Development 130, 1549-1564

doi:10.1242/dev.00364

Genetic analysis of zebrafish *gli1* and *gli2* reveals divergent requirements for *gli* genes in vertebrate development

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Accepted 14 November 2002

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

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 target genes 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,

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 Hedgehog 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 $Gli1^{-/-};Gli2^{-/+}$ mice show ventral patterning defects not found in $Gli2^{-/+}$ 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* (*syu*), 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 chiasm 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^{tm276}, dtr^{te370} and dtr^{ts269}) were identified

previously in mutant screens (Brand et al., 1996; Karlstrom et al., 1996; van Eeden et al., 1996b). For mapping, dtr^{ts269} 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^{ts269}) allele. Other mutant strains used were *smooth muscle omitted* (smu^{b641}) and *you-too* (yot^{ty119}, yot^{ty17}).

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 from 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 *dtr*. Finer mapping, using embryos obtained from pairwise matings of heterozygous *dtr* parents in a WIK background, identified two other closely linked markers (z4910, z4950). The 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^{ts269} wild-type siblings; (2) dtr^{ts269} mutants; (3) dtr^{te370} mutants; and (4) dtr^{tm276} 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^{ts26} fish, a mutant-specific reverse primer designed for the dtr^{ts269} allele (ts269Mu.rv: 5′-TGGGATCATGTTGCCCA) was used with a forward primer (dtr8.fw: 5′-GTCTAAAGGCTAAATATGCAGC) to amplify a mutant-specific 560 bp product from homozygous mutants and heterozygotes. A wild-type reverse primer (ts269Wt.rv: 5′-TGGGATCATGTTGCCCG) served as an amplification control. To genotype yot^{ty17} fish, two primers flanking the mutation site (yot33.fw: 5′-CCACCTAGCATATCAGAGAAC, yot28.rv: 5′-CTTGCTCACCGATATTCTGAC) were used to amplify a 589 bp product which was then digested using the *Nla*IV restriction enzyme. The yot^{ty17} mutation eliminates a *Nla*IV 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 *Bam*HI using the T7 promoter. Other probes used were zebrafish *gli2* (Karlstrom et al., 1999), *lim3* (Glasgow et al., 1997), *myoD* (Weinberg et al., 1996), *nk2.2* (Barth and Wilson, 1995), *shh* (Krauss et al., 1993), *ptc1* (Concordet et al., 1996) and *pax6* (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 uninjected 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 from 1-15 ng of MO diluted in 1× Danio solution (Westerfield, 1993). z*fgli1* (5′-CCGACACACCCGCTACACCCACAGT) and z*fgli2* MO (5′-GGATGATGTAAAGTTCGTCAGTTGC), and a random control MO (5′-CCTCTTACCTCAGTTACAATTTATA) 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 *yot/gli2* 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 *gli* gene in the pcDNA3.1-His cloning vector (Invitrogen) in combination with a reporter plasmid containing luciferase inserted downstream of 8×Gli 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 2×10^5 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 dtr^{ts269} and dtr^{te370} are more severe than dtr^{tm276} (R. O. K., unpublished results), we focused our analysis on dtr^{ts269}. 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

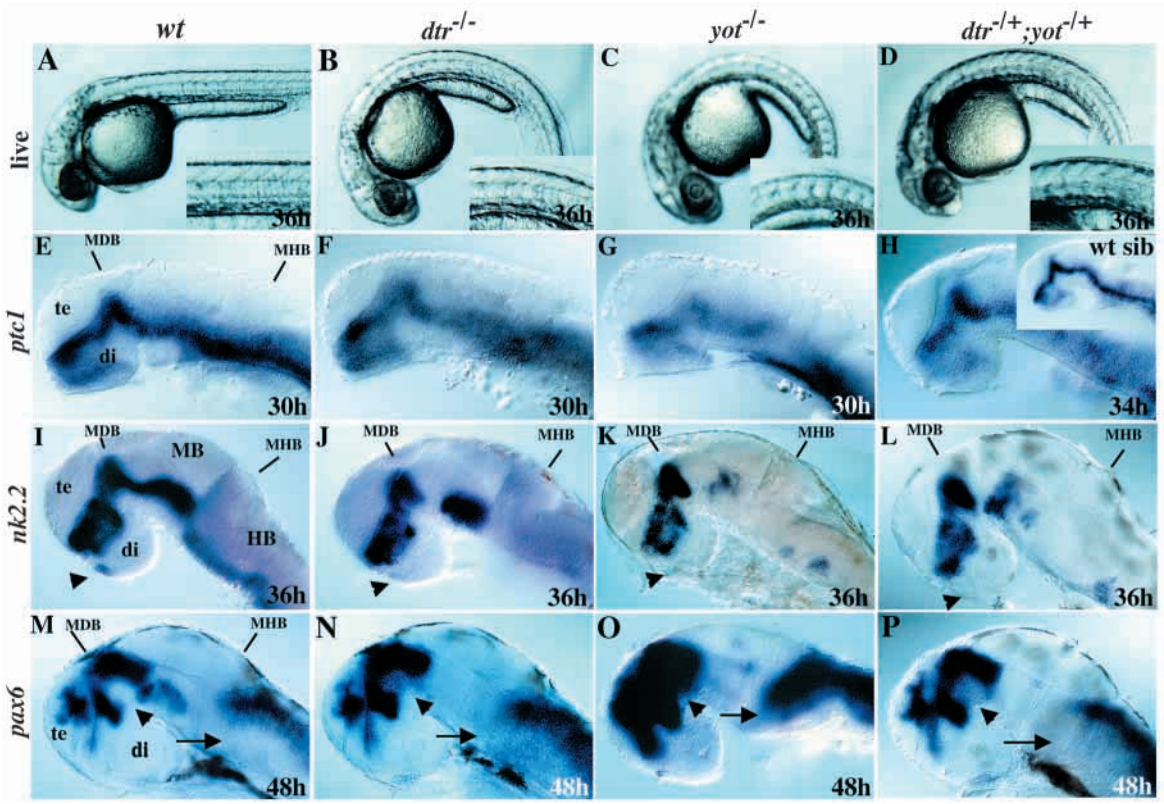

Fig. 1. $dtr^{-/-}$, $yot^{-/-}$ and $dtr^{-/+}$;$yot^{-/+}$ embryos have defects in body axis formation and expression of Hh target genes in the brain. (A-D) Examination of live 36-hour embryos reveals curled body axes in $dtr^{-/-}$, $yot^{-/-}$ and $dtr^{-/+}$;$yot^{-/+}$ mutant embryos. U-shaped somites, indicative of defects in slow muscle cell differentiation, are seen only in $yot^{-/-}$ and $dtr^{-/+}$;$yot^{-/+}$ embryos, $dtr^{-/-}$ 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.

