requires Shh signaling. This would be consistent with the finding that Shh signaling can activate *Gli1* transcription. To examine whether *Shh* could be required for the initial transcriptional activation of endogenous *Gli1*, we compared the expression patterns of *Shh* mRNA with *Gli1-lacZ*.

Expression of *Shh* starts at E7.5 in the midline mesoderm of the head process (Echelard et al., 1993). Shortly thereafter, *Shh* expression expands posteriorly along the midline into the node. By the headfold stage, *Shh* expression is initiated in the anterior midbrain of the neural ectoderm. As somites form, *Shh* expression expands anteriorly into the ventral forebrain and posteriorly along the spinal cord. By E9.5, *Shh* expression can be detected in both the notochord and floor plate throughout the anterior/posterior (AP) axis, along the sulcus limitans in the forebrain, and in the gut endoderm (Fig. 3A-D) (Echelard et al., 1993). Expression of *Gli1-lacZ* was found to follow closely the expression pattern of *Shh*. *Gli1-lacZ* expression can first be detected weakly at the late streak stage (E7.5), close to the midline in the head process (Fig. 3E). It then followed *Shh* expression by extending into the area surrounding the node (Fig. 3F). When *Shh* is expressed throughout the AP axis in the notochord and floor plate, *Gli1-lacZ* was also detected in mesodermal cells surrounding the notochord and in cells next to the floor plate, as well as in the gut (Fig. 3G,H).

These expression studies showed that *Gli1* expression is complementary to *Shh*, but it was not possible to determine whether *Gli1* is actually transcribed before or after *Shh*.

