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# *Gli1* can rescue the in vivo function of *Gli2*

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## SUMMARY

**In mice, three Gli genes are thought to mediate sonic hedgehog (Shh) signaling collectively. Mis-expression studies and analysis of null mutants for each gene have indicated that the Gli proteins have different functions. In particular, Gli1 appears to be a constitutive activator, and Gli2 and Gli3 have repressor functions. To determine the precise functional differences between *Gli1* and *Gli2*, we have expressed *Gli1* in place of *Gli2* from the endogenous *Gli2* locus in mice. Strikingly, a low level of *Gli1* can rescue all the Shh signaling defects in *Gli2* mutants; however, only in the presence of a wild-type *Shh* gene. These studies demonstrate that only the activator function of Gli2 is actually required, and indicates that in specific situations, *Shh* can modulate the ability of Gli1 to activate target genes. Furthermore, expression of both copies of *Gli1* in place of *Gli2* does not disrupt spinal cord patterning, but does result in new gain-of-function defects that lead to lethality. We show that the defects are enhanced when *Gli3* function is reduced, demonstrating that an important difference between *Gli1* and *Gli2* is the ability of *Gli1* to antagonize *Gli3* function.**

Key words: Gli3, Neural patterning, Mouse, Mutants

## INTRODUCTION

Inductive signals play crucial roles in patterning and development of the central nervous system (CNS). One key signaling molecule, sonic hedgehog (Shh), is initially expressed in the axial mesoderm, including the notochord and prechordal plate that underlie the neural plate (Echelard et al., 1993). Shortly after the neural tube closes, Shh also is expressed in the floor plate, a structure that lies at the ventral midline of the spinal cord and much of the brain. Loss-of-function and gain-of-function studies have demonstrated that Shh is a signal that is both required to specify most ventral neuronal cell fates and sufficient to induce them (Chiang et al., 1996; Ericson et al., 1996; Pierani et al., 1999; Roelink et al., 1995). However, inappropriate activation of the Shh pathway has been implicated in tumors such as basal cell carcinoma and medulloblastoma (Goodrich et al., 1997; Grachtchouk et al., 2000; Nilsson et al., 2000; Oro et al., 1997; Reifenberger et al., 1998; Xie et al., 1998).

In vitro explant assays have shown that Shh is able to induce the formation of distinct ventral cell types in a concentration dependent manner; floor plate cells are induced at high concentrations, and motoneurons and three distinct classes of ventral interneurons (V1-V3) at progressively lower concentrations (Ericson et al., 1997). Recent evidence further suggests that this induction is achieved by Shh differentially regulating the expression of cell intrinsic determinants, such as induction of class I homeobox transcription factors and repression of class II transcription factors (Briscoe et al., 2000; Stone and Rosenthal, 2000). However, it is not clear at the molecular level how *Shh* functions to regulate differentially the expression of these transcription factors.

Shh belongs to the broader Hedgehog (Hh) family of signaling molecules. The molecular mechanisms of Hh signaling pathway have been worked out in greatest detail in *Drosophila* (Hammerschmidt et al., 1997; Ingham, 1998). At the end of the pathway is the zinc-finger-containing transcription factor Cubitus interruptus (Ci), which appears to be required for all aspects of Hh signaling in *Drosophila* (Methot and Basler, 2001). Hh signaling controls Ci protein activity at the post-translational level, such that in the absence of Hh signaling, Ci is processed into a truncated repressor form, $Ci^R$, which can inhibit some Hh target genes (Aza-Blanc et al., 1997; Robbins et al., 1997). In the presence of Hh signaling, the proteolytic processing of Ci is inhibited, and furthermore, Hh stimulates the maturation of Ci into a labile activator, $Ci^A$ (Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998). Loss of *hh* function results in all Ci being converted into $Ci^R$, thus resulting in a more severe phenotype in *hh* mutants than in *ci* mutants, which do not have $Ci^R$ (Methot and Basler, 2001).

Three homologs of *Ci*, *Gli1*, *Gli2* and *Gli*, have been identified in human and mouse (Hui et al., 1994; Ruppert et al., 1990). The Gli proteins share high homology in their five zinc-finger domains, but limited homology outside of this region (Matise and Joyner, 1999). Gain-of-function studies in

embryos have been used in an attempt to compare the properties of the three Gli genes in vivo. Ectopic expression of *Gli1*, but not *Gli2* or *Gli3*, can induce the expression of a floor plate marker, HNF3β (Foxa2 – Mouse Genome Informatics) in the dorsal CNS of mouse and frog embryos (Hynes et al., 1997; Lee et al., 1997; Marine et al., 1997; Park et al., 2000; Sasaki et al., 1999). When expressed in flies, Gli1 also functions as an activator in Hh signaling (Aza-Blanc et al., 2000; von Mering and Basler, 1999). When *Gli2* or *Gli3* are injected together with *Gli1*, they inhibit the ectopic activation function of Gli1 (Ruiz i Altaba, 1998). This may reflect that Gli2 and Gli3 have N-terminal repressor domains (Dai et al., 1999; Sasaki et al., 1999; Yoon et al., 1998). Gli2 has also been shown to function as a weak activator in transgenic flies, and in frog embryos, Gli2 can induce motoneuron development (Aza-Blanc et al., 2000; Ruiz i Altaba, 1998). Gli3, however, has been shown to function as a repressor in Hh signaling in fly and frog embryos (Aza-Blanc et al., 2000; Ruiz i Altaba, 1998; von Mering and Basler, 1999). Of the three Gli genes, only *Gli1* expression is dependent on and can be activated by Shh (Grindley et al., 1997; Hynes et al., 1995; Lee et al., 1997; Litingtung and Chiang, 2000; Marigo et al., 1996; Rowitch et al., 1999). Taken together, the mis-expression studies suggest that the three Gli proteins have primarily different functions.

Biochemical studies have shown that Gli3 can be processed like Ci into an N-terminal form, both in fly imaginal disc cells and mouse and chick limbs (Aza-Blanc et al., 2000; Wang et al., 2000). Furthermore, the proteolytic processing of Gli3 was found to be regulated by Hh signaling, such that in the presence of Hh signaling this processing is blocked (Aza-Blanc et al., 2000; Wang et al., 2000). Interestingly, even though Gli2 contains both activator and repressor domains, the processing of frog Gli2 in fly imaginal disc cells was found to be independent of Shh (Aza-Blanc et al., 2000), whereas when *Gli2* was over-expressed in COS cells, no processing was detected (Wang et al., 2000). By contrast, Gli1 does not appear to be processed in mouse extracts, or when ectopically expressed in COS7 cells or fly imaginal discs (Aza-Blanc et al., 2000; Dai et al., 1999; Park et al., 2000).

Null mutations in the three mouse Gli genes have been identified or generated. Surprisingly, mice that lack *Gli1* function are phenotypically normal (Park et al., 2000) (C. B. B. and A. L. J., unpublished). Mice with mutations in *Gli2*, however, die at birth and have defects in floor plate and V3 interneuron development, as well as many abnormalities in many other tissues including the skeleton and lungs (Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Motoyama et al., 1998; Park et al., 2000). The lack of floor plate in *Gli2* mutants indicates *Gli2* acts downstream of *Shh* to induce at least some ventral genes. By contrast, mice with a mutation in *Gli3* (extra toes, $Gli3^{Xtj}$) have dominant dorsal brain defects and polydactyly, but have a relatively normal spinal cord (Ding et al., 1998; Theil et al., 1999). As the *Gli3* mutant defects are similar to *Shh* gain-of-function defects, Gli3 may functions to repress Shh targets. Indeed, in *Shh;Gli3* double mutants, motoneurons develop in the spinal cord, indicating that a crucial function of Shh is to inhibit the repressor activity of Gli3, to allow motoneuron development (Litingtung and Chiang, 2000).

Interestingly, *Gli1;Gli2* double homozygous mutant mice have only slightly more severe CNS and lung phenotypes than either mutant alone, suggesting that *Gli1* and *Gli2* have few overlapping functions (Park et al., 2000). *Gli2;Gli3* mutant analysis revealed overlapping functions of the two genes in skeletal and lung development, probably reflecting similar repressor functions of the two proteins (Hardcastle et al., 1998; Mo et al., 1997; Motoyama et al., 1998). By contrast, *Gli1;Gli3* double mutants do not appear to have an enhanced *Gli3* phenotype (Park et al., 2000). Because of redundancy in some aspects of gene function and differences in the gene expression patterns of the three *Gli* genes, the mutant analysis in mice does not provide a direct in vivo comparison of the different *Gli* functions.

