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Cloning of zebrafish *nkx6.2* and a comprehensive analysis of the conserved transcriptional response to Hedgehog/Gli signaling in the zebrafish neural tube

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Abstract

Sonic Hedgehog (Shh) signaling helps pattern the vertebrate neural tube, in part by regulating the dorsal/ventral expression of a number of homeodomain containing transcription factors. These Hh responsive genes have been divided into two classes, with Class II genes being activated by Hh signaling and Class I genes being repressed by Hh signaling. While the transcriptional response to varying Hh levels is well defined in chick and mouse, it is only partially described in zebrafish, despite the fact that zebrafish has emerged as a powerful genetic system for the study of neural patterning. To better characterize the Hh response in the zebrafish neural tube, we cloned the zebrafish Class II Hh target genes *nkx2.9* and *nkx6.2*. We then analyzed the expression of a number of Class I and Class II Hh responsive genes in wild type, Hh mutant, and Hh over-expressing zebrafish embryos. We show that expression of Class I and Class II genes is highly conserved in the vertebrate neural tube. Further, ventral-most Class II gene expression was completely lost in all Hh pathway mutants analyzed, indicating high levels of Hh signaling are blocked in all of these mutants. In contrast, more dorsally expressed genes were variably affected in different Hh pathway mutants, indicating mid-levels of Hh signaling are differentially affected. This comprehensive expression study provides an important tool for the characterization of Hh signaling in zebrafish and provides a sensitive assay for determining the degree to which newly identified zebrafish mutants affect Hh signaling.

Keywords

dbx1a; dbx2; hlx1; hlx6.2; irx1a; irx3a; irx5; nkx2.2a; nkx2.9; nkx6.1; nkx6.2; pax3; pax6a; pax7; chameleon; detour; slow muscle omitted; you-too; cyclopamine; floorplate; motor neurons

1. Results and Discussion

Secreted proteins of the Hedgehog (Hh) family are well known for their role in patterning the vertebrate and invertebrate embryo (Ingham and McMahon, 2001). During development, Hh functions in a concentration dependent manner to influence cell differentiation (Ericson et al., 1997a). Hh can also act as a survival factor (Miao et al., 1997) or a mitogen (Dahmane et al., 2001) to help shape the embryo. The morphogen activity of Hh has been well studied in the neural tube of chick and mouse (Ericson et al., 1995; Wijgerde et al., 2002), where Hh secreted from the notochord induces floorplate and ventral neural fates based on the concentration of Hh seen by a neural progenitor cell (Echelard et al., 1993). Cells respond to Hh through a

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complicated signaling system that includes the membrane proteins Patched and Smoothened. Signaling is relayed to the nucleus via zinc-finger containing transcription factors of the Gli family that either activate or repress the transcription of Hh-responsive genes. These Gli transcription factors form a gradient of Gli activator function in the neural tube that reflects the Hh concentration gradient (Lei et al., 2004; Stamatakis et al., 2005).

Neural progenitor cells within the spinal cord respond to the concentration of Hh/Gli activity by expressing different combinations of homeodomain containing transcription factors (Ericson et al., 1997). A majority of these Hh responsive transcription factors belong to the *nkx* and *pax* families, which guide cell differentiation and patterning in a number of embryonic tissues (reviewed in Lang et al., 2006; Stanfel et al., 2005). Within the neural tube, these Hh-regulated transcription factors have been divided into two classes based on the transcriptional response elicited by Hh (Briscoe et al., 2000). Class II genes (including *nkx2.2*, *nkx2.9*, *nkx6.1*, and *nkx6.2*) are transcriptionally activated by Hh while Class I genes (including *pax6*, *pax7*, *irx3*, *dbx1*, and *dbx2*) are transcriptionally repressed by Hh. The combinatorial expression of Class I and II genes defines five domains of neural differentiation in the ventral half of the spinal cord, with the ventral-most domain producing V3 interneurons and motor neurons (MN), and more dorsal domains producing V0-V2 interneurons (Briscoe et al., 2000; Ericson et al., 1997a; Pierani et al., 1999).

In the mid 1990s, large-scale genetic screens identified a number of zebrafish mutations that subsequently have been shown to affect different components of the Hh signaling cascade (Brand et al., 1996; Karlstrom et al., 1996). The first of these mutants to be cloned was clairvoyantly named *sonic-you* (*syu*) and encodes Sonic Hedgehog (Shh) itself (Schauerte et al., 1998). Zebrafish *detour* (*dtr*) encodes Gli1, with *dtr* alleles blocking Gli1 mediated Hh signaling (Karlstrom et al., 2003). Zebrafish *youtoo* (*yot*) encodes dominant repressor forms of Gli2 (Gli2DR) that block both Gli1 and Gli2 mediated Hh signaling (Karlstrom et al., 2003). *Slow-muscle omitted* (*smu*) encodes Smoothened, and *smu* (*smo*) mutants appear to completely lack Hh activity (Chen et al., 2001; Varga et al., 2001). The zebrafish mutant *chameleon* (*con*) encodes Dispatched 1 (*disp1*), a membrane protein that is needed for Hh secretion and processing (Nakano et al., 2004). More recently identified mutants include *iguana* (*igu*), which encodes a novel protein in the Hh pathway called dZip (Sekimizu et al., 2004; Wolff et al., 2004), and *you(you)*, which encodes the secreted EGF repeat containing protein Scube2 that regulates long range Hh signaling (Kawakami et al., 2005; Woods and Talbot, 2005).

