

Roles of Hedgehog pathway components and retinoic acid signalling in specifying zebrafish ventral spinal cord neurons

Samantha England^{1,2,*}, Manuel F. Batista^{2,*}, John K. Mich³, James K. Chen⁴ and Katharine E. Lewis^{1,2,†}

SUMMARY

In mouse, Hedgehog (Hh) signalling is required for most ventral spinal neurons to form. Here, we analyse the spinal cord phenotype of zebrafish maternal-zygotic *smoothened* (*MZsmo*) mutants that completely lack Hh signalling. We find that most V3 domain cells and motoneurons are lost, whereas medial floorplate still develops normally and V2, V1 and V0v cells form in normal numbers. This phenotype resembles that of mice that lack both Hh signalling and Gli repressor activity. Ventral spinal cord progenitor domain transcription factors are not expressed at 24 hpf in zebrafish *MZsmo* mutants. However, pMN, p2 and p1 domain markers are expressed at early somitogenesis stages in these mutants. This suggests that Gli repressor activity does not extend into zebrafish ventral spinal cord at these stages, even in the absence of Hh signalling. Consistent with this, ectopic expression of Gli3R represses ventral progenitor domain expression at these early stages and knocking down Gli repressor activity rescues later expression. We investigated whether retinoic acid (RA) signalling specifies ventral spinal neurons in the absence of Hh signalling. The results suggest that RA is required for the correct number of many different spinal neurons to form. This is probably mediated, in part, by an effect on cell proliferation. However, V0v, V1 and V2 cells are still present, even in the absence of both Hh and RA signalling. We demonstrate that Gli1 has a Hh-independent role in specifying most of the remaining motoneurons and V3 domain cells in embryos that lack Hh signalling, but removal of Gli1 activity does not affect more dorsal neurons.

KEY WORDS: Smoothened, V1, V2, Floor plate, Gli3, Interneuron, Zebrafish

INTRODUCTION

In mouse, Hedgehog (Hh) signalling is required for the formation of most ventral spinal cord motoneurons (MNs) and interneurons (Ericson et al., 1996; Litington and Chiang, 2000; Wijgerde et al., 2002; Lupo et al., 2006; Lewis, 2006). This was determined by analysing sonic hedgehog (*Shh*) mutants as well as mosaic embryos that contained smoothened (*Smo*) mutant cells. *Smo* is an essential component of the Hh signalling pathway and it is required for signalling by all Hh proteins (reviewed by Ingham, 2008). In mouse *Shh* mutants, V3 cells and MNs are lost and V2, V1 and V0v cells are severely decreased (Litington and Chiang, 2000; Wijgerde et al., 2002). Some ventral interneurons may persist owing to Indian hedgehog signals emanating from the gut (Wijgerde et al., 2002), although this has not been directly tested. Consistent with this hypothesis, in mosaic mouse embryos that contained *Smo* mutant cells, none of the mutant cells developed into V3, MN, V2 or V1 cells, although a small number of *Smo* mutant V0v cells were observed in the ventralmost part of the spinal cord (Wijgerde et al., 2002). However, the spinal cord phenotype of embryos that completely lack Hh signalling has not yet been reported in any vertebrate.

In mouse, *Smo* mutants die too early for ventral neurons to be examined (Wijgerde et al., 2002; Zhang et al., 2001) and there have been no reports of spinal cord patterning in compound *Hh* mutants.

Hh signalling is mediated by Gli transcription factors and compound Gli mouse mutants have been analysed (e.g. Jacob and Briscoe, 2003; Lei et al., 2004). However, as some Gli transcription factors repress, as well as activate, downstream targets of Hh signalling, these do not have the same effect as just losing Hh signalling. In zebrafish, several laboratories have investigated the effects of severely reducing Hh signalling on the expression of spinal cord ventral progenitor domain markers (e.g. Cheesman et al., 2004; Guner and Karlstrom, 2007; Park et al., 2002; Park et al., 2004; Schauerte et al., 1998) and the formation of MNs (Chen et al., 2001; Lewis and Eisen, 2001; Park et al., 2004; Varga et al., 2001) and V3 domain cells (Schäfer et al., 2007), but none of these studies has investigated whether loss of Hh signalling affects V2, V1 or V0v cells.

The zebrafish genome contains a single *smo* gene, which is expressed both maternally and zygotically (Chen et al., 2001; Lewis and Eisen, 2001; Varga et al., 2001; Mich and Chen, 2011). However, unlike in mouse, in zebrafish even maternal-zygotic *smo* (*MZsmo*) mutants survive until after spinal cord neurons have developed. This provided us with a unique opportunity to examine spinal cord patterning in a vertebrate embryo that completely lacks Hh signalling. To our surprise, we found that, unlike in mouse, normal numbers of V2, V1 and V0v cells form in zebrafish in the absence of Hh signalling. We investigated possible mechanisms that might explain this difference. We also tested various hypotheses about what induces ventral neurons in the absence of Hh signalling. In both cases, we discovered a key role for Gli transcription factors.

¹Biology Department, Syracuse University, 107 College Place, Syracuse, NY 13244, USA. ²Department of Physiology, Development and Neuroscience, Anatomy Building, Downing Street, Cambridge, CB2 3DY, UK. ³Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, USA. ⁴Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305, USA.

*These authors contributed equally to this work

†Author for correspondence (kelewi02@syr.edu)

In amniotes, retinoic acid (RA) signalling has been implicated in the specification and differentiation of at least V1 cells, V0v cells and MNs, suggesting that it might be the signal that specifies ventral spinal neurons in the absence of Hh signalling. For example, RA is sufficient to induce V1 and V0v cells in vitro, and vitamin A-deficient quail embryos lack V1 neurons in the anterior spinal cord (Pierani et al., 1999; Wilson et al., 2004). In addition, amniote embryos that lack RA signalling have fewer spinal neurons and the expression of many, although not all, spinal cord progenitor domain transcription factors is reduced (Diez del Corral et al., 2003; Novitsch et al., 2003; Wilson et al., 2004; Lupu et al., 2006). Therefore, we also investigate whether RA signalling is required for the formation of ventral spinal neurons in zebrafish, in either the presence or the absence of Hh signalling.

MATERIALS AND METHODS

Zebrafish lines

Zebrafish (*Danio rerio*) embryos were obtained from wild-type (WT) (AB, TL, or AB/TL hybrids) adults or identified carriers heterozygous for *smo*^{b641}, a point mutation in *smo* (Lewis and Eisen, 2001; Varga et al., 2001), or *detour* (*dtu*^{ts269}), a point mutation in *gli1* (Karlstrom et al., 2003). Both mutants are probably null alleles. Maternal zygotic *smo* (*MZsmo*)^{hi1640} mutant embryos were generated as described (Mich et al., 2009; Mich and Chen, 2011). *smo*^{hi1640} is a proviral insertion into the first coding exon of *smo* that also creates a null allele (Chen et al., 2001). All zebrafish lines (except those used to generate *MZsmo* mutants and associated controls) were maintained at the University of Cambridge or Syracuse University. *MZsmo* and associated control embryos were generated at Stanford University and shipped as fixed embryos to

Cambridge or Syracuse for analysis. Embryos were staged by hours post-fertilisation (hpf) at 28.5°C and confirmed using morphological criteria (Kimmel et al., 1995).