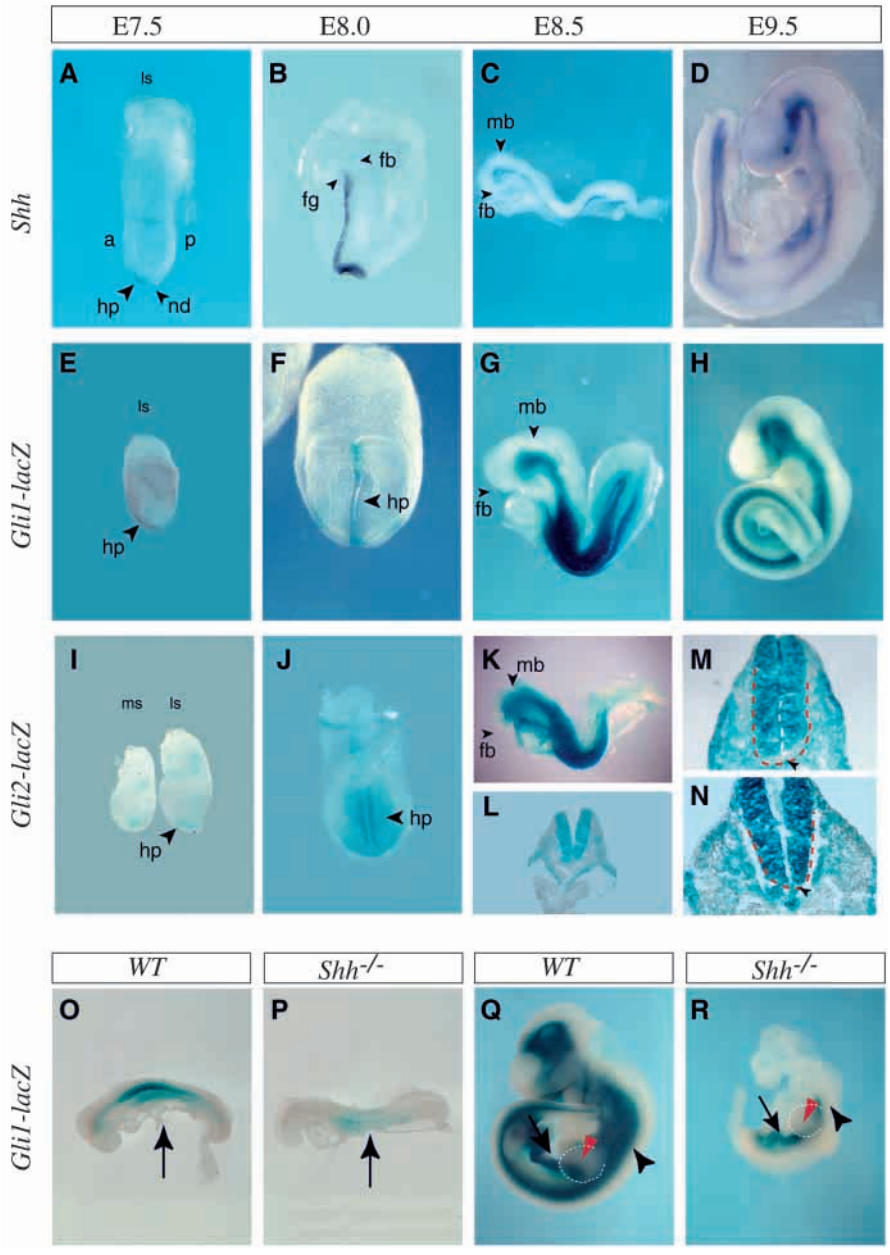

Fig. 3. *Shh* is required for transcription of *Gli1*. (A-D) RNA in situ hybridization of *Shh* at E7.5, E8.0, E8.5 and E9.5 in wild-type embryos. (E-H) X-gal staining of *lacZ* in *Gli1*$^{lz/+}$ embryos at E7.5, E8.0, E8.5 and E9.5. Note the brownish weak X-gal staining of *Gli1-lacZ* at E7.5. The embryo in E is slightly older than the embryo in A. (I-N) X-gal staining of *Gli2-lacZ* from E7.5 to E9.5. Note, one embryo in I is at mid-streak stage (ms), and the other embryo is at the late-streak stage (ls). (L) Spinal cord section of an E8.5 embryo showing *Gli2-lacZ* in the ventral midline. (M,N) Spinal cord section of an E9.5 embryo at an anterior (M) or posterior (N) position. Arrowhead indicates floorplate cells. Spinal cord is outlined by broken red lines. a, anterior; hp, head process; fb, forebrain; fg, foregut; ls, late-streak; mb, midbrain; ms, mid-streak; nd, notochord; p, posterior. (O-R) X-gal staining of *lacZ* in *Gli1*$^{lz/+}$ embryos (O,Q) or *Gli1*$^{lz/+}$;*Shh*$^{-/-}$ embryos (P,R) at E8.5 (O,P) and E10.5 (Q,R). Limb buds in Q and R are outlined by broken white lines. At E8.5, *Gli1-lacZ* expression can be detected in the CNS and the gut (indicated by an arrow) in wild-type embryos (O), whereas it can only be detected weakly in the gut in *Shh* mutant embryos (P). At E10.5, *Gli1-lacZ* is strongly expressed in the gut (arrow), posterior limb bud (red arrowhead) and CNS (black arrowhead) in wild-type embryos (Q). In *Shh* mutant embryos, the expression in the posterior limb bud and the CNS cannot be detected (R).

Therefore, to address whether transcription of *Gli1* is dependent on Shh signaling, we examined *Gli1-lacZ* expression in wild-type and *Shh* mutant embryos at E8.5 and E10.5. In wild-type embryos at E8.5, *Gli1-lacZ* is strongly expressed in the ventral CNS, some mesodermal cells surrounding the notochord and in the gut (Fig. 3O). This expression pattern remains the same but becomes more intensified at E10.5 with additional expression in the forelimbs (Fig. 3Q). Significantly, in *Shh* mutant embryos, *Gli1-lacZ* was not detected at E8.5 in the ventral CNS (Fig. 3P). The expression in mesodermal cells and gut was greatly reduced, but not completely lost, which is probably due to expression of *Ihh* in the gut (Bitgood and McMahon, 1995). By E10.5 in *Shh* mutants, *Gli1-lacZ* could only be detected in the gut that expresses *Ihh*, but not in the CNS or limbs (Fig. 3R). These studies show that *Gli1* transcription is absolutely dependent on Hh signaling.

In contrast to *Gli1*, *Gli2* transcription is not dependent on *Shh* (Bai and Joyner, 2001). To address whether *Gli2* is capable of mediating the initial Shh signaling, we examined the expression of *Gli2-lacZ* at early stages. *Gli2-lacZ* can be detected at the mid-streak stage, prior to *Shh* transcription (Fig. 3I). At the late-streak stage and neural plate stage (E8.0), *Gli2-lacZ* can be detected in a domain complimentary to *Shh* (Fig. 3I,J). At E8.5, *Gli2-lacZ* was still expressed in the ventral midline of the neural tube at the time the floorplate is being induced (Fig. 3L). At E9.5, *Gli2-lacZ* cannot be detected in floorplate cells in the anterior spinal cord (Fig. 3M), but can still be detected in the ventral midline of the tail region (Fig. 3N). Therefore, the temporal and spatial pattern of *Gli2* expression is consistent with Gli2 mediating the initial Shh signaling in the ventral CNS. Indeed, loss of *Gli2* results in defects in floorplate induction (Ding et al., 1998; Matise et al., 1998).

Gli2, but not *Gli1*, is involved in transducing ectopic activation of the Shh pathway in *Ptc* mutants

Ptc is the receptor for Shh and has been found to be a negative regulator of the pathway in mice and flies (Stone et al., 1996; Marigo et al., 1996). Humans with Gorlin's syndrome have heterozygous mutations in *PTC* and are born with many developmental defects. Later in life, they can develop medulloblastoma and basal cell carcinomas (Gorlin, 1987). Furthermore, in many basal cell carcinomas caused by hereditary or sporadic mutations in *PTC*, loss of heterozygosity of *PTC* is seen (Gailani et al., 1992; Unden et al., 1996; Levanat et al., 1996). Consistent with activation of the Shh pathway due to loss of a negative regulator, *GLI1* is expressed in both types of tumors (Dahmane et al., 1997; Dahmane et al., 2001).

