Mouse *Gli1* mutants are viable but have defects in SHH signaling in combination with a *Gli2* mutation

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SUMMARY

The secreted factor Sonic hedgehog (SHH) is both required for and sufficient to induce multiple developmental processes, including ventralization of the CNS, branching morphogenesis of the lungs and anteroposterior patterning of the limbs. Based on analogy to the *Drosophila* Hh pathway, the multiple GLI transcription factors in vertebrates are likely to both transduce SHH signaling and repress *Shh* transcription. In order to discriminate between overlapping versus unique requirements for the three *Gli* genes in mice, we have produced a *Gli1* mutant and analyzed the phenotypes of *Gli1/Gli2* and *Gli1/3* double mutants. *Gli3^{xt}* mutants have polydactyly and dorsal CNS defects associated with ectopic *Shh* expression, indicating GLI3 plays a role in repressing *Shh*. In contrast, *Gli2* mutants have five digits, but lack a floorplate, indicating that it is required to transduce SHH signaling in some tissues. Remarkably, mice homozygous for a *Gli1^{zfd}* mutation that deletes the exons encoding the DNA-binding domain are viable and appear normal. Transgenic mice expressing a GLI1 protein lacking the zinc fingers can not induce SHH targets in the dorsal brain, indicating that the *Gli1^{zfd}* allele contains a hypomorphic or null mutation. Interestingly, *Gli1^{zfd/zfd};Gli2^{zfd/+}*, but not *Gli1^{zfd/zfd};Gli3^{zfd/+}* double mutants have a severe phenotype; most *Gli1^{zfd/zfd};Gli2^{zfd/+}* mice die soon after birth and all have multiple defects including a variable loss of ventral spinal cord cells and smaller lungs that are similar to, but less extreme than, *Gli2^{zfd/zfd}* mutants. *Gli1/Gli2* double homozygous mutants have more extreme CNS and lung defects than *Gli1^{zfd/zfd};Gli2^{zfd/+}* mutants, however, in contrast to *Shh* mutants, ventrolateral neurons develop in the CNS and the limbs have 5 digits with an extra postaxial nubbin. These studies demonstrate that the zinc-finger DNA-binding domain of GLI1 protein is not required for SHH signaling in mouse. Furthermore, *Gli1* and *Gli2*, but not *Gli1* and *Gli3*, have extensive overlapping functions that are likely downstream of SHH signaling.

Key words: Mouse, *Gli1*, Sonic Hedgehog, Anteroposterior patterning, Polydactyly, CNS

INTRODUCTION

The developing embryo is dependent on numerous genetic interactions that specify cell fates within tissues and along the body axes. In recent years, a growing number of interesting mouse developmental mutants have been made by gene targeting and the genes responsible for some spontaneous developmental mutants have been cloned (Gossler and Balling, 1992; Melton, 1994; Roths et al., 1999). Phenotypic analysis of many of these mutants has been instrumental in identifying genes required for particular developmental processes. Studies of double mutants have become critical in order to decipher complex genetic pathways and uncover overlapping functions of gene family members. One very interesting genetic pathway conserved across species and required for many patterning

processes is the signaling cascade initiated by the Hedgehog (HH) family of secreted factors.

The *Drosophila hh* gene was the first family member identified, and was shown to be required for many developmental events, including anteroposterior patterning of the body segments and imaginal discs (Basler and Struhl, 1994; Mohler, 1988; Tabata et al., 1992; Tabata and Kornberg, 1994). Patched (Ptc) was identified as a novel transmembrane protein that is a Hh receptor and acts as a constitutive repressor of the Hh pathway in the absence of Hh protein (Ingham et al., 1991; Johnson et al., 1995). Genetic and biochemical studies in *Drosophila* have shown that *cubitus interruptus (ci)* encodes a zinc-finger-containing transcription factor that is required for transduction of Hh signaling (Alexandre et al., 1996; Dominguez et al., 1996; Orenic et al., 1990; Schwartz et al.,

1995). Furthermore, *ci* has been shown to interact genetically with *fused* (*fu*), which encodes a serine-threonine kinase, and *costal2* (*cos2*), which encodes a kinesin-like molecule (Forbes et al., 1993). Recently, Ci protein has been found to form a cytoplasmic complex, with Su(fu) and Cos2, that tethers Ci to the cytoskeleton (Monnier et al., 1998; Robbins et al., 1997; Sisson et al., 1997). Ci has both activator and repressor activities and the molecular basis for this involves post-translational modification of Ci (Aza-Blanc et al., 1997; Hepker et al., 1997; Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998). An N-terminal processed form of Ci is present in the nucleus of cells not receiving Hh signaling, and it acts as a repressor of *hh* and some *hh* target genes. In contrast, the full-length protein is present mostly in the cytoplasm of cells near sources of Hh and it acts as an activator of Hh targets, including *ptc* and *dpp*.

There are three *Hh* genes in mice, *Sonic hedgehog* (*Shh*), *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*) (Bitgood et al., 1996; Bitgood and McMahon, 1995; Echelard et al., 1993). Mutant studies in various vertebrates have defined some of the developmental processes dependent on SHH signaling. For example, during CNS development *Shh* is first expressed in the notochord and then in the floorplate and misexpression of SHH in the dorsal spinal cord induces floorplate genes (Echelard et al., 1993; Hynes and Porter, 1995; Lee et al., 1997). Likewise, *Shh* is expressed in the zone of polarizing activity (ZPA) in the posterior limb bud and can induce mirror-image duplications when expressed in the anterior limb (Liu et al., 1998; Riddle et al., 1993). *Shh* null mutants lack all ventral CNS cell types and anteroposterior patterning of the limbs (Chiang et al., 1996), demonstrating *Shh* is required for these two processes. In addition, branching of the lungs is greatly reduced in *Shh* mutants (Litingtung et al., 1998; Pepicelli et al., 1998) and there are abnormalities in the induction of epaxial muscles (Borycki et al., 1999) and hair follicle morphogenesis (Chiang et al., 1999; St-Jacques et al., 1998).

The vertebrate *ci*-related genes, *Gli1, Gli2* and *Gli3,* encode proteins with five tandem zinc-finger motifs that can bind DNA in a sequence-specific manner (Kinzler and Vogelstein, 1990; Pavletich and Pabo, 1993). Interestingly, these genes are expressed in embryonic regions that also express the three *Hh* genes with *Gli1* being restricted to cells flanking sources of *Hh* and *Gli2* and *Gli3* expressed at a progressive distance (Hui et al., 1994; Platt et al., 1997). In all tissues, a vertebrate homologue of *ptc*, *Ptc1*, is co-expressed with *Gli1* (Marigo et al., 1996b; Platt et al., 1997).

Unlike *ci* in *Drosophila*, the *Gli* genes are transcriptionally regulated by HH protein. Ectopic expression of SHH induces *Ptc* and *Gli1*, whereas it represses *Gli3* (Grindley et al., 1997; Hynes and Porter, 1995; Lee et al., 1997; Marigo et al., 1996a; Platt et al., 1997). Interestingly, GLI1, but not GLI3 or GLI2 can induce floorplate development in the dorsal CNS (Hynes et al., 1997; Lee et al., 1997; Marine et al., 1997; Ruiz i Altaba et al., 1995a). Recently *Xenopus Gli2* was shown to be induced by SHH in the dorsal CNS and to be capable of inducing motor neuron differentiation (Ruiz i Altaba, 1998). However, in mouse lungs, *Gli2* is not induced by SHH (Bellusci et al., 1997). Taken together, these results indicate that GLI1 and GLI2 could be involved downstream of SHH signaling in controlling patterning of the developing vertebrate embryo.

In order to determine the requirement for the various *Gli* genes in SHH signaling, we have produced mice with mutations in *Gli1* and *Gli2,* and identified XT^J mutants as having a deletion in *Gli3* (Hui and Joyner, 1993; Mo et al., 1997). *Gli3* mutant mice have many abnormalities including CNS and lung defects and limb polydactyly (Franz, 1994; Grindley et al., 1997; Grove et al., 1998; Hui and Joyner, 1993; Schimmang et al., 1992). Aspects of these phenotypes are similar to SHH gain-of-function effects and, indeed, *Shh* has been found to be expressed ectopically in the anterior limb buds and dorsal neural tube of $Gli3^{xtJ}$ mutants (Büscher et al., 1997; Ruiz i Altaba, 1998), indicating a role for GLI3 in repressing *Shh* expression. $Gli2^{zfd}$ mutants with a deletion of the exons encoding zinc fingers 3-5 have numerous skeletal defects (Mo et al., 1997) and abnormal lungs (Motoyama et al., 1998). Similar to *Shh* mutants, $Gli2^{zfd}$ mutants lack a floorplate and adjacent ventral intermediate region (VIR) cells in the spinal cord, providing direct evidence that *Gli2* functions downstream of *Shh* (Ding et al., 1998; Matise et al., 1998). In contrast to *Shh* mutants, however, $Gli2^{zfd}$ mutants develop motoneurons. *Gli2/Gli3* double mutants have skeletal and lung defects that are more extreme than either homozygous phenotype, showing that *Gli2* and *Gli3* have some overlapping functions (Mo et al., 1997; Motoyama et al., 1998).

