INTRODUCTION

Members of the Hedgehog (Hh) family of intercellular signaling molecules control a variety of developmental processes, ranging from segment patterning in Drosophila to forebrain development in humans (reviewed by Ingham and McMahon, 2001). Hedgehog signals are transduced by binding and antagonizing the membrane protein Patched (Ptc), leading to the activation of the membrane protein Smoothened (Smo). In Drosophila, all Hh signaling is mediated by post-translational modulation of Cubitus interruptus (Ci) activity. Ci is a transcription factor of the Gli family that can be both an activator and a repressor of Hh target genes. In the absence of Hh signaling, proteolytic cleavage results in a Ci isoform that is a transcriptional repressor, consisting of an N-terminal repressor domain and the zinc finger DNA binding domain (Aza-Blanc et al., 1997; Wang and Holmgren, 1999). Upon activation of Hh signaling, cleavage is inhibited and a full-length activator form of the molecule predominates (Aza-Blanc et al., 1997; Wang and Holmgren, 1999). Because of the dual function of Ci, Ci null mutants do not have the same phenotype as hh mutants (see Methot and Basler, 2001). hh mutants display a loss of expression of all Hh target genes, whereas loss of Ci leads to both the inappropriate derepression of some Hh targets and the loss of expression of other Hh-regulated genes.

In vertebrates, additional complexity in Gli function is caused by the presence of at least three gli genes, gli1, gli2, and gli3. The functions of the different gli genes have been analyzed using mouse mutants and mis- and overexpression in Xenopus, Drosophila and cultured cells (reviewed by Ingham and McMahon, 2001; Koebernick and Pieler, 2002; Ruiz i Altaba et al., 2002). While the in vivo relevance of some of these studies remains to be established, current evidence suggests the following roles for Gli proteins. Gli1 appears to be an activator of Hh target genes, but in contrast to Ci, gli2 has minor roles as a repressor or activator of Hh targets.

SUMMARY

Gli proteins regulate the transcription of Hedgehog (Hh) target genes. Genetic studies in mouse have shown that Gli1 is not essential for embryogenesis, whereas Gli2 acts as an activator of Hh target genes. In contrast, misexpression studies in Xenopus and cultured cells have suggested that Gli1 can act as an activator of Hh-regulated genes, whereas Gli2 might function as a repressor of a subset of Hh targets. To clarify the roles of gli genes during vertebrate development, we have analyzed the requirements for gli1 and gli2 during zebrafish embryogenesis. We report that detour (dtr) mutations encode loss-of-function alleles of gli1. In contrast to mouse Gli1 mutants, dtr mutants and embryos injected with gli1 antisense morpholino oligonucleotides display defects in the activation of Hh target genes in the ventral neuroectoderm. Mutations in you-too (yot) encode C-terminally truncated Gli2. We find that these truncated proteins act as dominant repressors of Hh signaling, in part by blocking Gli1 function. In contrast, blocking Gli2 function by eliminating full-length Gli2 results in minor Hh signaling defects and uncovers a repressor function of Gli2 in the telencephalon. In addition, we find that Gli1 and Gli2 have activator functions during somite and neural development. These results reveal divergent requirements for Gli1 and Gli2 in mouse and zebrafish and indicate that zebrafish Gli1 is an activator of Hh-regulated genes, while zebrafish Gli2 has minor roles as a repressor or activator of Hh targets.

Key words: Forebrain patterning, Hedgehog signaling, Adaxial cells, floor plate, cyclopamine, Morpholino
Gli1 activity is not regulated post-translationally but transcriptionally by Hh-mediated gene activation (Epstein et al., 1996; Marigo et al., 1996a; Hynes et al., 1997; Lee et al., 1997; Dai et al., 1999). Both N- and C-terminal domains of Gli1 are necessary for its activation function (Ding et al., 1999; Ruiz i Altaba, 1999). Despite its apparent activator function, Gli1 is not essential for normal mouse development (Park et al., 2000; Bai and Joyner, 2001; Bai et al., 2002). In contrast, mouse Gli2 mutations are perinatal lethal and result in the down-regulation of Hh target genes (Ding et al., 1998; Matise et al., 1998), supporting the idea that Gli2 is a Hh-dependent activator. The C-terminal region of Gli2 appears to be essential for its activation function because C-terminally truncated Gli2 inhibits Hh target genes (Ruiz i Altaba, 1999; Sasaki et al., 1999). Since a C-terminally truncated form of Gli2 might be generated by proteolytic processing, it has been suggested that Gli2 also has repressor activity (Ruiz i Altaba, 1999; Sasaki et al., 1999; von Mering and Basler, 1999; Aza-Blanc et al., 2000). Similarly, Gli3 appears to be processed to a C-terminally truncated repressor of Hh target genes (Ruiz i Altaba, 1999; Sasaki et al., 1999; Shin et al., 1999; Aza-Blanc et al., 2000; Wang et al., 2000). Accordingly, Gli3 mouse mutants display ectopic activation of Hh targets (Masuya et al., 1995; Ruiz i Altaba, 1998; Litingtung and Chiang, 2000; Tole et al., 2000). Hh signaling is thought to repress Gli3 transcription and Gli3 processing (Marigo et al., 1996a; Ruiz i Altaba, 1998; Dai et al., 1999; von Mering and Basler, 1999; Aza-Blanc et al., 2000; Wang et al., 2000). The full-length form of Gli3 has been postulated to act as an activator of Hh targets (Dai et al., 1999; Sasaki et al., 1999; Borycki et al., 2000; Litingtung and Chiang, 2000), but direct in vivo evidence is currently not available to support this hypothesis.

Misexpression and cell culture studies give insights into potential Gli functions, but the exact requirement for vertebrate Hh signaling and Gli genes has been studied in most detail during neural patterning in mouse mutants. Sonic hedgehog is expressed in the notochord and floor plate (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ekker et al., 1995) and is essential for the induction of floor plate, motor neurons and most classes of ventral interneurons in the spinal cord (Chiang et al., 1996; Ericson et al., 1996). Gli2 is required to mediate some aspects of Hh signaling in the ventral neural tube. Whereas motor neurons and most interneurons develop normally in Gli2 mutants, the floor plate does not form (Ding et al., 1998; Matise et al., 1998). In contrast, Gli1 mutant mice have an apparently normal spinal cord, indicating that Gli1 is not essential for interpreting Hh signals in the ventral CNS (Park et al., 2000). Double mutant analysis suggests, however, that Gli1 can contribute to Hh signaling since Gli1<sup>−/−</sup>;Gli2<sup>−/−</sup> mice show ventral patterning defects not found in Gli2<sup>−/−</sup> mice (Park et al., 2000). Moreover, expression of low levels of Gli1 in place of Gli2 can rescue Gli2 mutants (Bai and Joyner, 2001). Taken together, these results support the idea that Gli1 and Gli2 are positive mediators of Hh signaling. In contrast, Gli3 appears to be involved in the repression of Hh targets in the dorsal CNS (Litingtung and Chiang, 2000; Tole et al., 2000).