Gene mis-expression studies can provide a quick assay to analyze the potential function of a protein, but the results do not necessarily determine what the gene normally does during development. For example, ectopic expression of *Gli1* has been shown to be able to induce floor plate development, and yet *Gli1* is not required in vivo for floor plate development. Conversely, ectopic *Gli2* expression does not induce floor plate formation, but *Gli2* is required for floor plate development in the mouse CNS. Given the many essential roles the Shh pathway plays in mammalian development and disease, it is key to test directly in vivo whether *Gli1* and *Gli2* have different functions in transducing Shh signaling. We addressed this question directly by replacing the *Gli2*-coding sequences with those of *Gli1* using a gene targeting knock-in approach (Hanks et al., 1995) that ensures that *Gli1* is expressed in precisely the same way as *Gli2* in the right places and at the right times during embryonic development and postnatally. Given the different biochemical properties and genetic functions of Gli1 and Gli2, a prediction was that replacement of *Gli2* with *Gli1* would result in activation of *Shh* targets in *Gli2*-expressing cells, resulting in the formation of excess floor plate in the CNS. Strikingly, our studies show that a low level of *Gli1* can replace all *Gli2* functions and rescue the embryonic lethality of *Gli2* mutations. When both copies of *Gli2* are replaced with *Gli1*, however, lethality occurs due to new gain-of-function defects, despite CNS patterning being normal. We show that the defects are at least in part due to *Gli1* interfering with *Gli3* function. Finally, as Gli1 can act as a constitutive activator, we tested whether expression of *Gli1* in place of *Gli2* can rescue the *Shh* mutant phenotypes and found *Gli1* in this context was insufficient to rescue the ventral spinal cord.

## MATERIALS AND METHODS

### cDNA constructs

Mouse *Gli1* (Park et al., 2000) and *Gli2* (Hughes et al., 1997) cDNAs corresponding to the coding regions were tagged at their 5′ ends with a FLAG epitope (Sigma) by PCR and confirmed by sequencing. These FLAG tags could be detected in transfected cells but could not be detected by western blot or by immunostaining in mouse embryos containing the targeted alleles. The cDNA for nuclear localized β-galactosidase (*lacZ*) was excised from *pnLacF* (Mercer et al., 1991). The full-length mouse *Gli2* 3′ UTR was identified by 3′ RACE and subcloned into the 3′ end of the above cDNAs. To ensure proper processing, three tandem repeats of 250 bp of SV40 polyA signal were added to the 3′ end of all constructs.

### Generation of knock-in mice

Genomic DNA containing the first three exons of *Gli2* was isolated

from a 129SvEv genomic phage library (Stratagene). To make the targeting vectors, a 3.2 kb *Bam*HI fragment (from a 5′ *Bam*HI to 74 bp before the AUG) was used as a 5′ arm and a 6 kb *Cla*I fragment (from 39 bp after the ATG to a 3′ *Cla*I site) was used as a 3′ arm (Fig. 1A). *1ki*, *2ki* and *lzki* constructs (Fig. 1B) were then inserted into the targeting vector. The *neo* cassette is in the opposite orientation compared with the endogenous *Gli2* gene. W4 ES cells (Auerbach et al., 2000) were grown, electroporated and subjected to double selections as described (Matise et al., 2000). Targeted clones were identified by *Bam*HI restriction enzyme digestion and Southern blot analysis with a 3′ external probe, giving a wild-type band of >13 kb and a mutant band of about 11 kb (Fig. 1C). The same blot was then reprobed with a 5′ internal probe, giving a >13 kb wild-type band and 4.7 kb (*1ki*), 10.3 kb (*2ki*) and 3.2 kb (*lzki*) target locus bands. Three targeted cell lines for each vector were then injected into C57 BL/6J blastocysts (Papaioannou and Johnson, 2000). Chimeras were bred with Black Swiss Webster outbred mice and 129S6/SvEv inbred mice (Taconic) to establish $F_1$ heterozygotes. Two independent germline transmitting mouse lines were established for each knock-in construct. PCR analysis was used to routinely genotype knock-in animals or embryos (see Fig. 1 for primer locations). The primers used were: P1, ATGGAGACTTCTGCCCCAGCCCCTGCACTG; P2. ATAAACCC-AGCGTGCCTCCCAGATGACAGG; P3, AGACTGCCTTGGG-AAAAGCG; P4, GTCTTGCCTGAATAGACTGTGGGTGAATCC.

Breeding and genotyping of *Gli2*$^{zfd}$ and *Shh* mutants were as described (Chiang et al., 1996; Mo et al., 1997). *Gli3*$^{Xtj/+}$ mice were obtained from Jackson Laboratory and heterozygous mice were identified by their characteristic limb phenotypes (Hui and Joyner, 1993). Mice were kept and analyzed on an outbred Swiss Webster background.

### Immunohistochemistry and RNA *in situ* analysis

Embryos were fixed in 4% paraformaldehyde for 20 minutes at 4°C before embedding. Frozen sections were cut at 12 μm as described previously (Matise et al., 1998). Monoclonal antibodies (obtained from the Developmental Studies Hybridoma Bank, University of Iowa) for the following proteins were used: Shh, HNF3β, Nkx2.2, Isl1/2, Pax6, Pax7 (Ericson et al., 1996). Cy3-conjugated anti-mouse secondary antibody was obtained from Jackson ImmnunoResearch and used at 1:500. Images were captured via a Hamamatsu cooled CCD camera and colors were assigned.

Whole-mount RNA in situ hybridization and X-gal staining were performed as previously described (Matise et al., 1998). The in situ probes used were for *Gli1* and *Gli2* (Hui et al., 1994).

## RESULTS

### Generation of three lines of *Gli2* knock-in mice

A powerful approach to test whether two proteins have similar biological functions is to determine whether one protein can compensate for the lack of the other in vivo. In mice, this can be done by using the knock-in gene targeting technique that involves replacing the coding sequence of one gene with that of another (Hanks et al., 1995). To determine whether *Gli1* can compensate for a lack of *Gli2* function, 56 bp of *Gli2* exon 2, including the ATG, were replaced with a FLAG-tagged *Gli1* cDNA (Fig. 1A). To ensure proper translation and processing of the *Gli1* fusion transcript, *Gli2* 3′ UTR sequences and three copies of an SV40 polyadenylation signals were placed downstream of the *Gli1* cDNA-coding sequences. We have previously shown that this Gli1 protein construct is translated in COS7 cells like other Gli1 proteins (Hynes et al., 1997; Sasaki et al., 1999), and can induce HNF3β in dorsal midbrain cells in transgenic mice (Park et al., 2000). cDNAs encoding *lacZ* and a FLAG-tagged Gli2 protein were also targeted into the same site in the *Gli2* locus, using the same targeting strategy (Fig. 1B). The targeting vector used in the experiments contained a floxed neomycin-resistant gene (*neo*) downstream of the inserted cDNA for positive selection.

Following electroporation of each vector into W4 ES cells (Auerbach et al., 2000), Southern blot analysis using 5′ and 3′ probes identified correctly targeted clones (Fig. 1C and see Materials and Methods). At least two independent targeted alleles were transmitted through the germline for each allele following ES cell chimera formation (Papaioannou and Johnson, 2000). Since all the initial mice that were made had a *neo* cassette, they were designated with an n (for example *Gli2*$^{n1ki}$, for *Gli1* knock-in into *Gli2* with *neo*). The *neo* gene in the targeted alleles was then removed by crossing the mice to *NLS-CRE*-expressing transgenic mice. The resulting mice lacking the *neo* cassette were designated without an n (for example, *Gli2*$^{1ki}$).

### Homozygous *Gli2* knock-in mice (*Gli2*$^{2ki}$) are normal and *lacZ* knock-in mutants (*Gli2*$^{lzki}$) have defects similar to *Gli2*$^{zfd}$ mutants

It was expected that insertion of an exogenous cDNA into the second exon of the *Gli2* genomic locus would result in the cDNA being expressed precisely in the same manner as the endogenous *Gli2* gene. Furthermore, it was expected that the strong polyadenylation signal downstream of the inserted cDNA would terminate transcription after the inserted *Gli2* 3′ UTR. Insertion of a *lacZ* cDNA into the *Gli2* genomic locus should therefore generate a null *Gli2* allele, whereas inserting a *Gli2* cDNA should result in a normal allele.

*Gli2*$^{zfd}$ mutants, which lack the exons encoding the zinc-finger DNA-binding domains 3-5, have no floor plate, small lungs that lack the accessory lobe, a lack of notochord regression and skeletal abnormalities (Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Motoyama et al., 1998; Park et al., 2000). To address whether the new *Gli2*$^{lzki}$ allele produced the same phenotypes as the *Gli2*$^{zfd}$ allele, homozygous *Gli2*$^{lzki}$ embryos were examined and compared with *Gli2*$^{zfd}$ mutants. In wild-type embryos, Shh and HNF3β are both expressed in the notochord and floor plate. In the spinal cord of *Gli2*$^{lzki/lzki}$ E10.5 embryos, as in *Gli2*$^{zfd/zfd}$ embryos, no cells were present that expressed Shh and HNF3β (Fig. 2G,H) (Matise et al., 1998). In addition, the number of Nkx2.2 expressing V3 interneurons that are normally adjacent to the floor plate was greatly reduced in both *Gli2* mutants (Fig. 2I) (Matise et al., 1998). With the absence of floor plate cells and V3 interneurons, the Isl1/2-expressing motoneurons occupied the ventral midline of the two *Gli2* mutant spinal cords (Fig. 2J) (Matise et al., 1998). The expression of dorsal markers of the spinal cord, such as Pax6 and Pax7, however, were largely unaffected in both *Gli2* mutants (Fig. 2K,L) (Matise et al., 1998). Furthermore, the lungs of E12.5 *Gli2*$^{lzki/lzki}$ embryos, as in *Gli2*$^{zfd/zfd}$ embryos, were reduced in size and the accessory lobe was missing. In addition, the notochord was situated close to the spinal cord in both *Gli2* mutants (data not shown).