Here we report the generation of mice with a $Gli1^{zfd}$ mutation that deletes the exons encoding the zinc fingers. In contrast to *Gli2* or *Gli3* mutants, mice homozygous for the $Gli1^{zfd}$ allele appear normal. Since in transgenic mice a mutant *Gli1* cDNA lacking the zinc fingers does not induce SHH targets, the $Gli1^{zfd}$ allele contains a loss-of-function mutation. A role for the GLI1 zinc-finger DNA-binding domain and, possibly, the complete GLI1 protein, in SHH signaling was demonstrated by generating *Gli1/Gli2* double mutants: all $Gli1^{zfd/zfd};Gli2^{zfd/+}$ double mutants have multiple defects that are similar to, but milder than, *Gli2* mutants and *Gli1/Gli2* double homozygous mutants have more extreme defects. In contrast, $Gli1^{zfd/zfd};Gli3^{xt/+}$ mutants are viable and appear identical to $Gli3^{xt/+}$ mutants. Most striking is our finding that mice carrying zinc-finger deletions in both *Gli1* or *Gli2* retain many processes requiring SHH signaling. Comparison of the *Gli1/Gli2* and *Shh* mutant phenotypes provides an assay for identifying those tissues in which SHH is dependent on normal GLI1 and GLI2 proteins. These results raise the question of whether GLI3 can play a critical role in transducing HH signaling, despite the lack of evidence for overlapping functions between *Gli1* and *Gli3* in double mutants. Alternatively, other proteins might act downstream of SHH. Recent biochemical studies have indicated that mouse GLI2 and GLI3 have activator and repressor activities, like Ci, whereas GLI1 only has activator activity and may not be processed (Dai et al., 1999; Sasaki et al., 1999). Our finding that mouse $Gli1^{zfd/zfd}$ mutants have normal HH signaling provides the first in vivo evidence that GLI1 functions differently from GLI2 and GLI3.

MATERIALS AND METHODS

Generation of *Gli1* mutant mice

RI ES cells (Nagy et al., 1993) were electroporated (Matise et al., 2000) with a targeting construct and following gancyclovir and G418 selection, three targeted colonies were obtained out of approximately

200 colonies screened. The *Gli1* mutant allele was identified by Southern blot hybridization using an 800 bp 5′ external *Xho*I-*Eco*RI probe (Fig. 1A) and restriction enzyme digestion of DNA with *Xho*I-*Spe*I giving a wild-type 13 kb and mutant 6 kb band (data not shown). Additional restriction enzyme digests were performed to confirm the structure of the mutant allele using two 3′ probes, a *Bam*HI-*Eco*RI genomic probe (a) and an external cDNA probe (see Fig. 1A). The targeted cell lines were used to generate chimeras by aggregation with diploid CD1 morulae (Wood et al., 1993) and one cell line was transmitted through the germ line. Chimeras were bred with CD1 outbred mice to obtain heterozygous mutant offspring. Homozygotes were generated by interbreeding heterozygotes, and then breeding homozygotes to either homozygous or heterozygous animals. To routinely genotype *Gli1* mutants, Southern blot analysis was performed using a 500 bp 3′ external *Nco*I fragment from the 3′ end of a *Gli1* cDNA (Hui et al., 1994) and restriction enzyme digestion of DNA with *Xba*I giving a wild-type 10.5 kb and mutant 4.5 kb band (Fig. 1B). The *Gli2* mutant allele was identified using PCR with *Gli2* sense and antisense primers and a primer to the *neo* (Mo et al., 1997). For genotyping embryos, genomic DNA was isolated from tails or yolk sacs using a PCR Template Purification Kit from Boehringer-Mannheim.

RT-PCR analysis

Poly(A)+ RNA was isolated as below. cDNAs were generated using a reverse transcription kit from BRL (Superscript II). 2 µl of the reverse transcription reaction were used as the template for PCR. The primers used to generate the 5′ end of mouse *Gli1* were: a 5′ primer based on human *GLI1* (CGA CCG AAG GTG CGT CTT GAG) (Kinzler et al., 1988) and a 3′ primer based on mouse zinc finger 3 sequences (AGC CCT GGA CCA CGC ATC) (Walterhouse et al., 1993). The PCR reaction was carried out as follows: 94°C for 1 minute, 56°C for 1 minute, 72°C for 2 minutes for 35 cycles. For analysis of the *Gli1* mutant allele, additional primers were generated flanking the deleted zinc-finger region: a 5′ sense probe in exon 6 (CAG CAG AGC CCC GGA GCG CA) and a 3′ antisense probe in exon 12 (AGC AGC ATG AGC TCT GCT TAC A). PCR products were purified from an agarose gel and cloned using the TA Cloning Kit (Invitrogen). These clones were then sequenced using M13 and T7 primers (ABI Sequencer). The major 5′ RT-PCR product from normal mRNA was 1.1 kb and sequence analysis confirmed that it contained the predicted cDNA. A minor 1.3 kb product was isolated that contained a 300 bp intron between exons 4 and 5. The two RT-PCR products both contained an additional 5′UTR exon (see Fig. 1A) that is not present in the one published human *GLI1* cDNA sequence (Walterhouse et al., 1993), although it is present in the human *GLI1* genomic locus in a similar position to mouse *Gli1* exon 2 (Liu et al., 1998).

Protein expression analysis

A full-length *Gli1* cDNA was generated by ligating 5′ and 3′ cDNA fragments using the common *Eco*RI site. It was tagged with FLAG at its N terminus by PCR and then confirmed by sequencing. A *Gli1* zinc-finger-deletion vector (*Gli1ᶻᶠᵈ*) was prepared by removing the cDNA fragment between the *Eco*RI and *Bam*HI sites, which deletes amino acids 272-423 that encodes zinc fingers 2-5 and leaves the reading frame intact. Both *FLAG-Gli1* and *FLAG-Gli1ᶻᶠᵈ* were subcloned into the pSRα vector (Takebe et al., 1988). Transfections of COS7 cells were performed using the Fugene 6 Transfection Reagent (Roche), as recommended by the manufacturer. 48 hours after transfection, cells were lysed. Cleared lysates were then separated on a 6% SDS-polyacrylamide gel. Western blot analysis was performed by using anti-FLAG M1 antibody (Sigma) and developed with ECL reagent (Amersham).

Generation of transgenic embryos

The *Wnt1-Gli1* and *Wnt1-Gliᶻᶠᵈ* transgenes were prepared by blunt end ligation of *FLAG-Gli1* and *FLAG-Gli1ᶻᶠᵈ* constructs into the *Eco*RV site of a modified Wexp3 vector (Epstein et al., 1996), which includes two copies of the chick β-globin insulator (kindly provided by D. Rowitch) and *Wnt1* regulatory sequences. Transgenic embryos were generated and collected at E14.5 as described (Hogan et al., 1994). PCR genotyping was performed using two primers specific for the *lacZ* tag (5′-CCG AAC CAT CCG CTG TGG TAC and 3′-CAT CCA CGC GCG CGT ACA TC). Expression of the transgene was monitored by whole-mount RNA in situ hybridization of tails with a *lacZ* probe.

Northern blot hybridization

Total RNA was isolated from whole 13.5 dpc wild-type (WT) and mutant embryos using Trizol reagent (BRL) and poly(A)+ RNA was purified using magnetic beads (Promega). Approximately 3 µg of RNA was loaded in each lane and run on a formaldehyde gel (Sambrook et al., 1989) and northern blot hybridization was carried out as described (Joyner et al., 1985). Probes used were: the 1.1 kb 5′ cDNA fragment produced by RT-PCR, a 500 bp *Nco*I 3′ cDNA fragment, a zinc-finger probe made using PCR from a *Gli1* cDNA using a 5′ primer in zinc finger 2 (ATA CAT GCT GGT GGT GCA CA) and a 3′ primer downstream of zinc finger 5 (GCA TCG GGA CCA TGC ACT GT), which was subcloned using the TA Cloning Kit (Invitrogen) and a 1 kb fragment of *neo*.

RNA in situ expression analysis

Whole-mount RNA in situ hybridization was performed as described (Parr et al., 1993) with modifications (Knecht and Harland, 1997). BM Purple was used as a substrate for alkaline-phosphatase-coupled anti-DIG antibodies (Boehringer-Mannheim). Lungs were dissected out and fixed overnight in 4% PFA at 4°C, as previously described (Grindley et al., 1997).

Fixed frozen sections were used for section in situ analysis as previously described for ³⁵S-labeled probes (Hui et al., 1994) with modifications as described (Toresson et al., 1999). The following DIG-labelled probes (Boehringer-Mannheim) were as previously published: *Gli1, Gli2, Gli3* (Hui et al., 1994), *Shh* (Echelard et al., 1993), *Ptc* (Goodrich et al., 1996) and *Nkx2.1* (Toresson et al., 1999). For immunohistochemistry, embryos were fixed for 1 hour at 4°C and washed in PBS overnight. Frozen sections were prepared and secondary Cy3- and fluorescein-coupled anti-mouse and anti-rabbit antibodies used were as described (Matise et al., 1998). Primary antibodies used were Islet1/2 (Ericson et al., 1992) Nkx2.2 and Hnf3β (Ruiz i Altaba et al., 1995b). Hoechst staining was performed as described (Matise et al., 1999).

Histology

Adult mice were perfused with 10% formalin and then stored in fix. Fixed tissue was paraffin embedded and then stained with Hematoxylin for 5 minutes and Eosin for 2 minutes. Slides were dehydrated in xylene and coverslipped with Permount.

Lung measurement

The individual lobes of E18.5 *Gli1ᶻᶠᵈ/ᶻᶠᵈ;Gli2ᶻᶠᵈ/+* lungs were separated and measured in millimeters (Fig. 3A). The variances of *Gli1ᶻᶠᵈ/ᶻᶠᵈ;Gli2ᶻᶠᵈ/+* lung measurements were compared to *Gli1ᶻᶠᵈ/+;Gli2ᶻᶠᵈ/+* lungs (WT) using the *f*-test and a significant difference was observed only in the length of the accessory lobe, suggesting a variability in the mutant phenotype. Using the *t*-test for unequal variances, the length of the accessory lobe was significantly different from WT ($P=0.01$). For all other measurements, variances were considered equal and the *t*-test revealed a significant difference in length of the right cranial lobe ($P=0.02$), the width of the accessory lobe ($P=0.013$) and the width of the left lobe ($P=0.03$) in eight embryos. At this stage, the size of the whole embryo did not appear significantly reduced.