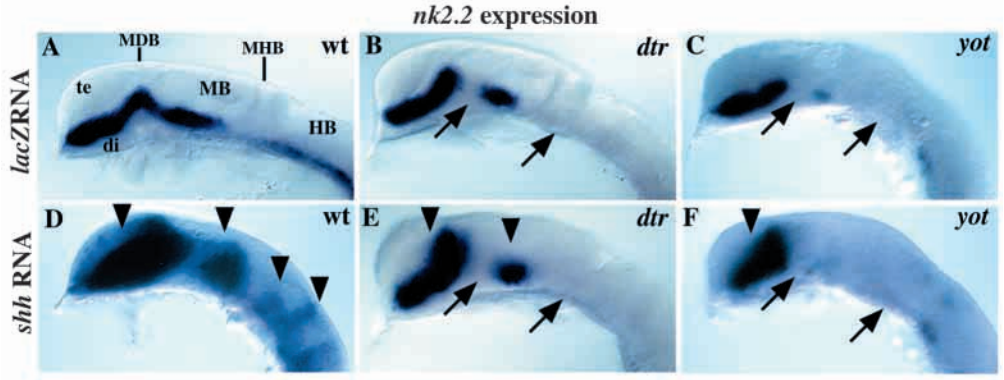

Fig. 2. Zebrafish *gli* mutations block Hh signaling. (A) Wild-type expression of the Hh target gene *nk2.2* is unaffected by injection of *lacZ* mRNA. (B,C) *nk2.2* expression is regionally absent in *dtr* and *yot/gli2* mutant embryos (arrows). (D) Injection of *shh* mRNA leads to an expansion of *nk2.2* throughout the CNS in wild-type embryos (arrowheads). (E,F) Over expression of *shh* does not activate *nk2.2* expression in defective regions of *dtr* and *yot/gli2* mutants (arrows), but *nk2.2* expression is expanded in unaffected regions (arrowheads). All panels show lateral views of 20-somite (19 hour) embryos, anterior to the left, eyes removed. 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 s*yu/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 (Fig. 3). Sequence analysis revealed point mutations in *gli1* in all three *dtr* alleles (Fig. 3B). Two of the identified point mutations (dtr^{te370} and dtr^{ts269}) introduce premature stop codons that are predicted to result in C-terminally truncated Gli1 proteins. The third point mutation (dtr^{tm276}) 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^{tm276}, but not dtr^{te370} and dtr^{ts269}, enhanced reporter gene activation by wild-type Gli1. Interestingly, despite its defective DNA binding domain, dtr^{tm276} 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-

Table 1. *gli1* MO injection into wild-type embryos phenocopies *dtr nk2.2* defects

gli MO injected	Very strong	*dtr* phenocopy	Partial phenocopy	wt *nk2.2*	Total
Control (6-13 ng)	0	0	0	150 (100%)	150
0.2-1 ng	0	0	44 (43%)	58 (57%)	102
2 ng	0	32 (60%)	14 (26%)	7 (13%)	53
3 ng	38 (26%)	48 (33%)	33 (23%)	17 (12%)	146
5-7 ng	37 (52%)	21 (30%)	13 (18%)	0	71
11 ng	37 (76%)	12 (24%)	0	0	49

Very strong: *nk2.2* extremely reduced in malformed embryos.
Partial phenocopy: *nk2.2* reduced but not absent.