To further confirm that the *Gli2*$^{lzki}$ allele and the previously generated *Gli2*$^{zfd}$ allele have the same defects, compound *Gli2*$^{lzki/zfd}$ mutant embryos were generated and analyzed at E10.5 and E12.5. As expected, the floor plate, the lungs and the notochord had the same phenotype as either mutant alone

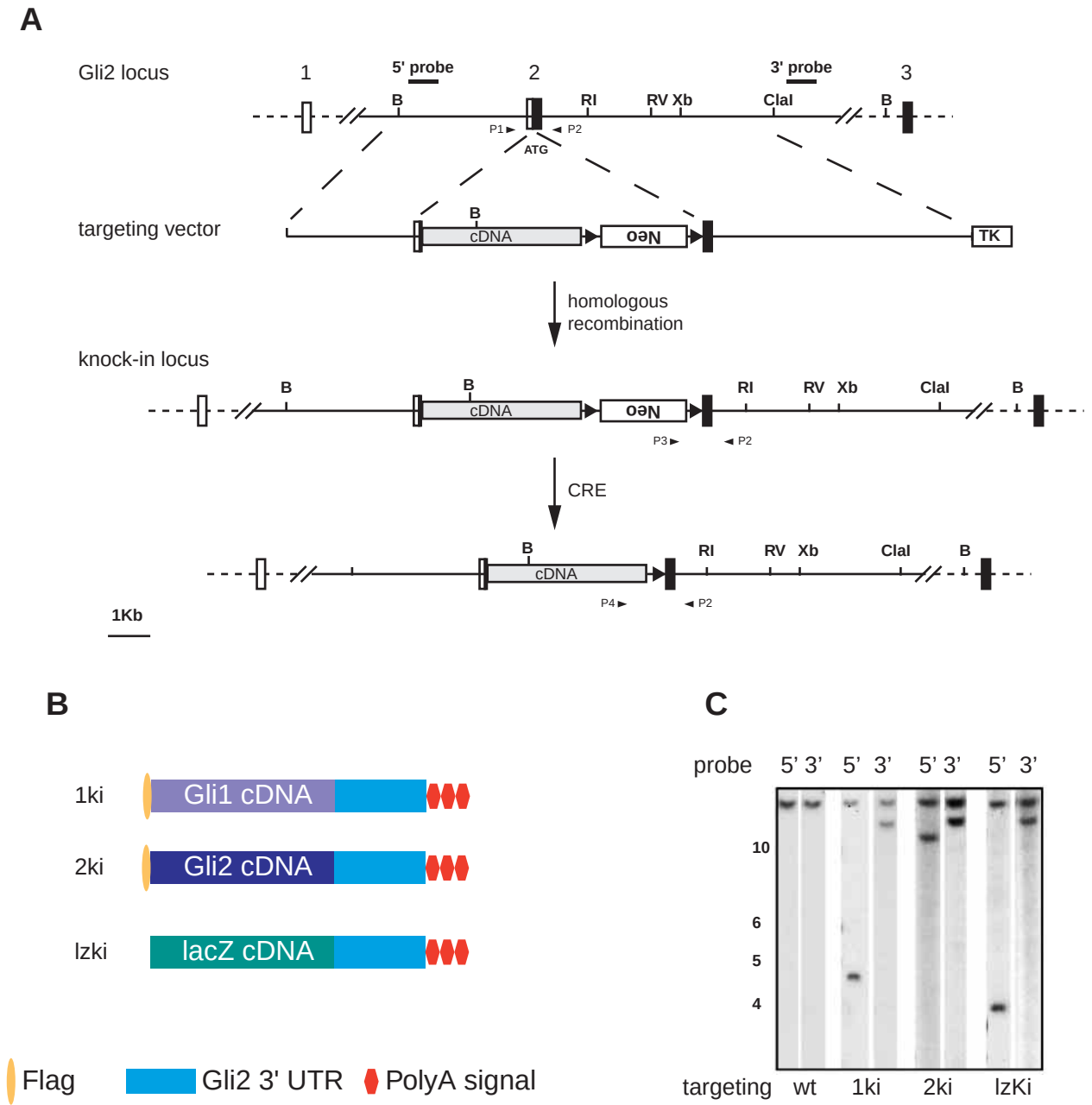


**Fig. 1.** Knock-in gene targeting strategy. (A) The *Gli2* locus, targeting vector and knock-in alleles (third and fourth diagrams) with and without *neo*. The first three exons of *Gli2* are shown as boxes, with white boxes representing untranslated exons and black boxes representing translated exons. The cDNA represents either the *1ki*, *2ki* or *lzki* (shown in B). CRE-mediated recombination was used to remove the *neo* cassette in the knock-in alleles in mice. P1-P4 represent primers for PCR genotyping. (B) Knock-in cDNA constructs. All cDNAs contain the *Gli2* 3′ UTR followed by three SV40 polyA signals. (C) Typical ES cell Southern blot analysis. Genomic DNA from ES cells was digested with Bam*HI* and probed with 5′ and 3′ probes. Different knock-in constructs gave different hybridization bands. Size in kb is shown on the left.

(Fig. 2M-R) (Matise et al., 1998; Park et al., 2000). These studies, together with the design of the $Gli2^{lzki}$ allele, suggest that both $Gli2^{zfd}$ and $Gli2^{lzki}$ represent null alleles.

Expression of *lacZ* from the *Gli2* allele in $Gli2^{nlzki}$ or $Gli2^{lzki}$ embryos exactly replicated the normal *Gli2* expression pattern (data not shown and see Fig. 4, Fig. 5). To further test whether an inserted cDNA was expressed like endogenous *Gli2* using our targeting strategy, homozygous $Gli2^{2ki}$ mice expression a *Gli2* cDNA from the *Gli2* allele were generated to determine whether they were normal. Three lines of germline transmitting mice were established and maintained on both inbred and outbred backgrounds. Intercrosses of heterozygous mice produced homozygous knock-in mice at a normal Mendelian frequency, and these animals appeared normal in all respects (Table 1). Taken together, these studies demonstrated a successful knock-in gene targeting strategy with *Gli2* function being abolished by insertion of a cDNA into exon 2 and expression of the cDNA precisely in a *Gli2*-like manner.