RESULTS

Gli1^{zfd/zfd} mutants are viable and appear normal

In order to address what developmental processes are dependent on *Gli1*, homologous recombination in embryonic stem cells was used to disrupt *Gli1*. A gene targeting vector was used to replace exons 9 to 11 that encode part of zinc finger 1, zinc fingers 2-5 and 43 adjacent amino acids with the bacterial *neomycin* resistance (*neo*) gene (Fig. 1A and see Materials and Methods). The mutant **z**inc **f**inger **d**eletion allele (*Gli1^{zfd}*) was transmitted through the germline, heterozygous mice intercrossed and adults of all three genotypes (+/+; +/−; −/−) were detected (Fig. 1B) at the expected Mendelian ratio (20:51:24). Southern blot analysis of DNA from homozygous mutants using a zinc-finger-specific probe confirmed the deletion (Fig. 1C). *Gli1^{zfd}* mutant mice can breed and live past 1 year of age and show no obvious behavioral abnormalities.

The *Gli1^{zfd}* allele expresses an abnormal transcript without a DNA-binding domain that cannot activate SHH targets in the brain.

In order to determine whether the *Gli1^{zfd}* allele

transcribes a mRNA that could encode an aberrant GLI1 protein, we characterized the *Gli1* mutant transcripts. Northern blot analysis of mRNA from E13.5 WT and *Gli1^{zfd}* homozygous embryos using a *Gli1* cDNA probe 3′ to the zinc fingers detected two *Gli1* transcripts of approximately 4.0 and 4.4 kb in WT mRNA (Hui et al., 1994; Fig. 1D) and one broad
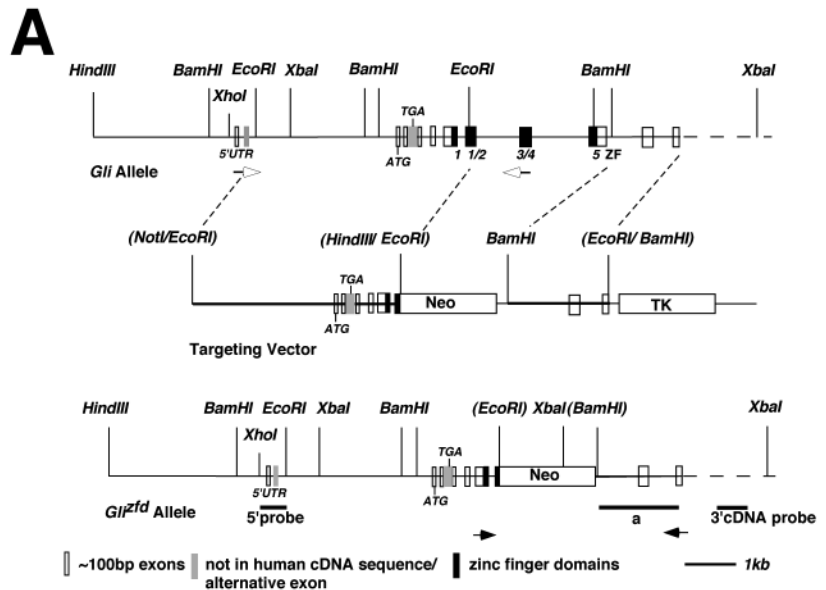
Fig. 1. Generation of *Gli1* zinc-finger-deletion mice by homologous recombination. (A) Schematic representation of the normal (top) and targeted (bottom) *Gli^{zfd}* locus highlighting the exons containing the conserved zinc-finger region (black and numbered 1-5ZF) and the exons not present in the human GLI1 cDNA (shaded), one being an alternatively spliced exon with a stop codon (TGA). In the targeting vector (middle), zinc fingers 2-5 were replaced with the *neo* gene. Probes used for screening and genotyping are indicated under the targeted allele. The position of the primers used to isolate the 5′ *Gli1* cDNA are indicated by open arrowheads and the aberrant *Gli1* transcript in mutant embryos are indicated by black arrows. (B) Genotyping of *Gli* mutants using Southern blot analysis of *Xba*I-digested tail DNA from WT, *Gli1* heterozygous and homozygous mutant animals using a 3′ cDNA probe b that detects a 10.5 kb WT and 4.5 kb mutant band. (C) A 300 bp zinc-finger probe was used in a Southern blot analysis to confirm the targeted deletion of the zinc-finger-coding exons in tail DNA from homozygous mutants compared to WT DNA digested with *Bam*HI (B), *Eco*RI (E), and *Xba*I (X). The arrows indicate the *Gli1* bands since the zinc-finger probe also likely binds to other *Gli* genes. (D) 3 μg of poly(A)+ whole embryo RNA was run on a formaldehyde gel for northern blot analysis. A *neo* probe does not detect any transcripts in WT RNA, but detects the expected 1.8 kb transcript expressed from the *PGK* promoter in the *neo* cassette in *Gli1* homozygous RNA. A 3′ cDNA probe, detects 4.4 and 4.0 kb transcripts in WT RNA, and a 3.8 kb transcript in mutant RNA. A 5′ cDNA probe detects only the larger transcript in WT RNA, and smaller aberrant transcripts in mutant RNA.
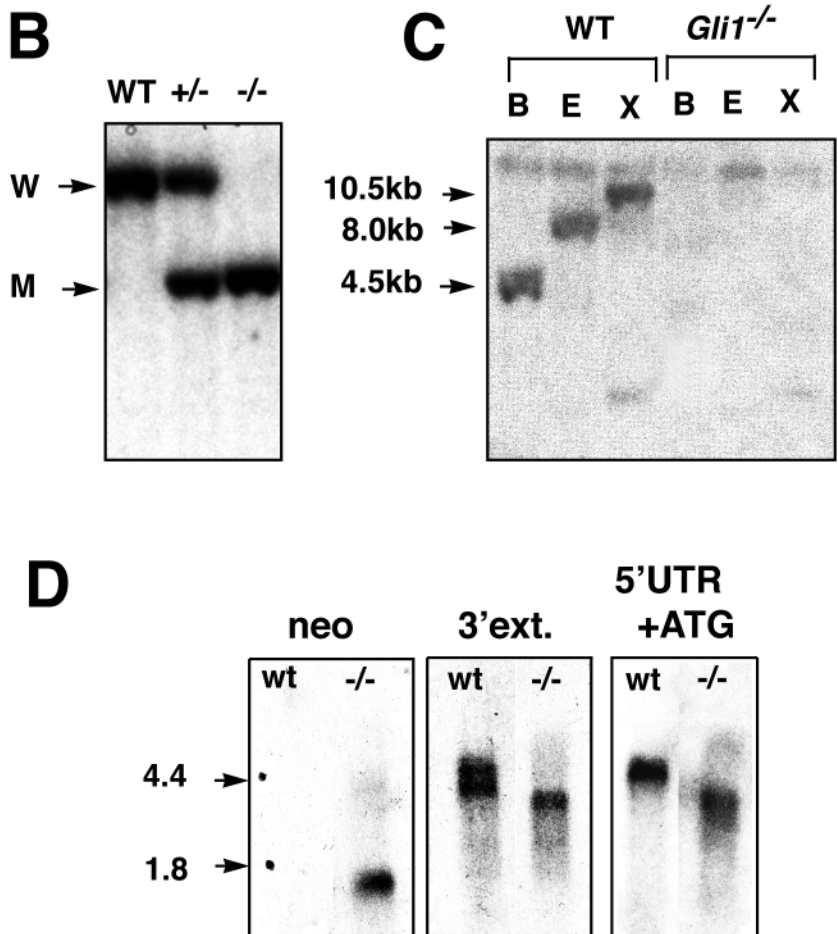
band that was slightly smaller in mutant mRNA (Fig. 1D). A 5′ probe or a zinc-finger probe only detected the 4.4 kb band in WT mRNA (Fig. 1D), suggesting the 4.0 kb transcript has an alternative 5′ end. As expected, the mutant ~4 kb band was not detected with the zinc-finger cDNA probe (data not shown) or with a *neo* probe, but a broad band of approximately 4 kb was detected with the 5′ probe (Fig. 1D).

If the *Gli1^{zfd}* transcript spliced around the *neo* insertion in the mutant locus from exon 7 to exon 11 this would generate a ~4 kb mRNA encoding an in-frame deletion of 182 amino acids, which would include most of the zinc fingers as well as 43 adjacent amino acids. To determine whether such a transcript was made, RT-PCR was performed with E13.5 *Gli1* mutant mRNA using a 5′ primer located in exon 6 and a 3′ primer in exon 12, 3′ of the deleted region (see open arrows in Fig. 1A). Sequence analysis of the ~0.5 kb RT-PCR product showed that a transcript was made that spliced around the *neo* insert from exon 8 into exon 12 (see Fig. 1A). Thus, the *Gli1^{zfd}* allele cannot encode for a protein with DNA-binding activity, but could produce a mutant protein lacking amino acids 269-437 that include most of the zinc fingers.