While a number of Hh responsive genes have been characterized in zebrafish, there has been no systematic analysis of Class I and Class II gene expression in the zebrafish spinal cord. We therefore cloned zebrafish homologs of the Class II genes *nkx2.9* and *nkx6.2* and analyzed the expression pattern of a large number of Class I and II genes in wild type embryos. We found that the transcriptional response to Hh signals in the CNS is largely conserved between mammals and teleosts. We then examined expression in the Hh pathway mutants *smu* (*smo*), *con* (*disp1*), *yot* (*gli2DR*), and *dtr* (*gli1*). The expression of ventrally expressed Class II genes was uniformly absent from these mutants, indicating that all of these mutations eliminate the highest levels of Hh activator function. In contrast, the expression of Class II genes *nkx6.1*, *nkx6.2*, *olig2* and the Class I genes *dbx1a* (*hlx1*), *dbx2*, *irx3a*, *pax7*, *pax3*, and *pax6a* were differentially affected, indicating that mid- and low-level Hh responses are disrupted to varying degrees in these Hh/Gli signaling mutants. Together, this collection of markers provides a means to thoroughly analyze the Hh response at all levels in the zebrafish spinal cord and allows for a more sensitive comparison of Hh signaling across vertebrate species.

1.1. Cloning of zebrafish *nkx2.9* and *nkx6.2*

Several Hh responsive transcription factors identified in other species have also been identified in zebrafish. These include homeobox containing Class I genes of the *dbx* (called *hlx1* in

zebrafish) (Seo et al., 1999), *iroquois* (*irx* genes) (Tan et al., 1999; Wang et al., 2001), and *pax* families (Krauss et al., 1991; Seo et al., 1998). Class II genes include homeobox containing *nkx* family members (Barth and Wilson, 1995; Cheesman et al., 2004) and *olig2* which encodes a bHLH transcription factor (Park et al., 2002). To allow for a more detailed analysis of the Hh transcriptional response in zebrafish, we cloned zebrafish homologues of two additional Class II genes of the *nkx* homeodomain transcription factor family. We designed PCR primers using zebrafish genomic sequences (www.sanger.ac.uk) similar to puffer fish (*Fugu rubripes*) *nkx2.9* (Santagati et al., 2001) and mouse *nkx6.2* (Komuro et al., 1993) and amplified these two *nkx* genes from zebrafish first strand cDNA. Sequence comparisons indicate that zebrafish *nkx2.9* (Accession # **DQ924560**) and *nkx6.2* (Accession # **DQ924561**) are most closely related to *Fugu nkx2.9* and mouse *nkx6.2*, respectively (Fig. 1). *nkx2.9* was subsequently identified in a microarray screen as a Hh responsive gene (Xu et al., 2006), but its expression during zebrafish embryogenesis was not documented. Assignment of homology was further verified by analysis of gene expression (see Fig. 2).

1.2. The expression of Hh responsive Class II genes is highly conserved across vertebrates

The newly cloned zebrafish *nkx2.9* gene is expressed in the *shh*-expressing domain of the ventral CNS (Fig. 2) in a pattern similar to chick and mouse *nkx2.9* (Briscoe et al., 2000; Pabst et al., 2003). In the brain, *nkx2.9* is expressed in the dorsal/anterior diencephalon, mid-diencephalon border, tegmentum, and ventral hindbrain (Fig. 2A). This expression is nearly identical to that of the well characterized class II gene *nkx2.2a* (Barth and Wilson, 1995), except that *nkx2.9* is not expressed in the adenohypophysis (Fig. 2A, see Karlstrom et al., 2003). *nkx2.9* is expressed in the ventral CNS as early as the tailbud stage (10 hpf) (Fig. 2A inset). Similar to *nkx2.9*, *nkx6.2* expression begins in the ventral CNS at the tailbud stage (10 hpf), but expression does not extend into the developing forebrain (Fig. 2B). *nkx6.2* expression terminates at the forebrain/midbrain border, clearly seen at 36hpf (Fig. 2B). The expression of zebrafish *nkx6.2* is most similar to the expression of *nkx6.2* in amniotes, including expression in the pancreas (Fig. 3B', Henseleit et al., 2005; Vallstedt et al., 2001).