Chemical treatments

Abrogation of Hh signalling was also achieved using cyclopamine, a small molecule that inhibits Smo (Chen et al., 2002a; Chen et al., 2002b; Cooper et al., 1998; Taipale et al., 2000). Cyclopamine was resuspended in ethanol and diluted with embryo medium (EM). We performed a dose-response experiment (supplementary material Fig. S1) and consequently treated embryos with 25 µM cyclopamine from the one-cell stage. Control embryos were treated with an equivalent concentration of ethanol.

RA signalling was abrogated using 4-diethylaminobenzaldehyde (DEAB; Fluka #39070). DEAB inhibits retinaldehyde dehydrogenase (Raldh), which is required to synthesize RA (Perz-Edwards et al., 2001; Russo, 1997). DEAB was resuspended in DMSO and diluted with EM. Embryos were incubated with 62.5 µM DEAB from 3.7 hpf in the dark at 28.5°C. This concentration is either similar to (Gribble et al., 2007; Kopinke et al., 2006; Reijntjes et al., 2007) or higher than (Begemann et al., 2004; Maves and Kimmel, 2005) that used in previous experiments, suggesting that it should eliminate RA signalling. Consistent with this, DEAB-treated embryos lost expression of *hoxb4a* and the posterior domain of *egr2b* as reported previously (Begemann et al., 2004; Gribble et al., 2007; Maves and Kimmel, 2005) (Fig. 13F,G) and morphologically resembled *neckless* (*raldh2*, now called *aldh1a2*) mutants (Begemann et al., 2004; Begemann et al., 2001). Control embryos were treated with an equivalent concentration of DMSO.

In situ hybridisation and immunohistochemistry

In situ hybridisation was performed as described (Concordet et al., 1996). Antisense RNA probes were prepared using the templates shown in Table 1.

Table 1. In situ hybridisation probes

In situ probe	Previous names	ZFIN ID	Source of probe plasmid
<i>col2a1a</i>	<i>coll2a1</i> , <i>col2a1</i> , <i>alpha-collagen</i>	ZDB-GENE-980526-192	Yan et al., 1995
<i>dbx1a</i>	<i>hlx1</i> , <i>hlx-1</i>	ZDB-GENE-000128-8	Gribble et al., 2007
<i>dbx1b</i>	<i>hlx2</i>	ZDB-GENE-000128-11	Gribble et al., 2007
<i>dbx2</i>	<i>hlx3</i>	ZDB-GENE-000128-13	Gribble et al., 2007
<i>egr2b</i>	<i>krox20</i> , <i>krox-20</i> , <i>egr2</i> , <i>krx20</i>	ZDB-GENE-980526-283	Oxtoby and Jowett, 1993
<i>eng1b</i>	<i>engrailed 4</i> , <i>eng4</i>	ZDB-GENE-980526-6	The 1.3 kb product encompassing the <i>eng1b</i> ORF was a kind gift of Drs Kikuchi and Westerfield, University of Oregon (Batista and Lewis, 2008)
<i>evx1</i>	None	ZDB-GENE-980526-364	Thaeron et al., 2000
<i>gata3</i>	<i>gta3</i>	ZDB-GENE-990415-82	Neave et al., 1995
<i>gli2a</i>	<i>you-too</i> , <i>yot</i> , <i>gli2</i> , <i>you too</i>	ZDB-GENE-990706-8	Karlstrom et al., 1999
<i>gli3</i>	None	ZDB-GENE-041111-162	Tyurina et al., 2005
<i>gsx1</i>	<i>gsh1</i>	ZDB-GENE-041008-135	Cheesman and Eisen, 2004
<i>hoxb4a</i>	<i>hoxb4</i> , <i>Z-17</i> , <i>zf13</i> , <i>ZF-13</i>	ZDB-GENE-990415-105	Mich and Chen, 2011
<i>irx3a</i>	<i>iro3-s2</i> , <i>ziro3</i> , <i>iro3-s1</i> , <i>iro3</i> , <i>irx3</i>	ZDB-GENE-991119-5	Lewis et al., 2005; Tan et al., 1999
<i>islet2a</i>	<i>zflsl-2</i> , <i>isl2</i> ,	ZDB-GENE-980526-562	Appel et al., 1995
<i>nkx2.2a</i>	<i>nk2.2</i> , <i>nkx2.2</i> , <i>nkx2-2</i>	ZDB-GENE-980526-403	Barth and Wilson, 1995
<i>nkx2.2b</i>	None	ZDB-GENE-050217-3	Schäfer et al., 2005
<i>nkx6.1</i>	None	ZDB-GENE-040718-178	Cheesman et al., 2004
<i>nkx6.2</i>	None	ZDB-GENE-070626-1	Hutchinson et al., 2007
<i>olig2</i>	None	ZDB-GENE-070626-1	Park et al., 2002
<i>pax2a</i>	<i>pax-b</i> , <i>pax2.1</i> , <i>pax2a1</i> , <i>no isthmus</i> , <i>Pax-2</i> , <i>noi</i> , <i>pax[zf-b]</i> , <i>paxb</i> , <i>Pax2.1</i>	ZDB-GENE-990415-8	Pfeffer et al., 1998
<i>pax7a</i>	<i>pax7e</i> , <i>pax7c</i> , <i>pax7d</i> , <i>pax7</i>	ZDB-GENE-990415-201	Seo et al., 1998
<i>tal2</i>	None	ZDB-GENE-040115-1	Pinheiro et al., 2004
<i>vsx1</i>	None	ZDB-GENE-990415-205	Passini et al., 1997
<i>vsx2</i>	<i>vsx-2</i> , <i>alx</i> , <i>chx10</i>	ZDB-GENE-001222-1	The 1 kb PCR product of the <i>vsx2</i> coding region was a kind gift of Drs Bonner and Dorsky, University of Utah (Batista et al., 2008)

For immunohistochemistry, rabbit anti-phospho-Histone H3 (Ser10; Millipore #06-570; 1/500) was revealed with Alexa Fluor 488 goat anti-rabbit (Molecular Probes; 1/1000). Embryos were treated with Image-iT Signal Enhancer (Invitrogen) for 30 minutes before primary antibody incubation.

Photographs were taken using a Zeiss Axio Imager M1, a Zeiss Axio Examiner, or an Olympus stereomicroscope and processed using Adobe Photoshop.

mRNA and morpholino (MO) injections

One-cell stage embryos were injected with 10 ng *gli1* MO + 3 ng *p53* MO, or 10 ng *gli2a* MO + 5 ng *gli3* MO + 6 ng *p53* MO, or 70 pg of *gli3R-EGFP* RNA. *gli1* MO, 5'-CCGACACACCCGCTACCCACAGT-3' (Huang and Schier, 2009; Karlstrom et al., 2003); *gli2a* MO, 5'-GGATGATGTAAGTTCGTCAGTTGC-3' (Karlstrom et al., 2003); *gli3* MO, 5'-ACAACCTGGCATTCTCAGAGCATC-3' (Tyurina et al., 2005); *p53* MO, 5'-GCGCCATTGCTTTGCAAGAATTG-3' (Robu et al., 2007). Previous studies have demonstrated that the Gli MOs specifically and efficiently knockdown Gli1, Gli2a and Gli3 (Huang and Schier, 2009; Karlstrom et al., 2003; Tyurina et al., 2005). The *p53* (now called *tp53*) MO reduces non-specific off-target effects that are often associated with MOs (Huang and Schier, 2009; Robu et al., 2007). The *gli3R* RNA encodes a C-terminally truncated form of Gli3 that functions as a repressor (Huang and Schier, 2009).