While mutant data indicate that Gli1 and Gli2 are activators and Gli3 is a repressor of Hh targets, seemingly contradictory results are surprisingly common in the analysis of Gli function. For instance, mis-expression studies in Xenopus have led to the suggestion that Gli1 specifies floor plate development in the neural tube while Gli2 restricts floor plate specification, but induces motoneuron development and patterns the mesoderm (Lee et al., 1997; Marine et al., 1997; Ruiz i Altaba, 1998; Ruiz i Altaba, 1999; Mullor et al., 2001). These proposals contradict the observations that mouse Gli2 mutants lack floor plate, but do not display defects in early mesoderm patterning, and that Gli1 is not required for ventral patterning (Ding et al., 1998; Matise et al., 1998; Park et al., 2000). These results might reflect the shortcomings of misexpression approaches or complications due to redundancy, but they might also be indicative of context-dependent differences in Gli function. For instance, depending on cell type or species, the requirements and activities of Gli genes might differ.

Genetic studies of Hh signaling in zebrafish complement mutant analysis in the mouse and provide an approach to test the conservation and divergence of Gli function in vertebrates. Loss of zebrafish Hh signaling leads to ventral spinal cord defects, deficiencies in ventral forebrain specification, absence of an optic chiasm due to retinal axon guidance defects, absence of slow muscle fiber types, malformations of the dorsal aorta, ventral curvature of the body and defects in pectoral fin development (Brand et al., 1996; Chen et al., 1996; Karlstrom et al., 1996; van Eeden et al., 1996b; van Eeden et al., 1996a; Schauerte et al., 1998; Karlstrom et al., 1999; Lewis et al., 1999; Barresi et al., 2000; Odenthal et al., 2000; Chen et al., 2001; Varga et al., 2001). Forward genetic screens have identified mutations that cause all or some of these phenotypes and affect components of the Hh signaling cascade. These include sonic-you (syn), which disrupts shh (Schauerte et al., 1998), slow-muscle-omitted (smu), which inactivates smoothened (smo) (Chen et al., 2001; Varga et al., 2001) and you-too (yot), which encodes C-terminally truncated forms of Gli2 (Karlstrom et al., 1999). Moreover, several molecularly uncharacterized mutants have a subset of hh loss-of-function phenotypes, suggesting that they might encode additional components or mediators of Hh signaling. For instance, the detour (dtr) mutant was originally isolated because of errors in retinal axon guidance (Karlstrom et al., 1996) and ventral curvature of the body (Brand et al., 1996). Axons that normally cross the midline of the diencephalon fail to do so in dtr mutants, and no optic chiasms forms (Karlstrom et al., 1996). In addition, lateral floor plate cells are absent, suggesting defects in Hh signaling similar to those seen in syu/shh, smu/smo and yot/gli2 (Odenthal et al., 2000). Cranial motor neurons also fail to differentiate in dtr mutant embryos (Chandrasekhar et al., 1999). Unlike syu/shh, smu/smo and yot/gli2, dtr does not appear to affect somite patterning, differentiation of slow muscle fibers, or formation of the dorsal aorta. Here we identify the dtr locus as gli1 and analyze the roles of gli1 and gli2 during zebrafish development. Our results reveal contrasting requirements for gli genes in mouse and zebrafish and suggest that gli1 is an essential activator of Hh-regulated genes, whereas gli2 has minor roles in activating or repressing Hh targets.

MATERIALS AND METHODS

Mutant and mapping strains

Three alleles of dtr (dtr<sup>me276</sup>, dtr<sup>rc370</sup> and dtr<sup>rc269</sup>) were identified...
previously in mutant screens (Brand et al., 1996; Karlstrom et al., 1996; van Eeden et al., 1996b). For mapping, dtr<sup>p1269</sup> was crossed to two polymorphic lines, the WIK line (Rauch et al., 1997) and the TL line. In situ and antibody analyses were performed with the stronger (dtr<sup>p650</sup>) allele. Other mutant strains used were smooth muscle omitted (smu<sup>664</sup>) and you-too (yot<sup>p112</sup>, yot<sup>p177</sup>).

**Genetic mapping and linkage analysis**

We determined the position of dtr on the zebrafish genetic map using centromere linkage analysis (Johnson et al., 1996; Postlethwait and Talbot, 1997). Gynogenetic diploid embryos were obtained from heterozygous females by early pressure treatment of eggs fertilized with inactivated sperm. Mutant and wild-type progeny were identified by visual inspection on day 1 or day 2. DNA prepared from individuals or pools of eight mutant or wild-type individuals was assayed by PCR using polymorphic markers (simple sequence length polymorphisms) (Knapik et al., 1998). This identified a genetic marker (z3581) on LG6 that was linked to polymorphisms (Karlstrom et al., 1998). This detailed genetic map in the region of the gli1 locus can be viewed online using the zebrafish information network (ZFIN) at http://zfin.org.

**Cloning the zebrafish gli genes**

Genomic clones were obtained by screening a gridded genomic bacterial artificial chromosome (BAC) library (Genome Systems) using radiolabeled probes for a mouse Gli2 cDNA at low stringency hybridization conditions. BAC DNA was prepared for positive clones and the BAC ends were sequenced using T7 and SP6 vector primers. SP6 end sequence of clone 152g22 showed homology to mouse Gli1. PCR primers based on sequence from the T7 end of clone 152g22 amplified a simple sequence length polymorphism (SSLP) detectable upon electrophoresis through 2% agarose gels. This SSLP was used to map the BAC end to LG6 and detect linkage to the dtr locus (0 recombinants in 83 meioses). A partial cDNA clone encoding gli1 was isolated from a 15- to 19-hour embryonic cDNA library (generously provided by Bruce Appel and Judith Eisen, University of Oregon, Eugene) using a radio-labeled PCR probe generated to sequence from the SP6 end of BAC 152g22. 5’ and 3’ RACE reactions (Invitrogen) identified cDNA fragments encoding the 3’ and 5’ portions of zebrafish gli1. These fragments were cloned into the pTOPO vector (Invitrogen) and their sequences assembled into the full gli1 coding region (GenBank accession no. AY173030).