As Ptc is a negative regulator of the Shh signaling pathway, loss of *Ptc* in mouse embryos results in a great increase in the Hh signaling output and results in early lethality (Goodrich et al., 1997). To determine whether *Gli1*, or *Gli2*, contributes to the embryonic phenotypes seen in $Ptc^{-/-}$ embryos, we removed each gene from *Ptc* mutants. In the spinal cord of *Ptc* mutants, activation of the Shh signaling pathway results in an expansion of ventral CNS cell fates dorsally. *lacZ* inserted into the *Ptc* mutant allele (Goodrich et al., 1997) can be used as a read-out of Shh signaling, as *Ptc* can be activated by *Shh* and *lacZ* is under the control of the endogenous *Ptc* promoter. In wild-type embryos at E8.5, *Ptc-lacZ* is expressed in the ventral CNS (Fig. 4A), similar to *Gli1-lacZ*. As expected, in $Gli1^{zfd/zfd};Ptc^{+/-}$ embryos, the expression of *Ptc-lacZ* remained the same as in wild-type embryos (Fig. 4B). By contrast, in $Gli2^{zfd/zfd};Ptc^{+/-}$ embryos at E8.5, the expression of *Ptc-lacZ* was greatly reduced (Fig. 4C), suggesting *Gli2* is normally required for most aspects of *Ptc* activation.

We then examined embryos lacking *Ptc* and a specific *Gli* gene. Consistent with activation of the Shh pathway in *Ptc* mutants, *Ptc* mutant embryos showed an elevated level of *Ptc-lacZ* expression throughout the embryo (Fig. 4D) (Goodrich et al., 1997). In addition, no $Ptc^{-/-}$ mutants were detected after E9.5. In $Gli1^{zfd/zfd};Ptc^{-/-}$ embryos, *Ptc-lacZ* was still expressed throughout the embryo, just as in *Ptc* mutant embryos (Fig. 4E), consistent with persistent activation of the Shh pathway. Furthermore, no $Gli1^{zfd/zfd};Ptc^{-/-}$ embryos were recovered after E9.5 when *Ptc* mutants die (data not shown). By contrast, in $Gli2^{zfd/zfd};Ptc^{-/-}$ embryos, the expression of *Ptc-lacZ* was reduced compared with *Ptc* mutant embryos, in particular in the forebrain and trunk mesoderm (Fig. 4F).

To better assay for a possible rescue effect of removing *Gli2*, we determined the frequency of all genotypes in $Gli2^{zfd/+};Ptc^{+/-}$ intercrosses at E10.5 and E11.5. As expected, no $Ptc^{-/-}$ embryos were recovered from two litters of 20 embryos at E10.5. By contrast, five $Gli2^{zfd/zfd};Ptc^{-/-}$ embryos were present in the two litters at E10.5, although the mutants had exencephaly (Fig. 5A-C). In 20 embryos at E11.5, no

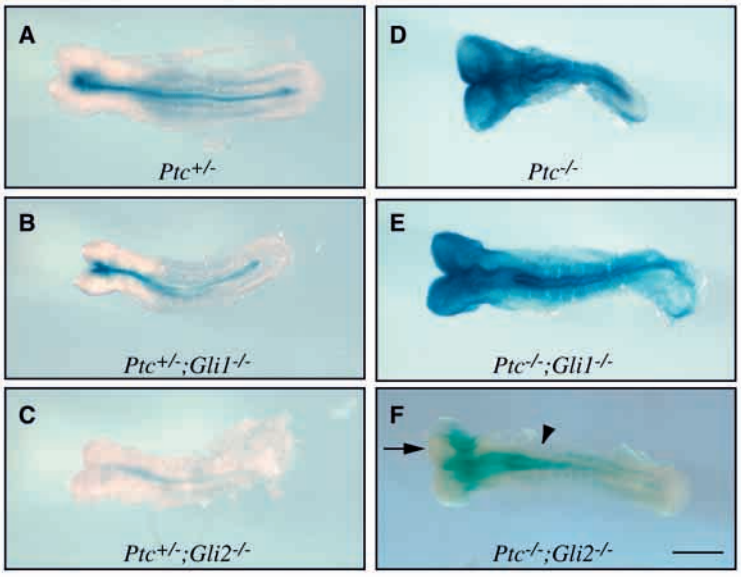

Fig. 4. *Gli2*, but not *Gli1*, is required for Shh signaling in *Ptc* mutants. X-gal staining of *Ptc-lacZ* in six- to eight-somite E8.5 embryos. In wild-type embryos, *Ptc-lacZ* is expressed in the ventral CNS and somites (A). The expression of *Ptc-lacZ* is not altered in $Gli1^{zfd/zfd};Ptc^{+/-}$ embryos (B), but the expression is downregulated in $Gli2^{zfd/zfd};Ptc^{+/-}$ embryos (C). Loss of *Ptc* function results in upregulation of *Ptc-lacZ* throughout the embryos (D). Removal of *Gli1* function in $Ptc^{-/-}$ embryos does not rescue the overexpression of *Ptc-lacZ* (E). However, removal of *Gli2* function in $Ptc^{-/-}$ embryos (F) reduces the overexpression of *Ptc-lacZ* in the head (arrow) and the trunk mesoderm (arrowhead). Scale bar: 0.5 mm.