Cell counts

Cell counts encompass both sides of a five-somite length of spinal cord adjacent to somites 6-10 and are an average of at least five different embryos; *n* values are provided in the figure legends. Cell row numbers are assigned ventral to dorsal [e.g. cells directly above the notochord are in row 1; see Batista and Lewis (Batista and Lewis, 2008) for a more detailed description of how row numbers are assigned]. The markers used in this study also have characteristic expression patterns in territories unaffected by our experiments. Only embryos with these expression patterns (e.g. in head mesendoderm or proctodeum) were selected for counting. For phospho-Histone H3 counts (Fig. 13E), DIC images were used to define *z*-stacks encompassing only the spinal cord. Labelled cells falling outside these limits were not counted.

RESULTS

Medial floorplate still forms in the absence of Hh signalling

We and others have previously shown that the medial floor plate still forms in zebrafish embryos under experimental conditions in which Hh signalling is at least severely reduced (e.g. Etheridge et al., 2001; Lewis and Eisen, 2001). However, in all of these experiments, a very small amount of Hh signalling might still have been present. Therefore, we examined medial floorplate in *MZsmo* mutants, which completely lack Hh signalling (Mich et al., 2009; Mich and Chen, 2011). We used *MZsmo* mutants because some Hh signalling persists in *smo* mutants due to maternally provided *smo* RNA and possibly protein (Chen et al., 2001; Lewis and Eisen, 2001; Varga et al., 2001; Mich and Chen, 2011). We found that even in *MZsmo* mutants medial floor plate is still present at 24 hpf (see Fig. 2A"). Therefore, although Hh signalling is required to maintain medial floor plate (Albert et al., 2003), it is not required for the initial formation of this structure.

In the absence of Hh signalling, most V3 and MN cells are lost, but V0v, V1 and V2 cells persist

In zebrafish, analysis of the role of Hh signalling in specifying postmitotic spinal neurons has so far been limited to MNs (Chen et al., 2001; Lewis and Eisen, 2001; Park et al., 2004; Varga et al., 2001) and neurons that form ventral to MNs in the V3 domain (Schäfer et al., 2007). We examined markers of various postmitotic neurons in zebrafish ventral spinal cord (Fig. 1, Table 1) in both *smo* mutants and *MZsmo* mutants. Consistent with previous

analyses in amniotes, there was a dramatic reduction of cells usually found in the V3 and MN domains. However, a small number of V3 cells and MNs persisted in the anterior spinal cord even in *MZsmo* mutants (Fig. 2B-D", Fig. 3A-C).

In striking contrast to these more ventral markers, we saw no change in the number of V2, V1 or V0v cells in either *smo* or *MZsmo* mutants at 24 hpf in either the anterior or posterior spinal cord, although these cells were located more ventrally in *MZsmo* and *smo* mutants than in WT embryos (Fig. 2D-I", Fig. 3C-H, Fig. 4B; data not shown).

Ventral progenitor domains are lost in the absence of Hh signalling at 24 hpf

In mice that lack Hh signalling, the loss of ventral postmitotic neurons is presumed to result from an earlier loss of ventral progenitor domain expression. Previous reports suggest that ventral progenitor domain markers are lost in zebrafish when Hh signalling is abrogated (e.g. Cheesman et al., 2004; Gribble et al., 2007; Guner and Karlstrom, 2007; Lewis et al., 2005; Park et al., 2002; Park et al., 2004; Schauerer et al., 1998). However, given that normal numbers of V2, V1 and V0v cells form in *MZsmo* mutants, we examined progenitor domain markers in *MZsmo* mutants. Consistent with previous studies, we observed a complete loss of expression of p3 and pMN genes (*nkx2.2b* and *olig2*, respectively) in *smo* and *MZsmo* mutants at 24 hpf (Fig. 5A-B"). However, a very small number of p2 and p1 domain (*nkx6.1*- and *nkx6.2*-expressing) cells persist even in *MZsmo* mutants (Fig. 5C-D", Fig. 6A,B). By contrast, p0 domain markers (*dbx1a*, *dbx1b*, *dbx2*) expand into almost all of the ventral spinal cord (Fig. 5F-H", Fig. 6D-F). The dP6 domain, and possibly also the dP5 domain, appears to expand ventrally by one to two rows in *MZsmo* mutants but, in the main, the dorsal spinal cord is unaffected (Fig. 5I-J", Fig. 6G,H; data not shown).

pMN, p2 and p1 domain genes are expressed at early somitogenesis stages, even in the absence of Hh signalling

Despite the severe reduction of pMN, p2 and p1 domains in 24-hpf *MZsmo* mutants, small numbers of MNs persist and V2, V1 and V0v cells form in normal numbers. Therefore, we investigated whether ventral progenitor domain transcription factors are

Progenitor Genes			Post-Mitotic Genes		
dP5		<i>gsx1</i>	<i>pax7a</i>		dI5
dP6					dI6
p0	<i>irx3a</i>	<i>dbx2</i>	<i>dbx1a</i>	<i>evx1</i> (V0v)	V0
p1				<i>eng1b</i>	V1
p2				<i>vsx1/2</i> (V2a)	V2
pMN	<i>olig2</i>	<i>nkx6.1</i>	<i>nkx6.2</i>	<i>gata3</i> (V2b)	MN
p3	<i>nkx2.2b</i>			<i>islet2a</i>	
				<i>tal2</i>	V3
				<i>gata3</i>	

Fig. 1. Genes expressed in progenitor and postmitotic ventral spinal cord domains. Schematic transverse sections of zebrafish ventral spinal cord (ventral bottom, dorsal top), showing domains that express transcription factor genes examined in this study. *dbx1b* (not shown) is expressed similarly to *dbx1a* but extends slightly more ventrally.