**Sequencing mutant alleles**

RT-PCR and cycle sequencing were used to sequence the three ENU-induced dtr alleles. RNA was isolated from the following pools of 40 embryos: (1) dtr<sup>p2306</sup> wild-type siblings; (2) dtr<sup>p269</sup> mutants; (3) dtr<sup>p650</sup> mutants; and (4) dtr<sup>p1269</sup> mutants. First-strand cDNA was made using Superscript reverse transcriptase (GIBCO). Fragments (500-1000 bp) were amplified from first strand cDNA by PCR using primers based on the deduced gli1 cDNA sequence. DNA fragments were then gel purified and cycle sequenced (Stratagene Cyclist). Sequences were compared between pools and to the gli1 cDNA sequence. The fragments containing the dtr point mutations were also subcloned using the TA cloning system (Invitrogen). DNA from two separately isolated clones was purified, and the mutant sequence was verified.

**PCR genotyping dtr/gli1 and yot/gli2 fish**

Embryos or fin clippings were placed in 50 μl lysis buffer (10 mM Tris pH 7.5, 50 mM KCl, 0.3% Tween 20, 0.3% NP40, 1 mM EDTA) and incubated for 10 minutes at 98°C. Tissue was then digested by adding Proteinase K (Roche) to 2 mg/ml and incubating 2 hours to overnight at 55°C. Proteinase K was then inactivated by incubation at 98°C for 10 minutes. For genotyping dtr<sup>p269</sup> fish, a mutant-specific reverse primer designed for the dtr<sup>p269</sup> allele (ts269Mu:rv: 5’-TGGATGATCAGTGGGCCCA) was used with a forward primer (dtr8fw: 5’-GTCTAAAGGCTCTAAATATCCAGTC) to amplify a mutant-specific 560 bp product from homozygous mutants and heterozygotes. A wild-type reverse primer (ts269Wlt:rv: 5’-TGGGATCATGTTGCCC) served as an amplification control. To genotype yot<sup>p112</sup> fish, two primers flanking the mutation site (yot33fw: 5’-CCACCTAGCTATACAGAGAAC, yot28rv: 5’-CTTGCTTACGGAATTTGTCG) were used to amplify a 589 bp product which was then digested using the NlaIV restriction enzyme. The yot<sup>0/17</sup> mutation eliminates a NlaIV restriction site in the amplified region, resulting in the appearance of a mutant-specific 363 bp band that can be visualized on an agarose gel.

**In situ hybridization and antibody labeling**

In situ labeling was performed as described previously (Schier et al., 1997). A 1.4 kb gli1 probe was synthesized using the 3’ RACE containing plasmid (dtr3’RACE.pCRII) linearized with BamHI using the T7 promoter. Other probes used were zebrafish gli2 (Karlstrom et al., 1999), lim3 (Glazzard et al., 1997), myoD (Weinberg et al., 1996), nkr2.2 (Barth and Wilson, 1995), shh (Krauss et al., 1993), ptc1 (Concordet et al., 1996) and parX (Krauss et al., 1991).

**mRNA and morpholino antisense oligonucleotide injections**

Embryos were pressure injected with 500 pl-1 nl of solution at the 1- to 4-cell stage. Embryos were injected in their chorions and held in agarose troughs (Westerfield, 1993). Injected, control injected and un.injected embryos were grown to ~80% epiboly at 28°C, then shifted to 22°C and grown to the 20-somite stage, fixed in 4% paraformaldehyde and processed for in situ hybridization. For morpholino antisense oligonucleotide (MO) injections, embryos were injected with 1–15 ng of MO dissolved in 1:1 Danio solution (Westerfield, 1993). zfgli1 (5’-CCGACACACCGGTACACCG-CGT) and zfgli2 MO (5’-GGATGATGTTAAGTGTCATGTCG) and a random control MO (5’-CCTTCTACCCGTTACAAAF) were synthesized by Gene Tools (Eugene, OR) and kept as 25 mg/ml stocks in 1× Danio solution. Specificity of these MOs is demonstrated by (1) the suppression of the zfgli2 repressor phenotype by the gli2 MO and (2) phenocopy of the dtr phenotype by the gli1 MO in wild-type embryos. Synthetic mRNA was made using the Message Machine kit (Ambion) and diluted in water to 1 mg/ml. shh mRNA was synthesized from a pT7TS plasmid containing shh (Ekker et al., 1995). Control, β-gal-encoding mRNA was synthesized from a pT7TS plasmid containing the lacZ gene.

**Cell culture analysis of transcriptional activity**

The rat neural stem cell line MNS70 (Nakagawa et al., 1996) was co-transfected with different plasmid constructs containing a gli1 gene in the pcDNA3.1-His cloning vector (Invitrogen) in combination with a reporter plasmid containing luciferase inserted downstream of 8xGli binding sites (Sasaki et al., 1997). Full-length gli1 and gli2 inserts were subcloned into the pcDNA vector from pBluescript (Stratagene). Mutant constructs were made by swapping the appropriate, mutation-containing DNA fragment, which was generated by RT-PCR from cDNA made from mutant embryos. One day before transfection, MNS70 cells were plated onto poly-D-lysine coated six-well plates at the concentration of 2×10<sup>5</sup> cells per well. Four hours before transfection, cells were re-fed with fresh medium. 1 μg (total) of plasmid DNA (0.4 μg of effector [0.2 μg each of two effectors indicated in figure], 0.5 μg of reporter and 0.1 μg of reference [SV-β-gal]) was transfected to a well by mixing with 6 μl of Fugene 6 transfection reagent (Roche) according to the manufacture’s protocol. Cell lysates were prepared 48 hours after transfection and assayed for luciferase and β-galactosidase activities as previously described (Sasaki et al., 1997). For western analysis, epitope-tagged proteins were detected using an Omni-probe antibody (Santa Cruz Biotechnology).
Cyclopamine treatments
2-4 cell embryos were treated with 100 μM cyclopamine (Toronto Chemical) (Incardona et al., 1998) by adding 10 μl of a 10 mM stock solution (in 95% ethanol) to 1 ml of egg water (0.3 g/l Instant Ocean Salt, 1 mg/l Methylene Blue). Control embryos were treated simultaneously with an equal volume (10 μl) of 95% ethanol (cyclopamine carrier) in 1 ml egg water. Treatments were carried out in 12-well plates (40 embryos/well) at 28.5°C. Embryos were grown to the 4-somite stage, dechorionated using 0.2 mg/ml (final) pronase (Sigma) in egg water, fixed with 4% paraformaldehyde, dehydrated in methanol, and processed for in situ hybridization.