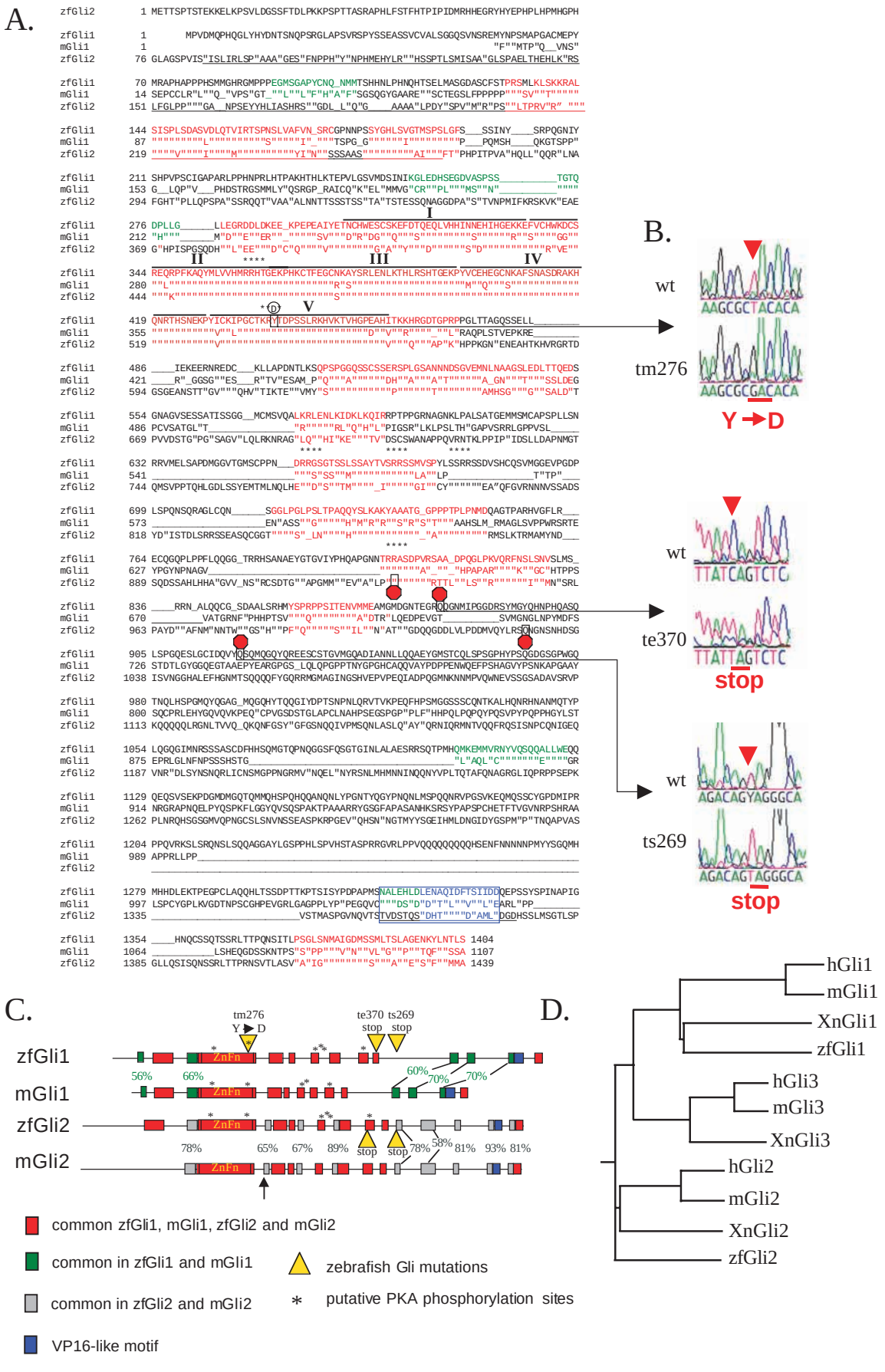
A.
B.
wt
tm276
Y→D
wt
te370
stop
wt
ts269
stop
C.
tm276
Y→D
te370 ts269
stop stop
zfGli1
mGli1
zfGli2
mGli2
ZnFn
56%
66%
60%
70%
70%
78%
65%
67%
89%
stop
stop
78%
58%
81%
93%
81%
common zfGli1, mGli1, zfGli2 and mGli2
common in zfGli1 and mGli1
common in zfGli2 and mGli2
VP16-like motif
zebrafish Gli mutations
* putative PKA phosphorylation sites
D.
hGli1
mGli1
XnGli1
zfGli1
hGli3
mGli3
XnGli3
hGli2
mGli2
XnGli2
zfGli2

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*^{tm276}, *dtr*^{te370} and *dtr*^{ts269}) and point mutations were found for each allele (boxes). The altered amino acid in *dtr*^{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*^{tm276} U 1633 is mutated to G, changing tyrosine 440 (UAC: Y) into an aspartic acid (GAC: D). In *dtr*^{ts269} C 2956 is mutated to U, changing glutamine 881 (CAG: Q) into a stop codon (UAG). In *dtr*^{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.

injected embryos (Fig. 5D-F). Taken together, these results establish that *dtr* disrupts *gli1* and demonstrate that Gli1 is essential for zebrafish development.

Regulation of *gli1* expression

Although the *gli1* loss-of-function phenotype in zebrafish is in marked contrast to the apparently normal phenotype of mouse *gli1* mutants, the regulation of *gli1* gene expression appears conserved (Fig. 6). Similar to mouse and frog *Gli1* (Hui et al., 1994; Hynes et al., 1997; Lee et al., 1997; Platt et al., 1997), zebrafish *gli1* is expressed in tissues responding to Hh signaling, including the ventral CNS, presomitic mesoderm, posterior fin buds and later in endodermal tissue (see Fig. 6 for details). As in other species, this expression closely parallels the expression of the Hh target gene *ptc1* (Concordet et al., 1996; Goodrich et al., 1996; Marigo et al., 1996b; Platt et al., 1997).

Ectopic Hh signaling is sufficient to activate *Gli1* expression in mouse, chick and frog (Epstein et al., 1996; Marigo et al., 1996a; Hynes et al., 1997; Lee et al., 1997; Ruiz i Altaba, 1998). Similarly, we find that *gli1* transcription is activated throughout the CNS by ectopic Shh expression (Fig. 7B). These results