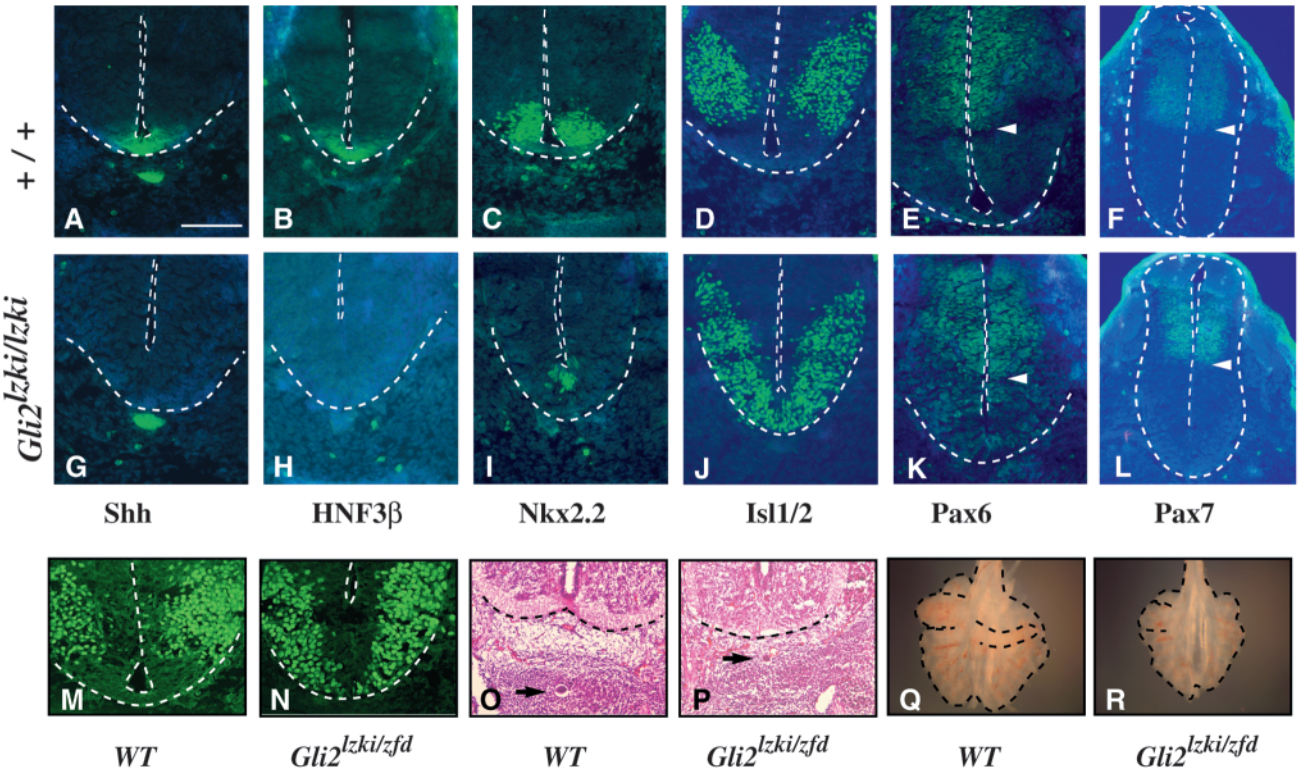


**Fig. 2.** Homozygous *Gli2*$^{lzki/lzki}$ embryos have defects in ventral spinal cord development at E10.5, similar to *Gli2*$^{zfd/zfd}$ mutants. In *Gli2*$^{lzki}$ mutant spinal cords (G-L), the floor plate cells that express Shh and HNF3β are lost (G,H). Nkx2.2-expressing V3 interneurons are greatly reduced in number(I). Loss of floor plate also results in a ventral expansion of Islet1/2- (J) and Pax6- (K) expressing cells. Pax7 expression is unaffected (L). White arrowhead represents ventral limit of Pax6 or Pax7 expression domain. (M,N) E10.5 spinal cord stained with an Isl1/2 antibody for motoneurons. The floor plate fails to develop in *Gli2*$^{lzki/zfd}$ embryos, and motoneurons occupy the ventral midline. (O,P) Hematoxylin and Eosin staining of E12.5 spinal cord. The notochord (indicated by black arrow) fails to regress in *Gli2*$^{lzki/zfd}$ embryos. (Q,R) E12.5 *Gli2*$^{lzki/zfd}$ embryos have smaller lungs without an accessory lobe. The margins of the spinal cord and spinal canal are outlined by broken white lines. The margin of lungs is outlined by broken black lines. Scale bar: 100 μm in A-E,D-K; 78 μm in F,L; 130 μm in M,N.

### *Gli1* can replace the embryonic requirement for *Gli2*

To test directly whether *Gli1* can substitute for *Gli2* function in vivo, the phenotypes of *Gli2* mice carrying the *Gli1* knock-in alleles were examined. As a first step to ensure that *Gli1* was expressed in place of *Gli2*, we examined the embryonic expression of *Gli1* by RNA in situ hybridization. At E10.5, *Gli1* is normally expressed in the ventral spinal cord only near the floor plate, and in the posterior limb bud mesenchyme

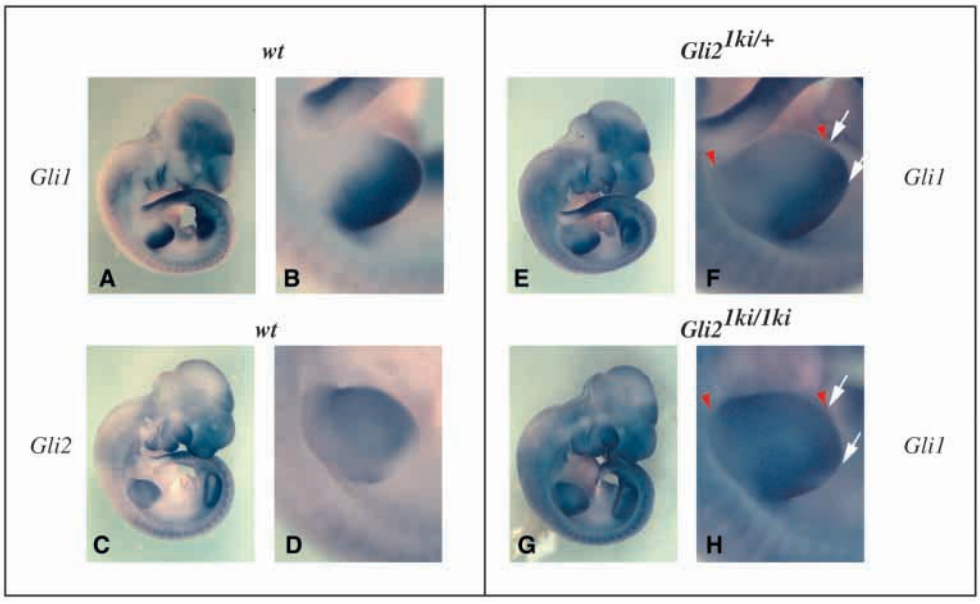


**Fig. 3.** *Gli1* is expressed in the *Gli2* domains from the knock-in allele. (A,C,E,G) Whole embryos and (B,D,F,H) high-power images of the limbs of the same embryos. (A,B) Wild-type E10.5 embryo stained with a *Gli1* probe. (C,D) Wild-type embryo stained with a *Gli2* probe. (E,F) *Gli2*$^{1ki/+}$ embryo stained with a *Gli1* probe. (G,H) *Gli2*$^{1ki/1ki}$ embryo stained with a *Gli1* probe. *Gli1* is expressed in the *Gli1* (white arrows in F,H) and *Gli2* (between red arrowheads in F,H) expression domains. Note the elevated *Gli1* expression in H compared with F.

**Table 1. Mouse breeding**

| Parental cross | Offspring genotype | | |
|---|---|---|---|
| | $Gli2^{ki/ki}$ | $Gli2^{ki/+}$ | $Gli2^{+/+}$ |
| $Gli2^{2ki/+} \times Gli2^{2ki/+}$ | 38 | 91 | 38 |
| $Gli2^{1ki/+} \times Gli2^{lzki/+}$ | 18‡ | 38† | 24 |
| $Gli2^{n1ki/+} \times Gli2^{n1ki/+}$ | 5 | 49 | 29 |
| $Gli2^{1ki/+} \times Gli2^{1ki/+}$ | 0 | 29 | 12 |

*ki represents either *1ki*, *n1ki*, *2ki* or *lzki* allele.
†$Gli2^{1ki/+}$ and $Gli2^{lzki/+}$.
‡$Gli2^{1ki/lzki}$.

surrounding the zone of polarizing activity (ZPA) (see Fig. 3A,B). By contrast, *Gli2* is expressed at E10.5 throughout the spinal cord, except for in the floor plate, and throughout the limb bud, except for in the ZPA (see Fig. 3C,D). In heterozygous *Gli1* knock-in embryos ($Gli2^{1ki/+}$), in addition to the normal *Gli1* expression domain in the posterior limb bud, ectopic weak *Gli1* expression was detected throughout the anterior limb bud in a similar pattern to that of *Gli2* (Fig. 3E,F). Expression of *Gli1* from the *Gli2* knock-in allele containing *neo* was lower (data not shown). Expression of *Gli1* in the *Gli2* domain in homozygous $Gli2^{1ki}$ embryos increased as expected, whereas the level of expression of endogenous *Gli1* in the posterior limb appeared unchanged (Fig. 3G,H). Furthermore, in sections of $Gli2^{1ki/lzki}$ skin, a *Gli2*-coding sequence cDNA probe did not detect any signal, whereas ectopic *Gli1* expression was detected in cells that normally express *Gli2* (C. B. B., Marc Fuccillo and A. L. J., unpublished).

To address whether one copy of *Gli1* can replace the function of *Gli2* during development, $Gli2^{n1ki/–}$ and $Gli2^{1ki/–}$ (where '–' indicates the null *lacZ* knock-in allele) embryos were generated. Whereas the floor plate is missing in E10.5 embryos lacking *Gli2* function, the floor plate appeared normal in *Gli2* knock-in embryos. Furthermore, Shh was expressed as in wild-type embryos in both the notochord and the floor plate of *Gli2* knock-in embryos (Fig. 4A and data not shown). The Isl1/2-expressing motoneurons and Nkx2.2-expressing interneurons also occupied the appropriate ventralateral area of the spinal cord in the $Gli2^{1ki/–}$ and $Gli2^{n1ki/+}$ mutant embryos instead of the midline area (Fig. 4B,C, and data not shown). In addition, the normal ventral domain of *Gli2* expression, as detected by X-gal staining for the $Gli2^{lzki}$ allele, was restored in $Gli2^{1ki/–}$ embryos, as indicated by an absence of blue staining in the floor plate of $Gli2^{1ki/–}$ embryos and staining throughout the ventral region in *Gli2* null mutants (Fig. 4D-F). As generation of the floor plate has been shown to depend on Shh signaling and *Gli2* function (Ding et al., 1998; Matise et al., 1998), the appearance of a floor plate in $Gli2^{1ki/–}$ embryos showed that aspects of Shh signaling had been restored in the spinal cord. Thus, one copy of *Gli1* in place of *Gli2* can replace Gli2 function.

