

Zebrafish Gli3 functions as both an activator and a repressor in Hedgehog signaling

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Received for publication 1 July 2004, revised 13 September 2004, accepted 7 October 2004

Available online 13 November 2004

Abstract

Hedgehog (Hh) signaling regulates cell differentiation and patterning in a wide variety of embryonic tissues. In vertebrates, at least three Gli transcription factors (Gli1, Gli2, and Gli3) are involved in Hh signal transduction. Comparative studies have revealed divergent requirements for Gli1 and Gli2 in zebrafish and mouse. Here, we address the question of whether Gli3 function has also diverged in zebrafish and analyze the regulatory interactions between Hh signaling and Gli activity. We find that zebrafish Gli3 has an early function as an activator of Hh target genes that overlaps with Gli1 activator function in the ventral neural tube. In vitro reporter analysis shows that Gli3 cooperates with Gli1 to activate transcription in the presence of high concentrations of Hh. During late somitogenesis stages, Gli3 is required as a repressor of the Hh response. Gli3 shares this repressor activity with Gli2 in the dorsal spinal cord, hindbrain, and midbrain, but not in the forebrain. Consistently, zebrafish Gli3 blocks Gli1-mediated activation of a reporter gene in the absence of Hh in vitro. In the eye, Gli3 is also required for proper *ath5* expression and the differentiation of retinal ganglion cells (RGCs). These results reveal a conserved role for Gli3 in vertebrate development and uncover novel regional functions and regulatory interactions among *gli* genes.

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Keywords: Sonic hedgehog; Gli; Morphogen; Retinal ganglion cells; C17 cell line; Cyclopamine; Morpholino

Introduction

Secreted morphogens of the Hedgehog (Hh) family direct a wide range of developmental events, from segmentation and wing patterning in *Drosophila* to nervous system and limb patterning in vertebrates (reviewed in Ingham and McMahon, 2001). In the vertebrate neural tube, evidence

strongly supports the idea that Shh works as a long-range morphogen to direct cell fates in a concentration-dependent manner. Cells closest to the ventral source of Hh adopt ventral fates, while cells more distant from the Hh source acquire progressively more dorsal fates (Briscoe and Ericson, 1999; Ericson et al., 1997; Marti et al., 1995; Wijgerde et al., 2002).

Members of the Hh family bind to the transmembrane receptor Patched (Ptc) resulting in the derepression of another transmembrane protein Smoothed (Smo) that regulates the activity of Gli family transcription factors (reviewed in Ingham and McMahon, 2001). In *Drosophila*, Hh signaling regulates the function of the single Gli homolog, Cubitus interruptus (Ci). Ci can either activate or repress Hh signaling, depending on posttranslational mod-

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ifications of the protein. In the absence of Hh signaling, Ci is cleaved to produce an N-terminal repressor isoform that represses Hh target gene expression. Activation by Hh suppresses this cleavage, leading to a full-length activator form of the protein. At least three Gli proteins are found in vertebrates. In the mouse, these proteins mediate all Hh-dependent patterning in the neural tube (Bai et al., 2004). Like Ci, all three vertebrate Gli proteins have five highly conserved zinc finger DNA binding domains and C-terminal activation domains, with Gli2 and Gli3 also having N-terminal repressor domains (Dai et al., 1999; Sasaki et al., 1999).

Based on genetic and misexpression analyses, complex overlapping and unique functions for Gli1 and Gli2 have been shown. Mouse mutant studies have shown that Gli2 is an Hh-dependent activator of the Hh response in the nervous system, limbs, and lung and is essential for early development (Ding et al., 1998; Matisse et al., 1998; Mo et al., 1997; Motoyama et al., 1998). Mice lacking Gli2 function fail to form a floor plate and have ventral neural tube defects (Ding et al., 1998; Matisse et al., 1998). While Gli1 is transcriptionally up-regulated by Hh and can activate the Hh

response, mutant analyses in mouse show that Gli1 is not required for neural patterning or early development (Park et al., 2000). However, Gli1 can substitute for Gli2 in mouse development, indicating overlapping activator roles for these two Gli proteins (Bai and Joyner, 2001). The situation is different in zebrafish, where Gli1 is the major activator of Hh target genes and Gli2 plays both activator and repressor roles in different regions of the embryo (Karlstrom et al., 2003). Consistent with these mutant analyses, in vitro reporter assays and overexpression experiments in frog embryos also suggest that Gli1 is an activator of Hh target genes while Gli2 has both activator and repressor functions (Aza-Blanc et al., 2000; Mullor et al., 2001; Regl et al., 2002; Ruiz i Altaba, 1998; Sasaki et al., 1997).

Like Ci, Gli3 can be processed to form a repressor isoform, and this proteolytic processing is inhibited by Hh signaling (Litingtung et al., 2002; Wang et al., 2000). Gli3 also functions like Ci as both an activator and repressor of Hh signaling in vivo. In mouse, it appears that Gli3 is the only Gli protein that functions as a repressor. This repressor function is seen in loss-of-function mutants, which have phenotypes resembling those seen after Shh activation (Hui

Table 1
Morpholino injections: repressor roles for Gli3 and Gli2

Probe/assay	Injected cross	MO-injected	Great dorsal expansion	Dorsal expansion	Wild-type	Mutant phenotype	Total	
<i>fkd4</i> , 19 h	wt	uninjected	0	0	43 (100%)	0	43	
		<i>gli3</i> [10 ng]	0	0	30 (100%)	0	30	
		<i>gli2</i> [10 ng]	0	0	13 (100%)	0	13	
		<i>gli2</i> + 3 [5 + 5] ng	0	0	35 (100%)	0	35	
		<i>lacZ</i> RNA [65 pg]	0	0	34 (100%)	0	34	
		<i>shh</i> RNA [65 pg]	27 (77%)	4 (23%)	0	0	31	
		<i>dtr(gli1)</i> ^{-/-}	uninjected	0	0	52 (73%)	19 (27%)	71
<i>gli3</i> [10 ng]	0		0	18 (72%)	7 (28%)	25		
<i>fkd4</i> , 24 h	wt	uninjected	0	0	100 (100%)	0	100	
		<i>gli3</i> [10 ng]	125 (74%)	23 (14%)	12 (12%)	0	170	
		<i>gli3'</i> [10 ng]	3 (14%)	17 (81%)	1 (5%)	0	21	
		<i>lacZ</i> RNA [65 pg]	0	0	35 (100%)	0	35	
		<i>shh</i> RNA [65 pg]	25 (83%)	5 (17%)	0	0	30	
		<i>dtr(gli1)</i> ^{-/-}	uninjected	0	0	71 (71%)	29 (29%)	100
			<i>gli3</i> [10 ng]	25 wt (56%)	2 wt (4%)	10 (22%)	8 (18%)	45
<i>gli2</i> + 3 [5 + 5] ng	<i>gli2</i> + 3 [5 + 5] ng	31 (35%)	28 (32%)	11 (13%)	17 (20%)	87		
	<i>syu(shh)</i> ^{-/-}	uninjected	0	0	61 (73%)	23 (27%)	84	
<i>smu(smo)</i> ^{-/-}	<i>smu(smo)</i> ^{-/-}	<i>gli3</i> [10 ng]	40 wt (70%)	7 {wt + mut} (13%)	7 (13%)	2 (4%)	56	
		uninjected	0	0	126 (74%)	45 (26%)	171	
		<i>gli2</i> [10 ng]	0	15 {mut only} (11%)	97 (68%)	30 (21%)	142	
		<i>gli3</i> [10 ng]	99 wt (54%)	16 {wt only} (9%)	29 (16%)	38 (21%)	182	
<i>gli2</i> + 3 [5 + 5] ng	<i>gli2</i> + 3 [5 + 5] ng	37 wt (43%)	8 {mut only} (9%)	23 (27%)	18 (21%)	86		
	<i>gli1</i> , 19 h	wt	uninjected	0	0	53	NA	53
<i>gli3</i> [12 ng]			0	0	51	NA	51	
<i>gli1</i> , 24 h	untreated wt	uninjected	0	0	42	NA	42	
		<i>gli3</i> [9 + 12 ng]	0	81	0	NA	81	
		uninjected	0	0	0	40 (= red. exp.)	40	
<i>axial</i> , 24 h	wt	<i>gli3</i> [9 + 12 ng]	0	114 (also red. exp.)	0	0	114	
		uninjected	0	0	52 (100%)	0	52	
<i>smu(smo)</i> ^{-/-}	<i>smu(smo)</i> ^{-/-}	<i>gli3</i> [10 ng]	0	0	16 (100%)	0	16	
		uninjected	0	0	32 (70%)	14 (30%)	46	
		<i>gli2</i> [10 ng]	0	10 {1–2cells} (12%)	56 (69%)	15 (19%)	81	
		<i>gli2</i> + 3 [5 + 5] ng	0	2 {1–2 cells} (8%)	17 (65%)	7 (27%)	26	

and Joyner, 1993), including a dorsal expansion of some ventral cell types in the spinal cord (Persson et al., 2002) and dorsal expansion of ventral telencephalic markers in the forebrain (Aoto et al., 2002; Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). This major role as a repressor is also uncovered by removing Gli3 function in embryos lacking Shh or Smo function. Removing Gli3 rescues digit formation and all but the most ventral spinal cord defects caused by loss of Hh signaling (Litingtung and Chiang, 2000; Rallu et al., 2002; Wijgerde et al., 2002), indicating that a major role of Hh signaling is to overcome Gli3-mediated repression of Hh target genes.

Besides this well-documented repressor function, Gli3 can act as a weak activator during spinal cord patterning in vivo (Bai et al., 2004), consistent with in vitro studies that indicate Gli3 can activate Hh target gene expression (Dai et al., 1999; Ruiz i Altaba, 1999; Sasaki et al., 1997; Shin et al., 1999). Using elegant knockout and knockin approaches, Bai et al. (2004) showed that Hh-dependent expression of *Gli1* is reduced in *Gli3* mutants and completely absent in *Gli3/Gli2* double mutants. This indicates that Gli3 functions with Gli2 to activate Hh signaling. In addition, expressing Gli3 in place of Gli2 weakly induces some ventral cell types, confirming a weak activator function for Gli3 in the ventral spinal cord (Bai et al., 2004). Activator function for Gli3 is also illustrated by the fact that Gli3 can contribute to ventral specification,

especially in the rostral neural tube (Motoyama et al., 2003). While these data confirm that Gli3 mediates Hh signaling in vivo, little is known about how Gli3 activator and repressor functions are spatially and temporally distributed, and how Gli3 functions with Gli1 and Gli2 to pattern the embryo.

Studies of Gli3 activator and repressor function are particularly important because mutations in human *Gli3* lead to a variety of diseases including Greig cephalopolysyndactyly syndrome (GCPS) (Kalff-Suske et al., 1999; Vortkamp et al., 1991; Wild et al., 1997), Pallister-Hall syndrome (PHS) (Kang et al., 1997), and postaxial polydactyly type A (PAP-A) (Radhakrishna et al., 1997). These human diseases are the result of Gli3 truncations, point mutations, and frame shifts. While no clear genotype–phenotype correlation has been uncovered (Ming and Muenke, 2002), the fact that polydactyly is caused by all of these mutations points to a major repressor role for human Gli3, at least in the developing limb.

