Gli1 can rescue the in vivo function of Gli2

Chunyang Brian Bai1 and Alexandra L. Joyner1,2,*

1Howard Hughes Medical Institute and Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
2Department of Cell Biology and Physiology and Neuroscience, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA

*Author for correspondence (e-mail: joyner@saturn.med.nyu.edu)

Accepted 12 September 2001

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, CiR, 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, CiA (Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998). Loss of hh function results in all Ci being converted into CiR, thus resulting in a more severe phenotype in hh mutants than in ci mutants, which do not have CiR (Methot and Basler, 2001).

Three homologs of Ci, Gli1, Gli2 and Gli3, 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 Altata, 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 Altata, 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 Altata, 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, Gli3Xo) 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 function 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 Gli3 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 pmLacF (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 BamHI fragment (from a 5′ BamHI to 74 bp before the AUG) was used as a 5′ arm and a 6 kb ClaI fragment (from 39 bp after the ATG to a 3′ ClaI site) was used as a 3′ arm (Fig. 1A). 1ki, 2ki and 1zki 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 BamHI 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 (1zki) 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 129SvEv/SvEv inbred mice (Taconic) to establish F1 heterozygotes. Two independent germine 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, ATGGAGACTTCTGCCCCAGCGCTTCGACT; P2, ATAAAACCCGGGCGCTTCCCAGA TGACAGG; P3, AGACTGCCTTGGGGGGTGAGACTTCTGCCCCAGCCCCTGCACTG; P4, GTCTTGGCTGATAGACTGGTGGTTGAATCC.

Breeding and genotyping of Gli22d and Shh mutants were as described (Chiang et al., 1996; Mo et al., 1997). Gli3Xtj/+ 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 ImmunoResearch (Iowa) to establish F1 heterozygotes. Two independent germine 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, ATGGAGACTTCTGCCCCAGCGCTTCGACT; P2, ATAAAACCCGGGCGCTTCCCAGA TGACAGG; P3, AGACTGCCTTGGGGGGTGAGACTTCTGCCCCAGCCCCTGCACTG; P4, GTCTTGGCTGATAGACTGGTGGTTGAATCC.

Breeding and genotyping of Gli22d and Shh mutants were as described (Chiang et al., 1996; Mo et al., 1997). Gli3Xtj/+ 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.

**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 signal 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 Gli21ki, 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, Gli21ki).

**Homozygous Gli2 knock-in mice (Gli22ki) are normal and lacZ knock-in mutants (Gli22zki) have defects similar to Gli22d 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. Gli22d 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 Gli22zki allele produced the same phenotypes as the Gli22d allele, homozygous Gli22zki embryos were examined and compared with Gli22d mutants. In wild-type embryos, Shh and HNF3β are both expressed in the notochord and floor plate. In the spinal cord of Gli22zki/2zki, E10.5 embryos, as in Gli22d/d mice, 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 Gli22zki/zki embryos, as in Gli22d/d 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 Gli22zki allele and the previously generated Gli22d allele have the same defects, compound Gli22zki/di zki 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.
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 expressing 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. 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 BamHI and probed with 5' and 3' probes. Different knock-in constructs gave different hybridization bands. Size in kb is shown on the left.
Gli1 rescues Gli2 function

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 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.
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<sup>1ki/+</sup>), 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 <i>neo</i> was lower (data not shown). Expression of Gli1 in the Gli2 domain in homozygous Gli2<sup>1ki</sup> 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<sup>1ki/ki</sup> 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. Bai, Marc Fuccillo and A. L. J., unpublished).

To address whether one copy of Gli1 can replace the function of Gli2 during development, Gli2<sup>n1ki</sup>- and Gli2<sup>1ki</sup>- (where ‘<sup>n</sup>’ 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 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 ventral lateral area of the spinal cord in the Gli2<sup>1ki</sup> and Gli2<sup>n1ki</sup> 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<sup>2ki</sup> allele, was restored in Gli2<sup>1ki</sup>- embryos, as indicated by an absence of blue staining in the floor plate of Gli2<sup>1ki</sup> 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; Matise et al., 1998), the appearance of a floor plate in Gli2<sup>1ki</sup>- embryos revealed 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<sup>1ki</sup>- and Gli2<sup>n1ki</sup>- embryos at E12.5. Indeed, the accessory lobe, which is absent in Gli2 mutants, was restored in Gli2<sup>1ki</sup>- knock-in embryos, and the notochord regressed just as in wild-type embryos (data not shown). Furthermore, Gli2<sup>n1ki</sup>- mice were born at a Mendelian frequency and appeared to be phenotypically normal throughout life (Table 1). Gli2<sup>1ki</sup>- mice also appear 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 <i>neo</i>, 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<sup>−/−</sup>;Shh<sup>−/−</sup> mutants, Gli2<sup>−/−</sup>;Shh<sup>−/−</sup> 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) patterning 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
Gli1 rescues Gli2 function

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/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. 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 (IJ) and sections at E10.5 (KL). DRG, dorsal root ganglion. The margins of the spinal cord are outlined by broken red lines. 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-/- mutant mice. 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-/-;Shh-/- embryos were similar to those in Gli2-/-;Shh+/- embryos (Fig. 5I,J). By E10.5, Gli2-/-;Shh-/- embryos are much smaller than Gli2-/-;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-/-;Shh-/- mutants, lacZ was still expressed throughout the spinal cord (Fig. 5L).