Within the spinal cord, zebrafish *nkx2.9* is expressed in a single row of cells corresponding to the Hh dependent lateral floor plate cells (Schauerte et al., 1998) on either side of the midline (Fig. 2C). In mouse, *nkx2.9* is initially expressed in the floorplate and later is expressed in the ventral-most interneuron progenitors (p3) (Pabst et al., 1998), while in zebrafish *nkx2.9* expression seems to be restricted to lateral floor plate cells, at least up to 24 hpf (Fig. 2C, compare to *nkx2.2a* expression in Fig. 2F). In contrast, zebrafish *nkx6.2* expression encompasses the ventral half of the spinal cord, including the floor plate (Fig. 2D, Fig. 4A). This expression is similar to that in amniotes, where the *nkx6.2* expression domain includes p1-p3 progenitor cells, but does not include the floor plate (Vallstedt et al., 2001). In amniotes, the closely related gene *nkx6.1* is expressed in a slightly smaller ventral domain that does not include p1 progenitors (Briscoe et al., 1999). Zebrafish *nkx6.1* (Cheesman et al., 2004) is similarly expressed in all but the dorsal-most row of the *nkx6.2* expressing cells (Fig. 2E, see schematic in Fig. 4). Zebrafish *nkx2.2a* is expressed in lateral floor plate cells, and in the next cells dorsally, the V3 interneuron progenitors (p3) that arise ventral to motor neuron progenitors (pMN) (Barth and Wilson, 1995; Placzek and Briscoe, 2005). In amniotes, *olig2* is expressed in pMNs, oligodendrocyte precursors, as well as in motor neurons (Fu et al., 2002; Novitsch et al., 2001). Zebrafish *olig2* is similarly expressed in MN and V3 progenitors (pMN, p3) (Fig. 2H, Park et al., 2002). Finally, motor neurons themselves express a number of genes including *hlxb9* (Arber et al., 1999; Flanagan-Steet et al., 2005; Tanabe et al., 1998; Wendik et al., 2004), and *islet* genes (e.g. Hutchinson and Eisen, 2006). Zebrafish *hlxb9*, and *islet1/2* are expressed within the *nkx6.1* and *nkx6.2* expression domains, consistent with the differentiation of motor neurons in this region (Cheesman et al., 2004) (Fig. 2D, E, and G).

To characterize zebrafish Class II gene regulation by Hh signaling, the expression patterns of these genes were analyzed in embryos with elevated Shh levels, as well as in *smu(smo)*, *con(disp1)*, *yot(gli2DR)*, and *dtr(gli1)* mutants. Expression of *nkx2.9* was dramatically expanded in embryos with ectopic Hh signaling, consistent with its classification as Hh induced Class II gene (Fig. 2C, Xu et al., 2006). The expression of the *nkx2.9* was absent in all of the Hh pathway mutants examined (Fig. 2C) suggesting high levels of Hh signaling are required for transcriptional activation of this gene.

Expression of the newly cloned zebrafish *nkx6.2* gene was expanded in *shh* mRNA injected embryos and reduced in Hh pathway mutants (Fig. 2D), consistent with it being a Class II Hh regulated gene. In contrast to the ventral-most Class II genes, (e.g. *nkx2.2a*, and *nkx2.9*) expression of *nkx6.2* was affected differentially in different Hh pathway mutants. *nkx6.2* expression was completely absent in *smu(smo)* mutants (Fig. 2D), showing that *nkx6.2* expression absolutely requires Hh signaling. In *con(disp1)* mutants, dorsal *nkx6.2* expression is absent, while low levels of expression remain in the ventral-most CNS (Fig. 2D arrowhead). *nkx6.2* expression was more mildly disrupted in *dtr(gli1)* and *yot(gli2DR)* mutants, with expression absent in the floor plate, but remaining in more dorsal cells similar to the unaffected dorsal expression domain of *nkx6.2* in Gli2 knockout mice (Lei et al., 2004). This remaining *nkx6.2* expression suggests that some Hh signaling is intact, consistent with the ability of motor neurons to differentiate in these mutants (Chandrasekhar et al., 1999; Vanderlaan et al., 2005) and suggesting redundancy in Gli activator function. Surprisingly, the expression of *nkx6.2* in *dtr(gli1)* and *yot(gli2DR)* was complementary to that seen in *con(disp1)* mutants, with expression remaining dorsally, but being absent ventrally (Fig. 2D). Like *nkx6.2*, expression of *nkx6.1* was expanded in the presence of ectopic Shh and absent following cyclopamine induced loss of Hh activity (Fig. 2E, Cheesman et al., 2004). *Nkx6.1* expression is similarly lost in mouse *Smo* mutants (Wijgerde et al., 2002). However, unlike *nkx6.2*, *nkx6.1* expression was largely unaffected in *dtr(gli1)* and *yot(gli2)* mutants (Fig. 2E), suggesting differential regulation of these two genes by Hh signaling.