To test whether a GLI1 protein lacking the zinc fingers could activate SHH targets, we produced transgenic embryos that expressed either a 4.0 kb FLAG tagged-*Gli1^{zfd}* cDNA lacking zinc fingers 2-5 or a full-length FLAG tagged-*Gli1* cDNA from a *Wnt1* enhancer (Epstein et al., 1999) in the dorsal CNS (see Materials and Methods). Western blot analysis of COS7 cells transfected with expression constructs containing the two cDNAs demonstrated that proteins of the expected sizes are produced by the cDNAs (Fig. 2A). Interestingly, the GLI1 protein did not appear to be processed. Three *Wnt1-Gli1* and four *Wnt1-Gli1^{zfd}* E14.5 transgenic embryos expressing the transgene were generated and analyzed in serial sagittal sections for histological abnormalities and activation of SHH target genes. The neural epithelium in the dorsal midline of the caudal forebrain and rostral midbrain region of *Wnt1-Gli1* transgenics was clearly abnormal and had a wavy nature, indicating overproliferation (Fig. 2B). In addition, sections in this region and the mid/hindbrain junction had ectopic patches of HNF3β (Fig. 2C) and *Ptc1*-expressing cells (data not shown) similar to *En2-Gli1* transgenic embryos (Hynes et al., 1997). In contrast, the *Wnt1-Gli1^{zfd}* transgenics had a normal morphology and no ectopic gene expression (Fig. 2E),

demonstrating that any aberrant *Gli^{zfd}* transcript can not activate SHH targets in vivo.

Most *Gli1^{zfd/zfd};Gli2^{zfd/+}* mice die at birth and have multiple defects

One possible explanation for the lack of defects in *Gli1* mutants is that there is a compensation in function by *Gli2* and/or *Gli3*, since there is overlap in their expression domains. Since *Gli1* expression is strongest more proximal to *Shh* than *Gli2* or *Gli3*, we analyzed expression of *Gli2* and *Gli3* in *Gli1^{zfd/zfd}* mutants to determine whether their expression was upregulated in the *Gli1* domain. The expression patterns of *Gli2* ($n=8$) and *Gli3* ($n=8$) in E9.5 *Gli1^{zfd/zfd}* mutants, however, appeared normal based on whole-mounts RNA in situ analysis and RNA in situ analysis of sections (data not shown). SHH signaling was also assessed in *Gli1^{zfd/zfd}* mutants, but expression of *Shh* ($n=8$) and the target gene *Ptc1* ($n=8$) also appeared normal (data not shown).

To further test possible overlapping functions between *Gli1* and *Gli2* or *Gli3*, *Gli1^{zfd}* homozygotes carrying one mutant allele of *Gli2* or *3* were produced. Whereas double *Gli1/Gli2* heterozygotes appeared normal, most *Gli1^{zfd/zfd};Gli2^{zfd/+}* mutants died soon after birth (Table 1) and the rare ones that survived to weaning were smaller than their littermates, had a hopping gait and were unable to right themselves. They were killed at 5-7 weeks of age and gross analysis of tissues following dissection revealed several defects including a smaller lung size than in WT littermates (5/6), undescended testes with incompletely developed external genitalia in males (4/4) and a distended gut (5/5). The cause of death in most mutants is not clear, but might result from the gut defect. In contrast, *Gli1^{zfd/zfd};Gli3^{xt/+}* double mutants were viable and had the polydactyly phenotype typical of *Gli3^{xt/+}* mutants. These results demonstrate that a normal GLI1 protein is in fact required for development of many tissues, but that this requirement is only revealed when one allele of *Gli2* is also mutant.

To determine whether *Gli1/Gli2* double homozygotes had a more severe phenotype than *Gli2^{zfd/zfd}* mice, we collected litters from double heterozygote intercrosses and genotyped the offspring at three embryonic stages: E12.5, E15.5 and E18.5. Whereas double homozygotes were detected at a Mendelian ratio at E12.5, they had a reduced viability at E18.5 (Table 1). In order to compare the embryonic phenotypes of *Gli1/Gli2*

Fig. 2. Ectopic expression of *Gli1* induces overgrowth in the dorsal midbrain while *Gli1^{zfd}* does not. (A) Western blot analysis of COS-7 cells transfected with *FLAG-Gli1* (G1F) and *FLAG-Gli1^{zfd}* (Zd). (B) Transgenic mice with *FLAG-Gli1* have extensive overgrowth and an abnormal wavy texture in the midbrain when compared with WT mice (D). (C) In an adjacent section, at higher magnification of the boxed area in B, HNF3β is induced in the dorsal midbrain of *FLAG-Gli1* transgenic mice but not in *FLAG-Gli1^{zfd}* mice (E).
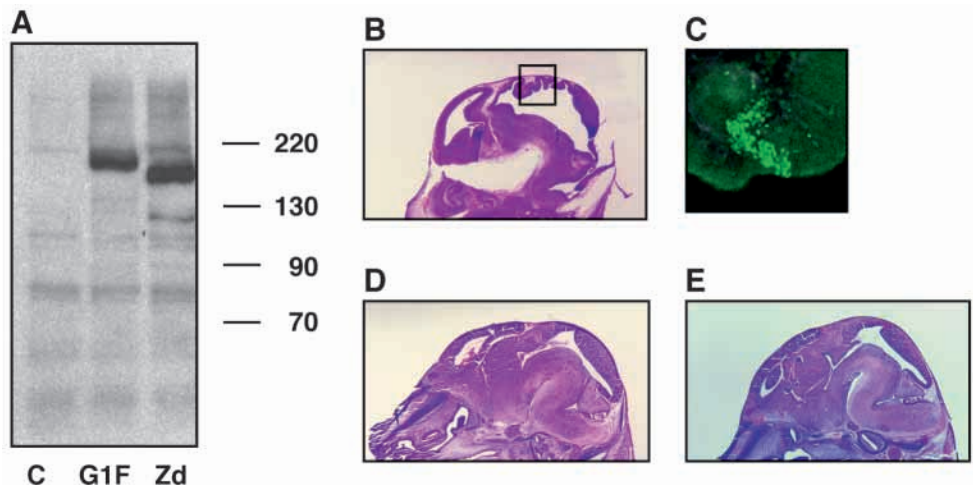
double mutants to *Gli2* and *Shh* mutants, we focused our analysis on the lungs, CNS and limbs where the function of *Shh* has been characterized in some detail.

Gli1 and *Gli2* have overlapping functions in lung lobe outgrowth.

Lung development begins at E9.5 as an outpocketing of the foregut in an asymmetric pattern that gives rise to four lobes off the right bronchus and one lobe from the left bronchus. All lobes are visible by E11.5 with the accessory lobe being the last lobe to form. Beginning at E11.5, *Shh* is expressed in the distal epithelium of the developing lung lobes and *Gli1*, *Gli2* and *Gli3* are expressed in the surrounding mesenchyme (Bellusci et al., 1997). At birth, *Shh* null mutants have very small lungs with one lobe and no visible branching of the airways (Litingtung et al., 1998; Pepicelli et al., 1998). At E18.5, $Gli2^{zfd/zfd}$ mutant lungs are much smaller than normal and the three remaining lobes on the right side appear fused (Motoyama et al., 1998 and see Fig. 3D). Since marker gene analysis of *Gli2* mutant lungs did not reveal any changes in gene expression, morphology was used to assess the phenotypes of *Gli1/Gli2* double mutants.

Whereas the lungs of $Gli1^{zfd/zfd}$ and $Gli2^{zfd/+}$ mutant adults or embryos appeared normal (data not shown), $Gli1^{zfd/zfd};Gli2^{zfd/+}$ E18.5 mutant embryos had a variable phenotype that was milder than *Gli2* homozygous mutants. The length of the accessory and right cranial lobes, and the width of the accessory and left lobes were reduced (Fig. 3A). In E12.5 and E18.5 $Gli1^{zfd/+};Gli2^{zfd/zfd}$ mutant embryos, the lungs appeared smaller than in $Gli2^{zfd/zfd}$ mutants and they had what appeared to be two lobes (Fig. 3H; data not shown). In $Gli1^{zfd/zfd};Gli2^{zfd/zfd}$ mutant embryos, only one small lobe was visible on each side at E12.5 (*n*=6) and 18.5 (*n*=2), although branching within each lobe was visible (Fig. 3G,C). In contrast, the lungs of *Shh* mutants at E12.5 had four small visible lobes (Fig. 3F) but, by E18.5, the lung lobes appeared fused and transparent due to a lack of branching and they were smaller than $Gli1^{zfd/zfd};Gli2^{zfd/zfd}$ mutants (Litingtung et al., 1998; Pepicelli et al., 1998). Since a study of *Gli2/Gli3* double mutants demonstrated that the genes have overlapping functions in lung development (Motoyama et al., 1998), *Gli3*

Table 1. Most $Gli1^{zfd/zfd};Gli2^{+/zfd}$ mice do not survive past birth

	E12.5 Actual number/%	E15.5	E18.5	P21	Expected %
WT	12/8.5	5/5.3	5/5.7	7/6.6	6.25
Gli1+/−	16/11.3	16/16.8	9/10.2	12/11.3	12.5
Gli1−/−	10/7	5/5.3	6/6.8	16/15.1	6.25
Gli2+/−	18/12.3	21/22.1	5/5.6	15/14.1	12.5
Gli2−/−	10/7	7/7.4	2/2.3	N/A	6.25
Gli1+/−;Gli2+/−	31/21.8	22/23.2	37/42	25/23.6	25
Gli1-/-;Gli2+/−	24/16.9	6/6.3	16/18.2	4/3.8*	12.5
Gli1+/-;Gli2−/−	12/8.5	8/8.4	7/8	N/A	12.5
Gli1-/-;Gli2−/−	9/6.3	5/5.3	2/2.2	N/A	6.25
Total number	142	96	88	79	100

$Gli1^{+/zfd};Gli2^{+/zfd}$ mice were interbred and yolk sacs or tail biopsies were taken at weaning for genotyping.

*All 4 mice were runted and abnormal (see text).

N/A, Not applicable ($Gli2^{zfd/zfd}$, $Gli1^{+/zfd};Gli2^{zfd/zfd}$ and $Gli1^{zfd/zfd};Gli2^{zfd/zfd}$ mice do not survive past birth.)

expression was analyzed in E12.5 $Gli1^{zfd/zfd};Gli2^{zfd/zfd}$ lungs (*n*=3), and found to appear normal in the distal mesoderm of each lung bud (data not shown).