The studies on Gli3 function have raised two major questions. First, previous studies have shown that the requirements for Gli1 and Gli2 have diverged between zebrafish and mouse. We therefore asked whether the role of Gli3 has also diverged or been conserved in zebrafish. Second, the regulatory interactions between Hh signaling and *gli* genes are still unclear. We have therefore used the

Table 2
Morpholino injections: activator role for Gli3

Probe/assay	Injected cross	MO-injected	<i>smu</i> -like	Greatly reduced	<i>dtr</i> -like	Generally reduced	Wild-type	Total
<i>nk2.2</i> , 19 h, forebrain	wt	uninjected	0	0	0	0	54 (100%)	54
		<i>gli3</i> [10 ng]	0	0	0	20 (77%)	6 (23%)	26
		<i>gli1</i> + 3 [3 ng + 7 ng]	4 (7%)	40 (73%)	9 (16%)	1 (2%)	1 (2%)	55
	<i>dtr(gli1^{-/-})</i>	uninjected	0	0	16 (33%)	0	33 (67%)	49
		<i>gli3</i> [10 ng]	1 (2%)	7 (17%)	3 (7%)	20 (47%)	11 (27%)	41
		<i>gli3'</i> [10 ng]	3 (5%)	10 (18%)	9 (16%)	10 (18%)	24 (43%)	56
		<i>gli1</i> + 3 [3 ng + 7 ng]	6 (8%)	39 (50%)	26 (33%)	4 (5%)	3 (4%)	78
	<i>syu(shh^{-/-})</i>	uninjected	0	0	13 (<i>syu</i> -like) (36%)	0	23 (64%)	36
		<i>gli3</i> [10 ng]	0	9 (20%)	0	0	36 (80%)	45
control [10 ng]		0	0	0	0	40 (100%)	40	
<i>nk2.1b</i> , 28 h	wt, di	<i>gli3</i> [10 ng]	0	0	0	44 (86%)	7 (14%)	51
		<i>gli2</i> + 3 [5 ng + 5 ng]	0	0	0	9 (45%)	11 (55%)	20
		control [10 ng]	0	0	8 (24%)	0	25 (76%)	33
	<i>dtr(gli1^{-/-})</i> , di	<i>gli3</i> [10 ng]	7 (30%)	0	10 (43.6%)	6 (26%)	1 (0.4%)	23
		<i>gli2</i> + 3 [5 ng + 5 ng]	6 (25%)	0	12 (50%)	6 (25%)	0	24
		control [10 ng]	0	0	0	0	90 (100%)	90
<i>ptc1</i> , 11 h, 19 h	wt	<i>gli1</i> [7 ng – 10 ng]	0	0	83 (90%)	0	9 (10%)	92
		<i>gli3</i> [10 ng]	0	0	0	62 (770%)	19 (23%)	81
		<i>gli1</i> + 3 [3 ng + 7 ng]	0	93 (74%)	33 (26%)	0	0	126
	<i>dtr(gli1^{-/-})</i>	uninjected	0	0	32 (24%)	0	99 (76%)	131
		<i>gli1</i> + 3 [3 + 7 ng]	0	25 (25%)	40 (40%)	16 (15%)	20 (20%)	101
		uninjected	NA	0	NA	0	20 (100%)	20
Zn-5, 48 h	wt	<i>gli3</i> [10 – 12 ng]	NA	5 (25%)	NA	8 (40%)	7 (35%)	20
		uninjected	NA	0	NA	0	50 (100%)	50
<i>ath5</i> , 34 h	wt	<i>gli3</i> [9 ng]	NA	15 (27%)	NA	28 (50%)	13 (23%)	56
		<i>gli3</i> [12 ng]	NA	27 (61%)	NA	13 (30%)	4 (9%)	44
		uninjected	NA	0	NA	0	50 (100%)	50

dtr phenotype is a general reduction in *ptc* expression and regionally absent expression of *nk2.2* and *nk2.1b*.

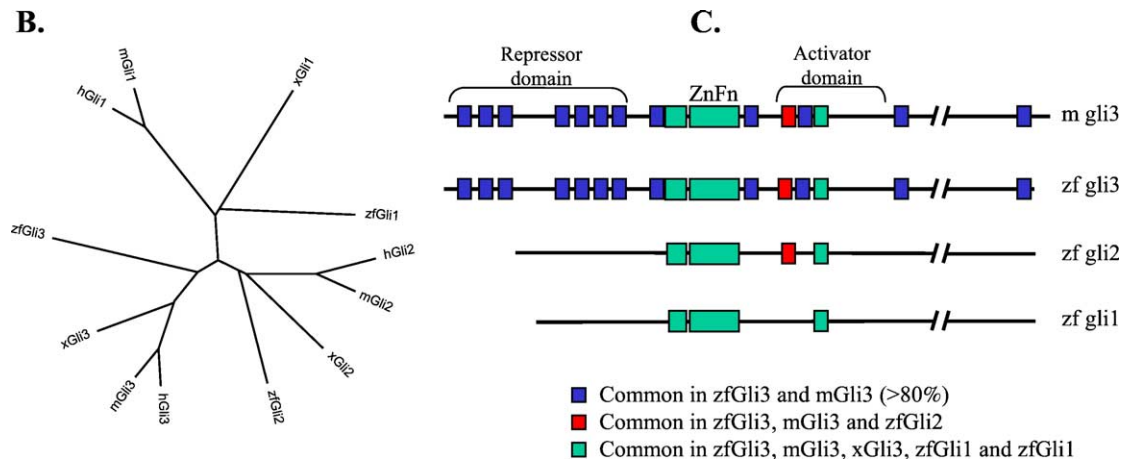


Fig. 1 (continued).

(RGC) differentiation. At later stages of CNS patterning, zebrafish Gli3 acquires a tissue- and target-specific repressor function that blocks the Hh response in the dorsal spinal cord and caudal brain. Our *in vivo* data help define how overlapping and distinct Gli1, Gli2, and Gli3 functions combine to integrate Hh signals.

Materials and methods

Cloning and mapping zebrafish gli3

We obtained a partial zebrafish *gli3* cDNA (containing the zinc finger and 3' regions) by screening a 15- to 19-h embryonic cDNA library (gift of B. Appel and J. Eisen) using a radio-labeled mouse *Gli2* probe and low stringency hybridization conditions. A 5' RACE reaction (Invitrogen) identified cDNA fragments encoding the 5' portion of zebrafish *gli3*. cDNA fragments were assembled to give the full Gli3-coding region that was sequenced using the UMass sequencing facility (Genbank accession no. AY377429). Sequence was subsequently verified by comparison to genomic sequence from the zebrafish genome project (Sanger Center: http://www.sanger.AC.uk/Projects/D_gerio/). Genomic clones containing zebrafish *gli* genes were obtained by screening a gridded genomic bacterial artificial chromosome (BAC) library (Genome Systems) using a *Gli2* probe (Karlstrom et al., 1999, 2003). *gli3*-containing BACs were then identified using *gli3*-specific PCR primers designed using cDNA sequence. These primers were then used to characterize four *gli3*-positive BACs (b156g24, b155o03, b51e17, and 164J21) and obtain genomic sequence. Subsequent PCR analysis indicated that none of the identified BACs contained the 5' end of the *gli3* cDNA. A single-strand conformation polymorphism (SSCP) was identified for SP6 end sequence derived from BAC 164J21, and this polymorphic fragment was used to map *gli3* to zebrafish linkage group 24 (genetic map of region can be viewed at <http://zfin.org>).

PCR genotyping *dtr(gli1)* fish

Embryos of *dtr^{ts269}* mutant clutch were genotyped as described in Karlstrom et al. (2003). Primer sequences were *ts269*Mu.rv: 5'-TGGGATCATGTTGCCCA and *dtr8*.fw: 5'-GTCTAAAGGCTAAATATGCAGC.

In situ hybridization

The *in situ* labeling was performed as previously described (Karlstrom et al., 2003). Probes used were *nk2.2* (Barth and Wilson, 1995), *nk2.1b* (Rohr et al., 2001), *fkf4* (Odenthal and Nusslein-Volhard, 1998), *axial (fkf1)* (Odenthal and Nusslein-Volhard, 1998; Strahle et al., 1993), *ptc1* (Maeda et al., 1993), *shh* (Krauss et al., 1993), and *ath5* (Masai et al., 2000). *gli3* probe was synthesized using T7 RNA polymerase from a *gli3*/pBluescript plasmid linearized with *Not1*. For double *gli3/shh* *in situ* staining, a fluorescein-labeled *shh* probe was used together with digoxigenin-labeled *gli3* probe. After incubation in an alkaline phosphatase-conjugated anti-digoxigenin antibody, embryos were labeled using the NBT substrate and fixed overnight. Embryos were then washed in PTw (PBS + 0.1% Tween 20) for 1 h, blocked as previously, and incubated in anti-fluorescein antibodies overnight at 4°C. After washing in PTw for 2 h and equilibration in 0.1 M Tris buffer (pH 9.5, 0.05 M MgCl₂, 0.1 M NaCl), a second color reaction was performed using the INT substrate as described in manufacturer's instructions (Roche).

Antibody labeling

Embryos injected with *gli3* morpholino (MO) and control MOs were grown to the 40-h stage, fixed in 4% paraformaldehyde for 30 min, and dehydrated in methanol for 30 min at room temperature. After rehydration in PTx (PBS + 0.1% Triton X-100), embryos were stained with the ZN-5 antibody as described in Karlstrom et al. (1996). After washing, embryos were incubated

with secondary FITC-conjugated goat anti-mouse antibodies (1:200 dilution) overnight at 4°C, washed in PTx, cleared in 75% glycerol, and visualized using a Zeiss confocal microscope.

mRNA and morpholino antisense oligonucleotide injections

Embryos were pressure-injected as described in Karlstrom et al. (2003). *zfgli1* (5'-CCGACACACCC-GCTACACCCACAGT), *zfgli2* (5'-GGATGATGTA-AAGTTCGTCAGTTGC) and two nonoverlapping *zfgli3* morpholino antisense oligos (5'-ACAACGGGCATTCTCAGAGCATC and 5'-GACAGGATACTCGTTGTTGT-GAAAC), and a random control oligo (5'-CCTCTTA-CCTCAGTTACAATTTATA) were synthesized by Gene Tools (Eugene, OR) and kept as 25 mg/ml stocks in 1× Danio solution (Westerfield, 1993). Three to twelve nanograms of different MOs or MO combinations were injected at the two-cell stage, and embryos were grown to the desired time points when they were fixed for in situ or antibody labeling (see Tables 1 and 2). At the higher concentrations, injection of *gli3* MOs led to convergence extension defects in 5–15% of embryos. Only embryos showing normal morphology were assayed for effects on CNS development. Embryonic phenotypes in *gli1*-, *gli3*-, and *gli1+gli3* MO-injected embryos were scored by three different members of the laboratory without knowledge of the experimental manipulation. Sorting of embryos based on *ptc1* expression levels was nearly identical in all three cases and corresponds to the results shown in Fig. 5.

For the cyclopamine experiments, embryos were treated with 100 μM cyclopamine (Toronto Chemical) by adding 10 μl of 10 mM stock solution (in 100% EtOH) to 1 ml of egg water (0.3 g/l Instant Ocean Salt, 1 mg/l methylene blue) starting at the shield stage. Control embryos were treated simultaneously with an equal volume of 100% EtOH (cyclopamine carrier) in 1 ml egg water.

For mRNA injections, plasmid DNA was linearized with the appropriate restriction enzyme, separated from uncut

DNA by running an agarose gel electrophoresis, and purified with GENE CLEAN II KIT (BIO 101 Systems, cat no. 1001-400). Synthetic mRNA was made using the Message Machine kit (Ambion) and diluted in water to 0.5–1 mg/ml. *shh* and control *lacZ* mRNAs were synthesized from pT7TS plasmids containing *shh* (Ekker et al., 1995) and *lacZ* (Karlstrom et al., 2003).

Cell culture analysis of transcriptional activity

Reporter assays were performed using the C17 neural cell line (Snyder et al., 1992) and a C17 cell line stably transfected with Shh-N (Feng et al., 2004). Twenty-four hours before transfection, 8×10^4 cells per well were plated into 24-well plates. Lipofectamine (Invitrogen) was used to transfect the reporter (0.2 μg $8 \times$ Gli-BS-luciferase; Sasaki et al., 1997), *Renilla luciferase*-expressing internal control (25 ng pRL-Null), and pcDNA constructs expressing Gli1, -2, -3, mutant *you-too*^{ty119} (Karlstrom et al., 1999), empty vector, or combinations as indicated in the Fig. 7 legend. Luciferase assays were performed 48 h after transfection using a dual-luciferase assay kit (Promega). Transfection results were normalized according to *Renilla luciferase* activity, performed with an *n* of 6 and duplicated.