To better correlate changes in *nkx* gene expression with changes in neural precursor fates, we next examined the expression of the motor neuron markers *hlxb9* and *olig2* in embryos with altered Hh signaling. *olig2* expression was expanded upon Hh overexpression and both *hlxb9* and *olig2* were absent in *smu(smo)* mutants or cyclopamine treated embryos (Fig. 2H, data not shown, Park et al. 2002). This confirms that zebrafish motor neurons require Hh signaling to differentiate (Park et al., 2002) and is consistent with the loss of motor neurons and MNR2/Hb9 expression in mouse *Smo*^{-/-} mutants (Wijgerde et al., 2002). Motor neurons do differentiate in *con(disp1)* (Nakano et al., 2004), *dtr(gli1)* (Chandrasekhar et al., 1999), and *yot(gli2)* (Vanderlaan et al., 2005) mutants. Consistently, *hlxb9* expression was highly reduced but present in *dtr(gli1)* and *yot(gli2DR)* mutant embryos (data not shown). Surprisingly, *hlxb9* expression was undetectable in *con(disp1)* mutants (Fig. 2G). The expression of *hlxb9* and *olig2* in Hh mutants *dtr(gli1)* and *yot(gli2)* at earlier stages (16–17hpf) was identical to that seen at 24hpf (data not shown).

This analysis of Class II gene expression in Hh pathway mutants supports the idea that *smu(smo)* mutants represent a complete loss of Hh signaling (Barresi et al., 2000; Lewis et al., 1999; Stickney et al., 2000), while *dtr(gli1)* and *yot(gli2DR)* mutants affect a subset of Hh signaling functions. In *con(disp1)* mutants, the expression of *nkx6.2* only in cells adjacent to the floor plate is consistent with a loss of Shh secretion and diffusion (Nakano et al., 2004). These data illustrate how these markers allow a more detailed and systematic analysis of the differences in Hh signal disruption in different Hh pathway mutants.

1.3. The Class I gene transcriptional response to Hh diverges slightly among vertebrate species

The Class I genes of the *pax* family encode paired box homeodomain proteins, some of which are expressed in different dorsal/ventral domains of the developing spinal cord (Briscoe et al., 2000; Ericson et al., 1997a). In teleosts and amniotes, *pax3* is the most dorsally expressed gene, encompassing the dorsal third of the CNS (Fig. 3A, Liem et al., 1995; Mansouri et al., 2001; Seo et al., 1998). *pax7* is also expressed dorsally in the spinal cord, but is excluded from the most dorsal cells and the roof plate (Fig. 3G, Briscoe et al., 1999; Ericson et al., 1997a; Seo et al., 1998). *pax6a* is the most broadly expressed of these *pax* genes in the neural tube with expression throughout most of the dorsal/ventral axis, with the exception of the floor plate and the roof plate (Fig. 3M, Karlstrom et al., 2003; Krauss et al., 1991). Ventrally, the *pax6a* expression domain includes V0-V2 interneuron and motor neuron progenitors (p0-p2, pMN) (Briscoe et al., 2000).

The Class I genes *dbx1a* and *dbx2* are expressed in horizontal bands in the middle of the neural tube (Fig. 3S, Y, Fjose et al., 1994). In amniotes, *dbx1* is expressed in V0 interneuron progenitors (Pierani et al., 1999; Sander et al., 2000), while *dbx2* is in these cells as well as V1 interneuron progenitors (Pierani et al., 1999; Sander et al., 2000). Finally, class I Hh responsive genes of the *iroquois* family are expressed in broad domains in the dorsal CNS (Lewis et al., 2005; Park et al., 2004). Medially, *irx3a* (Fig. 3Z) and *irx5a* (data not shown) are expressed in the dorsal half of the neural tube, while laterally they are expressed along the entire D/V axis (Fig. 3Z, Bosse et al., 2000; Bosse et al., 1997; Tan et al., 1999; Wang et al., 2001).

pax3 expression has been shown to be regulated by Hh in neural explants (Liem et al., 1995), and in zebrafish somites (Feng et al., 2006). To better characterize *pax3* regulation by Hh in the spinal cord, we examined expression in zebrafish Hh gain- and loss-of function scenarios. Consistent with *pax3* being a Class I Hh-regulated gene, over-expression of *shh* led to reduced *pax3* expression that was confined to the dorsal-most cells in the neural tube (Fig. 3B). While *pax3* expression was ventrally expanded in the midbrain of *smu(smo)* embryos (data not shown), spinal cord expression of *pax3* appeared unaltered in *smu(smo)* (Fig. 3C), *con* (*disp1*) (Fig. 3D), *yot(gli2DR)* (Fig. 3E), and *dtr(gli1)* (Fig. 3F) mutant embryos.

pax7 expression is known to be negatively regulated by Hh signaling in amniotes (Ericson et al., 1997a), but this regulation has not been examined in the teleost neural tube. As in amniotes, zebrafish *pax7* expression was reduced when Hh signaling was upregulated (Fig. 3H). However, a difference in Hh regulation across vertebrates was revealed by our analysis of *pax7* expression in the zebrafish Hh pathway mutants. Unlike the situation in mouse (Wijgerde et al., 2002), and similar to zebrafish *pax3* (Fig. 3C-F), the expression of zebrafish *pax7* was not expanded upon loss of Hh signaling (Fig. 3I-L). This may point to Hh-independent repression of *pax3* and *pax7* in zebrafish embryos that could arise from other signaling molecules, or from Hh independent Gli function.