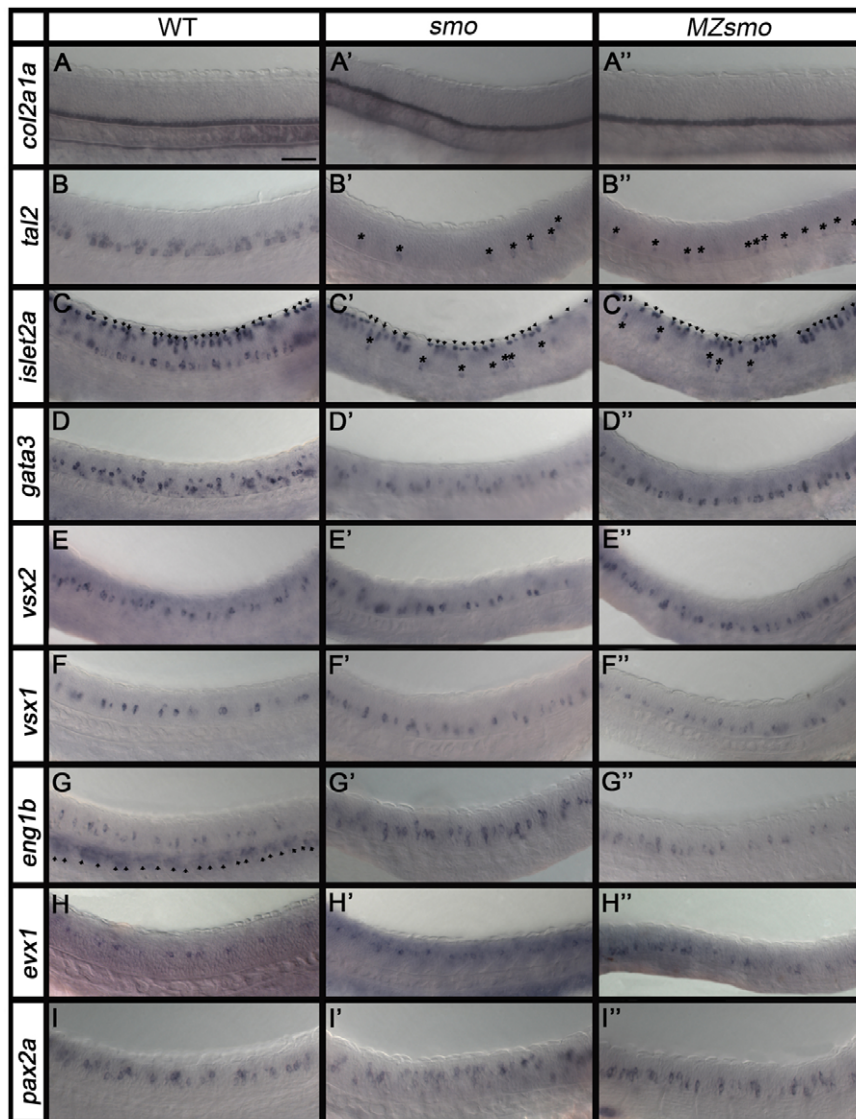


Fig. 2. V0v, V1 and V2 cells persist in the absence of Hh signalling. (A-I'') In situ hybridisation for the indicated marker genes in lateral views of 24-hpf trunk of WT, *smo* mutant and *MZsmo* mutant zebrafish embryos. Asterisks indicate *tal2*-expressing cells (B', B'') and *islet2a*-expressing MNs (C', C'') that still form in *smo* and *MZsmo* mutants. *islet2a* is also expressed in more dorsal Rohon-Beard cells (arrowheads in C-C''). Rohon-Beard cells are unaffected in *smo* or *MZsmo* mutants (C', C''). *eng1b* is also expressed in muscle pioneer cells ventral to the spinal cord in WT embryos (arrowheads in G). These cells are absent in *smo* or *MZsmo* mutants (G', G''). Scale bar: 50 μ m.

expressed in the spinal cord before 24 hpf in *MZsmo* mutants. There was no spinal cord expression of the p3 domain marker *nkx2.2b* at bud stage (10 hpf), 6 somites (12 hpf), 12 somites (15 hpf) or 20 somites (19 hpf) in *MZsmo* mutants (Fig. 7A-A'', E-E'', Fig. 8E; data not shown). By contrast, there was weak expression of the pMN domain marker *olig2* and slightly reduced, but still robust, expression of the p2 and p1 domain markers *nkx6.1* and *nkx6.2* at bud stage and 6 somites (Fig. 7B-D'', F-H''). *nkx6.1* and *nkx6.2* expression still persists at 12 and 20 somites, but by these later stages there is no longer any spinal cord expression of *olig2*, and by 20 somites expression of *nkx6.1* and *nkx6.2* is also very weak (Fig. 8F-H, R-T). This suggests that Hh signalling is required for the expression of p3 domain markers, but it is only needed to maintain the expression of pMN, p2 and p1 domain markers.

Ectopic Gli repressor activity reduces early expression, whereas reducing Gli repressor activity increases later expression, of pMN, p1 and p2 domain markers

One of the main roles of Hh signalling in the mammalian ventral spinal cord is to remove Gli3 repressor activity. In mice mutant for both *Shh* and *Gli3* or for *Smo* and *Gli3*, some MNs and substantial

numbers of V2, V1 and V0v cells still form (Litingtung and Chiang, 2000; Wijgerde et al., 2002). The similarity between these double-mutant phenotypes and the zebrafish *MZsmo* mutant phenotype suggested a possible hypothesis for why mouse *Shh* and zebrafish *MZsmo* mutants have such different phenotypes (Fig. 4). If Gli repressor activity is not present in *MZsmo* mutant ventral spinal cord until mid-somitogenesis stages, then this might be too late to repress the initial expression of progenitor domain genes and the formation of V2, V1 and V0v cells. Consistent with this, neither *gli3* nor *gli2a* [both of which encode Gli proteins with repressor activity in zebrafish (Huang and Schier, 2009; Karlstrom et al., 1999; Karlstrom et al., 2003; Mich and Chen, 2011; Tyurina et al., 2005)] is clearly visible in the spinal cord until after 6 somites (12 hpf) in WT embryos or embryos with abrogated Hh signalling, although this does not rule out the possibility that there might be low levels of expression that remain undetected by in situ hybridisation (supplementary material Fig. S2).

To test the hypothesis that ventral progenitor domain genes are still expressed in *MZsmo* embryos at early stages because Gli repressor activity is not present, we injected RNA for a truncated Gli3-EGFP fusion protein [Gli3R (Huang and Schier, 2009)] that has constitutive Gli repressor activity into embryos treated with