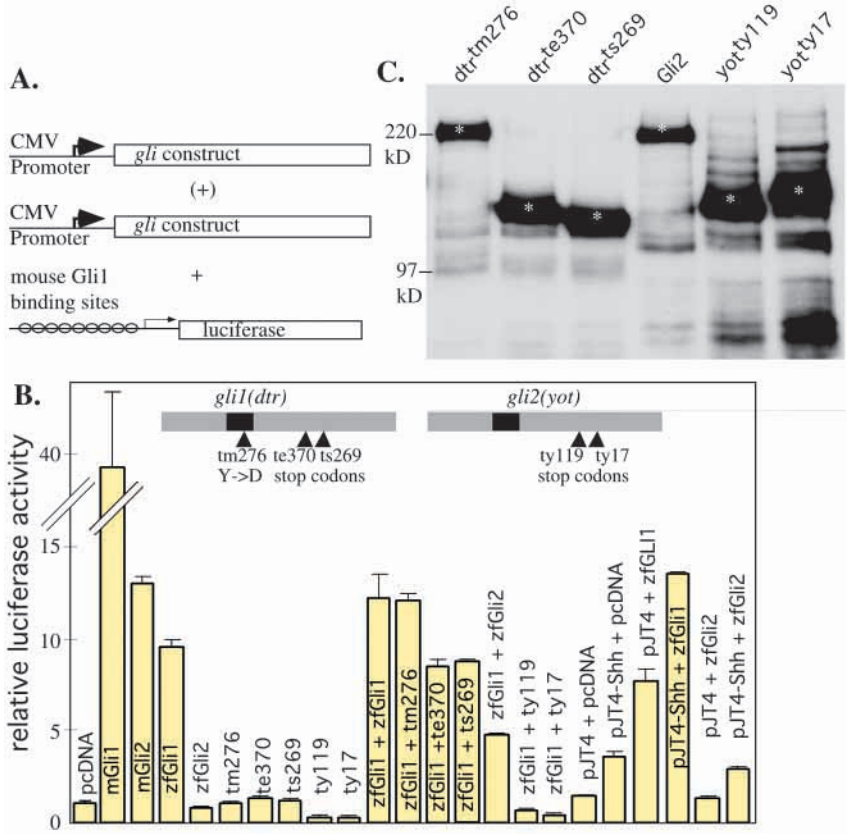

Fig. 4. Activity of Gli1, Gli2 and mutant Gli proteins in MNS70 cells. (A) Schematic of effector and reporter genes co-transfected into MNS70 cells. Different *gli* constructs were expressed under the control of a CMV promoter. Luciferase activity is induced in a reporter containing 8×Gli protein binding 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 *yot/gli2* (ty119, ty17) mutations show no activation. When co-transfected with full-length *gli1*, *dtr*^{tm276} (but not *dtr*^{te370} or *dtr*^{ts269}) 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 *yot* alleles result 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.

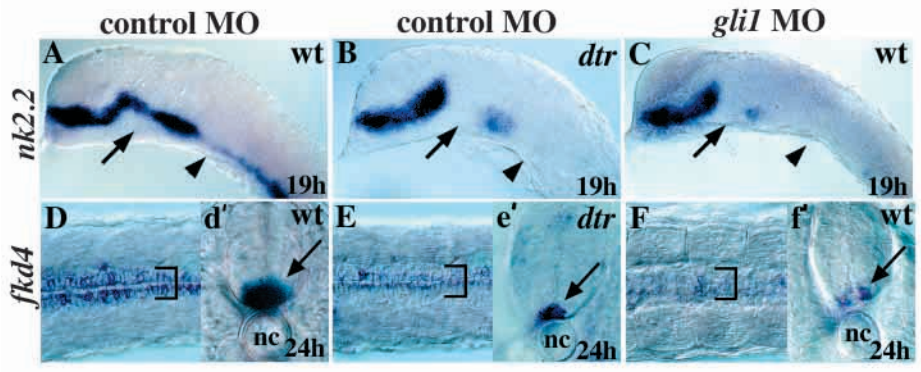

Fig. 5. *gli1* knockdown phenocopies *dtr/gli1*. (A) Expression of *nk2.2* is unaffected by injection of a control morpholino (MO). (B) *dtr/gli1* mutations eliminate *nk2.2* expression in some regions of the brain (arrow and arrowhead). (C) Injection of a *gli1*MO into wild-type embryos leads to a loss of *nk2.2* expression identical to that seen in *dtr/gli1* mutant embryos (compare arrows, see also Table 1). (D) Expression of *fkd4* in the medial and lateral floor plate is unaffected by control MO injections. (E) *fkd4* expression is extremely reduced in *dtr/gli1* mutants (compare bracket and arrow to those in D). (F) *fkd4* expression is similarly reduced in lateral floor plate cells after *gli1* MO injection (compare bracket and arrow to those in D). (d′, e′, and f′) show cross sections through the trunk at the level of the yolk plug. nc; notochord

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 *smoothened* 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 Smoothened 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 $yot^{-/+}$ 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 *yot/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 *yot* repressor hypothesis also predicts that the *yot/gli2* phenotype can be partially rescued, rather than phenocopied, by blocking the generation of the mutant proteins. Indeed, injection of *gli2* MOs into $yot^{-/-}$ 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 yot^{ty17} and yot^{ty119} 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 *yot* also interferes, at least in part, with Gli1 function in vivo. This model predicts that *yot/gli2* and *dtr/gli1* might genetically interact. Indeed, we found that $yot^{-/+}$; $dtr^{-/+}$ embryos display

Table 2. *gli2* MO injections into $yot^{-/+} \times yot^{-/+}$ crosses*

gli2 MO injected	$yot^{-/-}$ phenotype (⇩ *nk2.2*, no *myoD*)	$yot^{-/+}$ phenotype (wt *nk2.2*, ⇩ *myoD*)	Wild-type (*nk2.2*, *myoD*)	Total
Uninjected siblings*	62 (23%)	141 (51%)	73 (26%))	276
1 ng	3 (5%)	15 (24%)	45 (71%)	63
3-7 ng	0	18 (23%)	61 (77%)	79
10-15 ng	0	25 (14%)	148 (86%)	173
	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% $yot^{-/-}$, 50% $yot^{-/+}$, 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 *yot/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;