RESULTS
detour (dtr) mutations disrupt Hedgehog signaling
Previous studies established that dtr, syu/shh and yot/gli2 mutants share CNS and body shape phenotypes (Brand et al., 1996; Karlstrom et al., 1996; Schauerte et al., 1998; Odenthal et al., 2000) (see Fig. 1). This suggested that the dtr locus might encode a component of the Hh signaling pathway. To further test the relationship of Hh signaling and dtr, we carried out a detailed analysis of the dtr phenotype, focusing on the forebrain. Since previous studies of axon guidance defects in the three dtr alleles indicated that dtrts269 and dtrts370 are more severe than dtrts270 (R. O. K., unpublished results), we focused our analysis on dtrts269. In addition to the previously described ventral curvature of the body (Brand et al., 1996), lack of lateral floor plate (Odenthal et al., 2000) and abnormal ipsilateral projection of retinal axons (Karlstrom et al., 1996), we found that the expression of patched 1 (ptc1), an indicator of Hh signaling (see Goodrich and Scott, 1998), is reduced (Fig. 1F). Moreover, we found that expression of nk2.2, a Hh-induced marker for ventral neuroectoderm, is absent in the spinal cord and some regions of the ventral forebrain and midbrain and is reduced in the anterior pituitary anlage (Fig. 1J). The reduction of nk2.2 expression in dtr mutants resembles, but is not as severe as that seen in syu/shh (Sbrogna et al., 2003), smu/smo (Chen et al., 2001; Varga et al., 2001) or yot/gli2 mutants (Fig. 1K) (Karlstrom et al., 1999). The pax6 gene has been shown to be negatively regulated by Shh in zebrafish (Ekker et al., 1995; Macdonald et al., 1995). Consistent with a reduction of Hh signaling, pax6 expression is expanded in dtr mutant embryos (Fig. 1N). Taken together, the dtr forebrain

Fig. 1. dtrts269, yotyot and dtrts370;yotyot embryos have defects in body axis formation and expression of Hh target genes in the brain. (A-D) Examination of live 36-hour embryos reveals curled body axes in dtrts269, yotyot and dtrts370;yotyot mutant embryos. U-shaped somites, indicative of defects in slow muscle cell differentiation, are seen only in yotyot and dtrts370;yotyot embryos, dtrts269 embryos have wild-type somites (insets). (E-H) patched 1 (ptc1) expression is generally reduced in all three genotypes. In situ labeling was performed simultaneously and embryos were developed for the same amount of time in E, F and G. Inset in H shows wild-type sibling developed in same tube as this transheterozygote. (I-L) In all gli mutant embryos, nk2.2 expression is reduced or absent from the anterior pituitary anlage (arrowheads), as well as from different regions of the ventral midbrain and ventral hindbrain. (M-P) Expression of pax6, a gene known to be repressed by Hh signaling, is variably expanded in the MDB (arrowhead) and hindbrain (arrows). Expression of pax6 is expanded across the MDB expression domain of shh (not shown), ptc (E), and nk2.2 (I). All panels show lateral views, anterior to the left. Eyes were removed in E-P. Gene expression is indicated on the left. Di, diencephalon; HB, hindbrain; MB, midbrain; MDB, mid-diencephalon boundary; MHB, midbrain-hindbrain boundary; te, telencephalon.
phenotypes are similar to, but weaker than those seen in syu/shh, smu/smo and yot/gli2 mutants.

As an additional test for the role of dtr in Hh signaling, we analyzed the effect of Shh overexpression on nk2.2 transcription in wild-type, dtr and yot embryos. While nk2.2 was strongly expanded in wild-type embryos injected with Shh-encoding mRNA (Fig. 2D), the dtr and yot mutations strongly reduced ectopic activation of nk2.2 (Fig. 2E,F). These results indicate that dtr, like yot, acts downstream of Hh signals.

detour mutations disrupt zebrafish gli1

To determine if the dtr locus might encode a component of the Hh signaling pathway, we sought to clone the dtr gene. We mapped dtr to linkage group 6 (LG6) of the zebrafish genetic map. In parallel, we isolated gli-containing genomic clones and mapped several of these on the zebrafish genetic map. One clone (BAC 152g22) mapped to LG6 near the dtr locus and was tightly linked to dtr (0 recombinants in 83 meioses). Sequence obtained from the SP6 end of BAC 152g22 showed high sequence similarity to vertebrate Gli1 genes. We then isolated and sequenced a zebrafish gli cDNA corresponding to the gli sequence in BAC 152g22. Subsequent sequence analysis of this cDNA and a 5' RACE PCR product identified a full-length open reading frame of 1371 amino acids that is closely related to mouse Gli1.

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Subsequent sequence analysis revealed point mutations in gli1 in all three dtr alleles (Fig. 3B). Two of the identified point mutations (dtr<sup>le370</sup> and dtr<sup>le269</sup>) introduce premature stop codons that are predicted to result in C-terminally truncated Gli1 proteins. The third point mutation (dtr<sup>pm276</sup>) affects a conserved tyrosine residue in the DNA binding region of Gli1 known to contact target DNA (Pavletich and Pabo, 1993).

To test how the zebrafish dtr mutations affect Gli1 protein function, we used a cell culture assay for Gli transcriptional activity (Sasaki et al., 1997; Sasaki et al., 1999). We found that wild-type zebrafish Gli1 acted as an activator of a Gli-responsive reporter construct (Fig. 4). This activity was similar to, albeit weaker, than that of mouse Gli1. Co-transfection of zebrafish Gli1 with Shh resulted in roughly additive activation of the reporter, indicating that Shh did not significantly alter Gli1 activity in these cells (Fig. 4). The Gli1 proteins encoded by the three dtr alleles did not activate the reporter construct and did not interfere with activation mediated by wild-type Gli1 (Fig. 4, compare zfGli1, zfGli1 + dtr mutations, and zfGli1 + pJT4 vector). Consistent with the different allele strengths, dtr<sup>pm276</sup>, but not dtr<sup>le370</sup> and dtr<sup>le269</sup>, enhanced reporter gene activation by wild-type Gli1. Interestingly, despite its defective DNA binding domain, dtr<sup>pm276</sup> increased Gli1 activity as effectively as did wild-type Gli1. These results indicate that the dtr mutations are complete or partial loss-of-function alleles of gli1.