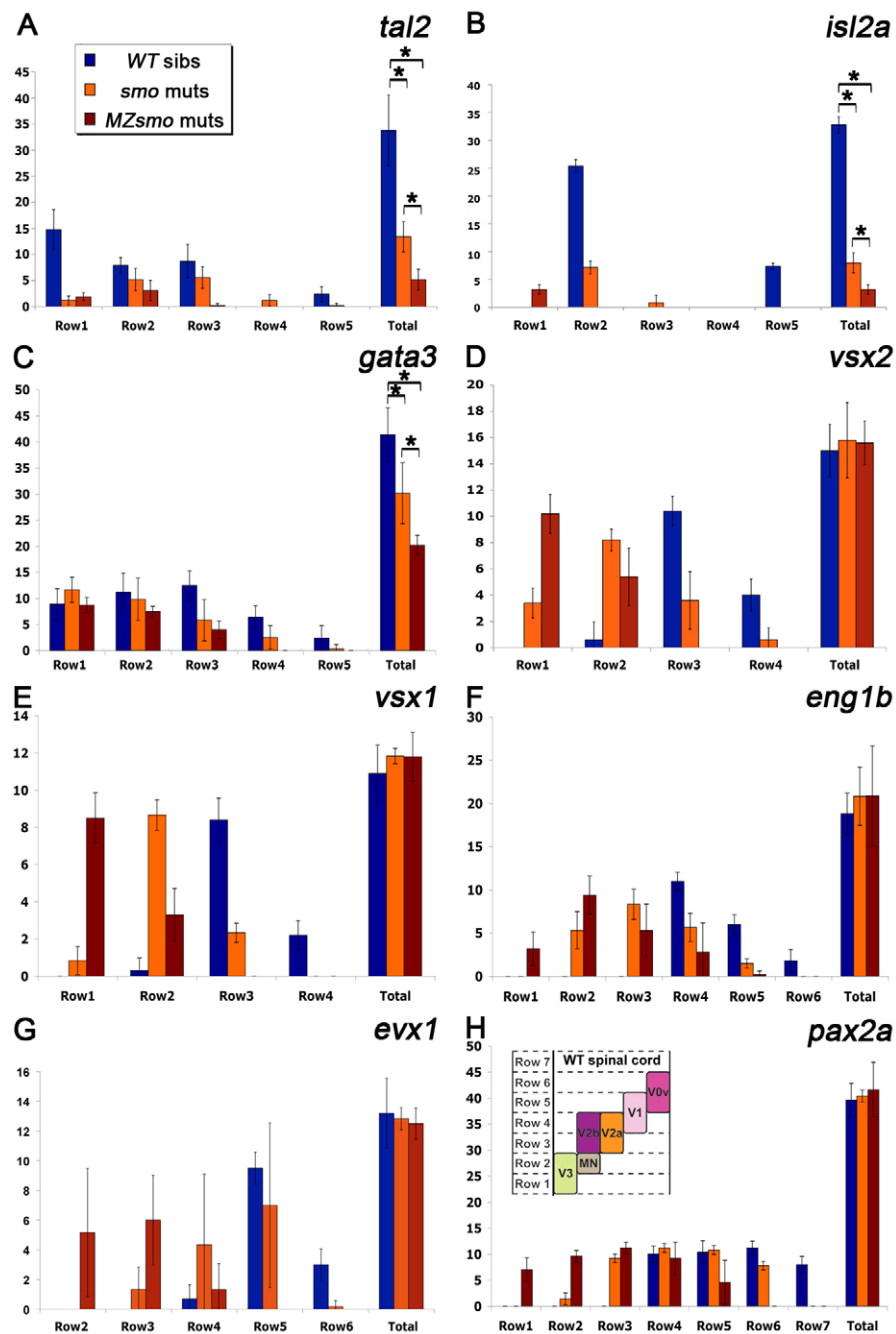


Fig. 3. V0v, V1 and V2 cells are found more ventrally in the absence of Hh signalling. (A-H) Average number ($n \geq 5$ embryos) of cells expressing markers of various postmitotic cell types at 24 hpf in spinal cord adjacent to somites 6-10. Results are presented both for individual rows and for the total number of cells. Cell row numbers are always assigned ventral to dorsal. Rows not shown have a cell count of zero. Error bars denote s.d. Statistically significant differences are indicated between total cell counts (*, $P < 0.05$, Student's t -test). A schematic indicating how postmitotic domains relate to row position in WT is shown in H. See also Fig. 4. (A) Some of the *tal2*-expressing cells that remain in the mutants are probably cells that normally form more dorsally, as occasional cells in row 5 express this gene in WT. (B) Cell counts are only shown for *islet2a*-expressing MNs and interneurons in ventral spinal cord; dorsal Rohon-Beard cells were not counted. (C) The reduction in *gata3*-expressing cells is presumably due to V3 cells (rather than V2b cells) being lost as there is no reduction in the number of cells expressing the V2 cell markers *vsx2* and *vsx1* (D,E).

cyclopamine. Unsurprisingly, we found that, just like in *MZsmo* mutants, there was no expression of *nkx2.2b* in these embryos at 6 somites (12 hpf) (Fig. 8M). However, the small amount of pMN domain (*olig2*) expression that remains in *MZsmo* mutants is now lost (compare Fig. 8N with Fig. 7F") and, even more strikingly, p2 domain expression (*nkx6.1*) is also lost and p1 domain expression (*nkx6.2*) is strongly reduced, particularly in the anterior spinal cord (compare Fig. 8O,P with Fig. 7G",H"). Consistent with these effects on progenitor domain genes, there were also fewer MNs in these embryos at 24 hpf than in *MZsmo* mutants (Fig. 9C, Fig. 10B). We observed only 2.0 ± 2.76 *islet2a*-expressing cells ($n=6$) along the whole length of the spinal cord, compared with 15.4 ± 2.41 cells in *MZsmo* mutants ($n=5$). Given the loss of the p2 domain marker *nkx6.1*, we also tested whether there was any reduction in the

number of V2 cells in these embryos at 24 hpf. Unlike in *MZsmo* mutants, in which the number of V2 cells is unchanged, we observed a significant reduction of *vsx1*- and *gata3*-expressing V2 cells (Fig. 9F,I, Fig. 10C,D). By contrast, there was still no change in the number of V1 (*eng1b*) or V0v (*evx1*) cells (data not shown).

To test this hypothesis further, we also reduced Gli repressor activity in embryos treated with cyclopamine by injecting MOs for *gli3* and *gli2a*. In these embryos, p3 domain *nkx2.2b* expression was still absent at 12 somites (15 hpf) (Fig. 8I). By contrast, a small amount of pMN domain *olig2* expression was recovered compared with *MZsmo* mutants (compare Fig. 8J with 8F) and this corresponded with an increase in the number of MNs at 24 hpf (Fig. 9J, Fig. 10B). The expression of the p2 and p1 domain markers *nkx6.1* and *nkx6.2* was also much stronger in these

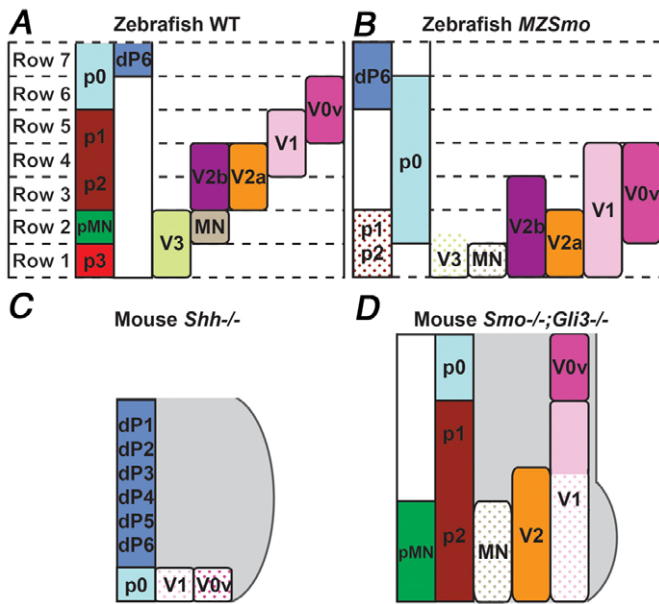


Fig. 4. Summary of mouse and zebrafish phenotypes.

(A,B) Schematic transverse sections of ventral spinal cord showing the relationship between domains and ventral-dorsal rows in zebrafish WT embryos (A) and *MZsmo* mutants (B). Many domains in zebrafish overlap slightly, presumably because of the small size of the zebrafish spinal cord. Dotted domains indicate that only a very small number of cells remain. Medial floor plate is not shown but is present (medial to row 1) in both WT and *MZsmo* mutants. The dorsal limit of the V3 domain is hard to determine precisely because both *tal2* and *gata3* are also expressed by more dorsal cells (*gata3* is expressed by V2b cells; it is currently unclear which other cells express *tal2*). (C,D) Schematics of mouse *Shh* mutant (C) and *Smo*;*Gli3* double-mutant (D) spinal cords are shown for comparison with zebrafish.

embryos than in *MZsmo* mutants and, unlike pMN domain *olig2* expression, which was still absent at 24 hpf, the increase in p2 and p1 domain marker expression persisted until 24 hpf (compare Fig. 8K,L with 8G,H and see Fig. 9D,G).