The outgrowth of the lobes may be dependent on medial-lateral and dorsal-ventral cues, and these seem to be perturbed in *Shh* and *Gli2/Gli3* mutant embryos (discussed in Hogan, 1999). In E12.5 *Gli2* homozygous mutants, the medial lobe does not extend ventrally and all three right lobes are aligned (Fig. 3I). In *Gli1/Gli2* homozygous mutant mice at E12.5, the two lung lobes are similar in size and appear to be planar (Fig. 3G). Our results suggest that *Gli1* and *Gli2* have overlapping functions in regulating the proper growth and patterning of lung lobes, but are not required for branching within the lobes.

Gli1 and *Gli2* are required for development of spinal cord ventral midline cells and regression of the notochord

To determine whether *Gli1* normally contributes to formation of the floorplate and VIR cells, we analyzed the spinal cords of $Gli1^{zfd/zfd};Gli2^{zfd/+}$ mutants for expression of the floorplate marker HNF3β, the motoneuron marker ISL1/2 and the VIR interneuron marker NKX2.2. Whereas, in the spinal cord of E10.5 $Gli2^{zfd/zfd}$ mutants, expression of NKX2.2 is reduced and HNF3β is absent (Matise et al., 1998), expression of these genes in most $Gli1^{zfd/zfd};Gli2^{zfd/+}$ mutants (3/5) and all $Gli1^{zfd/zfd}$ mutants (data not shown) appeared normal. In only two of the five $Gli1^{zfd/zfd};Gli2^{zfd/+}$ mutants, there was a variable loss of NKX2.2- and HNF3β-expressing cells in random patches in posterior regions of the spinal cord, and motoneurons expressing Islet1/2 were located closer to the midline than normal (Fig. 4; data not shown). Furthermore, in $Gli1^{zfd/zfd};Gli2^{zfd/zfd}$ mutants, we previously showed that motoneurons were induced in the ventral midline of the spinal cord, similar to $Gli2^{zfd/zfd}$ mutants (Fig. 4; Matise et al., 1998). These results indicate that *Gli1* plays only a limited role, and *Gli2* plays the primary role, in floorplate and VIR cell induction by SHH.

In $Gli2^{zfd/zfd}$ mutant embryos, the notochord does not move away from the ventral spinal cord, as it does in normal embryos beginning at E9.5 (Ding et al., 1998; Matise et al., 1998). In all E14 (*n*=4) and E12.5 (*n*=6) $Gli1^{zfd/zfd};Gli2^{zfd/+}$ mutants analyzed, the notochord had not regressed as in WT (Fig. 4G,H). Thus, both *Gli1* and *Gli2* are required for notochord regression and one normal allele of *Gli2*, in the presence of two $Gli1^{zfd}$ alleles is not sufficient for this process to occur normally.

Gli2 and *Gli1* are required for development of the pituitary and ventral diencephalon

Our previous analysis of $Gli2^{zfd/zfd}$ mutants showed that, while *Gli2* was required for the expression of HNF3β and SHH in the spinal cord, it was not required for their expression in the caudal diencephalon at E9.5 (Matise et al., 1998). However, after E9.5, *Shh* expression appears in the more anterior diencephalon in two domains that flank the ventral midline above the pituitary (Fig. 5; Echelard et al., 1993). *Shh*−/− mice have a loss of ventral cell types in the developing forebrain resulting in holoprosencephaly and ventral expansion of dorsal gene expression (Chiang et al., 1996). Morphological analysis of $Gli2^{zfd/zfd}$ forebrains at E12.5 showed no obvious defect in the striatum, but a variable
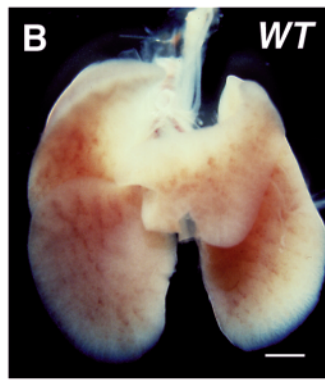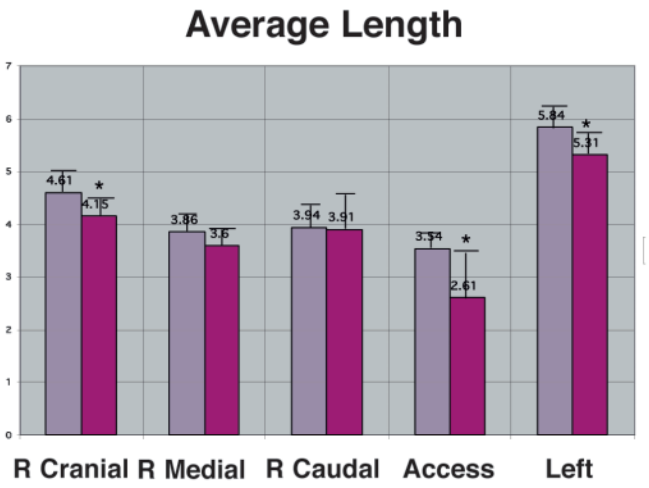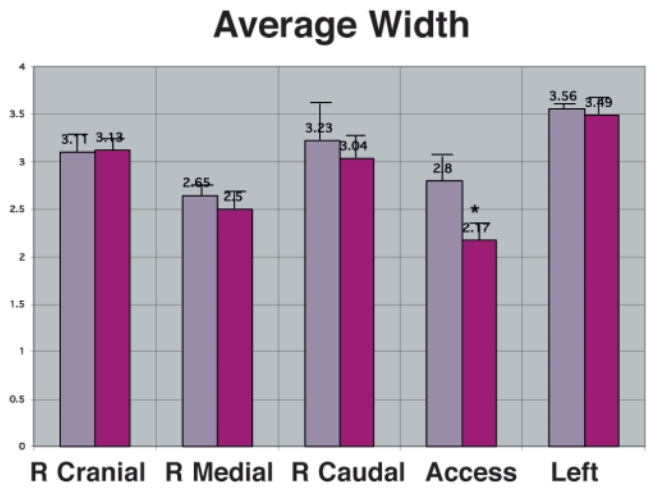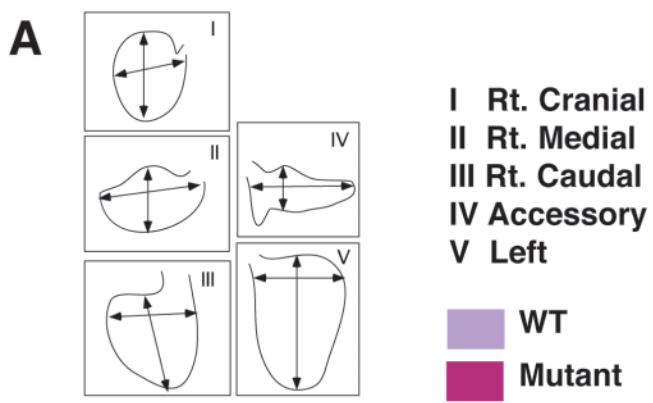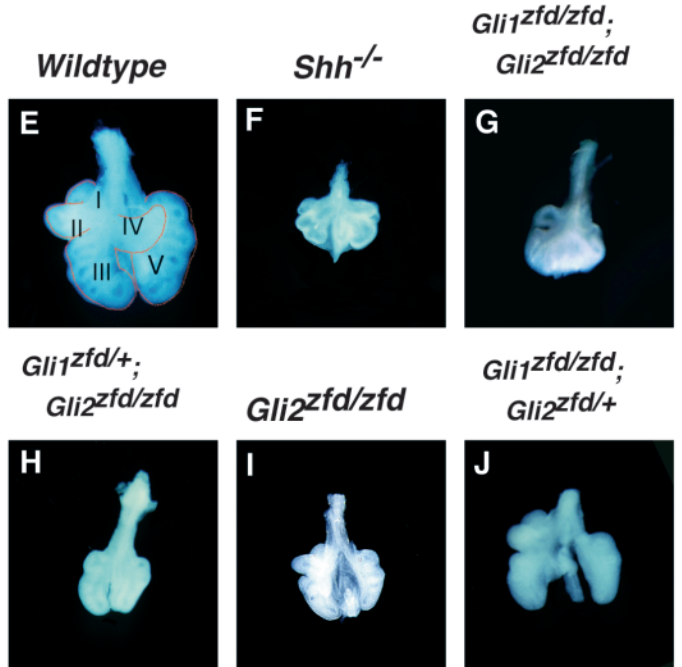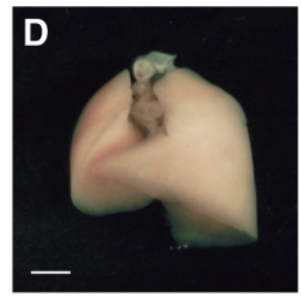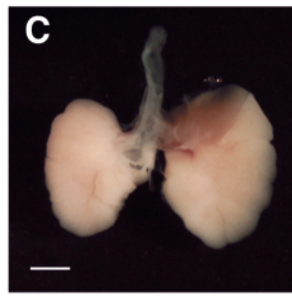
Fig. 3. *Gli1$^{zfd/zfd}$;Gli2 $^{zfd/+}$* mice have smaller lung lobes by E18.5. (A) The lung lobes of *Gli$^{zfd/+}$; Gli2$^{zfd/+}$* embryos appear WT, whereas *Gli1$^{zfd/zfd}$;Gli2 $^{zfd/+}$* lung lobes have abnormalities. Lung lobes were measured in length and width as shown by the arrows in the diagram in the top left. The averages of all measurements taken from eight different animals were plotted and standard deviations calculated for the graph (bottom left). The *t*-test (for either equal or unequal variances) was performed and the stars indicate the lobes that show a significant difference in size compared to WT. (B-D) E18.5 (E-J) E12.5 whole lungs. (B) WT lungs show the appearance of five lung buds and some branching beginning within each lobe. (C) *Gli1$^{zfd/zfd}$;Gli2$^{zfd/zfd}$* lungs appear smaller and form as two lobes. (D) *Gli2$^{zfd/zfd}$* lungs have an abnormal morphology with a fusion of the right lung lobes and a defect in left lobe outgrowth. (E) At E12.5 WT lungs have five lobes. (F) At E12.5, *Shh$^{-/-}$* lungs appear to have four tiny lobes, but form sac-like structures without any branching. (G) *Gli1$^{zfd/zfd}$;Gli2$^{zfd/zfd}$* lungs have a severe phenotype with two very small lobes, but with branching. (H) *Gli1$^{zfd/+}$; Gli2$^{zfd/zfd}$* lungs also have small buds and are more severely affected than (I) *Gli2$^{zfd/zfd}$* lungs, which are smaller and do not form an accessory lobe. (J) *Gli1$^{zfd/zfd}$;Gli2$^{zfd/+}$* lungs have a mild phenotype with a slight visible reduction in the accessory lobe at this stage.
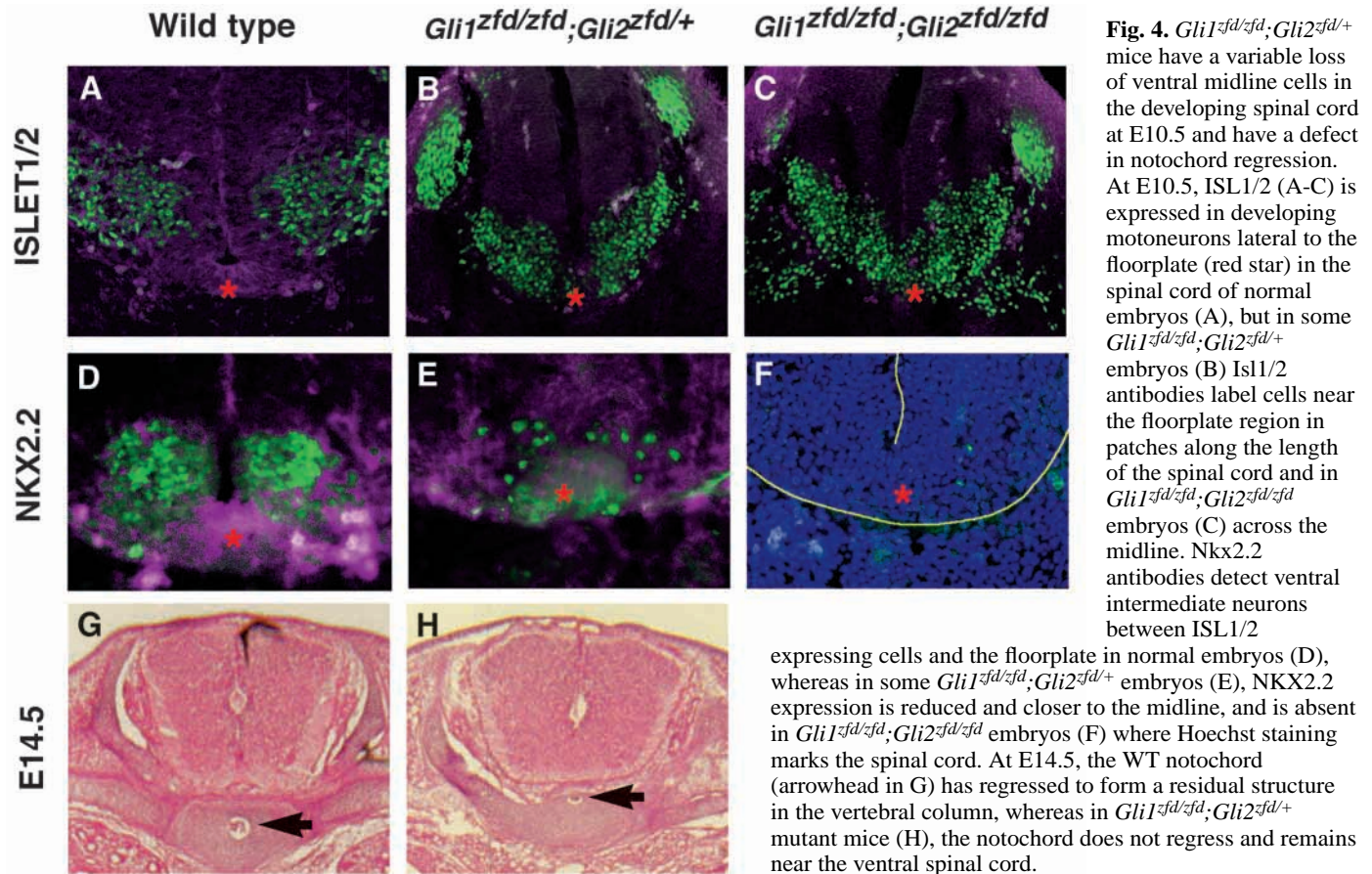
Fig. 4. *Gli1zfd/zfd;Gli2zfd/+*
mice have a variable loss
of ventral midline cells in
the developing spinal cord
at E10.5 and have a defect
in notochord regression.
At E10.5, ISL1/2 (A-C) is
expressed in developing
motoneurons lateral to the
floorplate (red star) in the
spinal cord of normal
embryos (A), but in some
Gli1zfd/zfd;Gli2zfd/+
embryos (B) Isl1/2
antibodies label cells near
the floorplate region in
patches along the length
of the spinal cord and in
Gli1zfd/zfd;Gli2zfd/zfd
embryos (C) across the
midline. Nkx2.2
antibodies detect ventral
intermediate neurons
between ISL1/2
expressing cells and the floorplate in normal embryos (D),
whereas in some *Gli1zfd/zfd;Gli2zfd/+* embryos (E), NKX2.2
expression is reduced and closer to the midline, and is absent
in *Gli1zfd/zfd;Gli2zfd/zfd* embryos (F) where Hoechst staining
marks the spinal cord. At E14.5, the WT notochord
(arrowhead in G) has regressed to form a residual structure
in the vertebral column, whereas in *Gli1zfd/zfd;Gli2zfd/+*
mutant mice (H), the notochord does not regress and remains
near the ventral spinal cord.