The expression of *pax6a* was ventrally expanded in the spinal cords of *smu(smo)*, *con(disp1)* (Nakano et al., 2004), *yot(gli2DR)*, and *dtr(gli1)* mutant embryos and was reduced in the presence of ectopic Hh signaling (Fig. 3N-R), as expected from a loss of Hh function. This result is consistent with the expansion of *pax6a* previously shown in the brain in *yot(gli2DR)* and *dtr(gli1)* mutant embryos (Karlstrom et al., 1999; Karlstrom et al., 2003) and matches the response of *pax6a* to the loss of Hh signaling in mammals (Caspary et al., 2002; Wijgerde et al., 2002).

The expression of *dbx1a* and *dbx2* in the middle of the neural tube (Fig. 3S, Y, Lu et al., 1992; Pierani et al., 1999; Seo et al., 1999; Shoji et al., 1996) suggests these genes may provide a sensitive assay for subtle defects in mid- to low-levels of Hh signaling, but this regulation

has not been shown in teleosts. Consistent with repression by Hh signaling, expression of both *dbx1a* and *dbx2* was reduced and shifted dorsally upon ectopic Shh induction (Fig. 3T, Y), with *dbx2* expression showing a more extreme dorsal shift (Fig. 3T, Y). In *smu(smo)* mutants, or with cyclopamine treatments that eliminate Hh signaling, *dbx1a* and *dbx2* expression were expanded ventrally (Fig. 3U, Y), similar to mouse *Smo* mutants (Wijgerde et al., 2002). In *con(displ)* mutant embryos the band of *dbx1a* expression was also expanded ventrally (Fig. 3V), showing that defects in Hh secretion affect the ability of Hh to repress expression of this gene. In contrast, *dbx1a* and *dbx2* expression appeared unaffected in *yot(gli2DR)* and *ddtr(gli1)* mutants (Fig. 3W-Y), suggesting that another Gli protein, possibly Gli3, might contribute to patterning in the intermediate regions of the spinal cord. Indeed, the expression of *dbx1a* was expanded dorsally in Gli3 mutant mice, indicating a regulatory role for Gli3 in the intermediate-dorsal spinal cord (Persson et al., 2002).

Finally, all three cloned members of the zebrafish *irx* gene family show regional regulation by Hh that is consistent with their classification as Class I genes. Medial *irx3a* (Fig. 3Z, Lewis et al., 2005; Park et al., 2004) and *irx5a* (data not shown) expression shifted dorsally with ectopic Hh signaling and ventrally with cyclopamine induced loss of Hh signaling, consistent with the ventral expansion of *irx3a* expression in mouse *smo*^{-/-} mutants (Wijgerde et al., 2002). *irx3a* expression appeared unaffected in *dtr(gli1)* and *yot(gli2DR)* mutants (Fig. 3Z). In summary, this careful analysis of class I gene expression provides a sensitive assay for subtle defects in Hh signaling, and points to slight differences in the Hh response across vertebrates.

1.4. Summary of the Hh transcriptional response in zebrafish

Figure 4 shows a summary of Hh responsive gene expression in the zebrafish spinal cord. As in mammals, Class I and Class II gene expression divides the zebrafish neural tube into distinct domains, each with unique combinations of homeodomain transcription factor gene expression that contribute to the differentiation of distinct neuronal cell types (Fig. 4A). In general, increasing Shh signaling ventralizes the spinal cord, while the loss of Gli mediated Hh signaling leads to dorsalization of these expression domains in the spinal cord (Caspari et al., 2002; Huang et al., 2002; Wijgerde et al., 2002). The loss of Hh signaling in *smu(smo)* mutants or cyclopamine treated embryos eliminated the activation of Class II gene (*nkx2.2a*, *nkx2.9*, *olig2*, *nkx6.1*, and *nkx6.2*) expression and eliminated ventral repression of Class I gene (*pax6a*, *dbx1a(hlx1)*, *dbx2*, and *irx3a*) expression (Fig. 4B). In *con(displ)* mutants, expression of the Class II gene *nkx6.2* was maintained in lateral floorplate cells that are adjacent to the Shh expressing medial floorplate (Fig. 4C). However, these ventral cells do not express other lateral floorplate or ventral precursor markers (Fig. 2, Nakano et al., 2004). This suggests that in *con(displ)* mutants, cells in direct contact with the floorplate receive diminished Hh signals that are capable of activating only *nkx6.2* expression, while more dorsal cells receive little or no Hh signaling (Fig. 4C). This is consistent with a loss of Shh secretion and diffusion due to a loss of *displ-1* function. Finally, the absence of Gli1 function (*dtr* mutants) or the presence of Gli2 dominant repressor proteins (*yot* mutants) eliminated expression of the ventral-most Class II genes *nkx2.2a* and *nkx2.9*, and reduced ventral aspects of the broader *nkx6.2* domain. The more ventral *nkx6.1* and *olig2* expression domains remained largely intact, while *hlxb9* expression was reduced but not absent (Figs 2, 4D). Interestingly, loss of Hh/Gli function in *dtr(gli1)* and *yot(gli2DR)* mutants expanded *pax6a* expression ventrally, but had no effect on the expression of the dorsally expressed Class I genes (*pax3*, *pax7*, *dbx1a(hlx1)*, and *dbx2*) or the dorso-laterally expressed *irx3a* (Figs. 3, 4D). Thus in the absence of either *gli1* or *gli2*, Hh signals appear to be generally reduced, but Hh signaling still influences gene expression in mid regions of the spinal cord (Fig. 4D). This indicates that Gli1 activator function is absolutely required for the ventral-most spinal cord progenitor cells in zebrafish, but that redundancy may exist in Gli function in more dorsal regions. This is consistent with the observation that motor