Gli1 has a Hh-independent role in V3 cell and MN formation but it is not required for V2, V1 or V0v cells

A recent report demonstrated that Gli1 can act independently of Hh to promote neurogenesis at the midbrain-hindbrain boundary, at least in the absence of Gsk3 β and PKA activity (Ninkovic et al., 2008), and low levels of *gli1* expression persist, even in zebrafish embryos that lack most Hh signalling (Karlstrom et al., 2003; Ninkovic et al., 2008). To determine if Gli1 might be responsible for specifying the remaining ventral neurons in the absence of Hh signalling, we injected *gli1* MO into cyclopamine-treated embryos. Notably, even though the reduction of V3 cells and MNs in embryos treated with cyclopamine was not as severe as in *MZsmo* mutants (Fig. 10, supplementary material Fig. S1), embryos that were injected with *gli1* MO and exposed to cyclopamine had a more severe loss of MNs and V3 cells than *MZsmo* mutants that lack all Hh signalling (Fig. 9E,K, Fig. 10B,C). This strongly suggests that these experiments removed a Hh-independent activity (that is still present in *MZsmo* mutants) in addition to removing most Hh signalling. By contrast, there was no reduction in the number of V2, V1 or V0v cells (Fig. 10D; data not shown).

To confirm these results, we treated *gli1* mutant (*detour*) embryos with cyclopamine. Again, these embryos had a more severe loss of MNs than *MZsmo* mutants (Fig. 9H, Fig. 10B). Consistent with this more severe reduction in MNs, the small amount of pMN (*olig2*) expression that remains in *MZsmo* mutants at 6 somites (12 hpf) is completely lost in embryos injected with *gli1* MO and exposed to cyclopamine (Fig. 9L).

Some V0v, V1 and V2 cells still form in the absence of Hh and RA signalling

As mentioned in the introduction, RA signalling has been implicated in the development of ventral spinal cord neurons, particularly in the V0v, V1 and MN domains (Diez del Corral et al., 2003; Novitch et al., 2003; Pierani et al., 1999; Wilson et al., 2004). To test whether RA might specify ventral neurons in the absence of Hh signalling, we treated *smo* mutants with DEAB, an Raldh inhibitor that prevents the synthesis of RA (see Materials and methods). These embryos had fewer cells expressing each of the ventral postmitotic markers that we examined. However, WT sibling embryos treated with DEAB had a similar decrease in the number of cells expressing these genes (Fig. 11A-F', Fig. 12A,B). In cases in which *smo* mutants had fewer cells than their WT siblings (e.g. cells expressing *tal2*, *gata3*), the phenotype of *smo* mutants treated with DEAB appeared to be additive (Fig. 12A).

To rule out the possibility that the small amount of Hh signalling that remains in *smo* mutants might be sufficient to specify the remaining postmitotic neurons, we treated *MZsmo* mutants with DEAB. However, even in these embryos, occasional MNs and several ventral interneurons still formed (Fig. 11N-P, Fig. 12A,B).

Consistent with reports in amniotes (Diez del Corral et al., 2003; Novitch et al., 2003; Wilson et al., 2004; Luo et al., 2006), we found that RA signalling is also required for the correct expression of many spinal cord progenitor domain transcription factors. Both ventral and dorsal progenitor genes were reduced in 24-hpf zebrafish embryos treated with DEAB (Fig. 11G-K', Fig. 12C). However, as has been observed in amniotes, some of these genes were more affected than others. We also examined the expression of the ventral progenitor genes *olig2* and *nkx6.1* at early somitogenesis stages. Consistent with the results at 24 hpf, these genes were also reduced at 6 somites in embryos treated with DEAB (Fig. 11L-M').

Loss of RA signalling causes a slight truncation of the body axis, so we were concerned that we might be counting cells over a shorter region of the spinal cord in DEAB-treated embryos. However, when we checked the number of cells per somite length of spinal cord there was no significant difference between WT embryos and embryos treated with DEAB (Table 2).

This widespread effect of DEAB led us to hypothesize that loss of RA signalling might be having a general effect on cell proliferation. To test this, we examined anti-phospho-Histone H3 staining and found that both WT and *smo* mutant embryos treated with DEAB had significantly fewer proliferating cells than the equivalent DMSO-treated control embryos (Fig. 13A-E).

DISCUSSION

Hh signalling is required for the formation of the vast majority of V3 domain cells and MNs but not for the formation of V2, V1 or V0v cells

We have shown that in zebrafish embryos that lack all Hh signalling most cells in the V3 domain do not form and there is a dramatic reduction, although not a complete elimination, of MNs. By contrast, medial floor plate still develops normally and V2, V1

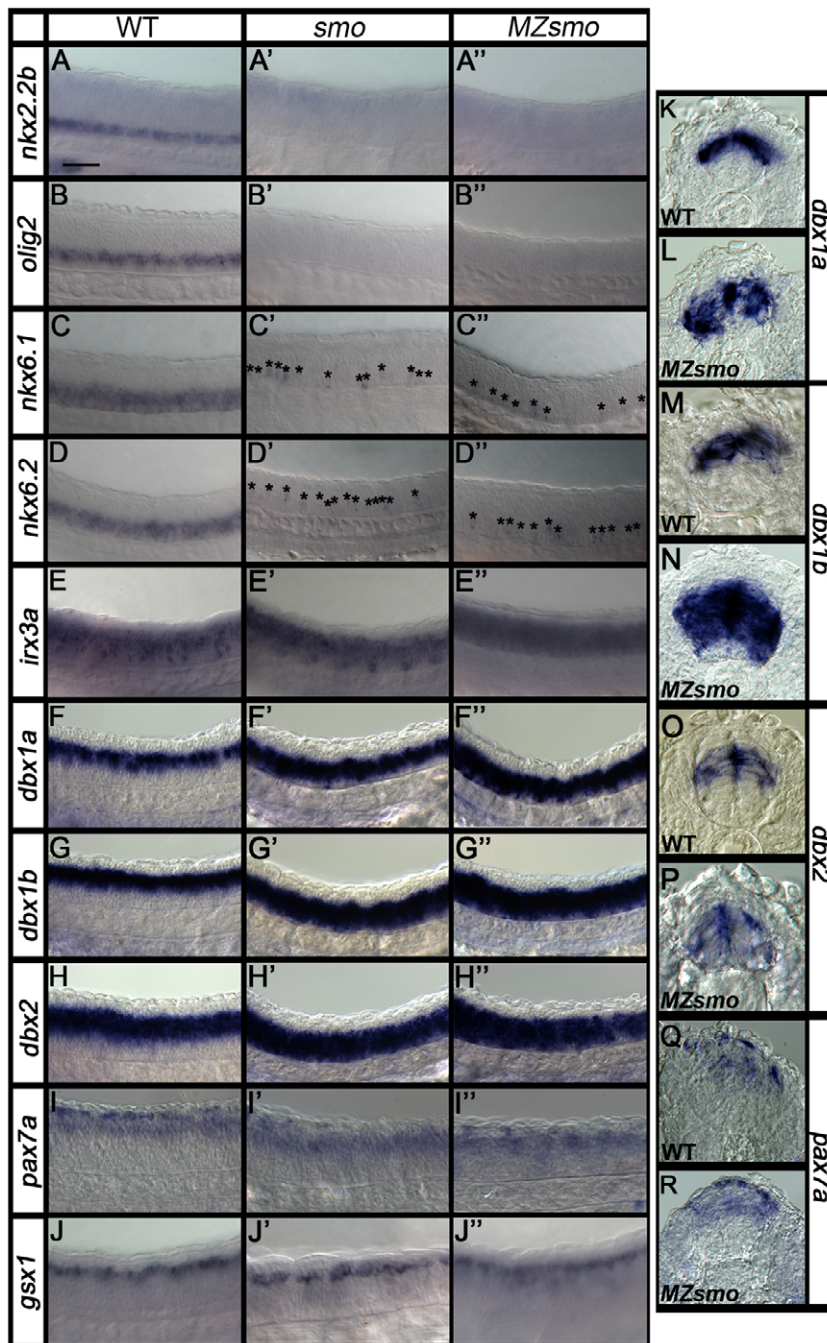


Fig. 5. Ventral progenitor domains are lost at 24 hpf in the absence of Hh signalling. (A-J'') Lateral views of zebrafish trunk and (K-R) transverse sections at 24 hpf. Asterisks (C', C'', D', D'') indicate weakly expressing cells. Scale bar: 50 μm in A-J''; 40 μm in K-R.