As an additional test to determine whether mutations in gli1 are responsible for the dtr phenotype, we knocked down Gli1 activity using an antisense morpholino oligonucleotide (MO) designed to interfere with gli1 translation. We found that injection of gli1 MOs into wild-type embryos phenocopied dtr spinal cord and forebrain defects. gli1 MO injection eliminated nk2.2 expression regionally in the forebrain in the same pattern as seen in dtr mutants, and eliminated spinal cord nk2.2 expression (Fig. 5B,C, Table 1). fkd4, a marker of medial and lateral floor plate cells in the spinal cord (Odenthal et al., 2000), was reduced similarly in dtr mutant and gli1 MO-

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<th>Table 1. gli1 MO injection into wild-type embryos phenocopies dtr nk2.2 defects</th>
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Very strong: nk2.2 extremely reduced in malformed embryos.
Partial phenocopy: nk2.2 reduced but not absent.
Fig. 3. Sequence of zebrafish Gli1 and identification of point mutations in the three dtr alleles. (A) The deduced amino acid sequence of zebrafish Gli1 (zfGli1) aligned with mouse Gli1 (mGli1) and zebrafish Gli2 (zfGli2). The entire coding region of gli1 was sequenced in each of the three ENU-induced dtr alleles (dtr\textsuperscript{tm276}, dtr\textsuperscript{ts269} and dtr\textsuperscript{te370}) and point mutations were found for each allele (boxes). The altered amino acid in dtr\textsuperscript{tm276} is shown above the box while nonsense mutations are indicated by red hexagons. Gli2 mutations found in you-too are from Karlstrom et al. (Karlstrom et al., 1999). The five zinc finger regions are indicated by lines and potential sites for phosphorylation by protein kinase A (PKA) are indicated by asterisks. A putative VP-16 activator-like domain is indicated by a blue box. Colored sections indicate regions of homology schematized in C. (B) Sequencing ferograms showing point mutations in the three dtr alleles. In dtr\textsuperscript{tm276} U 1633 is mutated to G, changing tyrosine 440 (UAC: Y) into an aspartic acid (GAC: D). In dtr\textsuperscript{ts269} C 2956 is mutated to U, changing glutamine 881 (CAG: Q) into a stop codon (UAG). In dtr\textsuperscript{te370} C 3073 is mutated to U, changing glutamine 920 (CAG: Q) into a stop codon (UAG). (C) Schematic representation of zebrafish and mouse Gli1 and Gli2 protein sequences showing the positions of the stop codons (arrowheads) in the zebrafish mutant alleles. The position corresponding to the site of cleavage that results in a repressor form of Ci is shown by an arrow. Red boxes indicate regions shared among all three sequences, green boxes indicate sequences shared in mouse and zebrafish Gli1 (with percentage amino acid identity indicated), while gray boxes show sequences shared between zebrafish and mouse Gli2 (with percentage amino acid identity indicated). The zinc finger region is marked by ZnFn. Blue box shows region of homology to the VP-16 activator domain, asterisks indicate potential PKA phosphorylation sites. (D) Cladogram showing similarity of mouse (m), frog (Xn) and zebrafish (zf) Gli sequences. Tree is based on ClustalW alignment of amino acid sequences. A search of zebrafish EST databases and genomic trace sequences using mouse Gli1 sequence did not reveal a sequence more similar than the zebrafish Gli1 sequence shown above.

Fig. 4. Activity of Gli1, Gli2 and mutant Gli proteins in MNS70 cells. (A) Schematic of effector and reporter genes co-transfected into MNS70 cells. Different gli constructs were expressed under the control of a CMV promoter. Luciferase activity is induced in a reporter containing 8Gli protein binding sites from the mouse HNF3β floor plate enhancer (see Sasaki et al., 1999). (B) pcDNA constructs encoding mouse Gli1 (mGli1) and mouse Gli2 (mGli2) both activate the luciferase reporter. A pcDNA construct encoding full-length zebrafish Gli1 (zfGli1) activates luciferase activity, while pcDNA constructs encoding zebrafish Gli2 (zfGli2) or the dtr/gli1 (tm276, te370, ts269) or you/gli2 (ty119, ty17) mutations show no activation. When co-transfected with full-length gli1, dtr\textsuperscript{tm276} (but not dtr\textsuperscript{ts269} or dtr\textsuperscript{te370}) enhances reporter gene activation by wild-type Gli1. In contrast, co-transfection of gli1 with constructs encoding full-length Gli2 or the C-terminally truncated you alleles results in the elimination of Gli1 mediated transcriptional activation. Transfection with a pJT4 plasmid encoding Shh activates luciferase activity. Co-transfection with pcDNA-zfGli1 and pJT4-shh has a roughly additive effect on luciferase activity. Co-transfection of pcDNA-gli2 with pJT4-shh reduces the luciferase activity induced by Shh alone. Averaged results of 2 experiments with standard errors. Relative luciferase activities are indicated by bars while protein schematics at top show the sites of the mutations encoded by each gli mutant construct. (C) Western analysis showing Gli proteins produced in cell culture. Asterisks indicate bands of predicted size for each transfected construct.
indicate that Hh signaling is sufficient to induce *gli1* gene expression. To test if Hh signaling is required for *gli1* transcription in zebrafish, as it is in mouse (Bai et al., 2002), we examined the expression of *gli1* in the *smoothed* mutant slow-muscle-omitted (*smu*) (Chen et al., 2001; Varga et al., 2001). *smu/smo* mutations severely block Hh signaling (Barresi et al., 2000). *gli1* expression is strongly, but not completely, reduced in *smu/smo* mutant embryos (Fig. 7B,C), indicating that Hh signaling is required for the full activation of *gli1* transcription. To determine whether *gli1* expression present in *smu/smo* mutant embryos is due to Hh signaling that results from maternal Smoothed function, we treated embryos with the alkaloid cyclopamine from the 2-cell stage throughout embryogenesis. Cyclopamine is thought to completely block Hh signaling at the level of Smoothened (Taipale et al., 2000). Low levels of *gli1* expression seen in cyclopamine-treated embryos were identical to those seen in *smu/smo* mutants, suggesting that Smoothened-mediated Hh signaling is not necessary to initiate weak *gli1* expression (Fig. 7E-G). These data suggest that low level *gli1* expression is independent of Hh signaling, and that *gli1* transcription becomes fully activated by Hh signals to mediate its effect on Hh target genes.