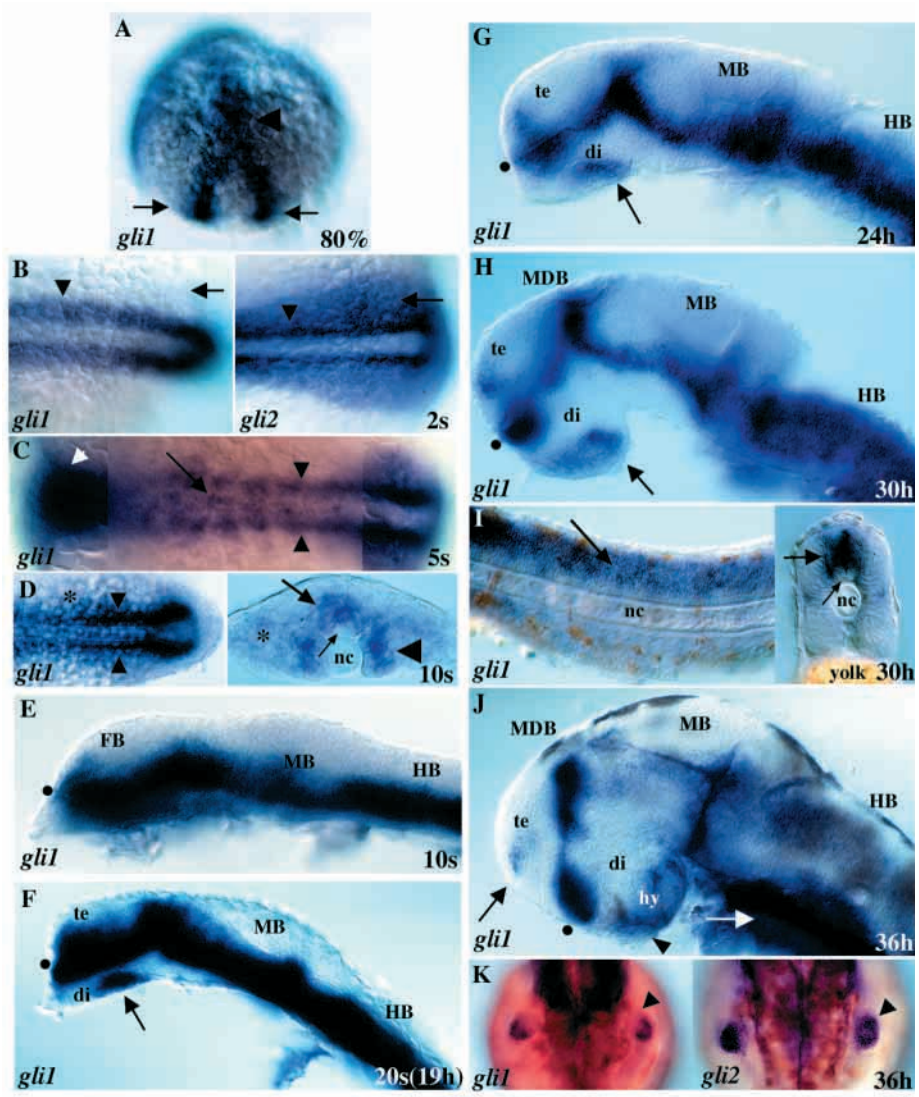

Fig. 6. Developmental expression of zebrafish *gli1*. (A) 80% epiboly. Transcripts for *gli1* are first detected in the anterior neural plate (arrowhead) and in pre-somitic mesoderm (arrows). (B) 2-somite stage. In the trunk, both *gli1* (left panel) and *gli2* (right panel) are expressed in adaxial cells (arrowheads) adjacent to the notochord. *gli1*, like *gli2*, is also expressed in paraxial mesoderm, with *gli2* expression extending more laterally (arrows). (C) 5-somite stage. *gli1* is expressed throughout the anterior neural plate (white arrowhead), in adaxial cells that give rise to slow muscle fibers (black arrowheads), as well as in the tailbud (out of focus). Some patchy expression is present in the developing spinal cord (arrow). (D) 10-somite stage, dorsal view (left) and cross section (right) of the trunk. *gli1* expression continues in adaxial cells (arrowheads) and spreads laterally into developing somites (asterisk). *gli1* is expressed ventrally in the spinal cord (larger arrow) but not in floor plate cells adjacent to the notochord (smaller arrow). (E-J) Lateral views of the brain, eyes have been removed. (E) 10-somite stage. *gli1* is expressed throughout the ventral forebrain, midbrain, hindbrain, and spinal cord (not shown). (F) 20-somite stage. In the brain, *gli1* is expressed in ventral regions in a pattern similar to that of *ptc1* (see Fig. 3). In the forebrain, *gli1* is primarily expressed in the diencephalon, but expression also extends into the ventral telencephalon dorsal to the optic recess (black dot). Expression is now absent in the ventral-most diencephalon, with the exception of a large patch in the posterior part of the developing hypothalamus (arrow). (G,H) 24 hours and 30 hours. *gli1* expression continues in the ventral CNS, including in the pre- and post-optic areas on either side of the optic recess (black dot) and in the patch in the posterior hypothalamus (arrow). (I) Expression in the trunk at 30 hours. *gli1* is strongly expressed in the spinal cord (arrows) and is more weakly expressed in somites. Cross section through trunk (right) shows spinal cord *gli1* expression (larger arrow) is absent from dorsal cells and ventral floor plate cells (smaller arrow). (J) 36 hours. By 36 hours, *gli1* is expressed predominantly along the diencephalon/telencephalon border and in the ventral hypothalamus, including the region of the anterior pituitary anlage (arrowhead). *gli1* is also expressed in a small patch in the telencephalon (arrow) and in endoderm (white arrow). (K) Expression in the fin bud at 36 hours. Both *gli1* (left) and *gli2* (right) are expressed in the pectoral fin buds (arrowheads). *gli1* expression is more limited than *gli2*, being predominantly in the posterior and distal mesenchyme, while *gli2* is expressed throughout the fin mesenchyme (compare arrowheads). (A-D) and (K) are dorsal views, (E-J) are lateral views. Anterior is to the left in all panels except (A) and (K), where anterior is up. di; diencephalon, FB; forebrain, HB; hindbrain, hy; hypothalamus, MB; midbrain, MDB; mid-diencephalon boundary, MHB; midbrain-hindbrain boundary, nc; notochord, te; telencephalon.

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.