loss of the pituitary with 3/6 embryos having no pituitary
tissue (Fig. 5B,H; data not shown). *Shh* and *Nkx2.1*
expression appeared normal in the subventricular zone of the
medial ganglionic eminences (Fig. 5A,B; data not shown),
whereas diencephalic expression was abnormal in the more
extreme mutants. *Shh* was expressed closer to, or in the
ventral midline, in regions above where the pituitary should
form (Fig. 5H) and the *Nkx2.1* domain of expression was
smaller and shifted ventrally (Fig. 5E). Animals lacking the
pituitary had more extreme alterations in the gene expression
patterns. The pituitary defect could result from abnormal
ventral hypothalamic development (Takuma et al., 1998),
and/or a loss of SHH signaling in the oral ectoderm.

To determine whether *Gli1* contributes to ventral midline
development in the hypothalamus, the brains of
Gli1zfd/zfd;Gli2zfd/+ and *Gli1/Gli2* double homozygous
mutants were examined. Marker gene and morphological
analysis of forebrains showed that *Gli1zfd/zfd;Gli2zfd/+*
embryos (*n*=6) were similar to WT, including the presence of
a pituitary (data not shown). In contrast, all *Gli1/Gli2* double
homozygous embryos (5/5) lacked a pituitary and had
alterations in *Shh* and *Nkx2.1* expression in the hypothalmus
that appeared more extreme than any of the *Gli2* mutants (Fig.
5F,I). *Shh* and *Nkx2.1* expressions were reduced or absent
in the ventral region, although ZLI expression of *Shh* was
maintained. In the region of the striatum, the medial and
lateral ganglionic eminences expressed *Shh* and *Nkx2.1*
appropriately, however, morphologically *Gli1/Gli2* double

mutants appeared abnormal in both the dorsal and ventral
regions of the telencephalon, possibly due to the loss of
diencephalic tissue (Fig. 5; data not shown). In addition, the
neural epithelium of all *Gli1/Gli2* double mutants appeared
thicker in these regions. In all *Gli2* (*n*=6) and *Gli1/Gli2* (*n*=5)
double mutants examined at E12.5, *Gli3* expression appeared
normal except in the ventral diencephalon where it was absent
in *Gli1/Gli2* double mutants likely due to loss of ventral
tissue (Fig. 5J-L; data not shown). These results show that
both *Gli1* and *Gli2* are required for aspects of ventral
forebrain development.