Results

Sequence and developmental expression of zebrafish gli3

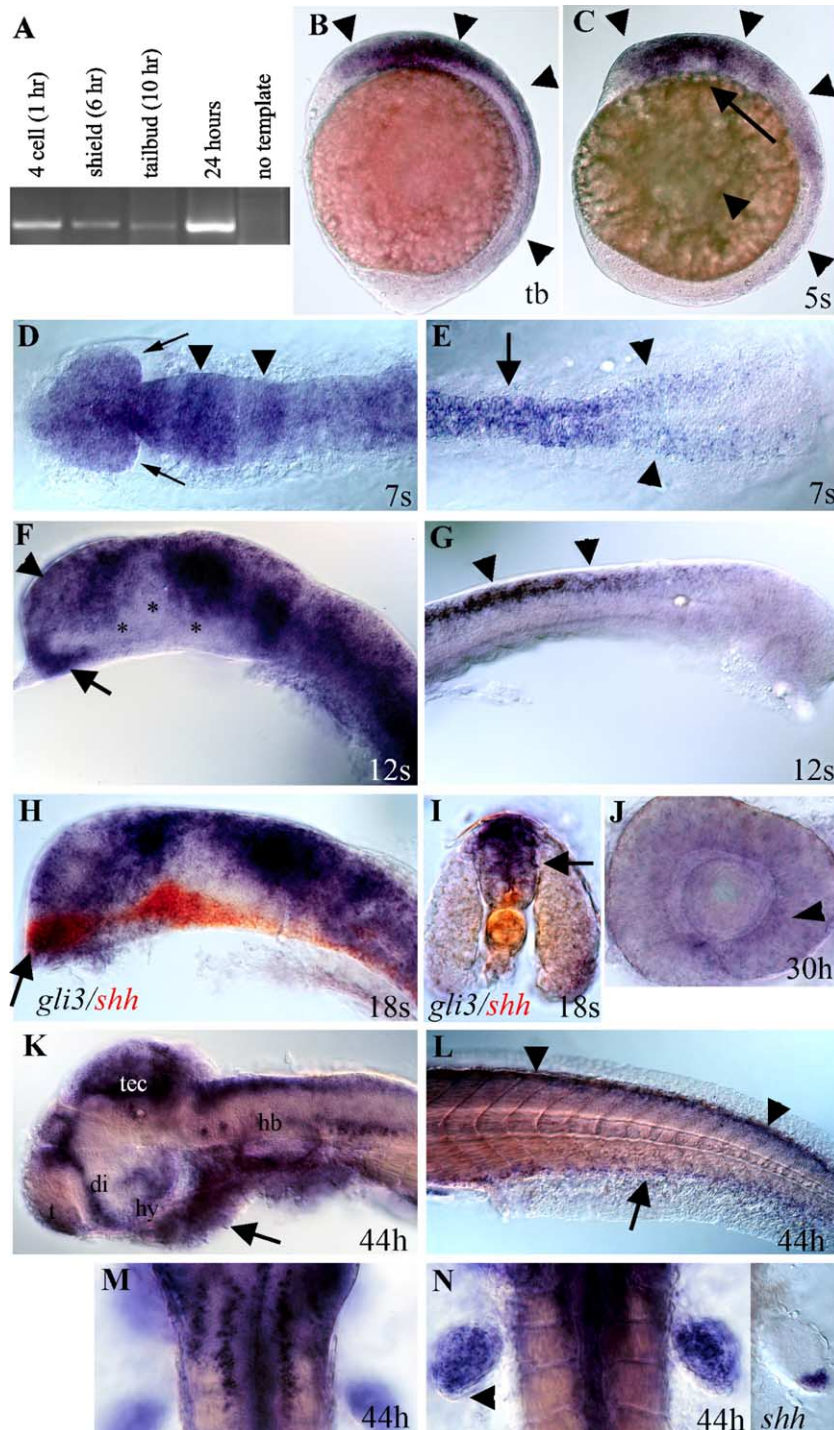
To better understand the in vivo function of vertebrate Gli3, we cloned and characterized the gene encoding zebrafish Gli3. Using a bacterial artificial chromosome (BAC) genomic library and an embryonic cDNA library, we identified several genomic and cDNA clones that contained *gli3*-like sequences (see Materials and methods). One *gli3*-containing BAC was mapped to linkage group 24 of the zebrafish genetic map by SSCP analysis. A RACE reaction was used to clone the 5' end of

Fig. 2. Developmental expression of zebrafish *gli3*. (A) RT-PCR analysis using *gli3*-specific primers shows that maternal *gli3* mRNA is present at the four-cell stage (prior to zygotic transcription), as well as at shield, tail bud, and 24-h stages, after zygotic transcription has begun. (B) Tail bud stage (10 h). *gli3* is first expressed in neural ectoderm (arrowheads) with expression most intense in the brain. (C) Five-somite stage (12 h). During early somitogenesis, *gli3* is expressed throughout the brain and spinal cord (arrowheads) with expression being lost in more ventral neural tissue (arrow). (D, E) Seven-somite stage (13 h). Dorsal view. (D) *gli3* is expressed throughout the embryonic brain and developing eyes (arrows) with more intense stripes of expression in the presumptive midbrain and midbrain/hindbrain border (arrowheads). (E) In the trunk, *gli3* is expressed in the spinal cord (arrow) and in the lateral neural plate prior to neural tube formation (arrowheads). (F, G) Twelve-somite stage (15 h). (F) At 15 h, *gli3* is expressed throughout the telencephalon (arrowhead) and anterior diencephalon (arrow) in the forebrain. (G) At 15 h, *gli3* is strongly expressed in the spinal cord and is restricted to the dorsal neural tube (arrows). (H, I) Eighteen-somite stage (17 h). (H) Dorsal expression of *gli3* (purple/blue) is largely complementary to the more ventral expression of *shh* (red) in the brain. However, in the most anterior diencephalon (arrow in H), *gli3* expression overlaps with *shh* expression. (I) Cross-section through the spinal cord shows that *gli3* (purple) is expressed in the neural tube dorsal to *shh* (red), which is in the floor plate (arrow) and notochord. (J) At 30 h, lateral view of dissected eye. *gli3* is expressed weakly in the eye in cells surrounding the lens. (K–N) At 44 h. (K) At later stages, *gli3* expression becomes restricted to the anterior telencephalon, dorsal diencephalon, hypothalamus, tectum, and dorsal hindbrain. Small clusters of cells also express *gli3* in the ventral hindbrain. Mesendoderm ventral to the brain also expresses *gli3* (arrow). (L) In the trunk, *gli3* expression is restricted to the most dorsal regions of the spinal cord (arrowheads) and ventral endoderm (arrow). (M) Dorsal view of hindbrain showing two rows of ventral cells (see K for lateral view) on either side of the midline that express *gli3*. (N) *gli3* is expressed throughout the fin bud with the exception of cells at the posterior margin (arrowhead). This expression overlaps with, but is much broader than, that of *shh* (right panel). (B, C, F–H, J–K) Side views, anterior to the left. (D, E, M, N) dorsal views. (I) Cross-section through trunk. (D–L) Yolks removed. (D, F, H) Eyes removed. Abbreviations: di; diencephalon, hb; hindbrain, hy; hypothalamus, tb; tail bud, t; telencephalon, tec; tectum.

zebrafish *gli3* and assemble a full-length cDNA of 4701 base pairs that encodes a protein of 1567 amino acids (aa) (Fig. 1A). Sequence of this Gli protein is more similar to mouse, human, and frog Gli3 than it is to zebrafish Gli1 and Gli2 (Fig. 1B). The N-terminal repressor domain (aa 1–397) (Dai et al., 1999; Sasaki et al., 1999) of zebrafish Gli3 shows 80% similarity with mouse, human, and *Xenopus* Gli3 proteins, and contains stretches of sequences with over 80% identity with mGli3 (Fig. 1C). The C-

terminal end of zfGli3 is less conserved, although the area that encodes a putative activator domain (aa 827–1132) (Dai et al., 1999) includes regions of more than 80% identity with Gli3 proteins of other species as well as zfGli1 and zfGli2 (Bai and Joyner, 2001; Ding et al., 1998; Karlstrom et al., 2003; Matise et al., 1998).

Further evidence that this gene encodes zebrafish Gli3 comes from expression analysis. Similar to *gli3* expression in other species, early *gli3* expression overlaps with



shh in all tissues examined, while later expression becomes complementary to *shh* expression. RT-PCR analysis showed that maternally derived *gli3* mRNA is present at the four-cell stage, prior to zygotic transcription (Fig. 2A). Neural *gli3* expression begins in the anterior part of the embryo (future brain) at tail bud stage, about 10 h postfertilization (Fig. 2B). During early somitogenesis, *gli3* is expressed throughout the brain and eyes (Figs. 2C, D), and later expression expands into the trunk (Figs. 2C, E, G). At 15–17 h, *gli3* is expressed in the intermediate/dorsal regions of the embryonic brain (Figs. 2F, H). In the forebrain, *gli3* expression extends more ventrally (Figs. 2F, H), overlapping with *shh* expression (Fig. 2H). In the trunk, *gli3* is first expressed throughout the neural plate and later becomes restricted to the dorsal spinal cord (Figs. 2E, G, I). This pattern is similar to *gli3* expression in mouse, chick, and frog (Lee et al., 1997; Meyer and Roelink, 2003; Sasaki et al., 1997). In the eye, *gli3* is weakly expressed at 28–30 h in retinal ganglion cells surrounding the lens (Fig. 2J). By 44 h, *gli3* expression is restricted to the most dorsal neural tube (Fig. 2L). In the forebrain, midbrain, and hindbrain, *gli3* transcripts can be detected in small clusters of cells throughout the brain (Figs. 2K, M), indicating a possible role in differentiation of these cells. *gli3* expression in the fin bud begins around 37 h of development, and by 44 h, *gli3* is expressed uniformly throughout the fin bud with expression in the posterior zone of polarizing activity (ZPA) overlapping with *shh* (Fig. 2N). This *gli3* expression pattern fits well with that of *gli3* in other species that have been reported (Lee et al., 1997; Sasaki et al., 1997) and shows details of overlapping expression not previously documented.

Transcriptional regulation of *gli3* by Hh signaling

Using both mutant and misexpression analyses, we next examined how *gli3* transcription is regulated by Hh signaling. We found that zebrafish *gli3* is negatively regulated by Hh signaling, similar to what has been suggested for mouse and frog *gli3* (Aoto et al., 2002; Wang et al., 2000; Wijgerde et al., 2002). In embryos that lack all Hh pathway activity [*smu(smo)* mutants] (Barresi et al., 2000; Chen et al., 2001; Varga et al., 2001; Wijgerde et al., 2002), *gli3* expression is expanded ventrally (Fig. 3B). Similar expansion was also detected in *syu(shh)* mutants (data not shown). Furthermore, ectopic Shh expression led to reduced *gli3* expression throughout the embryo (Fig. 3C). To determine whether other Gli proteins might regulate *gli3* expression, we analyzed the expression of *gli3* in *dtr(gli1)* and *yot(gli2)* mutants, and in wild-type embryos injected with *gli1* and *gli2* morpholino (MO) antisense oligonucleotides (Karlstrom et al., 2003). *gli3* expression was largely unaffected in *dtr* mutants and *gli1* morphants, suggesting that Gli1 does not play a major role in regulating *gli3* expression (data not shown). Interest-

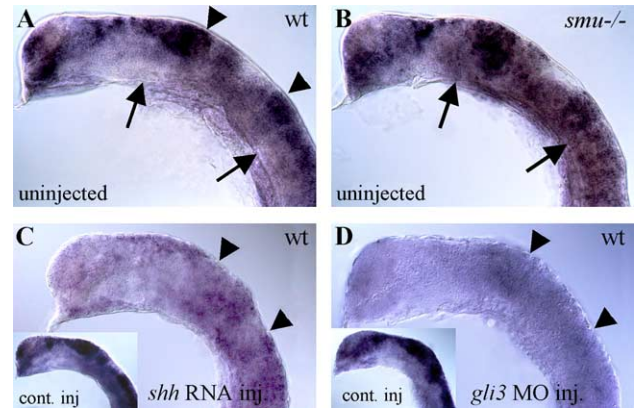


Fig. 3. Hh signaling negatively regulates *gli3* expression. (A) In 20-h wild-type embryos, *gli3* is expressed regionally in the dorsal neural tube, with strong expression in the tectum and midbrain/hindbrain boundary (arrowheads). (B) *gli3* expression is expanded ventrally in *smu(smo)* mutant embryos that lack Hh pathway activity (arrows). (C) *gli3* expression is generally reduced in *shh* mRNA-injected wild-type embryos (compare to *lacZ* mRNA-injected embryo in inset). (D) *gli3*MO injection also led to severely reduced *gli3* expression levels (compare to control MO-injected wild-type embryo in inset). All panels show *gli3* expression in 20-h zebrafish embryos. Lateral views of head, anterior to the left, eyes and yolk removed.

ingly, *gli2*MO injections and the presence of a dominant repressor form of Gli2 in *yot(gli2)* mutants have little or no effect on *gli3* expression (data not shown), indicating that the Hh-dependent repression of *gli3* in the ventral CNS is not mediated by Gli2 repressor activity, even at earlier ages when *gli2* is expressed more ventrally in the neural tube (Karlstrom et al., 1999, 2003).