neuron fates are only partially affected in the spinal cord of *dtr(gli1)* and *yot(gli2)* mutants (Chandrasekhar et al., 1999; Vanderlaan et al., 2005).

2. Experimental Procedures

2.1. Fish lines and cyclopamine treatments

Wild type and mutant zebrafish embryos were collected and maintained at 28°C as described in (Westerfield et al., 1993) and staged according to (Kimmel et al., 1995). Mutant lines used were *slow-muscle-omitted* (*smu^{b64l}*) (Barresi et al., 2000), a loss of function Smoothed allele (Chen et al., 2001; Varga et al., 2001), *detour* (*dtr^{ts269}*), a loss of function Gli1 allele (Karlstrom et al., 2003), *you-too* (*yot^{ty17}*), which encodes a dominant repressor form of Gli2, (Karlstrom et al., 1999), and *chameleon*, (*con^{15a}*), a loss of function *dispatched1* allele (Nakano et al., 2004). Wild type embryos were also treated with 100µM cyclopamine (Toronto Chemicals) in 1ml of embryo medium (2M MgSO₄, 2M KCl, 2M CaCl₂, 0.5M NaH₂PO₄, 5M NaCl) from a stock of 10mM cyclopamine, starting at shield stage or tailbud stage. Embryos were incubated in a 12-well plate at 28°C. Control embryos were treated with equal volumes of ethanol (cyclopamine carrier) as previously described (Sbrogna et al., 2003). 24hpf embryos were fixed overnight at 4 °C with 4% paraformaldehyde.

2.2. cDNA cloning

Fragments of zebrafish *nkx2.9* and *nkx6.2* coding regions were PCR amplified from an oligo dT primed zebrafish first strand cDNA (Superscript II RT kit, Invitrogen). Primers used were: *nkx2.9* forward (5' ATGGCTATTTCAAACAAGTTCAGT), *nkx2.9* reverse (5' CGGTTTGCCATCTCGAACC AAA), *nkx6.2* forward (5' CATGACCGAGATGAAGACCTC), and *nkx6.2* reverse (5' TTGGCTCTCGGTCATTCCCAG). PCR products were gel purified and cloned into pCR II-TOPO and pCR 2.1 vectors respectively, using the TA cloning kit (Invitrogen).

2.3. Whole-mount in situ hybridization

Whole mount in situ hybridization was performed as described (Karlstrom et al., 1999) using digoxigenin or fluorescein labeled probes (Roche). The *nkx2.9* probe was prepared by digesting pCR II-TOPO-*nk2.9* with Xho I and transcribing with Sp6 RNA polymerase. The pCR 2.1-*nkx6.2* was digested with SpeI and transcribed with T7. Other probes used were *nkx2.2a* (Barth and Wilson, 1995), *hlxb9* (a generous gift from Larry Moss), *pax6a* (Krauss et al., 1991), *pax7* (Seo et al., 1998), *hlx1* (Fjose et al., 1994), *pax3* (Seo et al., 1998), *irx1a* (Wang et al., 2001), *irx3a* (Tan et al., 1999), *irx5* (Wang et al., 2001). In situ probes for *olig2* (AF442964), *dbx2* (NM_131179), and *nkx6.1* (AY437556) were generated directly from PCR products that included a T7 RNA polymerase binding sequence at the 3' end. Primer sequences used were: *nkx6.1* forward, 5'TCTGCGTTTGTTCACGT T and reverse 5'TAATACGACTCACTATAGGGTTTTTCATCGTCCGAGTTTG, *olig2* forward, 5'ATCTTTTTCTGTCCGCCGTC and reverse 5'TAATACGACTCACTATAGGGTATAGTCGAGGG CTGAGGAA, *dbx2* forward, 5'AACAGACAGCACACACAGAG and reverse 5'TAATACGACTC ACTATAGGGTAAAACCCAACAGTCCACCT. Embryos were fixed in 4% paraformaldehyde overnight after the color reaction. Whole mount embryos were cleared in 75% glycerol and examined using DIC optics on a Zeiss axioskop.

2.4. mRNA Injections

The *shh/T7TS* plasmid (Egger et al., 1995) was linearized with BamHI and in vitro transcribed using T7 polymerase (mMessage mMachine-T7, Ambion kit). Wild type embryos were

injected with ~100 pg of *shh* RNA at the 1–2 cell stage. Injected embryos were incubated at 28°C until 24 hpf, fixed with 4% paraformaldehyde, and processed for in situ hybridization.