Gli1/Gli2 mutant limbs have five digits and a postaxial nubbin

Shh mutants have truncated limbs with no anteroposterior
patterning (Chiang et al., 1996). Surprisingly, all four limbs of
the one E18.5 and four E15.5 *Gli1zfd/zfd;Gli2zfd/zfd* mutant
embryos examined had five digits, with an extra postaxial
nubbin (Fig. 6). Furthermore, the limbs of E12.5
Gli1zfd/zfd;Gli2zfd/zfd mutant embryos (*n*=4) appeared to express
Ptc1 and *Gli3* normally (data not shown). In contrast, *Shh*
mutant limbs expressed *Ptc*, *Gli2* and *Gli3* diffusely throughout
the whole limb and *Gli1* was downregulated (Zuniga et al.,
1999; data not shown), showing that SHH signaling is
required to maintain their normal expression. These studies
demonstrate that, in contrast to other tissues, the limbs of
Gli1zfd/zfd;Gli2zfd/zfd mutants do not have defects that are similar
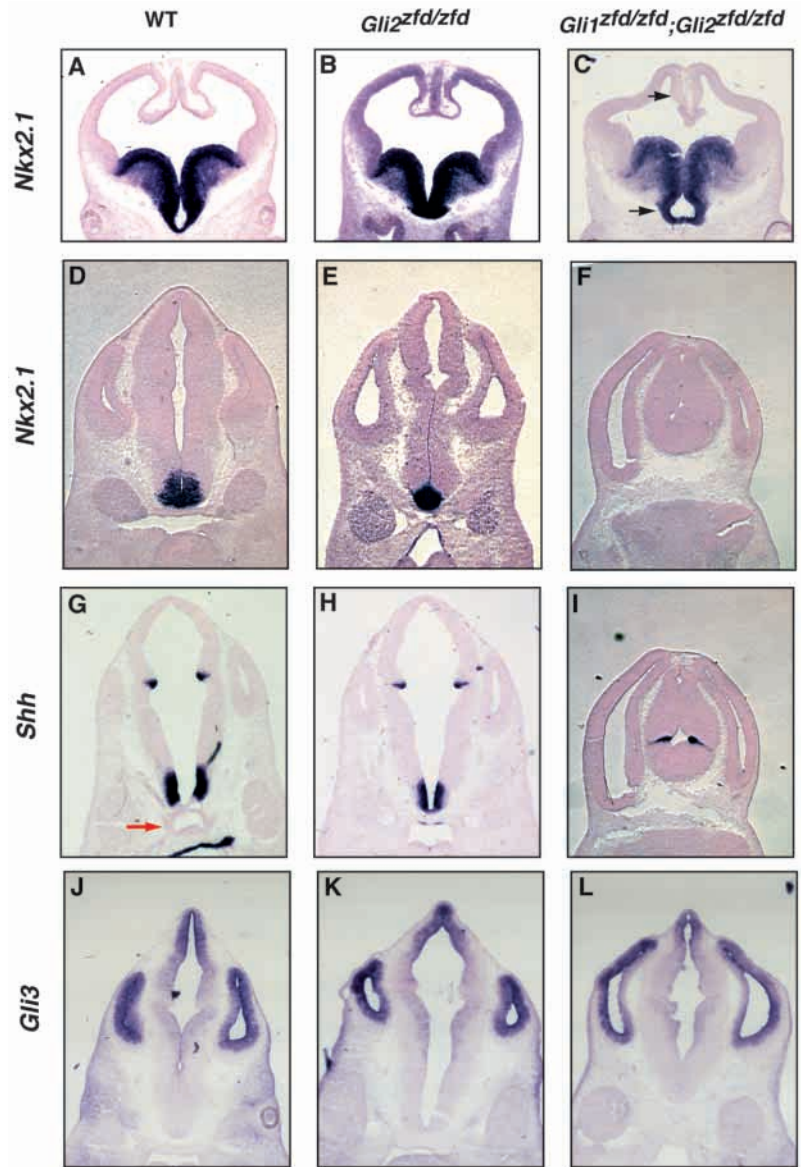to, and milder than, *Shh* mutants.

Fig. 5. Section RNA in situ analysis shows loss of midline cells in *Gli2^{zfd/zfd}* and *Gli1/Gli2* double homozygous mutant forebrains at E11.5-E12.5. *Nkx2.1* is expressed in the medial ganglionic eminence (MGE), and in the midline in the striatum region of the developing forebrain of WT (A), *Gli2^{zfd/zfd}* (B) and *Gli1^{zfd/zfd};Gli2^{zfd/zfd}* (C) embryos. In the rostral diencephalon, *Nkx2.1* is expressed in the ventral midline of the diencephalon in WT embryos (D) and *Gli2^{zfd/zfd}* (E), although in some embryos there appears to be a ventral shift in the domain of midline expression in mutants. In the severely affected *Gli1^{zfd/zfd};Gli2^{zfd/zfd}* animals (F), *Nkx2.1* expression is lost. In WT embryos (G), *Shh* expression splits in the region of the hypothalamus (caudal diencephalon) and forms two ventrolateral stripes flanking the midline. The pituitary anlage marked by a red arrow is visible at this stage. In the more severely affected *Gli2^{zfd/zfd}* embryos (H), *Shh* is expressed in the midline. In all *Gli1^{zfd/zfd};Gli2^{zfd/zfd}* embryos (I), no pituitary is visible and in the severely affected embryos, Shh expression is lost in the ventral midline but is maintained in the ZLI. *Gli3* is expressed in the dorsal regions of the caudal diencephalon and in the telencephalic vesicles in WT embryos (J) and in a similar pattern in *Gli2^{zfd/zfd}* (K) and *Gli1^{zfd/zfd};Gli2^{zfd/zfd}* (L) mutants. Also note the thickened neural epithelium in the *Gli1/Gli2* double mutants.
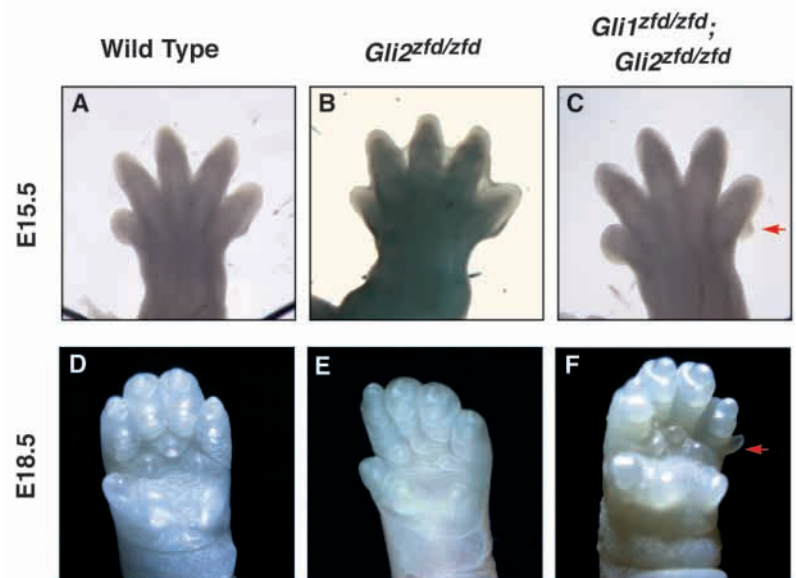

Fig. 6. *Gli1/Gli2* homozygous mutant late embryonic limbs have a postaxial nubbin. At E15.5, the five digits of the forelimb are clearly visible in normal (A) and *Gli2* mutant embryos (B), but *Gli1^{zfd/zfd};Gli2^{zfd/zfd}* limbs (C) have a small posterior outgrowth (red arrow) as well as 5 digits. At E18.5 in ventral view the limbs of *Gli1^{zfd/zfd};Gli2^{zfd/zfd}* (F) mutants have a clear postaxial nubbin (red arrow), whereas WT (D) and *Gli2* mutant (E) limbs do not.

DISCUSSION

We took a genetic approach to address the different requirements for the three *Gli* genes in developmental processes regulated by HH signaling in mammals. We demonstrate that the DNA-binding domain of GLI1 protein is not required for HH signaling, because mice homozygous for a *Gli1^{zfd}* mutation that deletes the zinc-finger encoding exons are viable and appear normal. A requirement for two normal *Gli1* alleles was found, however, in mice with one mutant *Gli2* allele that also deletes zinc-finger-encoding exons. Most *Gli1$^{zfd/zfd}$;Gli2$^{zfd/+}$* mutants die at birth and all have defects in a number of processes including floorplate development, notochord regression and lung lobe formation. Furthermore, mice with homozygous mutations in both *Gli1* and *Gli2* have more severe defects than *Gli2* homozygotes and have a mild limb polydactyly. Strikingly, in *Gli1/Gli2* double homozygous mutants aspects of many of the developmental defects seen in *Shh* mutants are normal, including formation of motoneurons, branching within the lung lobes and development of five digits in the limbs. These mutant studies raise the interesting possibility that GLI3 can play an essential role downstream of HH signaling and/or that there are transcription factors other than full-length GLI proteins that act downstream of HH signaling. Since, *Gli1$^{zfd/zfd}$;Gli3$^{xt/+}$* double mutants appear identical to *Gli3$^{xt/+}$* mutants, whereas *Gli2$^{+/-}$;Gli3$^{+/-}$* mutants are more severely defective, *Gli3* must primarily have overlapping functions with *Gli2* and not *Gli1*. The extent to which the overlapping functions include activation of HH targets in addition to repression of *Shh* targets, remains to be determined. Our studies have also revealed that two different signaling mechanisms function downstream of SHH. Based on a comparison of the phenotypes of *Shh* and *Gli1/Gli2* double mutants, one mechanism is active in tissues such as the ventral CNS and is dependent on full-length GLI1 and GLI2 proteins, whereas the other functions in organs like the limbs and does not require the proteins, despite SHH-dependent *Gli1* and *Gli2* expression in both tissues.

Gli1 and *Gli2* have different extents of overlapping function in each tissue

Interestingly, distinct tissues have different requirements for the number of normal *Gli1* or *Gli2* alleles. For example, the ventral spinal cord of most *Gli1$^{zfd/zfd}$;Gli2$^{zfd/+}$* mutants appears normal and *Gli1$^{zfd/zfd}$;Gli2$^{zfd/zfd}$* mutants have defects that are similar to *Gli2$^{zfd/zfd}$* mutants at E10.5. In contrast, the lung phenotypes are graded in severity as *Gli1* and *Gli2* alleles are deleted, except that *Gli1$^{zfd/zfd}$* mutants appear normal. The lack of regression of the notochord, which could be the result of a defect in somite patterning, appears to be the most sensitive phenotype, since all *Gli1$^{zfd/zfd}$; Gli2$^{zfd/+}$* mutants had a similar phenotype to *Gli2$^{zfd/zfd}$* mutants. These results could be taken to indicate that each tissue is sensitive to a certain dose of GLI1 plus GLI2 proteins. In every tissue, however, GLI2 seems to be the main limiting factor. Since the GLI protein levels can not be measured, this apparent difference based on genetic studies could reflect a difference in gene expression, rather than in protein functions.