**Zebrafish *yot* alleles encode dominant repressor forms of Gli2**

The finding that *gli1* is essential in zebrafish, but not in mouse, prompted us to extend our studies to determine the role of *gli2* in zebrafish. We first tested the activity of the previously identified *yot/gli2* mutations. The two available *yot/gli2* alleles contain point mutations that introduce premature stop codons in the C-terminus of the protein (Karlstrom et al., 1999). We speculated that these *yot/gli2* alleles might encode repressors of Hh signaling, because of the weak dominant muscle phenotype seen in *yor*/+ embryos (van Eeden et al., 1996b) and the similarities between these truncated proteins and cleaved Gli proteins known to act as repressors of Hh signaling (Ruiz i Altaba, 1999; Sasaki et al., 1999; Shin et al., 1999; von Mering and Basler, 1999; Aza-Blanc et al., 2000). In support of this hypothesis, co-transfection of the C-terminal *yor/gli2* truncations with *gli1* abolished Gli1-mediated transcriptional activation in cell culture (Fig. 4B). In contrast, transfection of wild-type *gli2* only partially blocks transcriptional activation by Gli1 or Shh (Fig. 4B). The *yor* repressor hypothesis also predicts that the *yor/gli2* phenotype can be partially rescued, rather than phenocopied, by blocking the generation of the mutant proteins. Indeed, injection of *gli2* MOs into *yor*/+ embryos effectively rescued *nk2.2* expression and partially suppressed defects in *myoD* expression (Fig. 8, Table 2; see below). These data provide evidence that the C-terminally truncated *gli2* proteins encoded by *yor*/+ and *yor*+/*yor* are potent repressors of Hh target genes.

Based on the repressive effects of C-terminally truncated Gli2 on Gli1-mediated activation in vitro, it is conceivable that *yor* also interferes, at least in part, with Gli1 function in vivo. This model predicts that *yor/gli2* and *dtr/gli1* might genetically interact. Indeed, we found that *yor*+/*yor*; *dtr*+/*dtr* embryos display

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**Table 2. *gli2* MO injections into *yor*+/*yor* crosses**

<table>
<thead>
<tr>
<th>gli2 MO injected</th>
<th>yor+ phenotype</th>
<th>yor+ phenotype</th>
<th>Wild-type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(wt <em>nk2.2</em>, no <em>myoD</em>)</td>
<td>(wt <em>nk2.2</em>, no <em>myoD</em>)</td>
<td>(wt <em>nk2.2</em>, no <em>myoD</em>)</td>
<td></td>
</tr>
<tr>
<td>Uninjected siblings*</td>
<td>62 (23%)</td>
<td>141 (51%)</td>
<td>73 (26%)</td>
<td>276</td>
</tr>
<tr>
<td>1 ng</td>
<td>3 (5%)</td>
<td>15 (24%)</td>
<td>45 (71%)</td>
<td>63</td>
</tr>
<tr>
<td>3-7 ng</td>
<td>0</td>
<td>18 (23%)</td>
<td>61 (77%)</td>
<td>79</td>
</tr>
<tr>
<td>10-15 ng</td>
<td>0</td>
<td>25 (14%)</td>
<td>148 (86%)</td>
<td>173</td>
</tr>
</tbody>
</table>

*nk2.1b* reduced in di. Partial *nk2.1b* rescue wt *nk2.1b*

Uninjected | 26 (30%) | 0 | 60 (70%) | 86 |

6 ng | 0 | 8 (21%) | 30 (79%) | 38** |

10 ng | 0 | 1 (2%) | 39 (98%) | 40** |

#: reduced; di: diencephalon.

*Expected genotypes: 25% *yor*+/*yor*, 50% *yor*+/*yor*, 25% homozygous wild type.

**All *gli2* MO-injected embryos showed expanded *nk2.1b* in the telencephalon.
phenotypes indicative of Hh signaling defects. Double heterozygotes have somite defects and curved body axes (Fig. 1D), reduction of nk2.2 and ptc1 expression (Fig. 1H, L) and expansion of pax6 expression (Fig. 1P). These phenotypes are more severe than in dtr/gli1 embryos, but less severe than in yol/gli2 embryos. Taken together, these results indicate that C-terminally truncated Gli2 proteins interfere with Hh signaling, in part by antagonizing Gli1.

Minor roles for full-length Gli2 in the activation of Hh target genes

Whereas the C-terminal truncation alleles of gli2 provide information about the effect of dominant repressors on Hh signaling in vivo, they do not address the requirement for Gli2 during embryonic development. Therefore, we characterized the phenotypes generated by injecting gli2 MOs into wild-type embryos. Surprisingly, knock down of Gli2 in wild-type embryos did not lead to significant defects in most structures affected by Hh signaling. In particular, ventral CNS (ptc1, nk2.2, fkd4) and somite (myoD) markers were expressed normally (Fig. 8A, D, G). In some embryos, ptc1 and fkd4 expression was slightly expanded (Fig. 8A and data not shown).

Previous studies have suggested that full-length Gli2 is a Hh-dependent activator of Hh target genes (Ding et al., 1998;
Matise et al., 1998; Ruiz i Altaba, 1998; Ruiz i Altaba, 1999; Aza-Blanc et al., 2000; Bai and Joyner, 2001), and that the C-terminus of Gli2 is required for this activity (Ruiz i Altaba, 1999; Sasaki et al., 1999). gli2 MO injection into wild-type embryos might still allow for some, albeit reduced, generation of full-length Gli2. We therefore analyzed in more detail embryos that produce no full-length Gli2 and express reduced levels of C-terminally truncated Gli2 by injecting gli2 MO into yot/gli2 mutants. Intriguingly, Hh targets in the nervous system such as ptc1 (Fig. 8C), nk2.2 (Fig. 8F) and fkd4 (not shown) are robustly expressed. These results suggest that full-length Gli2 is not required for Hh signaling in the zebrafish spinal cord.

The limited requirement for full-length Gli2 might be due to redundancy with other gli genes. To test if Gli2 and Gli1 have overlapping roles, we injected gli2 MOs into dtr/gli1 mutants (Fig. 8). Like dtr/gli1 mutants, these embryos display defects in nk2.2 expression in the brain and floor plate (Fig. 8K). Interestingly, a tegmental patch of nk2.2 expression that remains in dtr/gli1 mutants is eliminated by injection of gli2 MOs, suggesting Gli2 may act as an activator of Hh signaling in this region (Fig. 8K). In addition, myoD expression in adaxial cells is slightly but consistently reduced in gli2 MO; dtr/gli1 embryos (Fig. 8M), revealing overlapping roles of Gli1 and Gli2. Taken together, these data suggest that Gli2 plays a minor role in activating Hh target genes and is partially redundant with Gli1.