To determine whether one copy of *Gli1* can rescue other defects caused by loss of *Gli2* function, we examined the lungs and notochord of $Gli2^{1ki/–}$ and $Gli2^{n1ki/–}$ embryos at E12.5. Indeed, the accessory lobe, which is absent in *Gli2* mutants, was restored in *Gli2* knock-in embryos, and the notochord regressed just as in wild-type embryos (data not shown). Furthermore, $Gli2^{n1ki/–}$ mice were born at a Mendelian frequency and appeared to be phenotypically normal throughout life (Table 1). $Gli2^{1ki/–}$ mice also appear

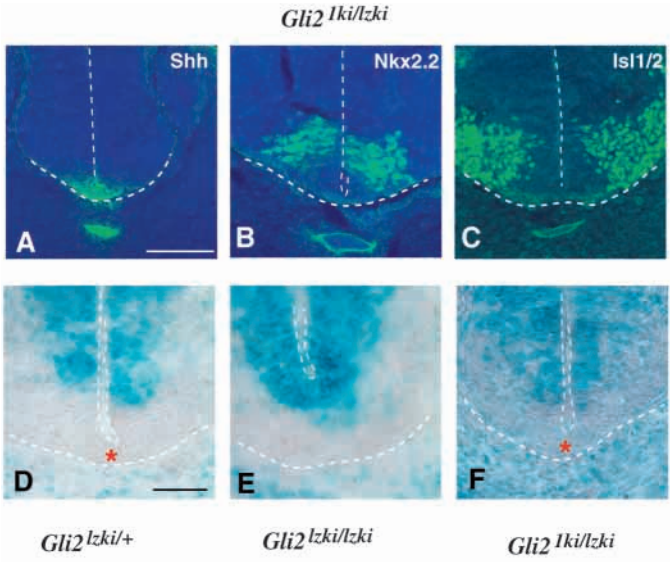


**Fig. 4.** Expression of one copy of *Gli1* can rescue the *Gli2* mutant floor plate defects. In *Gli2* mutant embryos, the floor plate cells are missing (Fig. 2G-L). At E10.5, ectopic expression of one copy of *Gli1* in the *Gli2* domain results in rescue of the ventral spinal cord defect, as assessed by immunohistochemical staining for Shh, Nkx2.2 and Islet1/2 (A-C) or by X-gal staining in $Gli2^{1ki/lzki}$ embryos (D-F). (A-C,F) $Gli2^{1ki/lzki}$ embryos, (D) $Gli2^{lzki/+}$ embryo and (E) $Gli2^{lzki/lzki}$ embryo. Red asterisk indicates floor plate that does not express *lacZ* from the *Gli2* allele. The margins of the spinal cord and spinal canal are outlined by broken lines. Scale bar: 100 μm.

phenotypically normal at weaning and were present at Mendelian ratio, but later develop a mild hair defect. As *Gli1* was only weakly expressed from the *Gli2* allele containing *neo*, these results show that a low level of *Gli1* is sufficient to compensate for all *Gli2* function during development and postnatally. Furthermore, as Gli1 is reported not to have repressor function (Sasaki et al., 1999), these results suggest that only the activator form of Gli2 is required during embryonic development.

As an additional test of whether any repressor form of Gli2 is required in Shh signaling, we examined whether removing *Gli2* rescues any of the *Shh* mutant defects. However, unlike $Gli3^{–/–};Shh^{–/–}$ mutants, $Gli2^{–/–};Shh^{–/–}$ E10.5 embryos ($n$=4) appeared similar to *Shh* mutant embryos, with cyclopia (Fig. 5C,G) and loss of ventral cell types including floor plate cells, interneurons and motoneurons in the spinal cord (Fig. 6C,G,K). Collectively, these results demonstrate that unlike Gli3, any Gli2 repressor form that is produced does not play a significant role in repressing *Shh* targets, at least in the presence of two normal *Gli3* alleles.

## Dorsoventral (D/V) pattering of the spinal cord appears normal in homozygous knock-in embryos expressing *Gli1* from the *Gli2* allele

In the developing vertebrate spinal cord, all dorsal progenitor cells express the homeobox transcription factors Pax3 and Pax7. In the ventral spinal cord, five distinct classes of progenitor cells express distinct combinations of transcription factors, probably as a response to different concentrations of Shh (Briscoe et al., 2000; Ericson et al., 1997). Furthermore, ectopic expression of human or mouse *Gli1* can induce *Shh*

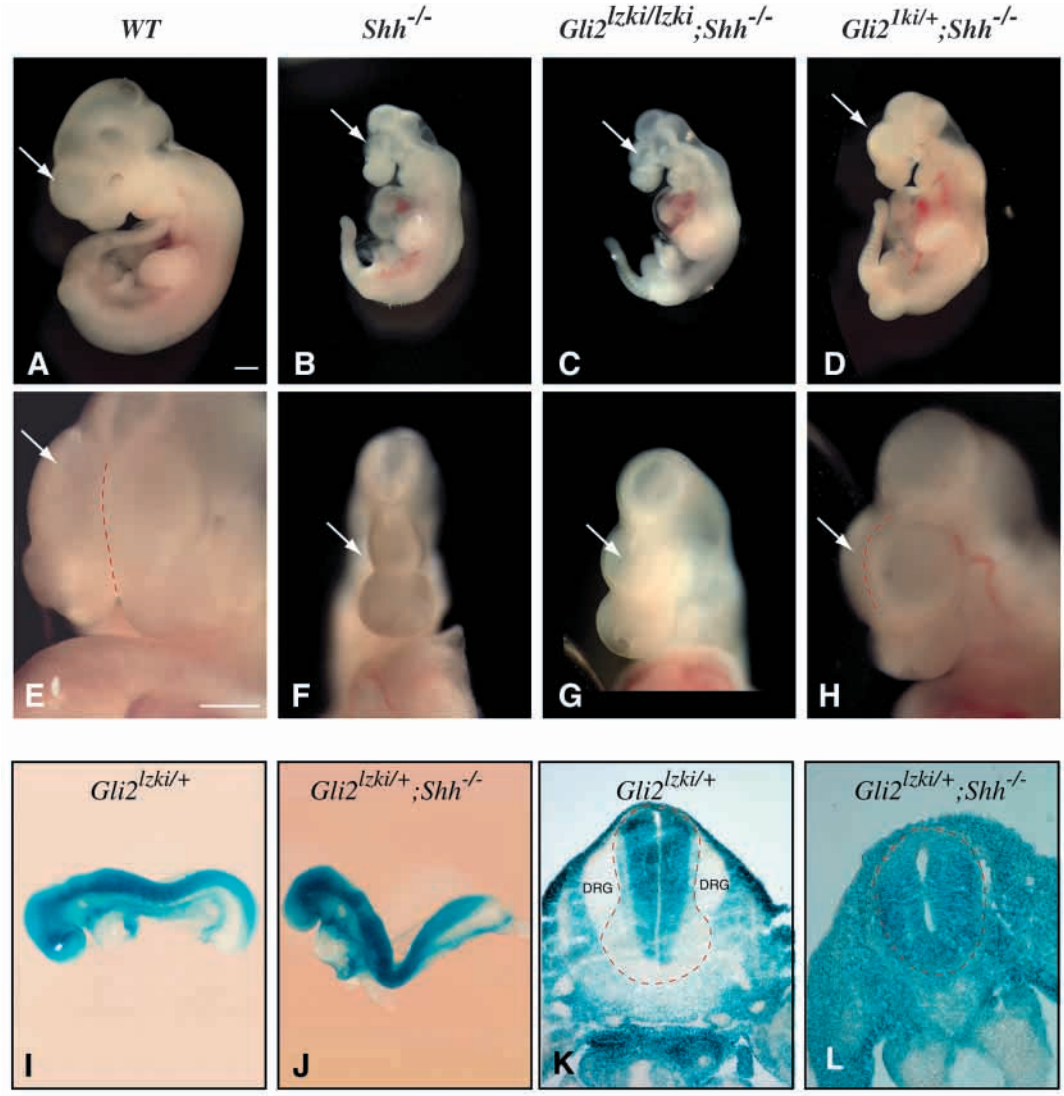


**Fig. 5.** Loss of *Gli2* does not rescue the *Shh* mutant defects and expression of *Gli1* from the *Gli2* allele can only partially rescue the *Shh* forebrain mutant defects at E10.5. (A,E) Wild-type embryos. (B,F) $Shh^{-/-}$ embryos. (C,G) $Gli2^{lzki/lzki};Shh^{-/-}$ embryos. (D,H) $Gli2^{1ki/+};Shh^{-/-}$ embryos. In $Shh^{-/-}$ embryos, the telencephalic vesicles are fused (arrows in B,F). In half the $Gli2^{1ki+/-};Shh^{-/-}$ embryos, the telencephalic vesicles are partially separated. The embryo in D represents the most fully rescued embryo. (E-H) Higher magnification views of the telencephalic regions of the same embryos as in (A-D), shown in frontal view. F is a frontal view, in order to visualize the lack of separation of the two vesicles, and the broken red lines in E,H indicate the midline. (I-L) *lacZ* is expressed in $Gli2^{lzki/+}$ and $Gli2^{lzki/+};Shh^{-/-}$ embryos at E8.5 (I,J) and sections at E10.5 (K,L). DRG, dorsal root ganglion. The margins of the spinal cord are outlined by broken red lines. Scale bar: 50 μm.

target genes in dorsal regions of the developing mouse brain (Hynes et al., 1997; Park et al., 2000; Sasaki et al., 1999). These facts prompted us to examine whether ectopic expression of *Gli1* throughout the spinal cord in homozygous knock-in embryos, which express a higher level of *Gli1* than in $Gli2^{1ki/-}$ embryos, has any effect on D/V patterning of the spinal cord.