It is interesting that some tissues, such as the limbs, of *Gli1$^{zfd/zfd}$;Gli2$^{zfd/zfd}$* mutants show no signs of loss of SHH signaling. In fact, in contrast to *Shh* mutants that lack digits, *Gli1$^{zfd/zfd}$;Gli2$^{zfd/zfd}$* mutants have a postaxial nubbin in all limbs. The basis of this phenotype is not clear, although it could reflect tissue overgrowth rather than altered patterning. Whether this is due to altered SHH signaling is not clear. The finding that some tissues do not require full-length GLI1 and GLI2 proteins for SHH signaling, whereas others do, provides evidence that two different molecular mechanisms can function downstream of SHH in different tissues. The basis of these mechanisms does not seem to involve differences in regulation of *Gli* expression in the different tissues since, in both the neural tube and limbs of *Shh* mutants, *Gli2* and *Gli3* are expressed throughout the tissue and *Gli1* is lost (H. L. P. and A. L. J., unpublished observations).

Molecular basis for the distinct phenotypes of the three *Gli* mutants

Previous studies showed that *Gli3$^{xt/xt}$* and *Gli2$^{zfd/zfd}$* mutants have distinct defects and we show here that *Gli1$^{zfd/zfd}$* mutants are apparently normal. Double mutant studies provided evidence that *Gli2* and *Gli3* have overlapping requirements in many developmental processes. Our present studies show that *Gli1* primarily has overlapping requirements with *Gli2*, and not *Gli3*. The apparent unique requirements for each of the *Gli* genes could be due to differences in gene expression patterns and/or biochemical properties of the three GLI proteins.

Recent biochemical studies and tissue culture transfection assays indicate that each GLI protein has a unique set of functions. Mouse GLI3 has been shown to have weak activator activity in tissue culture cells, as well as possessing an N-terminal repressor domain (Dai et al., 1999; Sasaki et al., 1999). Consistent with having a repressor activity, it appears that GLI3 can be processed similar to Ci in a SHH-dependent manner (Dai et al., 1999). Mouse GLI2 has similar activator and repressor domains and forms of GLI2 and GLI3 that contain an internal deletion in the N terminus can activate floorplate genes in the dorsal CNS of mice (Sasaki et al., 1999). In contrast, mouse GLI1 only appears to have an activation domain that does not depend on CBP binding and may not be processed (Dai et al., 1999; Fig. 2). Furthermore, a recent comparison of human GLI1 and GLI3 functions in transgenic flies has shown they have different functions (Mering and Basler, 1999) and protein studies indicate that the frog GLI proteins have different functions and may be cleaved (Ruiz i Altaba, 1999). Finally, in vertebrates, unlike *ci* in fly, *Gli1* mRNA expression is upregulated by SHH signaling and *Gli3* is repressed (Bellusci et al., 1997; Hynes and Porter, 1995; Lee et al., 1997; Platt et al., 1997).

Based on the different in vitro functions of the GLI proteins, it has been suggested that GLI2 and GLI3 are the GLI proteins that respond to, and are activated by, SHH signaling, whereas *Gli1* is a transcriptional target of activated GLI2 and GLI3 (Dai et al., 1999; Sasaki et al., 1999). Our mutant studies, which show that mice expressing at most a GLI1 protein lacking a DNA-binding domain have normal HH signaling, provide the first in vivo evidence that supports this suggestion. Furthermore, if the *Gli1$^{zfd/zfd}$* mutant allele is a hypomorphic allele and not a null, any function carried out by the abnormal GLI1 protein that is made must be novel and not involve DNA binding. Although it has been shown that N-terminal GLI1 protein can bind RNA, this also likely requires the zinc fingers (Graves et al., 1999).

GLI1 and GLI2 in Sonic Hedgehog Signaling

Our studies demonstrate that zinc-finger-containing GLI1 and GLI2 proteins are required downstream of SHH in only some cell types, since our *Gli1/Gli2* double homozygous mutants have a considerably milder phenotype than *Shh* mutants in all tissues. It is unlikely any remaining aberrant GLI1 protein can induce motor neurons and other tissues in *Gli1/Gli2* double mutants, as we have shown that GLI1 protein lacking zinc fingers cannot activate SHH targets in the dorsal brain. Although *Gli3* does not appear to be upregulated in cells adjacent to sources of SHH in *Gli1/Gli2* double mutants, it is possible that very low levels of GLI3 can activate appropriate targets in response to SHH when *Gli1* and *Gli2* are mutant. Since *Ptc* expression is reduced in *Gli2* mutants, this provides a second mechanism for inhibiting proteolysis of GLI3. In a similar way, *Gli2* might act upstream of *Shh* in some tissues in addition to acting downstream. The limb phenotypes of *Gli2/Gli3* double mutants are consistent with GLI2 functioning upstream of *Shh*, since *Gli2/Gli3* double heterozygous and homozygous/heterozygous mutants have a more extreme polydactyly than either *Gli3* mutant alone.

The finding that lung lobes are lost, in both *Gli1/Gli2* and *Gli2/Gli3* double mutant combinations, could be taken as evidence that all the genes act in the same manner in the Shh pathway. However, one striking finding that is not consistent with *Gli2* and *Gli3* functioning only downstream of SHH signaling in the lung is that *Gli2/Gli3* double homozygous mutants do not form lungs at all (Motoyama et al., 1998). This is a more extreme phenotype than in *Shh* mutants and might be due to a role of GLI2/GLI3 in repressing SHH targets in some cells in the lungs. If GLI1 is only an activator, and since the phenotype of *Gli1/3* mutants suggests little functional overlap between the two genes, GLI3 normally might not be required as an activator in many cells since GLI2 is present. Alternatively, *Gli1* and *Gli3* may have little overlap in expression and GLI3 might only share activator functions with GLI2 (see Fig. 7). The contributions of the two competing functions of GLI2 and GLI3 in various tissues remain to be determined.

Another possible explanation for why the *Gli1/Gli2* mutant phenotypes are milder than *Shh* is that there are two separate pathways downstream of SHH, only one of which is GLI protein-dependent. This hypothesis is supported by two studies that analyzed the regulatory sequences of Hh-responsive genes. In vertebrates, the *CoupTFII* gene is expressed in motoneurons and the gene can be induced by SHH. Sequences sufficient for SHH-dependent *CoupTFII* expression were identified, but they were found not to contain consensus GLI-binding sites (Krishnan et al., 1997). Similarly, regulatory sequences in the *Drosophila* HH target gene *wg* were identified that do not depend on Ci function (Lessing and Nusse, 1998). A recent analysis of expression of SHH targets in the chicken Talpid[3] mutant also suggests a bifurcation in the SHH signaling pathway (Lewis et al., 1999). Given our results, it is of interest to determine whether in cancers induced by inappropriate SHH signaling any of the GLI proteins are required for activation of genes involved in the tumor phenotype.

Finally, an additional explanation for the presence of tissues such as motoneurons in *Gli1/Gli2* mutants, but not in *Shh* mutants, is that in such tissues the primary function of SHH is to prevent repressor forms of GLI2 and GLI3 proteins from forming, rather than to induce GLI transcriptional activators. For example, in the developing spinal cord *Hnf3β* expression in the floorplate would be dependent on GLI-binding sites and an activator form of GLI protein. In *Shh* mutants, a repressor form of GLI2 would be present and inhibit *Hnf3β* transcription whereas, in *Gli2* mutants, no GLI2 protein would be produced and the low level of GLI1 present would not be sufficient to induce *Hnf3β*. Motoneuron genes, on the contrary, would not be dependent on GLI proteins for activation. In *Shh* mutants, however, a repressor form of GLI2 would be present and inhibit motoneuron gene expression, whereas in *Gli2* mutants such a repressor would not be present and additional transcription factors would be able to activate transcription. Studies of GLI protein processing in vivo and *Shh/Gli2* double mutants are required to distinguish between the various possibilities.
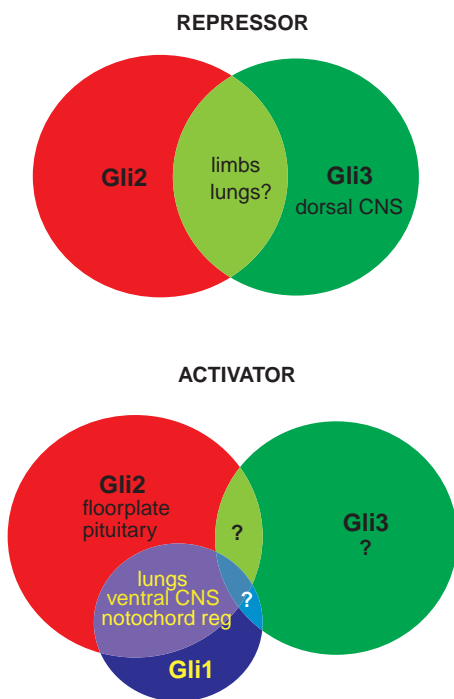

REPRESSOR

ACTIVATOR

Fig. 7. Model. The overlapping area between Gli2 and Gli3 indicates the redundancy between GLI repressor activity in tissues such as the limbs, the dorsal CNS and possibly the lungs. This diagram also demonstrates the overlapping activator functions of GLI1 and GLI2 in the lungs, ventral CNS, notochord regression and pituitary development. It is still unclear whether there is overlap in activator properties of GLI2 and GLI3 and, in addition, there appears to be little, if any, overlap in Gli1 and Gli3 function at all.

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