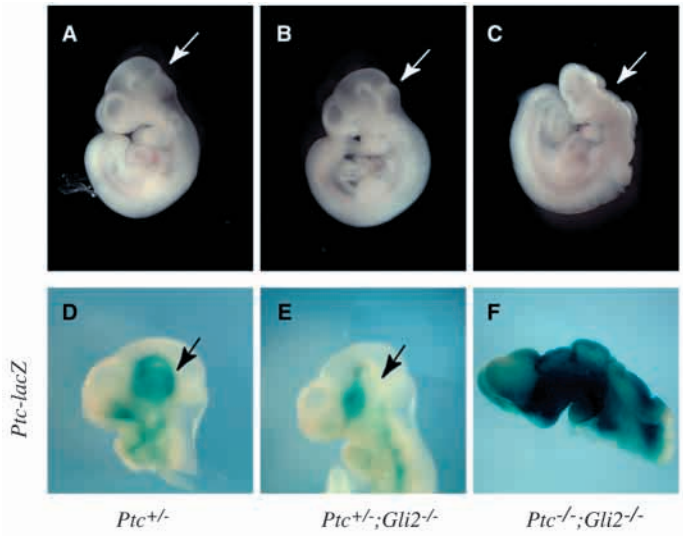

Fig. 5. *Gli2* can only partially rescue the *Ptc* mutant phenotype at E10.5. $Ptc^{+/-}$ (A,D), $Ptc^{+/-}$;$Gli2^{zfd/zfd}$(B,E) and $Ptc^{-/-}$;$Gli2^{zfd/zfd}$(C,F) embryos. $Ptc^{-/-}$;$Gli2^{zfd/zfd}$ embryos survive beyond E10.5, whereas $Ptc^{-/-}$ embryos die by E9.5. At E10.5, even though the spinal cords are closed, $Ptc^{-/-}$;$Gli2^{zfd/zfd}$ embryos show exencephaly (indicated by a white arrow). (D-F) X-gal staining of *Ptc-lacZ*. The expression of *Ptc-lacZ* is reduced in $Ptc^{+/-}$;$Gli2^{zfd/zfd}$ embryos (E, indicated by an arrow), when compared with $Ptc^{+/-}$ embryos (D). In $Ptc^{-/-}$;$Gli2^{zfd/zfd}$ embryos at E10.5, *Ptc-lacZ* expression is upregulated in the brain.

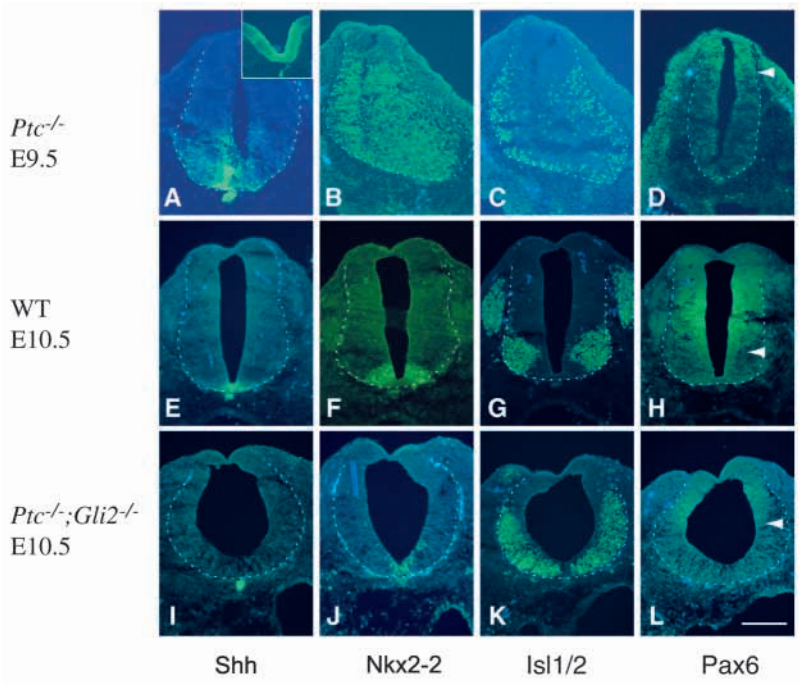

Fig. 6. The ventral spinal cord phenotype of *Ptc* mutants can be partially rescued by removing *Gli2* function. In *Ptc* mutants that survive to E9.5, ventral spinal cord markers expand dorsally (A-D) compared with wild-type embryos a day later at E10.5 (E-H). Insert in A is a more anterior spinal cord section. In *Ptc;Gli2* double homozygous mutants, Shh expression is lost in the spinal cord (I), Nkx2-2 expression is greatly reduced in the ventral midline (J), and the expansion of Isl1/2 and Pax6 seen in $Ptc^{-/-}$ mutants are shifted more ventrally (K,L). White arrowhead indicates ventral limit of Pax6 domain. Scale bar: 78 μm in A-D; 100 μm in E-L.