Consistently, injections of *gli1*MO alone and together with *gli2*MO also did not have any effect on *gli3* expression (data not shown). This lack of *gli3* transcriptional regulation by Gli1 and Gli2 is similar to what was seen in mouse *Gli2*^{-/-} and *Gli1*^{-/-};*Gli2*^{-/-} mice (Matise et al., 1998). We also designed *gli3*-specific MOs (see below) and examined the effect of reducing Gli3 function on *gli3* transcription. In contrast to Gli1 and Gli2, reducing Gli3 function led to a loss of *gli3* expression, suggesting that Gli3 may positively regulate its own transcription (Fig. 3D). While this is an attractive model, at this point, we cannot rule out the possibility that the loss in *gli3* mRNA in *gli3*MO-injected embryos is an artifact caused by destabilization of *gli3* mRNA upon MO binding. However, since no other *gli* MO shows a similar effect on its target message, mRNA destabilization does not appear to be a general artifact of MO binding.

Together, these experiments show that zebrafish *gli3* expression is negatively regulated by Hh signaling, similar to mouse and chick *gli3* (Aoto et al., 2002; Wang et al., 2000; Wijgerde et al., 2002), but that this regulation is independent of Gli1 and Gli2 activity. This suggests that a transcription factor other than Gli2 and Gli1 is involved in Hh-mediated repression of *gli3* transcription.

Gli3 repression of the Hh target gene fkd4 is temporally regulated and overlaps with Gli2 repressor function

To test the function of Gli3 during zebrafish embryogenesis, we designed two independent translation-blocking morpholino antisense oligonucleotides. Both morpholinos were effective based on changes in Hh target gene

expression, but the MO designed to cover the translational start site was most effective and was used for the majority of experiments presented. Similar to what has been shown in other vertebrates (Litingtung and Chiang, 2000; Meyer and Roelink, 2003; Persson et al., 2002; Wijgerde et al., 2002), we found that zebrafish Gli3 acts as a repressor of ventral gene expression in the dorsal CNS. Reducing Gli3 func-

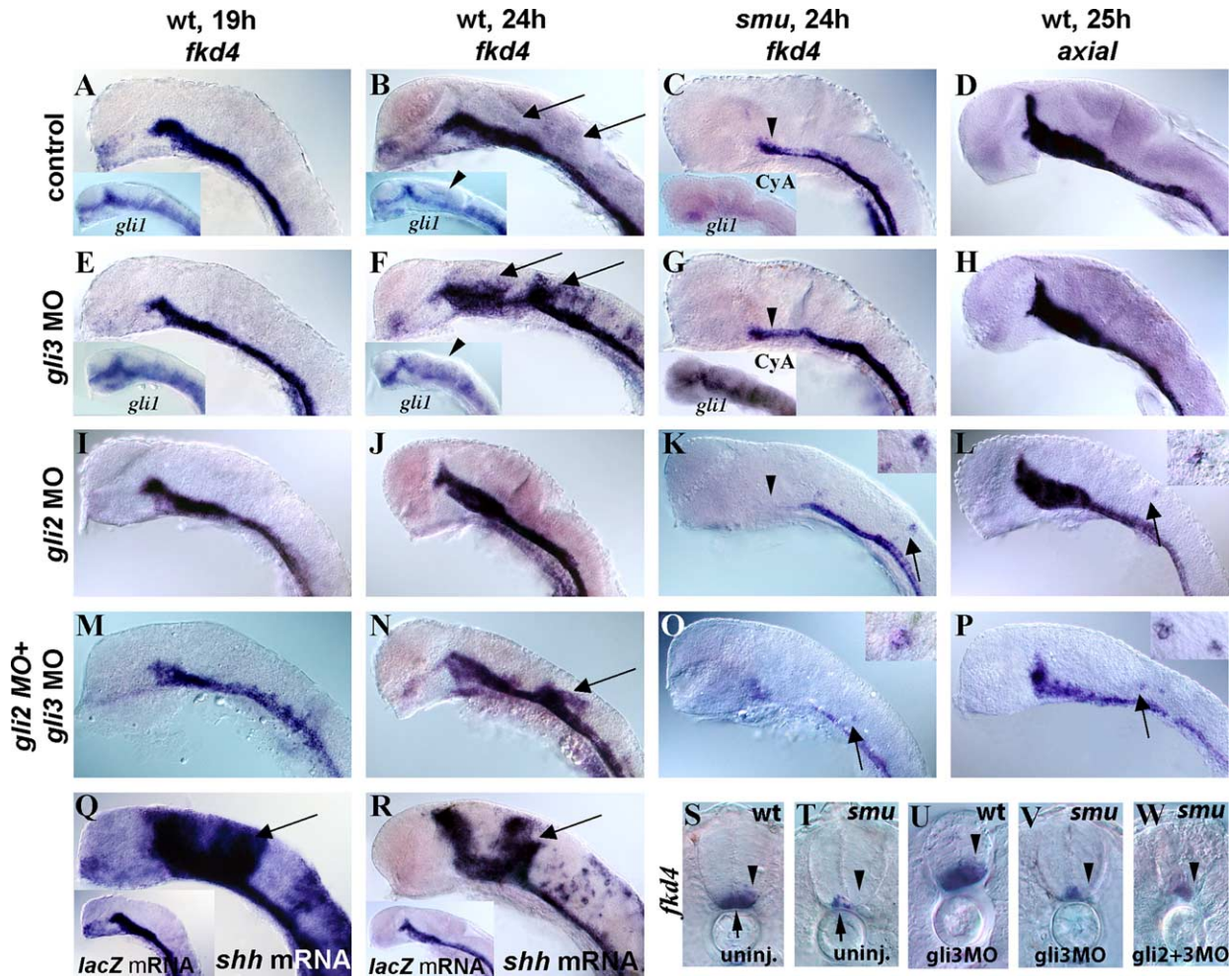


Fig. 4. Temporally regulated Gli3 repression of the Hh target gene *fkd4*. (A, B, D) Control MO-injected wild-type embryos with normal expression of *fkd4* (A, B) and *axial* (D) in the ventral brain, medial floor plate, and lateral floor plate. (E, F) *gli3*MO-injected wild-type embryos had normal expression of *fkd4* and *gli1* (inset) at 19 h (E) but had dorsally expanded *fkd4* (F, U) and *gli1* (F inset) expression at 24 h, revealing a temporally regulated repressor role for zebrafish Gli3. (C, G, T, V) *smu(sm)* mutant embryos had reduced *fkd4* expression (C, T, arrowheads), and *gli3*MO injections into *smu(sm)* mutant embryos did not lead to dorsal expansion of *fkd4* (G, V, arrowheads) indicating that active Hh signaling is necessary for this expansion. As in *smu(sm)* mutants (Karlstrom et al., 2003), cyclopamine treatment starting at shield stage led to an extreme reduction of *gli1* expression at 20 h (C inset). *gli3*MO injection resulted in a dramatic increase in *gli1* expression in cyclopamine-treated embryos (G inset). (D, H) No expansion of *axial* was detected in *gli3*MO-injected embryos (H), indicating target specificity for the Gli3 repressor. (I, J) *gli2*MO injection into wild-type embryos did not lead to expanded *fkd4* expression either at 19 h (I) or at 24 h (J). (K, L) In contrast, a few cells ectopically expressed *fkd4* in *smu(sm)* mutants (K) and *axial* in wild-type embryos (L) (see insets) after *gli2*MO injection, revealing a weak, Hh-independent, repressor function for Gli2. (M, N) Coinjection of *gli3*MOs and *gli2*MOs resulted in dorsal expansion of *fkd4* in wild-type embryos at 24 h (N) but not 19 h (M), similar to single *gli3*MO injections. (O) Double *gli2*MO + *gli3*MO injections also led to ectopic *fkd4* expression in a few dorsal neural cells in *smu(sm)* mutants at 24 h and expanded *axial* expression (P), similar to *gli2*MO injections alone. (Q, R) *shh* RNA-injected wild-type embryos had dorsally expanded *fkd4* expression at both 19 h (Q) and 24 h (R) compared to *lacZ* mRNA-injected embryos (inset), showing that Shh signaling is capable of inducing *fkd4* expression at both of these ages. (S) Cross-section shows *fkd4* expression in the medial (arrow) and lateral (arrowhead) floor plate cells. (T) In *smu(sm)* mutants, *fkd4* is expressed only in the medial floor plate (arrow) and is lost in lateral floor plate (arrowhead). (U) *gli3*MO injection into wild-type embryos led to dorsally expanded *fkd4* expression. (V) *gli3*MO injection into *smu(sm)* mutant embryos did not rescue of *fkd4* expression in lateral floor plate cells or expand *fkd4* expression dorsally. (W) Similarly, injection of *gli3*MOs and *gli2*MOs into *smu(sm)* mutants did not rescue *fkd4* expression in lateral floor plate or expand *fkd4* expression dorsally. (A–R) Side views of zebrafish embryos, yolks and eyes removed, anterior to the left. (S–W) Cross-sections of the trunk region, anterior up.

tion by injection of *gli3*MOs led to dorsal expansion of the lateral and medial floor plate marker *fkf4* (Figs. 4F, U, Table 1). This *in vivo* derepression of *fkf4* expression appears to depend on active Hh signaling, as no *fkf4* expansion was seen in *gli3*MO-injected *smu(smo)* mutant embryos (Figs. 4G, V).

A more careful temporal analysis demonstrated that this expansion of *fkf4* is developmentally regulated. While *gli3*MO injection led to dorsal expression of *fkf4* when injected embryos were assayed at 24 h (Fig. 4F), no dorsal expression was seen when assayed 5 h earlier at 19 h of development (Fig. 4E, Table 1). In fact, at this earlier time point, ventral *fkf4* expression was slightly reduced in *gli3*MO-injected embryos (Fig. 4E). To verify that dorsal neural cells are competent to express *fkf4* at both 19 and 24 h, we injected *shh* RNA into wild-type embryos and analyzed *fkf4* expression. Dorsal expansion of *fkf4* was detected at both 24 h (Fig. 4R, Table 1) and at 19 h (Fig. 4Q, Table 1), verifying that cells are competent to respond to Hh signaling at both of these time points. In addition, *gli3* is expressed in the dorsal neural tube at 19 h, a time when loss of Gli3 function fails to expand *fkf4* (see Figs. 2E–H). This indicates that other factors restrict *fkf4* expression to the ventral neural tube prior to 24 h. We wondered whether Gli2 might be this earlier repressor; however, removing both Gli2 and Gli3 function led to little or no expansion of *fkf4* at 19 h, with more robust expansion seen at 24 h (Figs. 4M, N).