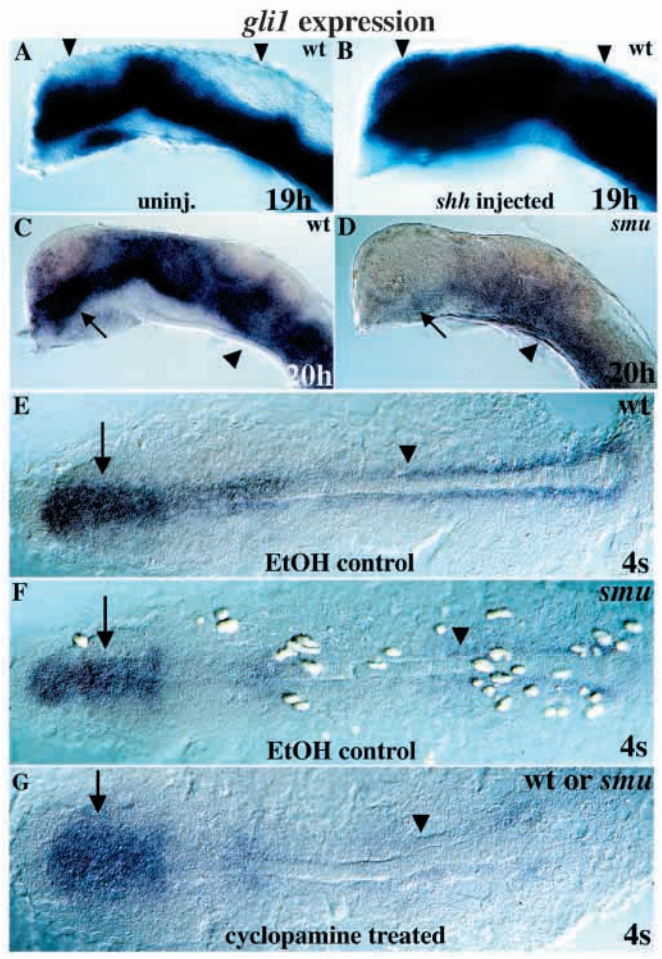

Fig. 7. Hh signaling regulates *gli1* expression. (A,B) Over expression of *shh* in wild type expands *gli1* expression dorsally throughout the embryo (compare arrowheads). (C,D) *gli1* expression is extremely reduced in Hh signaling-defective *smu/smo* mutant embryos relative to wild-type siblings, especially in the diencephalon (arrows). Some *gli1* expression remains in the ventral spinal cord and hindbrain (arrowheads). (E) Dorsal view of wild-type *gli1* expression in a 4-somite stage embryo; treated with ethanol (cyclopamine carrier). (F) In 4-somite stage *smu/smo* mutants, *gli1* expression is reduced in adaxial cells (arrowhead) and is less affected in the developing brain (arrows). (G) Similarly, cyclopamine treatment of wild-type or *smu/smo* embryos reduces but does not eliminate *gli1* expression. All 40 cyclopamine-treated embryos from a $smu^{-/+}$ incross showed the same *gli1* labeling pattern, indicating that the *smu/smo* mutation blocks Hh signaling as completely as cyclopamine, and that maternal *smu/smo* function is not responsible for low level *gli1* expression in *smu/smo* mutant embryos.

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 $dtr^{te370}$and dtr^{ts269} encode strong or complete loss-of-function versions of Gli1. The $dtr^{te370}$and dtr^{ts269} alleles lack a C-terminal activation domain and are inactive in cell culture, consistent with results obtained upon overexpression