$Gli2^{zfd/zfd}$;$Ptc^{-/-}$ embryos were recovered. *Ptc-lacZ* expression in the heads of the E10.5 embryos was also examined. In wild-type embryos at E10.5, *Ptc-lacZ* is expressed in the ventral CNS close to the source of Shh (Fig. 5D). As expected, in $Gli2^{zfd/zfd}$;$Ptc^{+/-}$ embryos at E10.5 *Ptc-lacZ* expression was severely downregulated (Fig. 5E). However, although $Gli2^{zfd/zfd}$ can rescue the early lethality of $Ptc^{-/-}$ embryos to E10.5, *Ptc-lacZ* was nevertheless upregulated in the brains of $Gli2^{zfd/zfd}$;$Ptc^{-/-}$ embryos. Furthermore, the dorsal brain failed to close in $Gli2^{zfd/zfd}$;$Ptc^{-/-}$ embryos, similar to the phenotype in $Gli3^{-/-}$ mutants, which also have a dorsal activation of the Shh signaling pathway (Fig. 5F).

To examine the expression of Shh targets in $Gli2^{zfd/zfd}$;$Ptc^{-/-}$ embryos, we analyzed dorsal/ventral (DV) patterning of the spinal cord in $Gli2^{zfd/zfd}$;$Ptc^{-/-}$ embryos as the spinal cord closes normally in these mutants. In $Ptc^{-/-}$ embryos at E9.5, Shh, Nkx2-2 and Isl1/2 were ectopically expressed in the spinal cord, resulting in overlapping expression of the three proteins in many ventral cells (Fig. 6A-D). In wild-type embryos at E10.5, Shh is expressed in the floorplate cells, and Nkx2-2 is expressed in adjacent V3 interneurons (Fig. 6E,F). More laterally, Isl1/2 is expressed in motoneurons and Pax6 is expressed at highest levels in the intermediate spinal cord (Fig. 6G,H). By contrast, in $Gli2^{zfd/zfd}$ embryos at E10.5, Shh is not expressed in the ventral spinal cord, Nkx2-2 is greatly reduced and Isl1/2-expressing motoneurons occupy the ventral spinal cord (see Fig. 7A-D) (Matise et al., 1998). In the rescued $Ptc^{-/-}$ embryos at E10.5 in which *Gli2* was removed, Shh was no longer expressed in the ventral spinal cord, even though it was still detected in the notochord (Fig. 6I). The number of V3 interneurons was greatly reduced compared with $Ptc^{-/-}$ embryos, and only a small number of V3 interneurons remained specifically in the ventral midline of the spinal cord (Fig. 6J). Interestingly, Isl1/2-expressing motoneurons occupied the majority, but not all, of the ventral midline in $Gli2^{zfd/zfd}$;$Ptc^{-/-}$ embryos (Fig. 6K). The expression of these three markers is similar to that of *Gli2* mutants. However, unlike *Gli2* mutants, more motoneurons were generated in the double mutants such that the motoneuron populations expanded into the dorsal half of the spinal cord. In addition, Pax6 was expressed at highest levels in the dorsal half of the spinal cord, instead of ventrally as in *Gli2* mutants (Fig. 6L). Therefore, loss of *Gli2* in $Ptc^{-/-}$ mutants results in a downregulation or loss of some Shh targets that are ectopically activated in the spinal cord in *Ptc* mutants. Taken together, these studies demonstrate that *Gli2* is the primary transcriptional regulator of Shh signaling in *Ptc* mutants, whereas *Gli1* is not.

Endogenous *Gli1* is not required in embryos in which Shh signaling is transduced through Gli1

The Shh signaling pathway can be artificially activated by loss of *Ptc* function, or it can be activated by ectopic expression of *Gli1*. For example, when *Gli1* is ectopically expressed in transgenic mice or frog embryos, *Shh* target genes are ectopically activated and over proliferation

occurs (Hynes et al., 1997; Lee et al., 1997; Park et al., 2000). Moreover, ectopic expression of *Gli1* in postnatal mouse skin can induce various tumors, including basal cell carcinomas and trichoepitheliomas (Nilsson et al., 2000). Recently, it was shown that in frog, transient activation of the pathway using human *GLI1* mRNA requires endogenous *Gli1* for hyperproliferation of the CNS to be induced (Dahmane et al., 2001). We therefore tested the requirement for endogenous *Gli1* in mouse embryos in which ectopic *Gli1* expressed from the *Gli2* locus (in place of *Gli2*) activates the Shh pathway.

We have previously shown that when *Gli1* is expressed from the *Gli2* locus at normal physiological levels, *Gli1* can mediate Shh signaling in place of *Gli2*, and patterning of the neural tube is normal (Bai and Joyner, 2001). To test whether activation of the endogenous *Gli1* gene is required for normal patterning in these mice, we removed endogenous *Gli1* in these knock-in mice ($Gli2^{1ki/1ki}$) and analyzed patterning of the spinal cord. When *Gli1* replaces endogenous *Gli2* in $Gli2^{1ki/1ki}$ embryos, the floor plate defects in $Gli2^{zfd/zfd}$ embryo are rescued, and Shh, HNF3β, Nkx2-2 and Isl1/2 are expressed normally (compare Fig. 7A-D with 7E-H) (Bai and Joyner, 2001). If endogenous *Gli1* is required for this artificial Shh signaling, then removal of *Gli1* in these embryos should disrupt patterning of the ventral spinal cord. However, we found that this is not the case, because in $Gli1^{lz/lz};Gli2^{1ki/1ki}$ embryos, patterning of the ventral spinal cord remained intact, as indicated by the domains of Shh, Nkx2-2 and Isl1/2 expression (Fig. 7I-K). Overall DV patterning of the spinal cord also appears normal, as indicated by Pax6 expression (Fig. 7L).