Since Gli1 is the main activator of Hh target genes in zebrafish (Karlstrom et al., 2003), we wondered whether expanded *fkf4* expression in *gli3*MO-injected embryos could be due to Gli1 activity. Indeed, *gli3*MO injections led to expanded *gli1* expression into the dorsal neural tube at 24 h but not 19 h of development (insets in Figs. 4E, F, Table 1). To directly test whether this expanded Gli1 expression is responsible for the dorsal expansion of *fkf4* expression, we next injected *gli3*MOs into *dtr(gli1)* mutant embryos. While *fkf4* expansion was seen in wild-type siblings, *fkf4* expansion was not seen in *gli3*MO/*dtr(gli1)* mutant embryos (Table 1). Thus, expansion of *fkf4* in *gli3*MO-injected embryos appears to be mediated by dorsally expanded *gli1* expression. We next examined whether Gli3-mediated repression of *gli1* transcription required Hh signaling activity. In this case, we blocked Smo-mediated Hh signaling using the alkaloid drug cyclopamine (CyA) (Incardona et al., 1998; Taipale et al., 2000). CyA treatment alone led to an extreme reduction of *gli1* expression (Fig. 4C inset), similar to the situation in *smu(smo)* mutants (Karlstrom et al., 2003). In contrast, injection of *gli3*MOs resulted in a dramatic increase in *gli1* expression in CyA-treated embryos (compare Fig. 4C and G insets, Table 1), indicating that inhibition of *gli1* transcription by Gli3 does not require Hh activity. Thus, Gli3 is acting as an Hh-independent repressor, similar to *Drosophila* Ci. Given the lack of *fkf4* expansion in *gli3*MO-injected *smu(smo)* mutant embryos (Fig. 4G), we conclude that expanded expression of *gli1* is not sufficient in itself for

the activation of Hh targets, but that Gli1 requires active Hh signaling to induce *fkf4* and presumably other Hh target genes.

In mouse embryos, the balance between Shh signaling and Gli3 repression was shown to pattern the neural tube (Litingtung and Chiang, 2000; Meyer and Roelink, 2003; Persson et al., 2002; Wijgerde et al., 2002). In *Shh*^{-/-}; *Gli3*^{-/-} double mutant mice, ventral neural cell types that are absent in *Shh*^{-/-} single mutants are partially restored (Litingtung and Chiang, 2000; Meyer and Roelink, 2003). This shows that Shh signaling is not necessary for the differentiation of some ventral cell types in the absence of Gli3 and implies that a major function of Hh signaling is to overcome Gli3 repression of ventral fates. To determine whether ventral cell types can form in the absence of both Hh and Gli3 function in zebrafish, we injected *gli3*MOs into *smu(smo)* mutants that lack Hh signaling activity. In *smu(smo)* mutants, *fkf4*-expressing lateral floor plate cells are missing, while *fkf4*-expressing medial floor plate cells are present either because they do not require Hh signaling activity to differentiate or due to maternal *smo* expression (Fig. 4T, Varga et al., 2001). Similar to the situation in mouse, differentiation of the most ventral cell types (*fkf4*-expressing lateral floor plate cells) was not rescued in *gli3*MO/*smu*^{-/-}(*smo*) embryos (Fig. 4V, Table 1). Unlike the situation in mouse, motoneuron differentiation, as assayed by *isl-2* expression, was not restored by injecting *gli3*MOs into *smu(smo)* mutants (data not shown).

In the trunk, *gli3* expression largely overlaps with *gli2* (Fig. 2 and Karlstrom et al., 1999). Since zebrafish Gli2 can also repress Hh target genes (Karlstrom et al., 2003), we wondered whether loss of both Gli2 and Gli3 function could rescue ventral cell types in *smu(smo)* mutants. Again, no rescue of *fkf4*-expressing lateral floor plate cells or *isl-2*-expressing motoneurons was seen in *gli2*MO/*gli3*MO/*smu*^{-/-}(*smo*) embryos (Fig. 4W), consistent with the idea that differentiation of these ventral cell types requires Gli1-mediated Hh signaling.

Interestingly, blocking Gli2 synthesis in *smu(smo)* mutants led to expanded *fkf4* expression in some dorsal hindbrain (Fig. 4K, Table 1) and spinal cord (not shown) cells, but had no effect on wild-type embryos (Figs. 4I, J, Table 1). Coinjections of *gli3*MO and *gli2*MO into the progeny of *smu(smo)* heterozygous parents led to dorsal *fkf4* expression in 37/60 wild-type and heterozygous embryos (43% of total injected embryos) (Fig. 4N, Table 1) and dorsal expression of *fkf4* in just a few cells in 8/26 mutants (9% of total injected embryos) (Fig. 4O, Table 1). These data show that Gli2 acts as a weak repressor in the dorsal hindbrain and spinal cord.

These experiments also uncovered an activator function for Gli2 in the ventral midbrain. In the midbrain, *fkf4* expression is eliminated after *gli2*MO injection into *smu(smo)* mutant embryos (Fig. 4K, arrowhead), indicating that Gli2 is needed for activation of this gene in this region, consistent with the earlier ventral expression of

gli2 (Karlstrom et al., 1999), and its minor role as an activator of Hh signaling in the ventral CNS (Karlstrom et al., 2003).

We also analyzed other Hh target genes expressed in the floor plate, including *nk2.2* and *axial*. To our surprise, expression of these markers did not expand in embryos injected with *gli3*MOs (Fig. 4H, Table 1 and data not shown). In combination with the fact that *shh* mRNA injection is capable of inducing *nk2.2* and *axial* expression (Hauptmann and Gerster, 1996), this suggests that Gli3 repression is not equivalent for all Hh target genes. One possibility is that our MO experiments do not completely block Gli3 function and that some Gli3 protein remains that is capable of repressing some but not all ventral genes. This model would suggest that Gli3 repression is more potent for some genes than others. Another possibility is that yet another Gli protein may act as a repressor of Hh targets in the caudal brain and spinal cord, and that the combination of Gli repressor activities leads to target-specific regulation. Indeed, in 10% of *gli2*MO- or *gli2*MO/*gli3*MO-injected embryos, *axial* expression was detected in a few cells in the dorsal neural tube (Figs. 4L, P, Table 1), suggesting a weak repressor role for Gli2 in regulating *axial* expression. In

either event, our data show that Gli3 and Gli2 repressor activities appear to be different for different target genes.

In summary, we have shown that the requirement for Gli3 repression of ventral gene expression is temporally regulated and that Gli2 and Gli3 have partially overlapping repressor functions that differ at different axial levels of the neural tube.

Gli3 functions with *Gli1* to activate ventral Hh target genes in the neural tube

To determine whether Gli3 also has a function as an activator of Hh signaling, we next examined the effect of *gli3* knockdown on Hh target genes expressed in the ventral neural tube. As mentioned above, *gli3*MO injections led to a reproducible but mild reduction in ventral *fkf4* expression in the midbrain and hindbrain (Fig. 4E) and *nk2.2* expression in the forebrain (Fig. 5D, Table 2), consistent with a weak activator function for Gli3. The fact that *gli1* and *gli3* expression initially overlap in the CNS prompted us to determine whether these two genes might functionally overlap in activation of the Hh response. To block both Gli1 and Gli3 function, we injected *gli3*MOs into *dtr(gli1)*

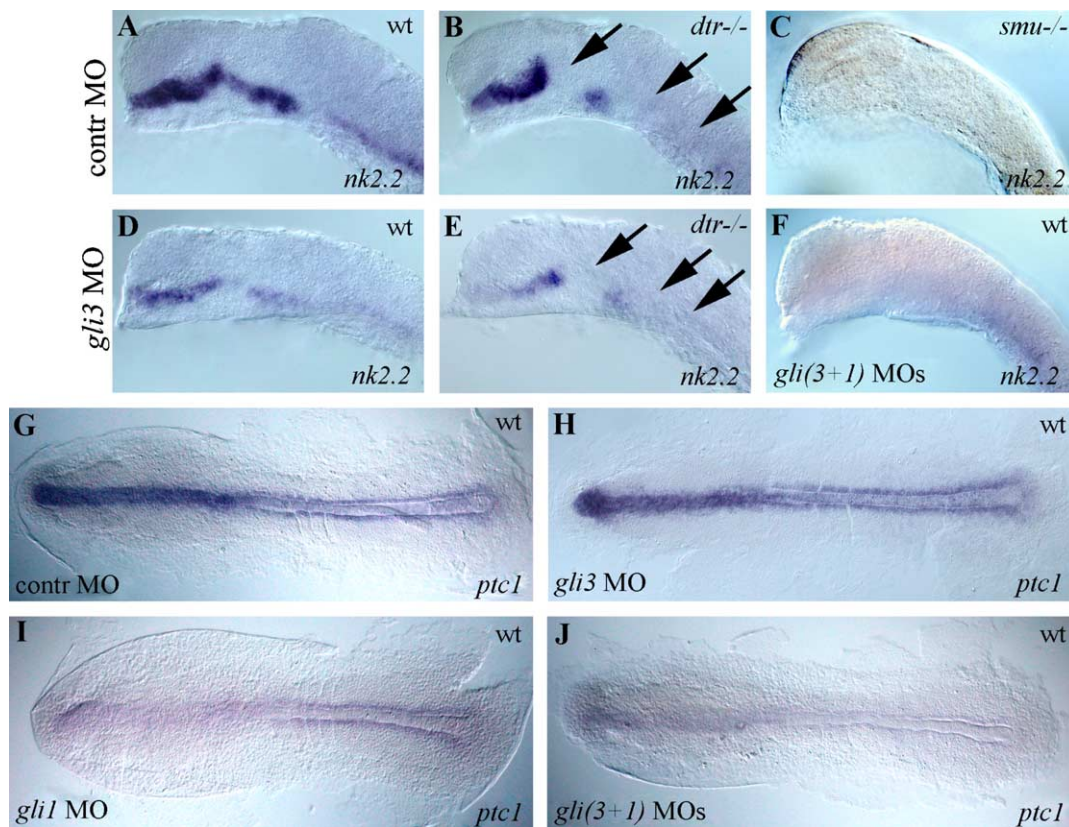


Fig. 5. Positive regulation of *nk2.2* and *ptc1* expression by Gli3 and Gli1. (A) Wild-type *nk2.2* expression in a control MO-injected zebrafish embryo. (B) In *dtr(gli1)* mutants *nk2.2*, expression is regionally absent (arrows). (C) *smu(sm)* mutants have no *nk2.2* expression at 19 h. (D, E) *gli3*MO injections generally decreased *nk2.2* expression in wild-type embryos (D) and further reduced *nk2.2* expression in *dtr(gli1)* mutants (E, arrows). (F) Coinjections of *gli3*MO and *gli1* MO produced a *smu(sm)*-like phenotype with no *nk2.2* expression. (G) *ptc1* expression was not affected by control MO injections. (H) *gli3*MO injections had little or no effect on *ptc1* expression. (I) *gli1*MO injections reduced expression of *ptc1*. (J) Coinjection of *gli3*MO and *gli1*MO reduced *ptc1* expression almost completely. (A–F) lateral views of 19-h embryos, eyes and yolks removed, anterior to the left. (G–J) Dorsal views of 11-h embryos, anterior to the left, yolks removed.

mutant embryos. In uninjected *dtr(gli1)* mutants, *nk2.2* expression is regionally absent (Fig. 5B and Karlstrom et al., 2003). Injections of *gli3MO* into *dtr(gli1)^{-/-}* embryos further reduced *nk2.2* expression (Fig. 5E, Table 2), and in some embryos, *nk2.2* expression was completely absent, similar to the phenotype caused by the complete loss of Hh signaling in *smu(smo)* mutants (compare Figs. 5C and F, Table 2). These results show that Gli3 and Gli1 have overlapping activator functions in the forebrain. We next examined the effect of reducing Gli1 and Gli3 function on the expression of the Hh target gene *ptc1*. Injection of *gli1MO* alone led to a minor general reduction in *ptc1* transcription (Fig. 5I), while *gli3MO* injections had little or no effect on *ptc1* expression (Fig. 5H). This is in contrast to the mild increase in *ptc1* expression seen after *gli2MO* injections (Karlstrom et al., 2003). Depletion of both Gli3 and Gli1 proteins led to a stronger reduction of *ptc1* expression than that seen in any single MO injection (Fig. 5J, Table 2), consistent with overlapping activator roles for Gli1 and Gli3.