Pax7, which is expressed by all dorsal progenitor cells in wild-type embryos, was still expressed only in the dorsal half of $Gli2^{1ki/1ki}$ embryos (Fig. 7L), suggesting that the generation of dorsal progenitor cells was not perturbed. The differentiation of the floor plate, ventral interneurons and motoneurons was then examined. In $Gli2^{1ki/1ki}$ embryos, expression of *Gli1* throughout the lateral and dorsal spinal cord did not result in excess floor plate differentiation, based on Shh and HNF3β expression (Fig. 7G,H). We further examined the Nkx2.2-expressing V3 interneurons and Isl1/2-expressing motoneurons, both of which lie dorsal to the floor plate cells and can be induced by low levels of Shh. Indeed, both V3 interneurons and motoneurons appeared to be generated correctly in $Gli2^{1ki/1ki}$ embryos (Fig. 7I,J). Taken together, these studies demonstrate that the expression of a higher level of *Gli1* throughout the spinal cord (except in the floor plate after E8.5, see Fig. 5K) at normal physiological levels for *Gli2* does not disturb the generation and patterning of lateral and dorsal neurons in any obvious manner. This suggests that the Shh signaling pathway was not ectopically activated and raised the possibility that Shh signaling might modulate the ability of Gli1 to function in the early ventral spinal cord.

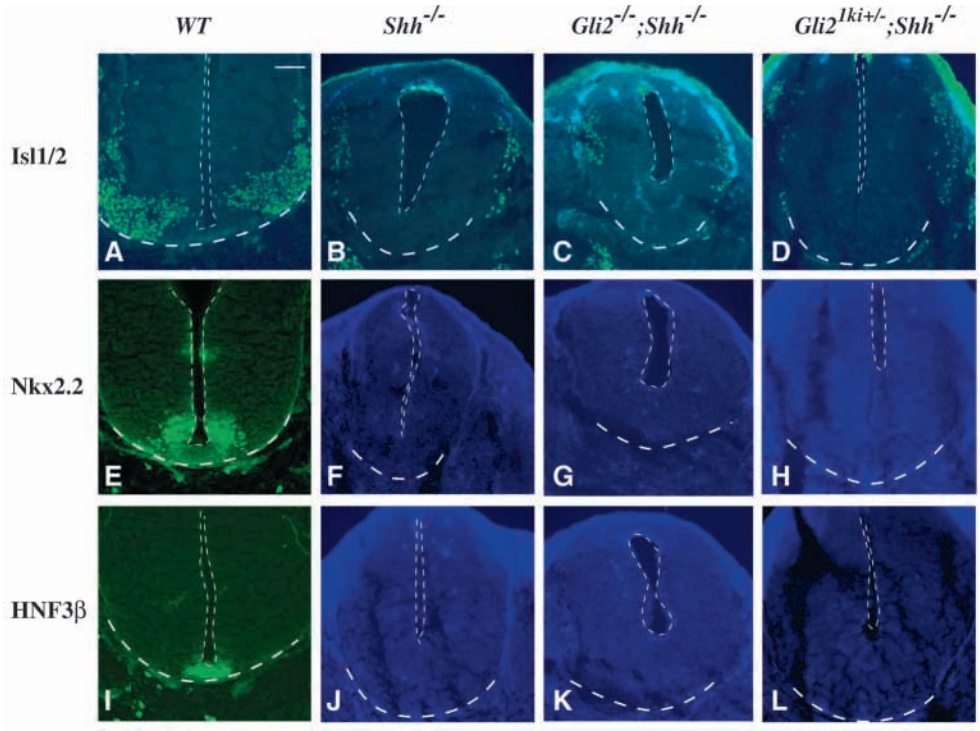


**Fig. 6.** Loss of *Gli2* does not rescue the *Shh* mutant defects and expression of *Gli1* from the *Gli2* allele in the neural plate does not rescue the ventral spinal cord defects in *Shh* mutant embryos. Antibody staining of E10.5 embryos for Isl1/2, HNF3β and Nkx2.2. In wild-type embryos, Nkx2.2-expressing interneurons and Isl1/2-expressing motoneurons are expressed in the ventralateral spinal cord (A,E), and HNF3β is expressed in the floor plate (I). These markers are not expressed in $Shh^{-/-}$ (B,F,J) or $Gli2^{-/-};Shh^{-/-}$ embryos (C,G,K). In addition, expression of one copy of *Gli1* in the place of *Gli2* in *Shh* mutant embryos does not rescue these ventral cell types (D,H,L). Scale bar: 50 μm.

## Expression of *Gli1* from the *Gli2* locus does not rescue the *Shh* mutant spinal cord defects

To test whether expression of *Gli1* from the *Gli2* locus requires *Shh* to induce a floor plate, as does *Gli2*, we examined spinal cord patterning in $Gli2^{1ki/+}$ mice that had a mutant *Shh* gene. If Gli1 has a constitutive activator function in vivo, then *Gli1* should be able to rescue the *Shh* mutant defects. We monitored the expression of *Gli2* in *Shh* mutants using the $Gli2^{lacZ}$ knock-in allele to ensure that *Gli1* would be expressed in the appropriate cells in the ventral neural plate of *Shh* mutants. *Gli2* expression normally starts at E7.5 in the embryonic ectoderm and mesoderm (Hui et al., 1994). By E8.5, *Gli2* expression can be seen in many ectoderm and mesoderm-derived tissues including the midline of the neural plate. At this stage, the *lacZ* domains in $Gli2^{lzki/+};Shh^{-/-}$ embryos were similar to those in $Gli2^{lzki/+};Shh^{+/-}$ embryos (Fig. 5I,J). By E10.5, $Gli2^{lzki/+};Shh^{-/-}$ embryos are much smaller than $Gli2^{lzki/+};Shh^{+/-}$ embryos, and have fused telencephalic vesicles (data not shown). Nevertheless *lacZ* was expressed in all tissues in *Shh* mutants. Furthermore, despite the loss of many ventral tissues in the spinal cord of $Gli2^{lzki/+};Shh^{-/-}$ mutants, *lacZ* was still expressed throughout the spinal cord (Fig. 5L).

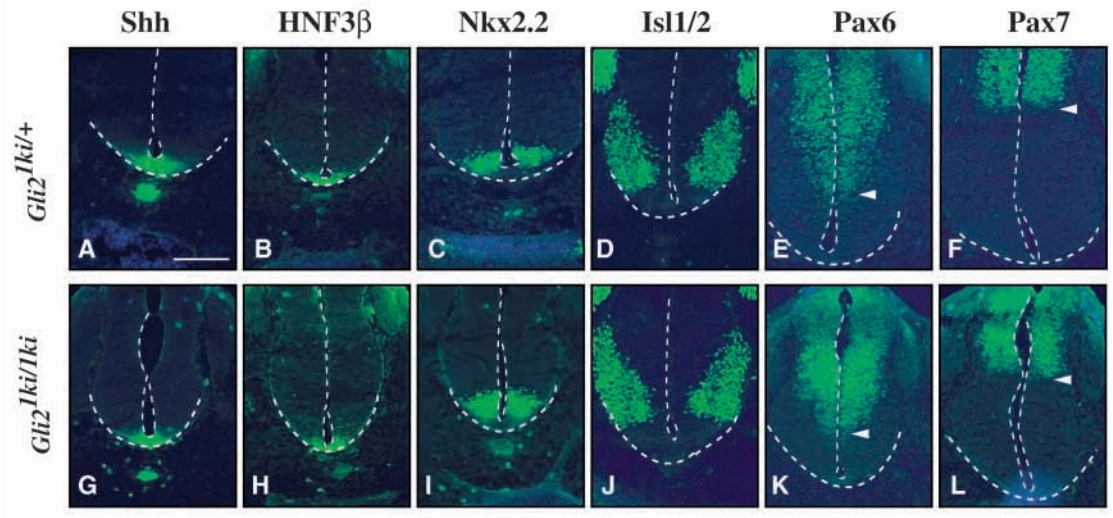


**Fig. 7.** Expression of two copies of *Gli1* from the *Gli2* allele does not result in spinal cord patterning defects. Immunoflurorescent staining of Shh, HNF3β, Nkx2.2, Isl1/2, Pax6 and Pax7 in E10.5 $Gli2^{1ki/+}$ embryos (A-F), or $Gli2^{1ki/1ki}$ embryos (G-L). The size of each mutant used for the analysis was slightly different. White arrowhead indicates the ventral limit of the Pax6 or Pax7 expression domains. Margins of the spinal cord and spinal canal are outlined by broken lines. Scale bar: 100 μm.