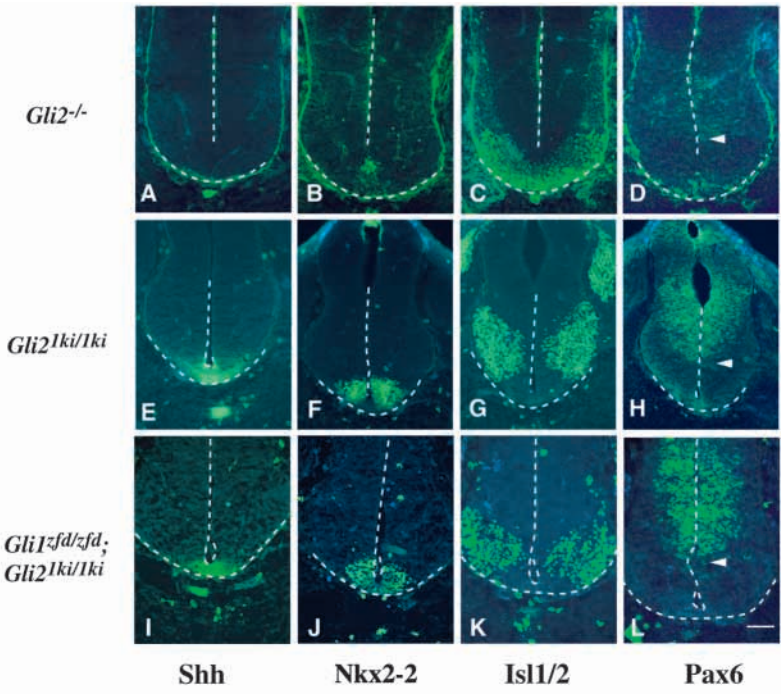

Fig. 7. Endogenous *Gli1* is not required when the Shh pathway is activated by ectopic *Gli1*. In *Gli2* mutant spinal cords (A-D), Shh expression cannot be detected, Nkx2-2 expression is greatly reduced in the ventral midline, and the expression domains of Isl1/2 and Pax6 are shifted ventrally. When *Gli1* is expressed from the endogenous *Gli2* locus in $Gli2^{1ki/1ki}$ embryos, the *Gli2* mutant defects are rescued and Shh signaling is restored (E-H). Removal of endogenous *Gli1* does not alter ventral spinal cord patterning in $Gli1^{lz/lz};Gli2^{1ki/1ki}$ embryos (I-L). Arrowhead indicates ventral limit of Pax6 expression domain. Scale bar: 50 μm.

DISCUSSION

Endogenous *Gli1* is not required for normal Shh signaling or development

In the present study, we undertook a number of genetic approaches to address the normal functions of *Gli1* in mouse development. First, we created a *lacZ* knock-in reporter allele of *Gli1* that removes all Gli1 protein, and definitively show that mice lacking *Gli1* function have a normal floorplate, are viable and show no obvious defects. We also compared the phenotype of the $Gli1^{lz}$ allele with our $Gli1^{zfd}$ allele that could be hypomorphic, in combination with a loss of one or two copies of *Gli2*. We found that these two alleles function indistinguishably and thus both act as null alleles. Second, we monitored the expression of the Shh target gene, *Ptc-lacZ*, in normal and *Gli1* mutant embryos, and found that *Gli1* is not required for Shh target gene expression. We further tested the requirement of endogenous *Gli1* in an artificial situation in which normal Shh signaling is transduced through *Gli1* instead of *Gli2*, and found that endogenous *Gli1* is not required for such Shh signaling. Together, our studies demonstrate that mouse *Gli1* is not required for embryonic development and normal Shh signaling, unless one copy of *Gli2* is defective.

Gli1 can not transduce the initial Shh signaling in tissues

Despite our finding that *Gli1* is not required in mouse development, Gli1 is capable of transducing Shh signaling. For example, Gli1 can activate a reporter construct containing tandem repeats of Gli-binding sites and an *Hnf3b* promoter in transfected cells (Dai et al., 1999; Sasaki et al., 1997). In addition, ectopic expression of *Gli1* in the CNS of transgenic mouse embryos, or RNA injected frog embryos also induces expression of Shh targets such as *Hnf3b* and *Ptc* (Hynes et al., 1997; Lee et al., 1997; Park et al., 2000). Finally, Gli1 is able to transduce Shh signaling when it is expressed in place of Gli2 from the *Gli2* locus (Bai and Joyner, 2001).