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 ventral 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.

Fig. 7. Expression of two copies of Gli1 from the Gli2 allele does not result in spinal cord patterning defects. Immunofluorescent staining of Shh, HNF3β, Nkx2.2, Isl1/2, Pax6 and Pax7 in E10.5 Gli2-/-/+ embryos (A-F), or Gli2-/-/+ 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 Gli21ki/+;Shh–/– embryos. Strikingly, no floor plate, V3 interneurons or motoneurons could be detected in Gli21ki/+;Shh–/– embryos (Fig. 6). Similar results were obtained when a Gli21ki/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.

**Homzygous Gli21ki mice die due to gain-of-function effects**

Although Gli21ki–/– 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 Gli21ki–/– animals that also lack Gli1 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, Gli21ki/+ animals also were found to develop a similar phenotype to Gli21ki–/– 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 Gli21ki/+ mice are normal, whereas Gli21ki/1ki mice develop a skin defect after 3 months of age (data not shown). As Gli1 is normally present in a repressor form. As Gli1 is not expressed from the Gli2 allele, a normal Gli1 gene is required for Gli1 to activate Shh target genes in the spinal cord.

**Gli1 antagonizes the normal repressor activity of Gli3**

The gain-of-function defects seen in Gli21ki/+ 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 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 Gli21ki/+ mice could lead to mutual antagonism.

To test whether any part of the Gli21ki+ gain-of-function phenotype is due to Gli1 interfering with Gli3 function, we compared the phenotype of Gli31ki–/– mice with that of Gli2ki/+;Gli31ki–/– mice. The most obvious phenotype in Gli31ki–/– mice is mild postaxial polydactyly of the forelimbs and preaxial polydactyly of the hindlimb (Hui and Joyner, 1993). Strikingly, Gli2ki1ki/+;Gli31ki–/– double heterozygous mice showed a severe hair loss that occurred throughout the body by six weeks of age (Fig. 8C), and the Gli31ki–/– 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 Gli2ki1ki/+;Gli31ki–/– double heterozygotes are reminiscent of the gain-of-function phenotypes seen in homozygous Gli21ki1ki 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 misexpression 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 Gli2 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+/– 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 pte 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 1 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, Gli2R should always be present. Given this, an important question was whether Gli2R plays a major role in development.

We addressed this question using two approaches. First, we asked whether removing Gli2R function alleviates the Shh mutant phenotypes, as does removal of Gli3 (Litingtung and Chiang, 2000). If Gli2R plays a role in repressing inappropriate targets in the absence of Shh, then removing Gli2R 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 Gli2/Gli3 double mutant analyses indicate that Gli2 does have a repressor function, our studies show that Gli2R 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<sup>1ki/+</sup> and Gli2<sup>1ki/–</sup> 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 CíR is also dispensable during fly embryogenesis (Method 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<sup>1ki/+</sup> animals that retain normal Gli2 function develop the same skin and hair defects as Gli2<sup>1ki/–</sup> 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 Gli3 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<sup>R</sup> (Aza-Blanc et al., 2000; Wang et al., 2000). Another possible mechanism for antagonizing Gli3<sup>R</sup> 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<sup>1ki/+</sup>) 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<sup>1ki/+</sup>;Gli3<sup>+/–</sup>), then the Gli2<sup>1ki/+</sup> hair phenotype becomes much more severe. In addition, the Gli3<sup>1ki/+</sup> polydactyl becomes worse. As Gli2<sup>1ki/+</sup>;Gli3<sup>1ki/+</sup> 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<sup>R</sup> 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<sup>1ki/+</sup>-Gli3<sup>1ki/–</sup> 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.

We thank R. Hill for the Gli2<sup>cDNA</sup>, R. Palmiter for the nuclear lacZ cDNA, and D. Stephens, Q. Guo, M. Fuccillo and N. Shalts for technical assistance. We are grateful to W. Auerbach for advice on ES cell techniques and for providing the NLS-Cre mice, and to the NYU/SoM transgenic/ES cell chimera facilities for making the chimeric mice. We also thank Drs. C. Loomis, G. Fishell, A. Schier and M. Zervas for critical reading of the manuscript. The monoclonal antibodies used in this study were obtained from the Developmental Studies Hybridoma Bank. C. B. is supported by an NRSA postdoctoral fellowship (F32 HD08585). A. L. J. is an investigator of the Howard Hughes Medical Institute. This research is funded by a grant from NIH.

**REFERENCES**