2.5. Sectioning

For cryosectioning, labeled embryos were rinsed with phosphate buffer (0.1 M PB) and placed in melted 1.2% low melting agarose (Sigma) (in 5% sucrose). Agar blocks were placed in 30% sucrose overnight at 4 °C, embedded in OCT compound (VWR Company), and 20 µm thick sections were cut using a Leica cryostat. Sections were collected on Superfrost slides (Fisher Scientific) and analyzed using DIC optics. Some embryos were hand-cut into 1-somite-thick sections using fire-sharpened tungsten wire tools. All sections shown represent the region of the spinal cord over the yolk plug (somites 7–9).

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Figure 1. Phylogenetic analysis of zebrafish Nkx2.9 and Nkx6.2

Un-rooted cladogram showing the relatedness of the protein sequences encoded by the newly cloned zebrafish *nkx* genes. Zebrafish Nkx2.9 is most closely related to *Fugu* and mouse Nkx2.9, while Nkx6.2 is most closely related to mouse Nkx6.2. Sequences for zebrafish *nkx2.9* and *nkx6.2* can be found in Genbank (accession numbers **DQ924560** and **DQ924561**, respectively). *Da*; *Danio rerio*, *Fu*; *Fugu rubripes*, *Mu*; *Mus musculus*.

Figure 2. Class II Hh regulated genes in zebrafish: *nkx2.9* and *nkx6.2* expression and regulation by Hh

(A, B) Lateral views of 36 hpf embryos, eyes removed, dot marks optic recess. (A) *nkx2.9* is expressed in the ventral hindbrain, ventral midbrain and diencephalon in a pattern nearly identical to that of *nkx2.2a* (see Karlstrom et al., 2003). No *nkx2.9* expression was seen outside the nervous system up to 36 hpf. Inset shows expression of *nkx2.9* in the CNS at the tailbud stage (10 hpf). (B) *nkx6.2* is expressed in the ventral hindbrain and ventral midbrain, but is not expressed in the forebrain. In the hindbrain, *nkx6.2* is expressed dorsally, presumably in cranial motor neurons (arrows). Upper inset shows *nkx6.2* expression in the ventral CNS at tailbud stage (10 hpf). (B') Side view of somites 4–7 in the trunk showing *nkx6.2* expression in the developing pancreas at 36 hpf. (C–H) Spinal cord cross-sections of 24 hpf embryos. (C) *nkx2.9* is expressed in lateral floor plate cells, but not in the medial floor plate (*), similar to *nkx2.2a* (F). *nkx2.9* expression is dorsally expanded in *shh* mRNA-injected embryos. The expression of *nkx2.9* is lost in *smu(smo)*, *con(displ1)*, *yot(gli2DR)*, and *dtr(gli1)* mutants. (D) *nkx6.2* is expressed in the ventral half of the CNS. In *shh* mRNA-injected embryos *nkx6.2* expression is expanded dorsally. *nkx6.2* expression is completely absent in *smu(smo)* mutants. In *con(displ1)* mutants, *nkx6.2* expression is absent dorsally but remains in the medial and lateral floor plate (arrowhead). In *yot(gli2DR)* and *dtr(gli1)* mutants, *nkx6.2* expression is absent from the floor plate (arrowheads), but expression remains more dorsally. (E) *nkx6.1* is expressed in lateral floor plate cells, in motor neuron precursors and in most ventral interneuron progenitors.

nkx6.1 expression is dorsally expanded in *shh* mRNA-injected embryos and is absent in cyclopamine treated embryos. The expression of *nkx6.1* is not affected in *yot(gli2DR)*, and *dtr(gli1)* mutant embryos. (F) *nkx2.2a* is expressed in the ventral-most cells of the CNS, including the lateral floor plate but not the medial floor plate (*). (G) *hlxb9* is expressed in developing motor neurons just dorsal to the floor plate. *hlxb9* expression is undetectable in *con(displ)* (inset) and *smu(smo)* mutants and highly reduced in *yot(gli2DR)*, and *dtr(gli1)* mutants (data not shown). (H) *olig2* is expressed in both motor neuron and interneuron precursor cells. The expression of *olig2* is dorsally expanded in *shh* mRNA-injected embryos and absent in cyclopamine treated embryos. *olig2* expression appears unaffected in *yot(gli2DR)*, and *dtr(gli1)* mutants. di; diencephalon, fb; forebrain, hb; hindbrain, mb; midbrain, nc; notochord, s; somite, t; telencephalon. * marks the medial floor plate cells. Double ended arrows in each panel show dorsal and ventral extent of gene expression. Scale bars: (A,B), 50 μ m, (C-H) 15 μ m in large panels, 24 μ m in smaller panels.