One possible reason why Gli1 is dispensable during mouse development is that *Gli1* is simply a target of the Shh signaling pathway and thus acts as a read-out of Shh signaling. Alternatively, it is possible that Gli1 normally transduces some Shh signaling, but in *Gli1* mutant embryos, this function is compensated for by other Gli genes. If Gli1 transduces Shh signaling, then Gli1 protein would need to be present when *Shh* is first expressed. We therefore examined the expression of *Gli1* in the absence of *Shh*. Of significance, no *Gli1* transcription was activated in the neural tube or limbs of E8.5 and E10.5 *Shh* mutant embryos. Thus, Gli1 is not normally present to transduce the initial Shh signaling. Two other *Gli* genes, *Gli2* and/or *Gli3*, could transduce the initial Shh

signaling, as both genes are expressed at the time *Shh* expression is initiated (Fig. 3I) (Hui et al., 1994). In support of Gli2 transducing the initial Shh signaling, we showed that transcription of the Shh target gene *Ptc* is greatly reduced in *Gli2* mutant embryos. Our finding that $Gli1^{lz/lz};Gli2^{zfd/+}$ mutant embryos have defects in Shh signaling (floorplate development), shows that Gli1 nevertheless normally contributes to propagation of Shh signaling once it is expressed.

Gli2 and not *Gli1* is required to transduce ectopic Shh signaling in *Ptc* mutants

Ectopic expression of *Gli1* can cause over proliferation of the dorsal neural tube and skin cancers. As many *Shh* target genes are activated in these situations, which resemble *Shh* gain-of-function phenotypes, we tested whether *Gli1* or *Gli2* contribute to ectopic *Shh* signaling in *Ptc* mutants. Since Ptc is a negative regulator of the Shh signaling pathway, loss of *Ptc* function results in ectopic activation of the Shh signaling pathway and transcriptional activation of *Gli1*.

We found that *Ptc;Gli1* double homozygous mutants have the same phenotype as *Ptc* mutants, showing that *Gli1* does not contribute a critical function to the ectopic Shh signaling in *Ptc* mutants. On the contrary, *Ptc;Gli2* double mutants showed a partial rescue phenotype when compared with *Ptc* mutants. The partial rescue phenotype included: (1) double mutant embryos survived a day longer than $Ptc^{-/-}$ embryos; (2) the floorplate marker Shh, which is expressed ectopically in the ventral spinal cord of *Ptc* mutants, was not expressed in double mutant embryos; and (3) the ventral interneuron marker Nkx2-2, which is expressed throughout the *Ptc* mutant spinal cord, is greatly reduced to a small patch in the ventral midline in double mutants. As *Ptc;Gli2* double mutant embryos have a ventral spinal cord phenotype similar to *Gli2* single mutants, this suggests that the function of the Shh signaling pathway in the ventral spinal cord of *Ptc* mutants is mediated primarily by *Gli2*. Given that the processing of Gli2 into an N-terminal repressor form is independent of Shh signaling, at least in fly embryos (Aza-Blanc et al., 2000), and that only the activator function of Gli2 is required during mouse embryonic development (Bai and Joyner, 2001), our studies provide further evidence that a primary function of Shh in development of the ventral spinal cord is to potentiate the activator function of Gli2.

Removal of *Gli2* in *Ptc* mutants does not completely rescue the *Ptc* mutant phenotypes. After an initial reduction of *Ptc-lacZ* expression in the brain and trunk mesoderm of six- to eight-somite embryos at E8.5 (Fig. 4F), more *Ptc-lacZ* expression is seen by the 10-12 somite stage (data not shown). By E10.5, *Ptc-lacZ* is expressed throughout the brain of double mutants (Fig. 5F). One possible explanation for this upregulation of *Ptc-lacZ* is the inhibition of the formation of a Gli3 repressor in *Ptc* mutant embryos. As one role of Shh signaling is to prevent the processing of Gli3 into a repressor form (Wang et al., 2000; Litingtung and Chiang, 2000), activation of the Shh pathway in *Ptc* mutants should result in loss of the Gli3 repressor. Indeed, the exencephaly phenotype of *Ptc;Gli2* double mutant embryos resembles the phenotype of *Gli3* mutants. In addition, contrary to our finding that some ventral cell types are not induced in *Ptc* mutants when *Gli2* is removed, there are more motoneurons in the double mutant spinal cords than in wild-type or *Gli2* mutants at E10.5. *Gli3* mutants have normal spinal cords, indicating that removal of the Gli3 repressor alone does not lead to an expansion of the motoneuron pool. It is possible, however, that the combination of loss of the Gli3 repressor and persistence of full-length Gli3 protein due to ectopic activation of the Shh pathway in *Ptc* mutants results in motoneurons developing at more dorsal levels than normal, in addition to the exencephaly phenotype. Our results therefore suggest that altered Gli3 activity, in addition to Gli2, contributes to the $Ptc^{-/-}$ mutant defects.

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REFERENCES

Auerbach, W., Dunmore, J. H., Fairchild-Huntress, V., Fang, Q., Auerbach, A. B., Huszar, D. and Joyner, A. L. (2000). Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. *Biotechniques* **29**, 1024-1032.

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

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

Bai, C. B. and Joyner, A. L. (2001). Gli1 can rescue the in vivo function of Gli2. *Development* **128**, 5161-5172.