When one copy of *Gli1* was expressed from the *Gli2* allele in E10.5 or E11.5 *Shh* mutant embryos, the head of the embryos was found to be only slightly larger than in $Shh^{-/-}$ embryos (10/10). Moreover, in only 50% of the embryos (5/10) development of the diencephalic vesicles was only slightly rescued, based on the finding that grossly the diencephalic vesicles were partially separated into two vesicles (Fig. 5E-H). The generation of a floor plate and ventral neurons in the spinal cord was then examined in E10.5 $Gli2^{1ki/+};Shh^{-/-}$ embryos. Strikingly, no floor plate, V3 interneurons or motoneurons could be detected in $Gli2^{1ki/+};Shh^{-/-}$ embryos (Fig. 6). Similar results were obtained when a $Gli2^{1ki/1ki};Shh^{-/-}$ embryo was examined. These results demonstrated that when *Gli1* is expressed from the *Gli2* allele, a normal *Shh* gene is required for Gli1 to activate *Shh* target genes in the spinal cord.

### Homozygous $Gli2^{1ki}$ mice die due to gain-of-function effects

Although $Gli2^{1ki/-}$ animals are found at the expected Mendelian frequency and live to old age and breed, they were found to develop a skin defect after 3 months of age (data not shown, but see Fig. 8A). As $Gli2^{n1ki/-}$ animals that also lack *Gli2* function but express a lower level of *Gli1* in place of *Gli2* do not develop a hair defect, this suggested the phenotype is due to a gain-of-function effect of *Gli1* mis-expression. Consistent with this, $Gli2^{1ki/+}$ animals also were found to develop a similar phenotype to $Gli2^{1ki/-}$ animals, which included a gradual loss of hair starting on the dorsal neck and proceeding caudally down the back after the animals reached about three weeks of age (Fig. 8A). The hair loss got worse with time, and at about 5 months, some mice had no hair around the neck (data not shown). By contrast, heterozygous *Gli1* knock-in mice with a *neo* cassette present in the knock-in allele ($Gli2^{n1ki/+}$) did not develop a hair defect (Fig. 8B).

The finding that the hair defect developed in $Gli2^{1ki/+}$, and not $Gli2^{n1ki/+}$ adult mice suggested that an increase in the level of *Gli1* in place of *Gli2* leads to skin defects. To further test this, $Gli2^{n1ki/+}$ mice were intercrossed to obtain homozygous $Gli2^{n1ki/n1ki}$ mice. While some homozygous $Gli2^{n1ki/n1ki}$ mice were obtained at weaning age, they were present at a reduced frequency (Table 1). Furthermore, homozygous mice that survived to adulthood had several defects, the most obvious of which was extreme loss of hair. Shortly after weaning in homozygous mice, when the second cycle of hair growth normally begins, hair loss was seen on the dorsal neck area and in many cases the skin became exposed (Fig. 8B). Most of the $Gli2^{n1ki/n1ki}$ mice that survived to weaning died by 3 months of age (5/5). At this age, surviving mice were about 40% smaller than their wild-type littermates, and they had a stiffness and lack of coordination of their hindlimb. Furthermore, when $Gli2^{1ki/+}$ mice lacking *neo* were intercrossed, no homozygous $Gli2^{1ki/1ki}$ mice were found at weaning (Table 1), showing that the phenotype becomes worse and leads to lethality as the *Gli1* expression level increases to the normal level produced from two *Gli2* alleles.

### Gli1 antagonizes the normal repressor activity of Gli3

The gain-of-function defects seen in $Gli2^{1ki/+}$ mice could be due to activation of *Shh* target genes in inappropriate cells, and/or due to Gli1 protein interfering with the function of another protein, in particular Gli3. As *Gli3* is normally expressed at a distance to *Shh*-expressing cells and the processing of Gli3 into an N-terminal truncated form in mouse embryos is inhibited by *Shh* (Wang et al., 2000), Gli3 is likely normally primarily present in a repressor form. As Gli1 is not cleaved and does not have repressor function, co-expression of Gli1 and Gli3 in the same cells in $Gli2^{1ki/+}$ mice could lead to mutual antagonism.

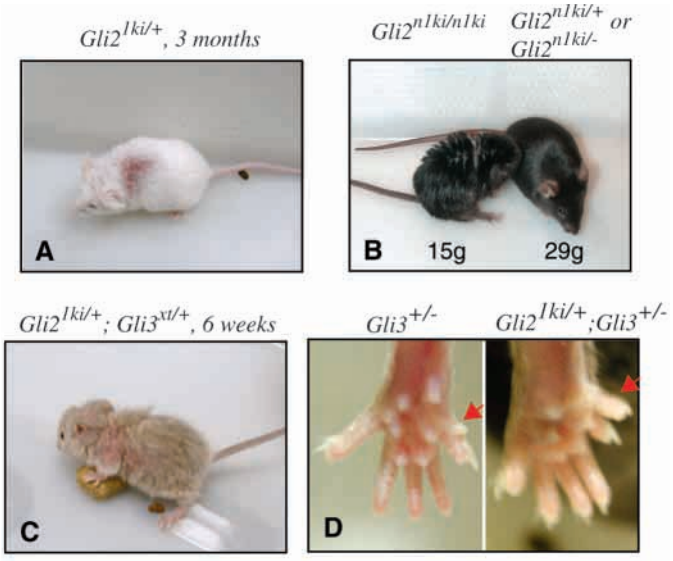


**Fig. 8.** *Gli2* knock-in mice expressing *Gli1* develop hair defects. (A) $Gli2^{1ki/+}$ mice develop hair defects at three months of age. (B) $Gli2^{n1ki/+}$ mice are normal, whereas $Gli2^{n1ki/n1ki}$ mice develop hair defects and are much smaller than $Gli2^{n1ki/+}$ littermates. Shown at 3 months of age, the $Gli2^{n1ki/n1ki}$ mouse weighed 15 g and the $Gli2^{n1ki/+}$ mouse weighed 29 g. (C) Removal of one copy of *Gli3* enhances the hair loss defect in $Gli2^{1ki/+};Gli3^{+/-}$ mice at 6 weeks of age. (D) Expression of one copy of *Gli1* from the *Gli2* allele enhances the polydactyly in $Gli2^{1ki/+};Gli3^{+/-}$ mice.

To test whether any part of the $Gli2^{1ki/+}$ gain-of-function phenotype is due to Gli1 interfering with Gli3 function, we compared the phenotype of $Gli3^{+/-}$ mice with that of $Gli2^{1ki/+};Gli3^{+/-}$ mice. The most obvious phenotype in $Gli3^{+/-}$ mice is mild postaxial polysyndactly of the forelimbs and preaxial polysyndactly of the hindlimb (Hui and Joyner, 1993). Strikingly, $Gli2^{1ki+/-};Gli3^{+/-}$ double heterozygous mice showed a severe hair loss that occurred throughout the body by six weeks of age (Fig. 8C), and the $Gli3^{+/-}$ polydactyly was enhanced (Fig. 8D). In addition, female double heterozygous mice died by six weeks of age ($n$=3), and all the male mice were sterile ($n$=5). As the enhanced phenotypes seen in $Gli2^{1ki/+};Gli3^{+/-}$ double heterozygotes are reminiscent of the gain-of-function phenotypes seen in homozygous $Gli2^{n1ki/n1ki}$ mice, these results provide genetic evidence that *Gli1* can antagonize the *Gli3* function.

## DISCUSSION

Based on the high degree of conservation of components of the Hh signaling pathway in different species, it is likely that collectively the Gli genes, like *Ci* in fly, are required to

transduce all Shh signaling. The situation in mice is more complicated, not only because there are three Gli genes, but also because these three Gli proteins also appear to have different biochemical properties and the genes are regulated at different levels. For example, at the transcriptional level, Shh activates transcription of *Gli1*, whereas it represses *Gli3* (Grindley et al., 1997; Hynes et al., 1997; Lee et al., 1997; Marigo et al., 1996). At the level of translational control at least, the human *GLI1* mRNA contains 3′UTR sequences that can inhibit translation (Jan et al., 1997). Finally, at the post-translational level, both Gli2 and Gli3, but not Gli1, can be processed into repressors (Aza-Blanc et al., 2000; Dai et al., 1999; Wang et al., 2000). A major challenge then, is to understand how each Gli protein participates in mediating Shh signaling.

### *Gli1* can replace all necessary *Gli2* functions

One of the key observations of this study is that *Gli1* can functionally substitute for *Gli2* in all processes. Unlike mis-expression studies, where it is difficult to control the timing, level and location of gene expression, we subjected *Gli1* to the same precise controls that regulate the expression of the endogenous *Gli2* gene and asked whether *Gli1* and *Gli2* have similar in vivo functions. To our surprise, we found that *Gli1* is able to rescue all the *Gli2* mutant phenotypes, including defects in floor plate, lung and notochord development. Furthermore, *Gli1* is able to rescue the embryonic lethality of *Gli2* mutants when expressed at low levels. These results are consistent with previous studies showing that Gli1 and Gli2 recognize a common set of target genes, including *HNF3β*, *Ptc* and *Gli1* itself. In cell culture, both Gli1 and Gli2 can activate transcription of reporter genes containing Gli-binding sites, and in transgenic mice, ectopic expression of *Gli1* or a form of *Gli2* with a deletion in the N terminus activates expression of the gene for HNF3β in the dorsal midbrain (Sasaki et al., 1999). In transgenic flies, it has also recently been shown that ectopic expression of both frog *Gli1* and *Gli2* can activate *dpp* and *ptc* expression (Aza-Blanc et al., 2000; von Mering and Basler, 1999). Interestingly, in each of these experiments, Gli1 and Gli2 activated the targets with different efficiencies.