The data above demonstrate that Gli1 and Gli3 have overlapping activator functions in the zebrafish ventral neural tube. In mouse, similar overlapping functions for Gli1 and Gli2 were demonstrated to be dosage-dependent (Park et al., 2000). To test whether the combined dosage of Gli1 and Gli3 is important for Hh target activation, we injected *gli3MOs* into *dtr(gli1)* heterozygous individuals and examined diencephalic expression of the Hh target gene *nk2.1b* (Rohr et al., 2001). Injection of *gli3MOs* into progeny of two *dtr(gli1)^{+/-}* heterozygous parents produced embryos that could be divided into three groups based on *nk2.1b* expression levels: (1) mildly reduced *nk2.1b* expression was detected in approximately 25% of embryos (presumed *dtr(gli1)^{+/+}*), corresponding to the reduction seen after *gli3MO* injection into known wild-type embryos (Figs. 6A, D), (2) a *dtr(gli1)^{-/-}* homozygous mutant-like reduction in *nk2.1b* expression was detected in 50% of the embryos, corresponding to *dtr^{+/-}* heterozygotes (Figs. 6B, E), (3) a dramatic reduction of *nk2.1b* expression resembling that seen in *smu(smo)^{-/-}* mutants was detected in 25% of the embryos, corresponding to *gli3MO*-injected *dtr(gli1)^{-/-}* homozygous mutants (Figs. 6C, F, see graph). Thus, while loss of one copy of Gli1 in *dtr(gli1)^{+/-}* heterozygotes had no effect on *nk2.1b* expression levels, reduction of Gli3 function in these same embryos led to reduced Hh signaling.

In contrast to this effect on diencephalic *nk2.1b* expression, reducing Gli3 function had little or no effect on *nk2.1b* expression in the telencephalon (arrows in Figs. 6D, E, F). We previously showed that Gli2 represses *nk2.1b* expression in the telencephalon (Karlstrom et al., 2003). Since the repressor functions of Gli3 and Gli2 overlap in the neural tube, we decided to test if they also overlap in the telencephalon. In embryos coinjected with *gli3MO* and *gli2MOs*, *nk2.1b* expression was expanded in the telencephalon to the same level as seen in embryos injected only

with *gli2MOs* (Karlstrom et al., 2003). In the diencephalon, *gli2MO* + *gli3MO* injections reduced *nk2.1b* expression to the levels seen after single *gli3MO* injection (arrowheads in Figs. 6G, H, I). Together, these data indicate that Gli2 and Gli3 do not have overlapping or opposing functions in the forebrain.

Shh regulates Gli3 repressor and activator function in a neural cell line

To further test how zebrafish Gli3 protein functions in relation to Shh and Gli1, we turned to a neural cell culture assay for Gli transcriptional activity (Karlstrom et al., 2003; Sasaki et al., 1997). Consistent with the late repressor function shown above for Gli3 in vivo, we found that zebrafish Gli3 reduced reporter activation by Gli1 in the absence of high levels of Shh (Figs. 7A, B). This is consistent with the repressor role shown for mouse and human Gli3 proteins (Sasaki et al., 1997, 1999). To determine whether the presence of Shh affects Gli3 function, we next repeated these reporter assays using a stably transfected cell line that expresses high levels of Shh (Feng et al., 2004). In the presence of Shh, Gli3 showed no activator function on its own (Fig. 7C). However, in the presence of Shh, Gli3 increased the ability of Gli1 to activate the Gli-responsive reporter construct (Fig. 7D). These results show that in the presence of Hh signaling, Gli3 can work with Gli1 to activate Hh target gene expression. The fact that Gli3 showed no activator function in the absence of Gli1 (Fig. 7C) indicates that this activator function depends on Gli1, as shown in mouse (Bai et al., 2004). These data are consistent with our in vivo data showing early and overlapping activator roles for Gli3 and Gli1 in the ventral neural tube, close to the source of Hh.

Gli3 and retinal ganglion cell differentiation

Hh signaling is required for differentiation of retinal ganglion cells (RGCs) in the developing eye, with a wave of Hh expression directing RGC differentiation starting at 28–30 h of development (Neumann and Nusslein-Volhard, 2000). This wave of cell differentiation requires *ath5* (Kay et al., 2001), a zebrafish homolog of the basic helix–loop–helix transcription factor atonal, which in turn requires Hh-mediated signaling, most likely from the midline (Masai et al., 2000; Stenkamp and Frey, 2003). The *gli* genes responsible for the activation of this Hh response in the eye have not been identified. Gli1 and Gli2 are not required in this process, as RGC differentiation is largely unaffected in *dtr(gli1)* and *yot(gli2DR)* mutant embryos (Karlstrom et al., 1996). We noticed that *gli3* is expressed in the eye at approximately the ages when RGC differentiation begins (Fig. 2J, data not shown) and wondered whether Gli3 might mediate this Hh-dependent differentiation event. To test this, we injected *gli3MOs* into wild-type embryos and assayed the differentiation of RGCs at 40 h and *ath5* expression at

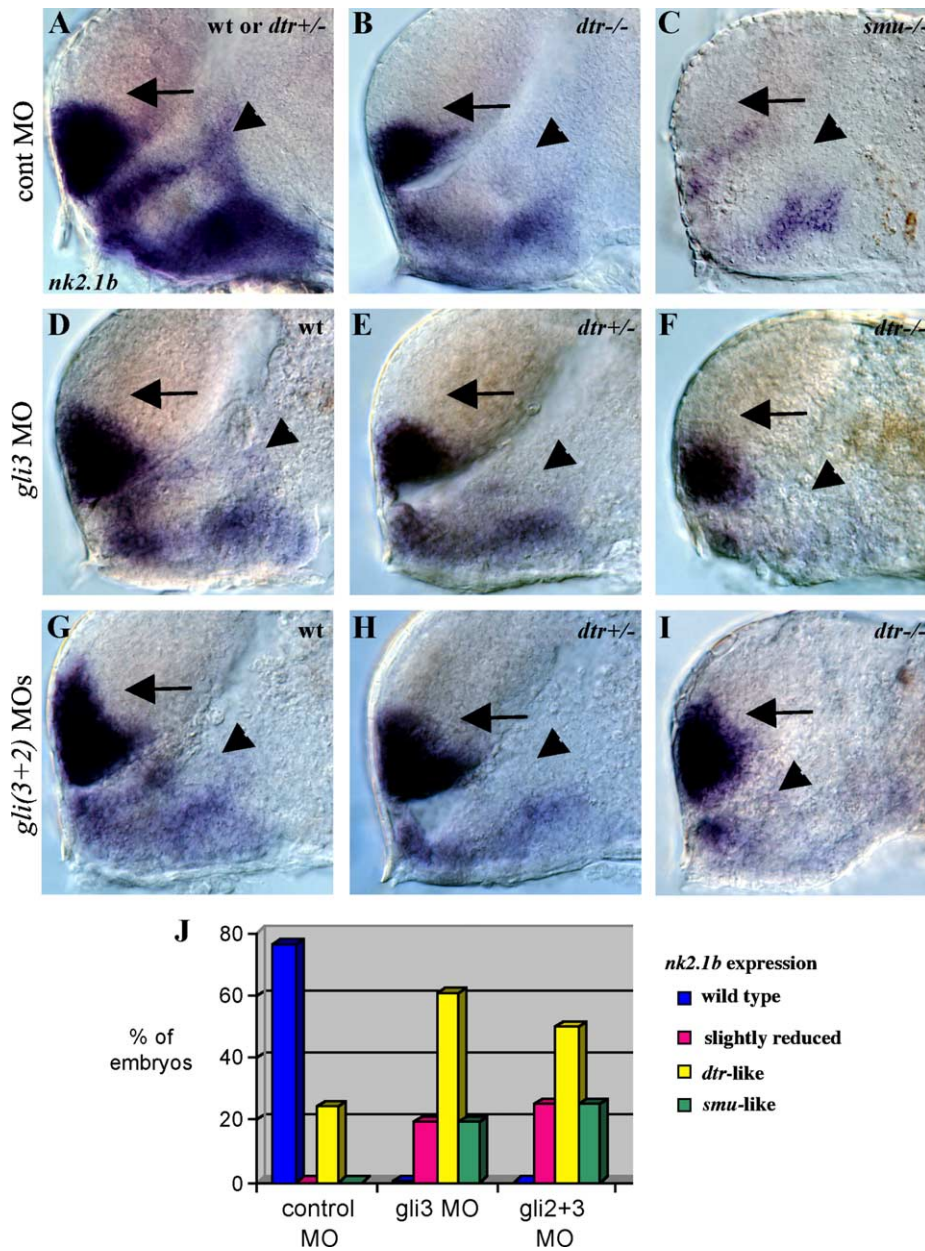


Fig. 6. Regulation of *nk2.1b* expression by *Gli3*, *Gli1*, and *Gli2*. (A) *nk2.1b* expression in the forebrain is identical in wild-type and *dtr(gli1)^{+/-}* heterozygous embryos. (B) *dtr(gli1)^{-/-}* mutants have reduced *nk2.1b* expression in the dorsal diencephalon adjacent to the telencephalon (arrowhead). (C) *smu(smo)* mutant with greatly reduced *nk2.1b* expression in both the telencephalon and diencephalon. (D, E, F) *gli3*MO (10 ng) injected into a clutch of embryos from *dtr(gli1)^{+/-}* heterozygous parents yielded 6 (26%) wild-type embryos which had a slight reduction of *nk2.1b* in the dorsal diencephalon (D, arrowhead), 10 (44%) heterozygous *dtr(gli1)^{+/-}* embryos which had a *dtr(gli1)* mutant-like loss of *nk2.1b* expression in the dorsal diencephalon (E, arrowhead), and 7 (30%) *dtr(gli1)^{-/-}* mutant embryos which had greatly reduced diencephalic *nk2.1b* expression (F, arrowhead) similar to phenotype seen in *smu(smo)* mutants. (G, H, I) Coinjection of *gli3*MOs and *gli2*MOs into a clutch of embryos from *dtr(gli1)^{+/-}* heterozygous parents resulted in a slight reduction of diencephalic *nk2.1b* expression in 6 (25%) embryos (G, arrowhead), a *dtr(gli1)^{-/-}* mutant-like reduction of *nk2.1b* expression in 12 (50%) embryos (*dtr(gli1)^{+/-}* heterozygotes) (H, arrowhead), and greatly reduced diencephalic *nk2.1b* expression in 6 (25%) embryos (*dtr(gli1)^{-/-}* mutants) (I, arrowhead). Double MO injections led to dorsal expansion of *nk2.1b* in the telencephalon (arrows in G, H, I), as shown previously for *gli2*MOs (Karlstrom et al., 2003). All panels show 30-h embryos, side views of the forebrain, eyes and yolk removed, anterior to the left. Genotype was determined by comparing defects in marker gene expression. (J) Graph showing percentages of injected embryos with different *nk2.1b* expression patterns.