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell- cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.

Buscher, D., Bosse, B., Heymer, J. and Ruther, U. (1997). Evidence for genetic control of Sonic hedgehog by Gli3 in mouse limb development. *Mech. Dev.* **62**, 175-182.

Dahmane, N., Lee, J., Robins, P., Heller, P. and Ruiz i Altaba, A. (1997). Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature* **389**, 876-881.

Dahmane, N., Sanchez, P., Gitton, Y., Palma, V., Sun, T., Beyna, M., Weiner, H. and Ruiz i Altaba, A. (2001). The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. *Development* **128**, 5201-5212.

Dai, P., Akimaru, H., Tanaka, Y., Maekawa, T., Nakafuku, M. and 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. and Hui, C. C. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. *Development* **125**, 2533-2543.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and 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. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Gailani, M. R., Bale, S. J., Leffell, D. J., DiGiovanna, J. J., Peck, G. L., Poliak, S., Drum, M. A., Pastakia, B., McBride, O. W., Kase, R. et al. (1992). Developmental defects in Gorlin syndrome related to a putative tumor suppressor gene on chromosome 9. *Cell* **69**, 111-117.

Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997).

Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.

Gorlin, R. J. (1987). Nevoid basal-cell carcinoma syndrome. *Medicine (Baltimore)* **66**, 98-113.

Grindley, J. C., Bellusci, S., Perkins, D. and Hogan, B. L. (1997). Evidence for the involvement of the Gli gene family in embryonic mouse lung development. *Dev. Biol.* **188**, 337-348.

Hui, C. C. and Joyner, A. L. (1993). A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat. Genet.* **3**, 241-246.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R. and 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.

Hynes, M., Stone, D. M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A. and Rosenthal, A. (1997). Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* **19**, 15-26.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

Kinzler, K. W., Bigner, S. H., Bigner, D. D., Trent, J. M., Law, M. L., O'Brien, S. J., Wong, A. J. and Vogelstein, B. (1987). Identification of an amplified, highly expressed gene in a human glioma. *Science* **236**, 70-73.

Lee, J., Platt, K. A., Censullo, P. and Ruiz i Altaba, A. (1997). Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. *Development* **124**, 2537-2552.

Levanat, S., Gorlin, R. J., Fallet, S., Johnson, D. R., Fantasia, J. E. and Bale, A. E. (1996). A two-hit model for developmental defects in Gorlin syndrome. *Nat. Genet.* **12**, 85-87.

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

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996). Biochemical evidence that patched is the Hedgehog receptor. *Nature* **384**, 176-179.

Masuya, H., Sagai, T., Wakana, S., Moriwaki, K. and Shiroishi, T. (1995). A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev.* **9**, 1645-1653.

Matise, M., Auerbach, W. and Joyner, A. L. (2000). Production of targeted embryonic stem cell clones. In *Gene Targeting: A Practical Approach* (ed. A. L. Joyner), pp. 101-132. New York: Oxford University Press.

Matise, M. P. and Joyner, A. L. (1999). Gli genes in development and cancer. *Oncogene* **18**, 7852-7859.

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

Methot, N. and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96**, 819-831.

Methot, N. and Basler, K. (2001). An absolute requirement for Cubitus interruptus in Hedgehog signaling. *Development* **128**, 733-742.

Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H., Chik, K. W., Shi, X. M., Tsui, L. C., Cheng, S. H. et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123.

Nilsson, M., Unden, A. B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P. G. and Toftgard, R. (2000). Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. *Proc. Natl. Acad. Sci. USA* **97**, 3438-3443.

Ohlmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* **396**, 749-753.

Papaioannou, V. and Johnson, R. (2000). Production of chimeras by blastocyst and morula injection of targeted ES cells. In *Gene Targeting: A Practical Approach*, (ed. A. L. Joyner), pp. 133-175. New York: Oxford University Press.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M. and Joyner, A. L. (2000). Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605.

Platt, K. A., Michaud, J. and Joyner, A. L. (1997). Expression of the mouse Gli and Ptc genes is adjacent to embryonic sources of hedgehog signals suggesting a conservation of pathways between flies and mice. *Mech. Dev.* **62**, 121-135.

Reifenberger, J., Wolter, M., Weber, R. G., Megahed, M., Ruzicka, T., Lichter, P. and Reifenberger, G. (1998). Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.* **58**, 1798-1803.

Ruiz i Altaba, A. (1997). Catching a Gli-mpse of Hedgehog. *Cell* **90**, 193-196.

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. and Vogelstein, B. (1988). The GLI-Kruppel family of human genes. *Mol. Cell. Biol.* **8**, 3104-3113.

Sasaki, H., Hui, C.-c., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3β 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. and 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.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H. et al. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**, 129-134.

Unden, A. B., Holmberg, E., Lundh-Rozell, B., Stahle-Backdahl, M., Zaphiropoulos, P. G., Toftgard, R. and Vorechovsky, I. (1996). Mutations in the human homologue of Drosophila patched (PTCH) in basal cell carcinomas and the Gorlin syndrome: different in vivo mechanisms of PTCH inactivation. *Cancer Res.* **56**, 4562-4565.

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