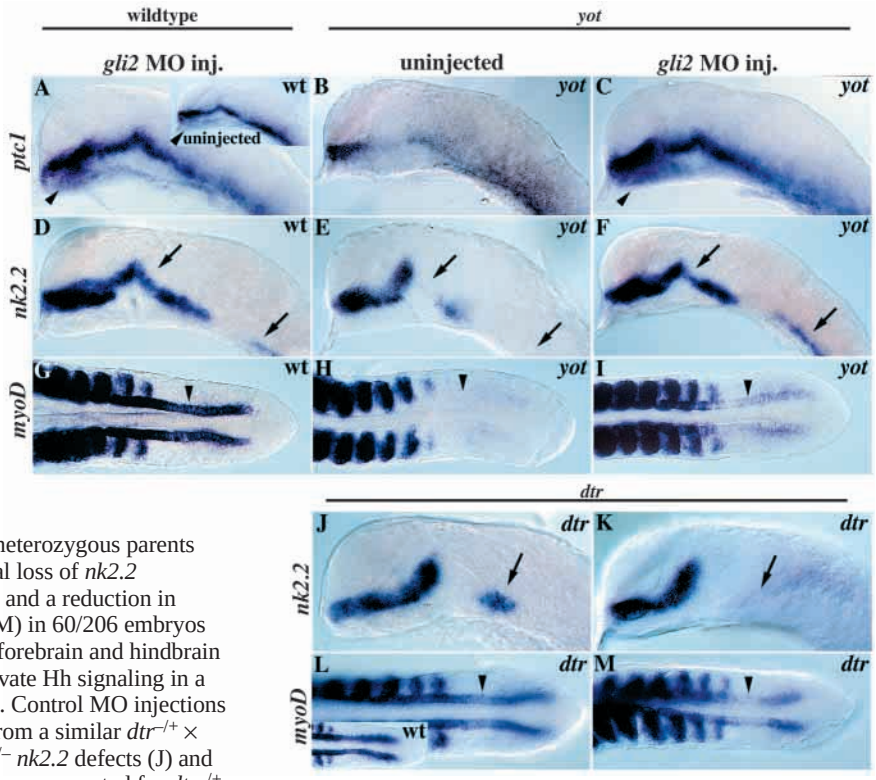

Fig. 8. *gli2* MO injection rescues *nk2.2* and *myoD* expression defects in *yot/gli2* mutants and reveals a weak activator role for Gli2. (A) *gli2* MO injection expands *ptc1* expression ventrally in the diencephalon (arrowheads) and causes a minor but consistent overall expansion of *ptc1* expression (compare to inset). (B) *yot/gli2* mutants have significantly reduced *ptc1* expression. (C) *gli2*MO injections rescue the *ptc1* defects seen in *yot/gli2* mutants and expand *ptc1* expression ventrally (arrowhead). (D) Injection of *gli2*MOs into wild-type embryos has no effect on *nk2.2* expression. (E,F) Injection of a *gli2*MOs into *yot*$^{-/-}$ mutant embryos can completely rescue *yot*-induced defects in *nk2*.2 expression (compare arrows). (G) *gli2*MO injection does not affect *myoD* expression in adaxial cells (arrowheads). (H,I) *gli2*MO injections partially rescue *yot*-induced defects in adaxial *myoD* expression (compare arrowheads). (J-M) Injection of 3-10 ng of *gli2*MO into embryos from a cross between *dtr*$^{-/+}$ heterozygous parents (25% *dtr*$^{-/-}$ mutants expected) resulted in an additional loss of *nk2.2* expression in the tegmentum (compare arrows in J,K) and a reduction in adaxial myoD expression (compare arrowheads in L,M) in 60/206 embryos (29%), all of which were *dtr*$^{-/-}$ mutants as judged by forebrain and hindbrain *nk2.2* expression defects. This suggests Gli2 may activate Hh signaling in a small area of the ventral midbrain and in adaxial cells. Control MO injections had no effect on *nk2.2* expression in 85/85 embryos from a similar *dtr*$^{-/+}$ × *dtr*$^{-/+}$ cross, with 25 embryos (29%) showing the *dtr*$^{-/-}$ *nk2.2* defects (J) and 60 embryos (71%) showing wild-type *nk2.2* expression as expected for *dtr*$^{-/+}$ and *dtr*$^{+/+}$ embryos. (A-F,J, and K) are lateral views of the head, eyes removed. (G-I,L, and M) are dorsal views of the tail region. All embryos are at the 20 somite (19 hour) stage. For *yot/gli2*, embryo genotypes were inferred by *myoD* expression in adaxial cells, then were verified by PCR (not shown, see Materials and Methods). D and G, E and H, F and I, J and L and K and M show the same individual labeled simultaneously with *nk2.2* and *myoD*.

of C-terminally truncated Gli1 in frog (Ruiz i Altaba, 1999). In vivo, *dtr*^{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*$^{-/+}$ 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*^{te370} and *dtr*^{ts269} mutants. Taken together, these results suggest that these mutants and *gli1* MO embryos lack all or most Gli1 activity.

The third point mutation (*dtr*^{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*^{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

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

	ptc1 vent. CNS	*nk2.2* forebrain	*nk2.1b* di.	*nk2.1b* tel.	*fkd4* lateral floorplate	*myoD* adaxial cells
dtr/gli1$^{-/-}$	⇩	⇩⇩	⇩	wt	⇩⇩	wt
gli1 MO	⇩	⇩⇩ (like *dtr*)	⇩	wt	⇩⇩	wt
yot/gli2$^{-/-}$	⇩	⇩⇩	⇩	wt	⇩⇩	⇩⇩
gli2 MO	wt/⇧	wt	wt/⇧	⇧	wt/⇧	wt
gli2 MO→*yot*$^{-/-}$	wt/⇧ (rescue)	wt (rescue)	wt (rescue)	⇧	wt/⇧ (rescue)	⇩ (rescue)
gli2 MO→*dtr*$^{-/-}$	nd	⇩⇩ (like *dtr*)	⇩ (like *dtr*)	⇧	nd	⇩
yot$^{-/+}$;*dtr*$^{-/+}$	⇩	⇩⇩*	nd	nd	⇩	⇩

⇩: reduced; ⇩⇩: strongly reduced; ⇧: increased/expanded; di: diencephalon; tel: telencephalon; mb: midbrain; nd: not determined; vent: ventral; wt: wild type.