34 h. *gli3*MO injection led to profound and variable defects in RGC differentiation. In the most severely affected embryos (approximately 23% of injected embryos), only a few ZN-5-labeled RGC cell bodies were present in the eye, and these were distributed in abnormal locations (Fig. 8B,

Table 2). Less severely affected embryos (approximately 38%) had reduced RGC numbers, with RGCs being patterned normally and extending axons correctly across the midline to form a thin optic nerve and chiasm (Fig. 8C, Table 2). These differences in defect severity are likely due

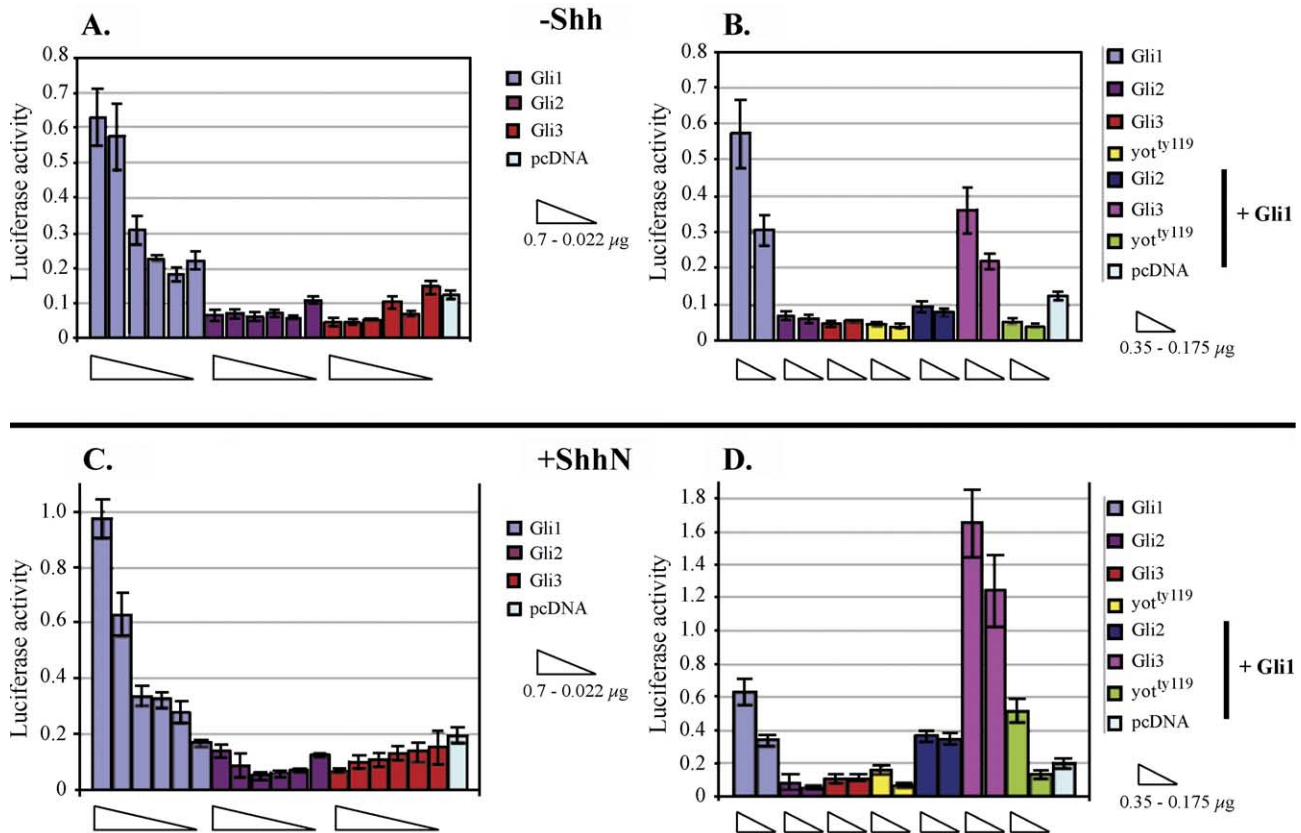


Fig. 7. Transcriptional activity of zebrafish Gli1, Gli2, and Gli3 in C17 neuronal cells. (A–D) pcDNA constructs encoding zebrafish Gli1, Gli2, Gli3, and the *yot^{ty119}* Gli2-dominant repressor (Gli2-DR) (Karlstrom et al., 2003) proteins were transfected into C17 cells along with a luciferase reporter gene under the transcriptional control of eight Gli1 binding sites (Sasaki et al., 1997). (A) In the absence of Shh, Gli1 (but not Gli2 or Gli3) can activate the reporter in a dose-dependent manner. (B) Cotransfection of multiple *gli* constructs in the absence of *Shh* shows that Gli2, truncated Gli2 proteins encoded by zebrafish *you-100* mutants, and Gli3 can all repress Gli1-mediated transcriptional activity. (C, D) The same experiments were performed in a cell line stably expressing Shh (Feng et al., 2004). (C) In the presence of Shh, Gli1 activator function is largely unaltered, and Gli2 and Gli3 do not activate the reporter construct. (D) In the presence of Shh, Gli3 expression enhances rather than represses Gli1-mediated reporter gene expression.

to reduced *gli3MO* concentrations and/or effectiveness at later stages of development (28 h) when Hh expression begins to induce RGC differentiation in the eye (Neumann and Nusslein-Volhard, 2000). The fact that RGC axons can cross the midline in embryos with extremely reduced RGC numbers suggests that midline guidance cues are expressed normally in the absence of Gli3 function at the midline. This is contrast to zebrafish Gli1 and Gli2, which are both involved in the formation of the diencephalic substrate for RGC axon growth (Karlstrom et al., 1999, 2003).

To determine whether the wave of cell differentiation caused by Hh signaling is initiated in the absence of Gli3 function, we examined the expression of *ath5* in *gli3MO*-injected embryos. Again, two classes of affected embryos were seen, with the severity of response being MO dose-dependent (Table 1). At higher doses of *gli3MO* (12 ng), *ath5* expression in the eye was completely eliminated in over 60% of injected embryos and severely reduced in 30% of embryos (Fig. 8E, Table 2). Since *ath5* expression and RGC differentiation are known to depend on Hh signaling (Kay et al., 2001; Masai et al., 2000; Neumann and Nusslein-Volhard, 2000; Stenkamp and Frey, 2003) and

since RGC differentiation is normal in zebrafish, *gli1* and *gli2* mutants and morphants (Karlstrom et al., 1996 and R.O.K. unpublished data), we conclude that *gli3* is the primary activator of Hh-mediated RGC differentiation in the developing eye.

Discussion

Dual roles for zebrafish Gli3 in activating and repressing Hh signals

We demonstrate that zebrafish Gli3 acts as both an Hh-dependent activator and an Hh-independent repressor in Hh signaling, functions that are conserved between mammals and fish. Early in development, Gli3 is an activator, combining with Gli1 to help pattern the ventral CNS. Consistent with this in vivo function, in vitro reporter assay analyses show that Gli3 cooperates with Gli1 in the presence of Hh to activate target gene transcription. Gli3 activator function is also required in the eye for the differentiation and patterning of retinal ganglion cells. We

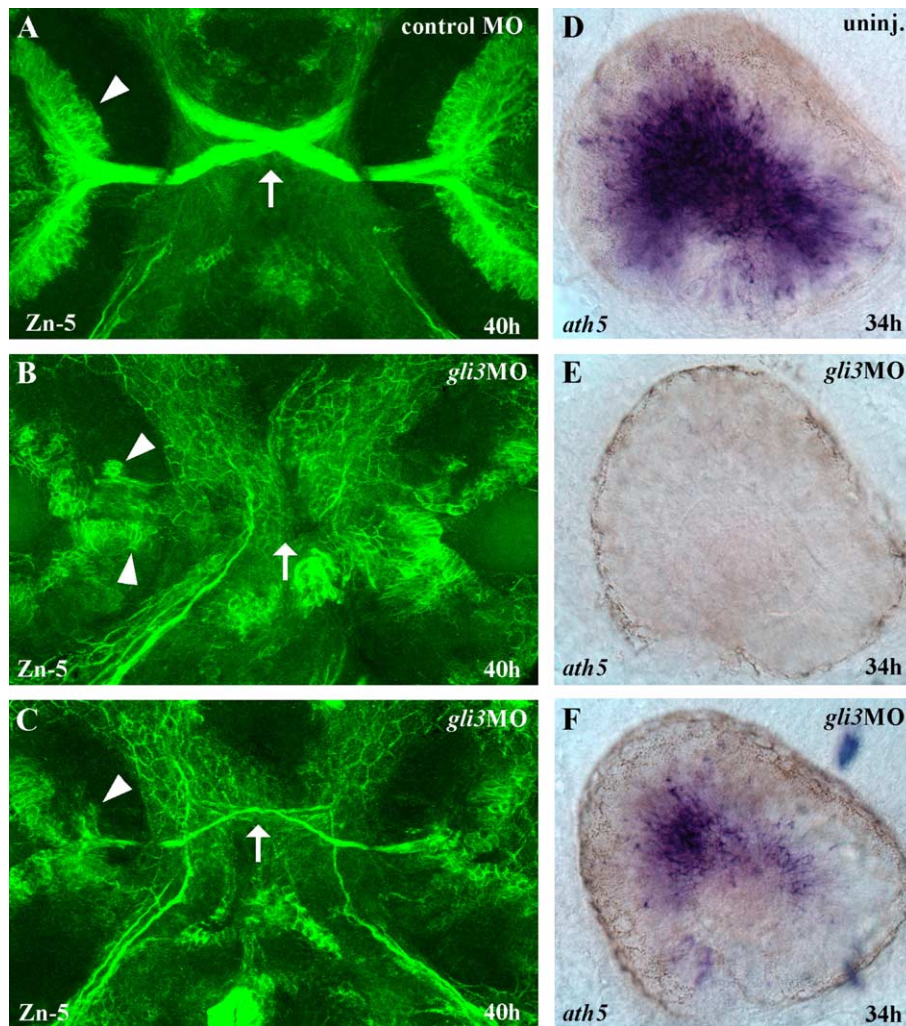


Fig. 8. Gli3 is required for RGC differentiation. (A) At 40 h of development, large numbers of Zn-5 labeled retinal ganglion cells (RGCs) (arrowheads) in the eyes have differentiated and extended axons across the midline to form the optic nerve and chiasm (arrow). (B) In severely affected *gli3MO* injected embryos, very few RGCs differentiated, and those that were present were distributed aberrantly in the eye (arrowheads). (C) In less severely affected *gli3MO* injected embryos, reduced numbers of RGCs differentiated in the correct location and extended axons across the midline to form a thin optic nerve and chiasm (arrow). (D) At 34 h, the zebrafish atonal homolog *ath5* is expressed throughout the central region of the differentiating retina. (E) In severely affected *gli3MO*-injected embryos, *ath5* expression was completely eliminated in the eye. (F) In less severely affected *gli3MO*-injected embryos, *ath5* expression was strongly reduced. (A–C) Fluorescent labeling of RGCs at 40 h using the Zn-5 antibody, ventral views of head, anterior up. (D–F) *ath5* in situ labeling of the eyes at 34 h, lateral views of the left eye, dorsal up, anterior to the left.

show that Gli3 function changes at late somite stages when Gli3 becomes required to repress expression of some but not all ventral Hh target genes. Consistent with a repressor role in tissues distant from the source of Hh, our *in vitro* analysis confirms that zebrafish Gli3 inhibits Gli1-mediated gene activation in the absence of Hh. Our data suggest that Gli3 blocks Hh target gene expression in part by restricting expression of Gli1, itself an Hh target gene, to the ventral neural tube.

Vertebrate Gli3 proteins as activators of Hh signaling

While Gli3 has been widely studied as a repressor of Hh signaling, several lines of evidence have recently come together to show that *gli3* can activate Hh signaling during

vertebrate embryogenesis. First, cell culture analyses show that full-length Gli3 can activate a reporter construct containing Gli1 binding sites (Dai et al., 1999). In addition, ectopic expression of N-terminally truncated forms of Gli3 shows that the Gli3 protein can activate Hh target genes *in vivo* (Ruiz i Altaba, 1998; Sasaki et al., 1999). These *in vitro/ectopic* expression results have recently been complemented by *in vivo* evidence for Gli3 activator function. The fact that *Gli2*^{-/-};*Gli3*^{+/-} double mutant mice have more severe skeletal, lung, and tracheal defects than *Gli2*^{-/-} mutants suggests overlapping activator functions for these two Gli proteins (Mo et al., 1997; Motoyama et al., 1998). Additionally, recent epistasis analyses of the *Ptc*, *Gli2*, and *Gli3* mutations in mouse show that Gli3 can activate the Hh response to help pattern the ventral neural tube (Motoyama

et al., 2003). Finally targeted knockin of mouse *gli3* into the *gli2* locus demonstrated that Gli3 can rescue some ventral spinal cord defects seen in Gli2 mutant mice, and that Gli3 acts as a Gli1-dependent activator of the Hh response (Bai et al., 2004).

Our double knockdown analysis has now shown that zebrafish Gli3 functions, in combination with Gli1, to help pattern the ventral neural tube. This Gli3 activator function is weaker than that of Gli1, as loss of Hh target genes is not seen when only Gli3 function is blocked. This is similar to the situation in mouse, where Gli3 activator function is weaker than that of Gli2, the primary activator in mammals (Bai et al., 2004). Our data show that the role of Gli3 as a secondary activator of Hh signaling has been evolutionarily conserved.