**Gli2 acts as a repressor of telencephalic nk2.1b expression**

Previous studies (Ruiz i Altaba, 1998; Sasaki et al., 1999; von Mering and Basler, 1999; Aza-Blanc et al., 2000) and our cell culture and in vivo data (Figs 4 and 8) indicate that Gli2 can act as a repressor of Hh target genes. In support of this, we found that in gli2 MO-injected embryos, expression of nk2.1b was expanded dorsally in the telencephalon and ventrally in the ventral diencephalon (Fig. 9A,B). This contrasts with the dramatic reduction in nk2.1b expression seen upon loss of Hh signaling in smu smo mutants (Fig. 9E). The expansion of nk2.1b expression caused by loss of Gli2 function is Gli1-independent, since gli2 MO injection into dtr/gli1 mutants leads to an expansion of nk2.1b in the ventral telencephalon (Fig. 9H). This suggests that one role of Hh signaling might be to overcome Gli2-mediated repression of nk2.1b. In this scenario, blocking Gli2 function should partially suppress the loss of nk2.1b in smu smo mutants. Indeed, injection of gli2 MO into smu smo mutants partially restored nk2.1b expression in the ventral telencephalon (Fig. 9F). These results suggest that Gli2 acts as a Hh-independent repressor of some Hh target genes.

**DISCUSSION**

**Essential role for gli1 in zebrafish Hh signaling and embryogenesis**

Our studies have identified a novel zebrafish gli gene that is orthologous to Gli1, based on three lines of evidence. First, zebrafish gli1 shares highest sequence similarity with frog and mouse Gli1. Second, both zebrafish and mouse Gli1 act as transcriptional activators in a cell culture assay. Third, both genes are expressed in regions in which the Hh signaling pathway has been activated. Moreover, Hh signaling is not only sufficient but also necessary for normal gli1 transcription in zebrafish and mouse (Bai et al., 2002). Interestingly, very weak expression of gli1 is still detected in the absence of Hh signaling in zebrafish, while no Gli1 gene expression has been detected in mouse Smo mutants.

Our analyses reveal that gli1 is disrupted in dtr mutants and indicate that dtr<sup>370</sup> and dtr<sup>pl269</sup> encode strong or complete loss-of-function versions of Gli1. The dtr<sup>370</sup> and dtr<sup>pl269</sup> alleles lack a C-terminal activation domain and are inactive in cell culture, consistent with results obtained upon overexpression.
of C-terminally truncated Gli1 in frog (Ruiz i Altaba, 1999). In vivo, dtr\textsuperscript{ts269} mutants are impaired in the upregulation of nk2.2 expression in response to ectopic Hh signaling in most regions of the CNS. In contrast to truncated zebrafish Gli2, truncated zebrafish Gli1 does not appear to act as a dominant repressor of Hh signaling; dtr\textsuperscript{-/+} embryos do not display any obvious phenotypes and truncated Gli1 does not interfere with gene activation by wild-type Gli1 in cell culture. Moreover, gli1 MO injection phenocopies dtr\textsuperscript{ts269} and dtr\textsuperscript{tm276} mutants. Taken together, these results suggest that these mutants and gli1 MO embryos lack all or most Gli1 activity.

The third point mutation (dtr\textsuperscript{tm276}) affects a conserved tyrosine residue in the DNA binding region of Gli1 known to contact target DNA (Pavletich and Pabo, 1993). On its own, this protein does not activate reporter gene expression in cultured cells, consistent with a potential defect in DNA binding. Interestingly, however, dtr\textsuperscript{tm276} activates transcription in the presence of wild-type Gli1. It is conceivable that the mutant protein forms a complex with the wild-type protein, thus being recruited to DNA and providing a transcriptional activation domain.

Together with previous studies (Brand et al., 1996; Karlstrom et al., 1996; Chandrasekhar et al., 1999; Odenthal et al., 2000), our results reveal that loss of gli1 function leads

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**Table 3. Comparison of defects in gli mutant and MO-injected embryos**

<table>
<thead>
<tr>
<th>ptc1</th>
<th>nk2.2</th>
<th>nk2.1b</th>
<th>fkd4 lateral floorplate</th>
<th>myoD adaxial cells</th>
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<td>tem</td>
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\(\text{\textsuperscript{\textcircled{o}}}\text{; reduced; }\text{\textsuperscript{\textcircled{\textbullet}}}; \text{strongly reduced; }\text{\textcircled{\textdagger}}; \text{increased/expanded; }\text{di}; \text{diencephalon; }\text{tel}; \text{telencephalon; }\text{mb}; \text{midbrain; }\text{nd}; \text{not determined; }\text{vent}; \text{ventral; }\text{wt}; \text{wild type.} \)
to ventral CNS patterning defects in zebrafish (summarized in Table 3). dtr/gli1 mutants lack the lateral floor plate and show reduced expression of markers for anterior pituitary and ventral diencephalon. These neural patterning defects are similar to, but weaker than those seen in smu/smo mutants or cyclopamine-treated embryos. For example, smu/smo mutant embryos show a more severe loss of ventral diencephalon and strong to complete reduction of ptc1, nk2.2, and nk2.1b expression (Chen et al., 2001; Varga et al., 2001). In addition, dtr/gli1 mutants appear normal with respect to somite development, pectoral fin formation and dorsal aorta differentiation, whereas smu/smo mutants show severe defects in these structures. These data indicate that gli1 is necessary for ventral CNS patterning, but that it is required in only a subset of cells responding to Hh signals.

Roles of Gli2 in zebrafish embryogenesis

Our results suggest that zebrafish Gli2 does not play a major role in the activation of Hh target genes in the CNS. For instance, floor plate marker expression is normal (or slightly expanded) and motor neurons develop in gli2 MO-injected embryos (Table 3 and data not shown). It is conceivable that gli2 MO injection reduces Gli2 protein levels insufficiently, allowing enough Gli2 activator to be made to mediate floor plate development. However, gli2 MO injection into yot/gli2 mutants also allows for floor plate development, rescuing floor plate defects caused by the truncated Gli2 proteins (Table 3). In this case, no full-length Gli2 (the putative activator form of Gli2) can be generated.

The phenotypic similarity between gli2 MO; dtr/gli1 and dtr/gli1 mutants also suggests a limited role for Gli2. Some overlapping functions of Gli1 and Gli2 are indicated by the reduction in myoD expression in somitic mesoderm and nk2.2 in the tectum in gli2MO; dtr/gli1 embryos. Overlapping roles of gli1 and gli2 are also evident in the loss of engrailed-expressing muscle cells upon reduction of both Gli1 and Gli2 (C. Wolff, S. Roy and P. Ingham, personal communication). These results suggest that Gli2 contributes as a positive mediator of Hh signaling to the activation of some Hh target genes. In contrast, telencephalic nk2.1b is expanded in gli2 MO embryos and expressed at reduced levels in smu/smo mutants. Blocking both Gli2 and Smo partially suppresses the smu/smo phenotype, indicating that Hh signaling relieves Gli2-mediated repression of nk2.1b. Importantly, neither expression nor expansion of nk2.1b are Gli1 dependent, indicating that Hh signaling might directly inhibit Gli2-mediated repression of nk2.1b. Taken together, these results suggest that zebrafish Gli2 can act as a Hh-dependent activator.