and V0v cells form in normal numbers (Fig. 4). The remaining MN, V2, V1 and V0v cells are found more ventrally than in WT embryos and they are more intermingled (Figs 2-4). However, there is still some residual dorsal-ventral patterning. For example, V2a cells are found mainly in row 1, with some cells in row 2, whereas V0v cells are found mainly in rows 2 and 3, with a few cells in row 4 (compare Fig. 3D with 3G and see Fig. 4B).

Our results differ from those obtained for mouse *Shh* mutants, in which V1 and V0v cells are severely decreased in number, only a few V2 cells remain and MNs are lost (Fig. 4C) (Chiang et al., 1996; Wijgerde et al., 2002). They also differ from mice that contain *Smo* mutant cells, where the only ventral neurons that formed from mutant cells were V0v cells (Wijgerde et al., 2002).

Instead, the phenotype of zebrafish *MZsmo* mutants is reminiscent of that of mice mutant for both *Shh* and *Gli3*, *Smo* and *Gli3*, or *Gli2* and *Gli3* (Fig. 4D) (Bai et al., 2004; Lei et al., 2004; Litingtung and Chiang, 2000; Persson et al., 2002; Wijgerde et al., 2002). In all of these double mutants, some MNs and substantial numbers of V2, V1 and V0v cells still form and V2 and V1 cells are found intermingled in the ventral spinal cord. These double-mutant results suggest that in mouse Hh signalling has two distinct functions. First, it is required to induce V3 cells and some MNs and to correctly separate ventral cell types into distinct domains. Second, it is required to remove *Gli3* repressor activity in the ventral spinal cord, allowing V2, V1 and V0v cells to form in normal numbers. By contrast, our results suggest that in zebrafish embryos Hh

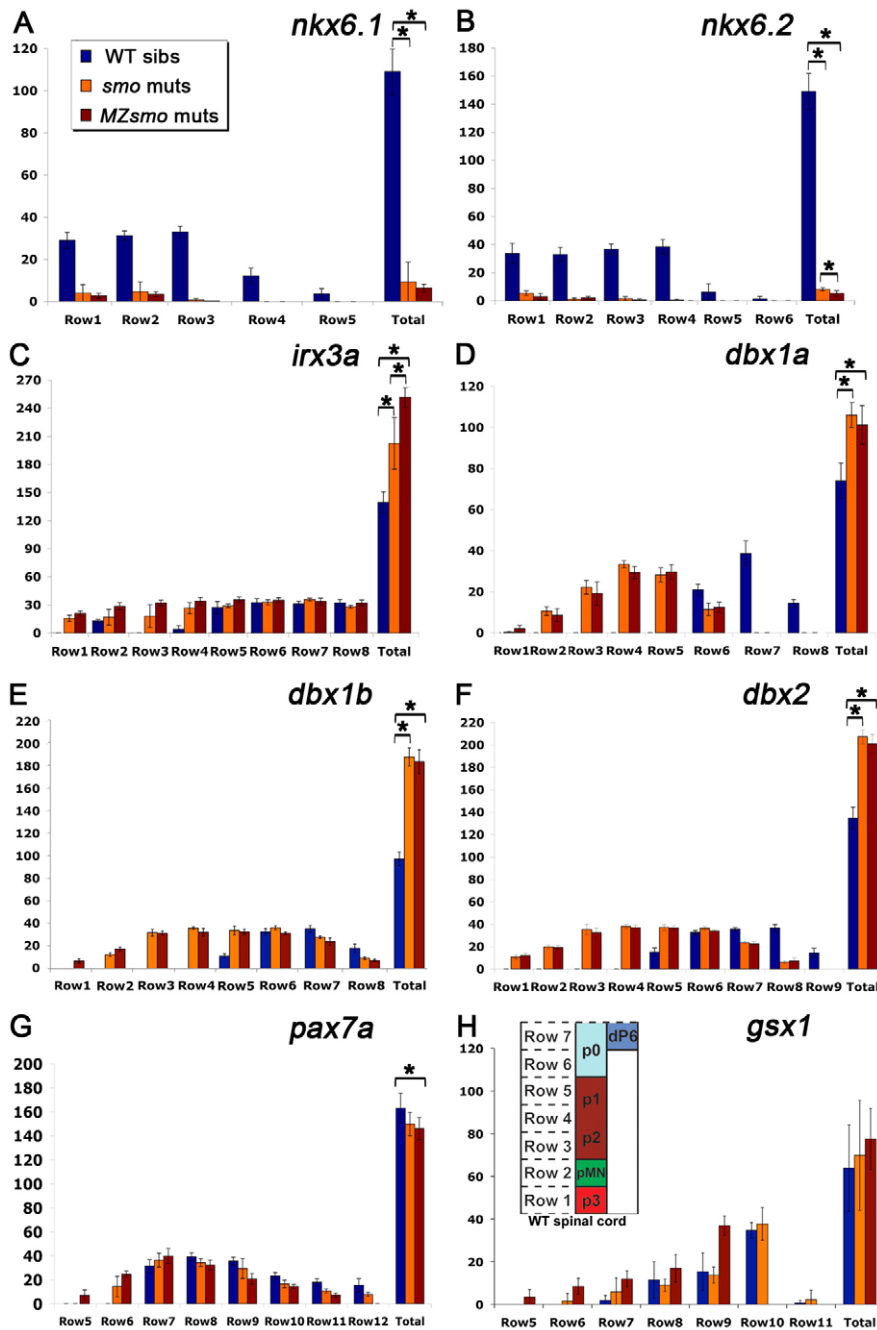


Fig. 6. p0 and dP6 are expanded ventrally at 24 hpf in the absence of Hh signalling. (A-H) Average number ($n \geq 5$ embryos) of cells expressing progenitor domain genes at 24 hpf in spinal cord adjacent to somites 6-10. Rows not shown have a cell count of zero. Error bars denote s.d. Statistically significant differences between total cell counts are indicated (*, $P < 0.05$, Student's t -test). There is no expression of *olig2* or *nkx2.2b* in either *smo* or *MZsmo* mutants at 24 hpf. A schematic indicating how progenitor domains relate to row position in WT is shown in H.

signalling has the first but not the second function: Hh signalling is not required (even indirectly, by antagonising Gli repressor activity) for the formation of V2, V1 or V0v cells.