Gli3 cooperates with Gli1 in the presence of Shh

Our in vitro reporter assay also provides new evidence that zebrafish Gli3 cooperates with Gli1 to activate Hh target genes. In the absence of Gli1, Gli3 shows no activator function in the C17 neuronal line. However, in the presence of Gli1, Gli3 promotes Gli1 activation of the reporter construct in an Shh-dependent manner. Human Gli3 was previously shown to activate a similar reporter construct in NIH3T3 and MNS70 cell lines, with Hh enhancing this activity while suppressing cleavage of Gli3 protein (Dai et al., 1999). Expression of Gli1 was not needed to see Gli3 activator function in these cells, and cooperativity with Gli1 was not examined. How can we explain the Hh and Gli1 dependence of Gli3 activity in C17 cells? A likely explanation for Hh dependence is that the processed repressor form of Gli3 predominates in C17 cells, and that Hh is needed to block this processing. It has been shown that Gli1 and Gli3 can bind the same DNA sequences in vitro (Ruppert et al., 1990; Sasaki et al., 1997; Vortkamp et al., 1995), suggesting that both proteins could be activating the reporter gene through the same promoter sequences. However, if Gli1 and Gli3 were simply binding to the same sequence, we would expect Gli3 alone to activate the Hh response. The fact that no activation is seen in C17 neuronal cells suggests that ectopically expressed Gli3 may be efficiently processed to a repressor form. The increased activation of the Gli reporter upon cotransfection of Gli1 and Gli3 suggests a cooperative interaction between Gli3 and Gli1 proteins resulting in enhanced activator function. The fact that cooperativity is seen only in cells transfected with Hh indicates that Hh is needed to confer this activator function on Gli3. Based on the known mechanisms by which Hh regulates Gli3 in vitro (Dai, 1999), this could occur by inhibition of cleavage and/or by activity enhancing modifications of the full-length protein.

Gli3 and retinal ganglion cell differentiation

Our studies have also uncovered a new role for *gli3* in the differentiation of retinal ganglion cells in the eye. MO

inhibition of Gli3 function led to a loss in *ath5* expression, a reduction in RGC numbers, and disrupted RGC organization in the eye. Hh signaling has been shown to be required for the wave of RGC and other cell differentiation in the eye (Neumann and Nüsslein-Volhard, 2000; Stenkamp and Frey, 2003), but it was not known which Gli protein was responsible for mediating this differentiation program. Our data indicate that Gli3 is the primary activator of Hh-mediated RGC differentiation in the eye, since we have previously shown that RGC differentiation is apparently normal in both *gli1(dtr)* and *gli2(yot)* mutant embryos (Karlstrom et al., 1996). The midline may be the original source of Hh signals that induces the first RGCs to differentiate (Masai et al., 2000; Stenkamp and Frey, 2003), with later (28–30h) eye-specific expression of Shh serving to propagate RGC differentiation as the eye develops (Neumann and Nüsslein-Volhard, 2000). *ath5* expression begins in the choroid fissure at approximately 25 h of development and spreads into the retina by 25 h (Masai et al., 2000), and *ath5* expression can be completely eliminated following injection of *gli3*MOs (Fig. 8E). *gli3* expression begins to be detected in the RGC layer at 26–30 h of development (Fig. 2J), suggesting that *gli3* expression in the eye could mediate this early *ath5* response. In addition, an increase in *gli3* expression in RGCs at 30 h indicates that *gli3* transcription may be up-regulated by Hh in these cells, consistent with a role in activating the Hh response. Whether Gli3 plays a role in later Hh-dependent retinal cell differentiation (Shkumatava et al., 2004) remains to be investigated.

Gli3 repressor activity: temporal regulation

A large amount of in vivo and in vitro data support a major role for Gli3 as a repressor of Hh signaling (reviewed in Ingham and McMahon, 2001). What remains unclear is exactly how this repressor function is spatially and temporally regulated in vivo. Our results demonstrate that the requirement for Gli3 repression in the hindbrain and spinal cord varies at different times in embryogenesis, with Gli3 being required to repress *fkf4* and *gli1* expression in the dorsal neural tube at 24 h, but not at 19 h of embryonic development (Fig. 4). Furthermore, we show that *gli3* is expressed in the neural tube at both ages and that *fkf4* can expand dorsally at 19 h in *shh* mRNA-injected embryos, indicating that Gli3 is present and cells are capable of ectopically expressing Hh genes at the earlier age. How then is *fkf4* expression restricted to ventral cells at early ages? One obvious possibility is that another Gli protein, most likely Gli2, acts as the primary repressor at early stages. However, reducing both Gli2 and Gli3 function did not lead to ectopic *fkf4* expression at 19 h. This may indicate that yet another Gli repressor may be involved in the early repression of Hh targets in the dorsal CNS. A fourth Gli-like sequence has been reported with expression similar to Gli2 that may perform

this function (Y.L. Yan and J.H. Postlethwait, personal communication).

How could Gli3 activate Hh signaling early in development but then repress Hh signaling at later ages? One possibility is that temporally regulated posttranslational processing of Gli3 may occur between these ages. While it remains to be shown that Gli3 is cleaved in the embryonic nervous system, several studies suggest that Gli3 repressor function is the result of a cleavage event that creates a truncated protein similar to the Ci repressor isoform (Dai et al., 1999; Shin et al., 1999). Indeed, it was demonstrated that human and mouse Gli3 proteins undergo cleavage to produce C-terminal repressors of Hh signaling when expressed in the fly wing imaginal disc (Aza-Blanc et al., 2000). Consistently, a truncated repressor form has been observed in cell culture assays (Ruiz i Altaba, 1999), and in mouse and chick limbs (Wang et al., 2000). In the mouse, analyses of *Shh*^{-/-} and *Shh*^{-/-};*Gli3*^{-/-} mutants demonstrated that Gli3 acts as a repressor in the absence of Hh signaling (Rallu et al., 2002). Our results raise the possibility that this cleavage could be temporally regulated.

Combinatorial Gli repressor functions

We found that Gli3 function is needed to repress *fkf4* expression but not to repress the floor plate markers *nk2.2* and *axial* (Fig. 4). Since Shh is known to regulate the expression of these genes, we predicted that another Gli protein must counteract Hh signaling in order to restrict *nk2.2* and *axial* to the ventral CNS. Since *gli3* and *gli2* expression patterns overlap in the neural tube (Fig. 2 and Karlstrom et al., 1999) and since Gli2 acts as a repressor in the telencephalon (Karlstrom et al., 2003), we examined whether Gli2 could be this second repressor. We found that Gli2 does indeed contribute to the repression of *axial* in the dorsal neural tube (Fig. 4L). Previous findings also indicate that Gli proteins can differentially regulate the expression of different Hh targets (Aza-Blanc et al., 2000; Mullor et al., 2001).

Unlike mouse where loss of Gli3 repressor function partially rescues the *Shh*^{-/-} phenotype (Litingtung and Chiang, 2000; Meyer and Roelink, 2003), loss of both Gli3 and Gli2 repressor functions in zebrafish embryos lacking Hh signaling activity [*smu(sm)* mutants] does not rescue lateral floor plate or motor neuron defects (Fig. 4O, Tables 1,2). Thus, repression of *gli3* by Hh is not sufficient for ventral spinal cord development in zebrafish as it is in mice. This could be because Gli1-mediated Hh signaling is directly needed for ventral differentiation. Alternatively, a Gli protein other than Gli2 and Gli3 may act to block Hh target gene expression in the dorsal neural tube.

Regulatory interactions among gli genes

The expression patterns of the different *gli* genes help define distinct cellular responses to Hh signals. Initially, all three *gli* genes are expressed in most regions of the CNS,

with expression becoming refined so that repressor *glis* (*gli2* and *gli3*) are expressed more dorsally, while expression of the primary activator *gli* (*gli1*) remains ventral. The presence of *gli3* transcripts prior to *gli1* and *hh* expression in frog animal caps, mouse and chick limb buds, and quail somites is consistent with an initial activator role for Gli3 in regulation of Gli1 in these tissues (Borycki et al., 2000; Lee et al., 1997). As in other species, expression of zebrafish *gli3* initially overlaps with *shh* and *gli1* in ventral neural tissue, consistent with their initial overlapping activator functions. The subsequent loss of *gli3* expression ventrally appears to be a direct result of Hh signaling, as loss of Hh signaling leads to a ventral expansion of *gli3*, while ectopic expression of Hh represses *gli3* expression throughout the neural tube (Fig. 3).

Our observation that *gli3*MO injection leads to a loss of *gli3* transcripts dorsally was surprising and suggests that Gli3 could positively regulate its own transcription, perhaps by inhibiting the transcription of another repressor. This would provide a feedback mechanism that might explain the later expression pattern. In this model, early Hh-independent *gli3* expression in the ventral neural tube would be repressed by Hh signals, while dorsal expression of *gli3* would directly or indirectly act to maintain *gli3* transcription there.

Our finding that *gli1* expression expands dorsally in *gli3*MO-injected embryos (Fig. 4F) indicates that *gli1* is itself a target of Gli3 transcriptional repression. The fact that this *gli1* expansion occurs in CyA-treated *gli3*MO-injected embryos (Fig. 4G) indicates that, like *Drosophila* Ci, Gli3 repression of Hh target genes is Hh-independent. Reducing Gli3 function does not completely restore normal levels of *gli1* in CyA-treated embryos, however, confirming that normal *gli1* expression levels require active Hh signaling. The final level of *gli1* expression in the zebrafish neural tube can thus be seen to result from a balance between Hh-dependent activation and Hh-independent Gli3-mediated repression, similar to the situation in mouse (Litingtung and Chiang, 2000; Meyer and Roelink, 2003). In the absence of Hh signals (CyA-treated and *smu(sm)* embryos), Gli3 repressor function predominates, resulting in a nearly complete repression of *gli1* expression (Fig. 4C). Since *gli3* is expressed broadly in the developing spinal cord (with the exception of the most ventral region, see Fig. 2H), this can partially explain the ventrally restricted *gli1* expression pattern; under normal conditions, Gli3 repression keeps *gli1* levels low except in the ventral cells that are exposed to Hh. The balance of Hh-dependent Gli1 activator and Hh-independent Gli3 repressor activities along the dorsal/ventral axis of the neural tube would ultimately result in graded expression of *gli1* activator function, contributing to a graded morphogen response.

Species-specific differences in Gli3 function

A major challenge in understanding Hh signaling is to determine how vertebrate Gli transcription factors work

together to interpret Hh signals in responding cells. In all vertebrate species examined, Gli1 seems to be primarily an activator of the Hh transcriptional response, while Gli2 and Gli3 can have both activator and repressor functions. Despite this conservation, Gli genes also have evolved distinct functions in different species. We previously showed that Gli1 is the major activator of Hh signaling in zebrafish, whereas Gli2 has minor activator and repressor roles that are tissue- and region-specific (Karlstrom et al., 2003). This contrasts with the situation in mouse, where Gli2 is the major activator of the ventral Hh response and Gli1 functions as a minor activator that is not essential for initial neural patterning. The dual roles of Gli3 as both a weak activator and repressor of Hh signaling seem to be conserved among vertebrate species. Besides similarities in function, we found that the transcription of *gli3* is regulated by Hh in a way that is similar to that in mouse (Fig. 3, Ruiz i Altaba, 1998; Wang et al., 2000). Thus, Gli3 is conserved as a complex, bifunctional transcriptional regulator of Hh target genes whose function is regulated at multiple levels, and in distinct ways in different tissues and at different developmental stages. Given that both gain-of-function and loss-of-function Hh signaling mutations lead to human disease, it is important to understand the rules governing Gli combinatorial function across different species.

Acknowledgments

We thank Noel Watkins and Jeanne Thomas for technical assistance, Emily Miller for help with zebrafish genotyping, Brendan Delbos for fish care, Michael Barresi for helping scoring “morphotypes,” Anandita Seth for RT-PCR analysis, and the rest of the Karlstrom laboratory for useful discussion. Thanks to John Postlethwait and Yi-Lin Yan for communicating data prior to publication, Will Talbot for advice on genetic mapping, and the Stenkamp laboratory and the rest of the zebrafish community for sharing *in situ* probes. A.F.S. is an Irma T. Hirsch Trust Career scientist, an established investigator of the American Heart Association, and supported by grants from the NIH. This work was supported in part by the Illinois Excellence in Academic Medicine Program (J.D.K.), NIH HD044745 (J.D.K.), and NIH NS39994 (R.O.K.).

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