Given that *Gli1* can replace *Gli2* in mice, it raises the question as to why endogenous *Gli1* does not compensate for a loss of *Gli2* in mutant mice. One likely reason is that transcription of *Gli1* is upregulated by Shh signaling (Grindley et al., 1997; Hynes et al., 1997; Lee et al., 1997; Marigo et al., 1996). If all *Gli1* transcription is actually dependent on Shh signaling, then in *Gli2* mutants, *Gli1* transcription would be decreased and unable to compensate for the lack of *Gli2*. Indeed, in *Gli2* mutant embryos, expression of *Gli1* is downregulated (Ding et al., 1998).

### When *Gli1* is expressed from the *Gli2* locus it does not induce *Shh* targets in the spinal cord in the absence of *Shh*

A surprising result of our studies was that *Gli1*, when expressed in the dorsal spinal cord from the *Gli2* locus, does not alter DV patterning or induce *Shh* targets in ectopic sites. Furthermore, when *Gli1* was expressed from one *Gli2* allele in *Shh* mutant embryos, a floor plate was not induced in the spinal cord. On the surface, the results of our studies appear different from previous transgenic studies in which a human *GLI1* cDNA or the same mouse *Gli1* cDNA as used in the present studies was found to induce *Shh* target genes when expressed in the dorsal midbrain, a region that lacks *Shh* expression (Hynes et al., 1997; Park et al., 2000; Sasaki et al., 1999). A likely explanation for the difference in results is that in the previous transgenic studies *Gli1* was expressed at higher levels. Our finding that ectopic expression of *Gli1* from the *Gli2* allele does cause dominant defects indicates that in some tissues the level of *Gli1* may be sufficient to activate target genes.

A possible reason why Shh is required for *Gli1* to induce a floor plate in our present study is that one function of *Shh* is to inhibit the processing of Gli3 into a repressor form in the ventral spinal cord (Litingtung and Chiang, 2000). This function may be required to allow Gli1 to activate appropriate targets when *Gli1* is expressed at low levels. An alternative, or additional reason why *Shh* could be required to induce a floor plate in $Gli2^{1ki/-}$ animals is that the Gli1 protein activator function is enhanced by Shh signaling and this enhancement is required when Gli1 is present at low levels. A recent study in transgenic flies supports this conclusion (Aza-Blanc et al., 2000). In anterior compartment cells that are far away from the AP border and do not receive Hh signaling, ectopic expression of frog *Gli1* was able to activate *dpp* and *ptc* transcription. However, in cells in the posterior compartment of the imaginal disc that receive Hh signaling, the ectopic frog Gli1 activity was enhanced.

### A Gli2 repressor form is not required during mouse development

Genetic analysis showed that $Gli2^{-/-};Gli3^{+/-}$ embryos have a more severe polydactyly phenotype than $Gli3^{+/-}$ mutants alone (Mo et al., 1997), suggesting that Gli2 and Gli3 have overlapping repressor functions. Indeed, transfection studies have shown that the N-terminal domain of Gli2 contains a repressor domain (Sasaki et al., 1999). In addition, when co-injected with *Gli1*, *Gli2* inhibits the activation function of *Gli1* (Ruiz i Altaba, 1998). Recent evidence in transgenic flies suggests that, unlike Ci in fly, some Gli2 protein is constitutively processed into a N-terminal repressor even in the presence of Hh (Aza-Blanc et al., 2000). By extrapolation, this would mean that in mouse embryos, in addition to full-length Gli2 being present, $Gli2^R$ should always be present. Given this, an important question was whether $Gli2^R$ plays a major role in development.

We addressed this question using two approaches. First, we asked whether removing $Gli2^R$ function alleviates the *Shh* mutant phenotypes, as does removal of *Gli3* (Litingtung and Chiang, 2000). If $Gli2^R$ plays a role in repressing inappropriate targets in the absence of Shh, then removing $Gli2^R$ should partially rescue the *Shh* mutant phenotypes. We found, however, that the phenotype of $Gli2^{-/-};Shh^{-/-}$ double mutant embryos was grossly indistinguishable from the $Shh^{-/-}$ phenotype. Thus, in spite of the fact that *Gl2;Gli3* double mutant analysis indicates that Gli2 does have a repressor function, our studies show that $Gli2^R$ does not in fact have a critical function, at least when *Gli3* is expressed normally. This finding may indicate that in mice, processing of Gli2 to a repressor form is indeed not regulated by Shh, whereas Gli3 is. We also took a more direct approach to the question by replacing *Gli2* with the simple activator *Gli1*, and analyzing the phenotype of embryos and mice on a *Gli2* mutant

background. Such embryos would not have the repressor function of Gli2 but would still have an activator function. We showed that *Gli2*$^{n1ki/-}$ and *Gli2*$^{1ki/-}$ mice are fully viable and develop a normal floor plate, notochord and lungs. Taken together, these two studies show that the repressor form of Gli2 is not required in mice. In this respect, it is interesting to note that Ci$^{R}$ is also dispensable during fly embryogenesis (Methot and Basler, 1999).

### Gli1 has a function not shared with Gli2

Our finding that *Gli1* causes new defects in a dose-dependent manner when expressed from the *Gli2* allele, shows that Gli1 possesses an activity different from Gli2. Our genetic studies demonstrate that the hair phenotype and other defects seen in adult *Gli2* knock-in mice are due to a gain-of-function effect of Gli1. First, *Gli2*$^{1ki/+}$ animals that retain normal *Gli2* function develop the same skin and hair defects as *Gli2*$^{1ki/-}$ animals. Second, *Gli2* homozygous mutant animals that express a low level of *Gli1* from the *neo* containing allele have no hair defects. Thus, *Gli1* can compensate for any function of *Gli2* in the skin and hair, only when *Gli1* is expressed at higher levels are defects seemed.

### Antagonism between Gli1 and Gli3

One direct mechanism by which Shh signaling inhibits Gli3 repressor activity is by inhibiting the proteolytic processing of Gli3 into Gli3$^{R}$ (Aza-Blanc et al., 2000; Wang et al., 2000). Another possible mechanism for antagonizing Gli3$^{R}$ would be for Shh to activate *Gli1* transcription, thus allowing Gli1 protein to compete with Gli3. Our data provide genetic evidence that the latter possibility occurs in vivo. Mice expressing one copy of *Gli1* from the *Gli2* allele (*Gli2*$^{1ki/+}$) have only a mild hair loss that becomes obvious around three months of age. However, if one copy of *Gli3* is removed from these mice (*Gli2*$^{1ki/+}$;*Gli3*$^{+/-}$), then the *Gli2*$^{1ki/+}$ hair phenotype becomes much more extreme. In addition, the *Gli3*$^{+/-}$ polydactyly becomes worse. As *Gli2*$^{+/-}$;*Gli3*$^{+/-}$ mice do not have an obvious hair phenotype or enhanced polydactyly, a plausible explanation is that Gli1 can antagonize Gli3 function when both proteins are expressed in the same cells in vivo. These data further demonstrate that Gli1 is not equivalent to Gli2, as *Gli2* and *Gli3* are expressed in the same cells and are not normally antagonistic.

Surprisingly, it appears that Gli1 can antagonize Gli3 function in some cell types, such as the skin, but not in others, such as cells in the dorsal spinal cord. It is possible that different tissues have different critical thresholds for Gli3$^{R}$ and/or that *Gli1* is ectopically expressed at different levels in the various tissues. Alternatively, Gli1 and Gli3 may not recognize all the same targets and the differences are specific to certain cell types.

A question arises as to whether such a mechanism functions in vivo during normal development. By E10.5 in mouse embryos, expression of *Gli1* and *Gli3* is largely non-overlapping, possibly because Shh signaling leads to repression of *Gli3* transcription, as well as activation of *Gli1*. Furthermore, if Gli1 normally antagonizes the repressor activity of Gli3, then it would be expected that *Gli1*$^{-/-}$;*Gli3*$^{+/-}$ double mutants would have a partially rescued *Gli3* phenotype. At least at a gross level, however, this is not the case (Park et al., 2000), suggesting that in normal development, transcriptional control mechanisms prevent *Gli1* and *Gli3* from being expressed in the same cells. Nevertheless it is possible that in some disease states, such as in individuals with Gorlin's that have heterozygous mutations in *PTC*, or in tumors that arise from mis-regulation of Shh signaling, that Gli1 and Gli3 are co-expressed and a mutual antagonism contributes to the phenotype.

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