Figure 3. Class I gene expression in zebrafish and regulation by Hh

(A) *pax3* is expressed in the dorsal-most cells of the spinal cord, including the roof plate. (B) *pax3* expression is absent or restricted dorsally in *shh* mRNA injected embryos. (C) In *smu(smo)* mutants, spinal cord *pax3* expression appears normal. (D-F) The expression of *pax3* is unaffected in the neural tubes of *con(displ)* (D), *yot(gli2DR)* (E), and *dtr(gli1)* (F) mutants. (G) *pax7* is expressed in the dorsal spinal cord, but not in the roof plate. (H) *pax7* expression is reduced or absent in *shh* mRNA-injected embryos. (I-L) The expression of *pax7* is unaltered in *smu(smo)* (I), *con(displ)* (J), *yot(gli2DR)* (K), and *dtr(gli1)* (L) mutant embryos. (M) *pax6a* is expressed throughout the spinal cord with the exception of the most ventral (arrow) and most dorsal cells. (N) *pax6a* is reduced in *shh* mRNA-injected embryos. (O-R) *pax6a*

expression is ventrally expanded in *smu(smo)* (O), *con(disp1)* (P), *yot(gli2DR)* (Q), and *dtr(gli1)* (R) mutants (arrows). (S) *dbx1a(hlx1)* is expressed in a band of cells in the middle of the D/V axis of the spinal cord. (T) *dbx1a(hlx1)* expression is reduced or absent after *shh* mRNA injection (arrow). (U-X) *dbx1a(hlx1)* expression is ventrally expanded in *smu(smo)* (U), and *con(disp1)* (V) mutants but is not altered in *yot(gli2DR)* (W), and *dtr(gli1)* (X) mutants. (Y) *dbx2* is expressed in the mid-dorsal cells of the spinal cord. (Y) The expression of *dbx2* is reduced and restricted to the dorsal most cells of the spinal cord in *shh* mRNA-injected embryos and *dbx2* expression is expanded ventrally in cyclopamine treated embryos. *dbx2* expression appears unaffected in *yot(gli2DR)* and *dtr(gli1)* mutants. (Z) *irx3a* is expressed in lateral and mid-dorsal cells. In *shh* mRNA-injected embryos, medial expression of *irx3a* is repressed while lateral *irx3a* expression remains unaffected. In cyclopamine treated embryos *irx3a* is expressed throughout the neural tube with the exception of the medial floor plate. The expression of *irx3a* appears unaffected in *yot(gli2DR)* and *dtr(gli1)* mutant embryos. All panels show spinal cord cross sections of 24 hpf embryos. Double-ended arrows show dorsal and ventral extent of gene expression. In general, labeling intensity appeared similar in wild type and mutant embryos. Scale bar: 15µm (33µm in Y, Z mutant panels).

Figure 4. Schematic of Class I, and Class II gene expression in the zebrafish spinal cord

(A) Schematic spinal cord cross section showing the overlapping expression domains of Hh responsive Class I (green bars) and Class II (red bars) genes. Progenitor cell positions are based on published data from mouse and chick, in which the expression domains of *olig2*, *nkx6.1*, *nkx6.2*, and *pax6a* overlap in p3 progenitor cells, and the expression domains of *nkx6.1*, *nkx6.2*, *hlxb9*, *olig2*, *irx3a*, and *pax6a* overlap in motor neuron (MN) precursors. The expression domains of *irx3a*, *pax6a*, *nkx6.1*, *nkx6.2*, and *alx(Chx10)* overlap in a few of the p2 progenitor cells (Kimura et al., 2006). The green triangle represents the level of Hh signaling, from high ventrally to low dorsally. (B) In *smu(smo)* mutant and cyclopamine treated embryos, most ventral Class II gene expression is lost and the expression of Class I genes expands ventrally (arrow extensions on red bars). The loss of Hh signaling is indicated by the absence of the green triangle. (C) In *con(displ)* mutant embryos, Class II gene expression is lost with the exception of *nkx6.2*, which remains only in the most ventral cells. The expression domains of the Class I genes *dbx1a(hlx1)* and *pax6a* expand ventrally, similar to the situation in *smu(smo)* mutants. The smaller triangle indicates reduced Hh signaling activity that extends only to cells adjacent to the Shh expressing floorplate. (D) In *yot(gli2DR)* and *dtr(gli1)* mutant embryos, expression of ventral-most Class II genes (*nkx2.2a*, *nkx2.9*) is absent, while the expression of *nkx6.2* is only absent ventrally. Expression of *olig2* and *nkx6.1* remains unaffected, while the expression of *hlxb9* is highly reduced. The expression of dorsally expressed Class I genes appears unchanged, while ventral *pax6a* expression expands into the ventral-most cells of the spinal cord. The thinner triangle indicates a general reduction in Hh

signaling levels throughout the ventral neural tube. Expression patterns shown are based on data in this paper, references mentioned above, as well as data contained in (Egger et al., 1995) for *shh*, (Odenthal and Nusslein-Volhard, 1998) for *axial(fkd1)/hnf3((foxa2) and fkd4*, and (Talbot et al., 1995) for *floating head (flh)*. ** *nkx6.1* expression is reduced in the ventral-most cells in *smu(smo)* mutants, but completely absent in cyclopamine treated embryos.