C-terminal truncations of Gli2 block Hedgehog signaling

Our results suggest that the C-terminally truncated Gli2 proteins encoded by yot/gli2 alleles encode dominant repressors of Hh signaling. In vitro, the truncated forms of Gli2 block Gli1-mediated transcriptional activation, resembling the activity of C-terminally truncated mouse and frog Gli2 proteins.
in the repression of floor plate markers. Although our results provide evidence for an essential role of zebrafish Gli2 in nk2.1b repression, this activity of Gli2 is not simply achieved by repressing Gli1, as proposed in Xenopus. In addition, we have found no evidence for a requirement of Gli2 in motor neuron induction or early mesoderm patterning. The apparent differences between zebrafish and Xenopus gli gene function might be due to species-specific roles. Alternatively, they might reflect the difficulty of comparing results gained in studies that test the requirement for gene function using loss-of-function approaches with studies that assign potential gene functions using gain-of-function strategies. Further clarification of the potential differences in zebrafish and Xenopus Gli function will require loss-of-function approaches in frog and gain-of-function studies in zebrafish.

**Comparison to mouse**

Our analyses in zebrafish suggest surprisingly divergent requirements for Gli1 and Gli2 in zebrafish and mouse. Genetic studies in mouse have shown that Gli1 is dispensable for development, whereas Gli2 is a major mediator of Hh signaling during neural development (Matise et al., 1998; Park et al., 2000; Bai and Joyner, 2001). Two lines of evidence suggest that mouse Gli2 acts predominantly as a transcriptional activator of Hh target genes. First, replacing Gli2 with Gli1 in a knock-in approach results in normal development (Bai and Joyner, 2001). Second, Shh;Gli2 double mutants have the same phenotype as Shh mutants (Bai and Joyner, 2001). These results suggest that Shh signaling requires Gli2 to activate Hh-regulated genes and does not de-repress Hh target genes by counteracting a putative Gli2 repressor form. In clear contrast to these conclusions, zebrafish Gli1 is an essential activator of Hh target genes during neural development, while Gli2 appears to have only minor activator roles and acts as a repressor of the Hh target gene nk2.1b in the telencephalon. It is unlikely that these differences are simply the result of allele variations. In the case of Gli1, strong (dtr^269, dtr^2370, gli1 MO) or even partial (dtr^m279) loss of Gli1 function results in nervous system defects not seen in mouse Gli1 null alleles. In the case of Gli2, loss of a putative activator form of Gli2 or partial reduction of Gli2 activity does not result in the CNS phenotypes attributed to the loss of an activator form of Gli2 in mouse.

The differences between orthologous gli genes are surprising in light of the overall conservation of sequence, expression, regulation and transcriptional activity in cell culture. Both overlapping functions of gli genes and subtle differences in Gli activity or expression might underlie the divergent requirements. In the case of gli2, it is possible that another gli gene compensates for reduction in Gli2 activity. For instance, Gli2 and Gli3 have partially overlapping roles in mouse foregut, tooth and skeletal development (Mo et al., 1997; Motoyama et al., 1998). It is thus possible that another Gli protein masks the role of Gli2 in zebrafish development. gli2 MO injection into dtr/gli1 mutants leads to only a minor enhancement of the dtr/gli1 mutant phenotype, suggesting that a gli gene other than gli1 might compensate for reduction in Gli2 activity.

We speculate that one of the major roles of Gli1 is to act as an amplifier of vertebrate Hh signaling. In this model, Gli1 activity is required in zebrafish, but not in mouse, because Hh target genes are insufficiently activated by initial Hh signaling.
in zebrafish. This model is based on the kinetics of gli gene activation. It has been shown that Gli1 is a transcriptional target of Hh signaling (Epstein et al., 1996; Marigo et al., 1996a; Lee et al., 1997; Dai et al., 1999) and thus acts as a delayed activator of Hh targets. In contrast, Gli2 and Gli3 protein activity can be post-translationally regulated (Ruiz i Altaba, 1999; Sasaki et al., 1999; von Mering and Basler, 1999; Aza-Blanc et al., 2000; Wang et al., 2000; Bai et al., 2002) and in the case of Gli3 has been shown to be directly modulated by Hh signaling (von Mering and Basler, 1999; Aza-Blanc et al., 2000; Wang et al., 2000). Hence, Hh signaling is thought to be initially mediated by Gli2 and Gli3, leading to the activation of downstream genes such as Gli1 and Ptc1 (Ingham and McMahon, 2001). Subsequently, Hh signaling can be maintained or amplified by Gli1. In some contexts, this amplification might be essential for full activation of Hh target genes. This model suggests that in the zebrafish CNS, the initial activation of Hh target genes by Gli2, Gli3 or other Gli proteins might be quite weak or short lived, requiring further enhancement by Gli1. In contrast, in the mouse CNS, Hh-mediated modulation of Gli2 and Gli3 activity is sufficient for Hh target gene activation. Interestingly, in Gli1–/–;Gli2–/+ mice, reduction of the levels of Gli2 leads to a requirement for Gli1 in Hh target gene activation (Park et al., 2000; Bai et al., 2002). According to the Gli1 amplifier model, Gli1 becomes essential because initial Hh-mediated signaling by Gli2 is weaker in Gli2–/+ than wild-type embryos. In this scenario, Gli2–/+ mouse embryos resemble zebrafish wild-type embryos, requiring Gli1 for full Hh target gene activation. It is conceivable that direct mediators of Hh signaling are less potent or expressed at lower levels in zebrafish than mouse or negative regulators might be more active or more highly expressed in zebrafish than mouse. In both cases, Gli1-mediated amplification would be required to allow full Hh target gene activation in zebrafish.

We thank Eric Jacobson for help with gli1 sequence analysis, Rory Feeney for fish care, Jiaojiao Zhang and Fiza Singh for technical assistance, the zebrafish community for providing probes and reagents, Rebecca Burdine, Gerd Fishell, Eric Jacobson, Alex Joyner and Debbie Yelon for comments on the manuscript, and Christian Feeney for fish care, Jiaojiao Zhang and Fiza Singh for technical assistance.

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