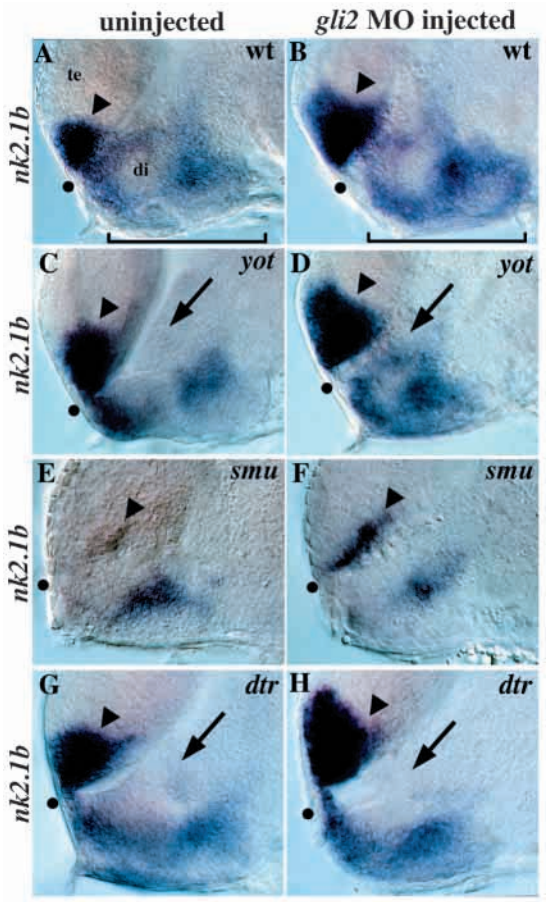

Fig. 9. Regulation of *nk2.1b* by *gli2*, *yot/gli2*, *smu/smo* and *dtr/gli1*. (A) *nk2.1b* is normally expressed in the anterior/ventral telencephalon (arrowhead) and in the diencephalon (bracket). (B) *gli2*MO injection into wild-type embryos leads to a dorsal expansion of telencephalic *nk2.1b* expression (arrowhead), as well as an increase in expression in the hypothalamus (compare brackets). This expansion was seen in 70/72 wild-type embryos injected with 10 ng of *gli 2*MO. (C) *yot*$^{-/-}$ embryos have reduced *nk2.1b* expression in the diencephalon adjacent to the first ventricle (arrow). (D) *gli2* MO injection into *yot*$^{-/-}$ embryos rescues the diencephalic *nk2.1b* expression defect (compare arrows in C and D, Table 2), and also leads to expanded expression in the telencephalon (compare arrowheads). (E) *nk2.1b* expression is extremely reduced in *smu/smo* mutants, with small patches of expression remaining in the diencephalon and telencephalon (arrowhead). (F) Injection of 10 ng of *gli2* MO into embryos from a cross of two *smu*$^{+/-}$ parents resulted in telencephalic *nk2.1b* expansion (arrowhead) in 89/89 embryos, including 18 *smu*$^{-/-}$ embryos (20%) and 71 wild-type and heterozygous siblings (80%). This shows that Gli2 repression of this Hh target gene is independent of Hh signaling. No *nk2.1b* expansion was detected in 49/49 embryos injected with 10 ng of control MO. (G) *dtr*$^{-/-}$ embryos have reduced *nk2.1b* expression in the diencephalon adjacent to the first ventricle (arrow) similar to the *yot/gli2* phenotype. (H) *gli2* MO injection does not rescue diencephalic *nk2.1b* expression in *dtr/gli1* mutants, but does expand *nk2.1b* expression in the telencephalon (arrowhead). Injection of 3-7 ng of *gli2* MO resulted in telencephalic *nk2.1b* expansion in 64/64 embryos, including 6 embryos (10%) that were clearly homozygous *dtr*$^{-/-}$ mutants based on diencephalic *nk2.1b* defects. The remaining 58 siblings (90%) also had expanded telencephalic *nk2.1b* expression. All panels show 30-hour embryos, lateral views of the forebrain, eyes removed, anterior to the left. All panel pairs show sibling embryos from the same experiment. Dot shows the optic recess, the anterior edge of the border between the diencephalon (di) and telencephalon (te).

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 tegmentum in *gli2*MO; *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

(Ruiz i Altaba, 1999; Sasaki et al., 1999). In vivo, *yot/gli2* mutations reduce Hh signaling (Karlstrom et al., 1999). Expression of Hh target genes such as *ptc1* and *nk2.2* is reduced and several structures that depend on Hh signaling (lateral floor plate, horizontal myoseptum, pectoral fins, dorsal aorta) do not form. Injection of *gli2* MO into *yot/gli2* embryos rescues most of the mutant phenotypes, demonstrating the antimorphic nature of the *yot/gli2* alleles. In addition, *yot/gli2* heterozygotes have subtle defects in somite patterning (van Eeden et al., 1996b; Karlstrom et al., 1999). These results suggest that zebrafish *yot/gli2* mutations turn Gli2 into a constitutive repressor of Hh-regulated genes. Precedence for this scenario has been provided by human *GLI3* mutations that result in C-terminally truncated repressor forms of GLI3 (Kang et al., 1997; Radhakrishna et al., 1997; Shin et al., 1999) and by the fact that truncated Gli proteins can act as dominant repressors in cell culture (Sasaki et al., 1999) or when ectopically expressed in embryos (Ruiz i Altaba, 1999).

Interestingly, embryos that are heterozygous for both *dtr/gli1* and *yot/gli2* have a phenotype that is intermediate between the two homozygous mutant phenotypes (Fig. 1). This result indicates that truncated Gli2 blocks Gli1-mediated activation of Hh targets and uncovers roles for *gli1* during somite development not revealed in *dtr/gli1* mutants. Gli1 cannot be the only factor antagonized by *yot*, since *yot/gli2* mutants have a more severe phenotype than *dtr/gli1* mutants.

The finding that truncated Gli2 acts as an in vivo repressor of Hh target genes has potential medical implications. Previous studies have shown that decreased Hh signaling can result in congenital defects such as holoprosencephaly (reviewed by Wallis and Muenke, 2000). Our results in zebrafish suggest that C-terminal truncations of Gli2 are candidates for the molecular basis of some cases of holoprosencephaly. In addition, C-terminally truncated Gli2 could be employed to repress the ectopic expression of Hh target genes in human cancers such as Basal Cell Carcinoma or medulloblastoma (reviewed by Ruiz i Altaba et al., 2002).

Species-specific roles of *Gli* genes

Vertebrate Gli function has been studied predominantly in *Xenopus* using gain-of-function approaches and in mouse using loss-of-function strategies. Our loss-of-function study in zebrafish suggests that *gli* genes might not have identical roles in all vertebrates.

Comparison to *Xenopus*

Based on mis- and overexpression studies, multiple roles for *Xenopus Gli* genes have been proposed. Gli1 has been considered to activate floor plate and motor neuron differentiation in the spinal cord and induce ventral cell types in the forebrain (Lee et al., 1997; Ruiz i Altaba, 1998; Ruiz i Altaba, 1999). Our results reveal an essential role for zebrafish Gli1 during lateral floor plate induction (Fig. 5) (Odenthal et al., 2000), but do not indicate a requirement in motor neuron induction (Brand et al., 1996) or telencephalic *nk2.1b* forebrain expression (Fig. 8). *Xenopus* Gli2 has been proposed to restrict floor plate development, repress *nk2.1b* expression in the forebrain, promote motor neuron formation and pattern mesoderm (Marine et al., 1997; Ruiz i Altaba, 1998; Ruiz i Altaba, 1999; Brewster et al., 2000; Mullor et al., 2001). Our studies reveal only a minor and variable role for zebrafish Gli2 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^{ts269}; dtr^{te370}; *gli1* MO) or even partial (dtr^{tm276}) 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, Gord Fishell, Eric Jacobson, Alex Joyner and Debbie Yelon for comments on the manuscript, and Christian Wolff, Sudipto Roy and Phil Ingham for communicating data prior to publication. This study was supported by NIH 1F32NS10185 (R.O.K.), NIH RO1NS39994 (R.O.K.), ACS IRG-93-033-07 (R.O.K.) and NIH RO1NS37801 (A.F.S.). A.F.S. is a Scholar of the McKnight Endowment Fund for Neuroscience, an Irma T. Hirschl Trust Career Scientist, and an Established Investigator of the American Heart Association.

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