Our analyses of progenitor domain genes suggest that these distinct zebrafish and mouse phenotypes are caused by differences in spinal cord patterning at gastrulation and/or early somitogenesis stages. Previous reports in mouse have shown that Hh signalling is required for the expression of ventral spinal cord progenitor domain transcription factors, which are then themselves required to specify corresponding postmitotic cell types (e.g. Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997). At 24 hpf, the expression of progenitor domain genes in zebrafish *MZsmo* mutants is very similar to that in mice that lack or have dramatically reduced Hh signalling: the p3-p1 domains are lost (with the exception of an extremely small number of p2 and p1

cells) and the p0 domain extends ventrally in the spinal cord (Fig. 5 and see Fig. 4B). However, at earlier stages, the pMN domain is still present (although heavily reduced) and the p2 and p1 domains initially appear to be normal (Fig. 7B-D"). Strikingly, the amount and duration of progenitor domain expression remaining at these early stages correlate with the number of postmitotic cells at later stages. In *MZsmo* mutants, we see no spinal cord expression of the p3 domain marker *nkx2.2b* at any of the stages we examined and very few, if any, V3 domain cells form. We see reduced expression of the pMN marker *olig2* at bud stage and expression is lost by 12 somites. Consistent with this, only a few MNs form. By contrast, expression of the p2 and p1 domain markers *nkx6.1* and *nkx6.2* is almost normal at bud stage and is not completely lost even at 24 hpf and this correlates with the formation of normal numbers of V2 and V1 cells. These results demonstrate that, although Hh

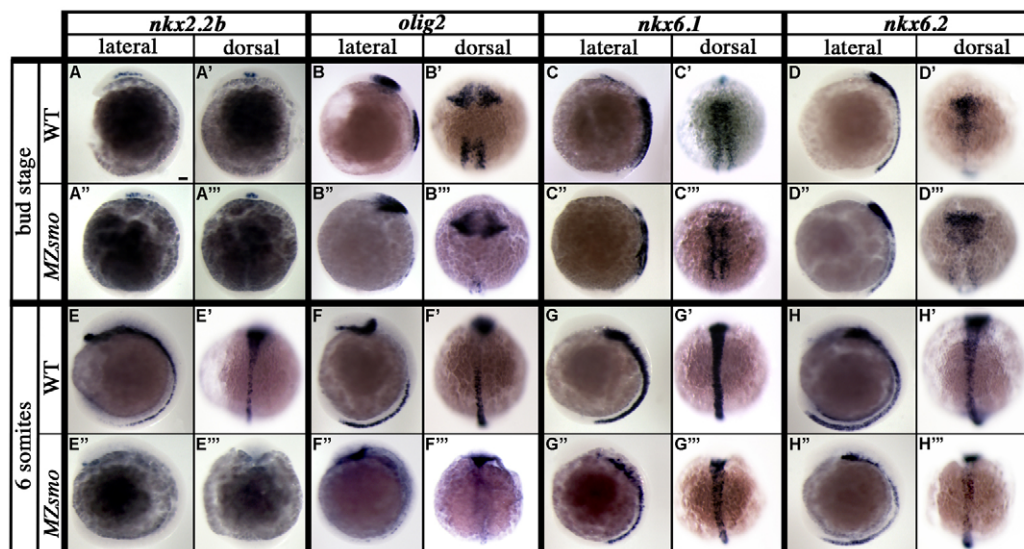


Fig. 7. *nkx6.1*, *nkx6.2* and *olig2* are expressed in the spinal cord at early somitogenesis stages in the absence of Hh signalling. (A-H'') Lateral and dorsal views of *nkx2.2b*, *olig2*, *nkx6.1* and *nkx6.2* expression in WT and *MZsmo* mutant zebrafish embryos at bud stage (10 hpf) and 6 somites (12 hpf). Scale bar: 50 μ m.

signalling is required for all p3 and most pMN expression, it is not required for p2 and p1 expression at early stages, although it is required for the maintenance of the p2, p1 and remaining pMN expression. These data also suggest that this remaining expression of pMN, p2 and p1 genes is sufficient to specify the postmitotic cells that we observe in the zebrafish ventral spinal cord in the absence of Hh signalling. Our results are consistent with data from Dessaud and colleagues (Dessaud et al., 2010) that suggest that in amniotes the duration of Shh signalling is as important as the levels of signalling for specifying distinct ventral spinal cord fates, although our data suggest that it is continued inhibition of Gli repressor activity (see below) that is important, rather than Hh signalling per se.

Taken together, the simplest model for reconciling these different mouse and zebrafish phenotypes is that, in zebrafish, Gli repressor activity is not present in the ventral spinal cord until the early to mid-somitogenesis stages and that this is too late to repress the initial expression of p2 and p1 domain transcription factors and the formation of V2 and V1 cells, although it is sufficient to repress p2 and p1 domain expression at later stages. Consistent with this, we show that ectopic expression of Gli3R can repress p2 and p1 domain expression at early stages and that this results in a reduction of V2 cells (Fig. 8O,P, Fig. 9F,I, Fig. 10C,D). By contrast, knocking down Gli repressor activity has no effect on p3

domain markers and very little effect on pMN domain markers, but results in more substantial expression of p2 and p1 domain markers at later stages (Fig. 8I-L, Fig. 9A,D,G). Therefore, our data are consistent with the hypothesis that, unlike in mouse, Gli repressor activity is not present early enough in zebrafish spinal cord to inhibit V2 cell formation in the absence of Hh signalling.

This difference between mouse and zebrafish could just reflect the faster development of zebrafish spinal neurons. Alternatively, it could result from an evolutionary change in Gli expression, either in the vertebrate lineage leading to zebrafish or the lineage leading to mouse. To distinguish between these possibilities it would be necessary to examine other vertebrate embryos that have completely lost Hh signalling. Although we cannot, given our results, rule out the possibility that Gli repressor activity cannot inhibit V1 or V0v fates in zebrafish, it is likely that we just were not able to ectopically express Gli3R at sufficient levels to inhibit the specification of these more dorsal cell types.

Gli1 can specify a small number of MNs and V3 cells in the absence of Hh signalling

Interestingly, we found that a small number of cells in the V3 and MN domains can still form, even in the absence of Hh signalling. This suggests that a Hh-independent mechanism might specify a small number of these cells. This might have been missed in

Table 2. Number of cells per somite length of spinal cord

Somite/treatment	Row 1	Row 2	Row 3	Row 4	Row 5	Overall average
WT somite 6	6.4±0.54	7.0±1.0	8.2±0.45	8.4±1.14	9.0±0.70	7.8±0.32
WT+DEAB somite 6	6.8±0.84	6.8±0.84	7.8±0.84	8.8±0.84	8.6±0.55	7.76±0.43
WT somite 10	6.2±0.84	6.2±0.84	7.2±0.84	8.4±0.89	8.6±0.89	7.32±0.36
WT+DEAB somite 10	6.6±0.55	7.4±0.55	7.4±1.14	8.6±1.34	7.8±0.84	7.56±0.36
WT average (somite 6+10)	6.3±0.68	6.6±0.97	7.7±0.82	8.4±0.97	8.8±0.79	7.56±0.41 [†]
WT+DEAB average (somite 6+10)	6.7±0.68	7.1±0.74	7.6±0.97	8.7±1.06	8.2±0.79	7.66±0.39 [†]

The average number of cells per somite length of spinal cord in the five most ventral rows of the spinal cord in somite 6 and somite 10 and averaged over both of these somites. WT embryos are compared with WT embryos treated with DEAB. Shown is the mean value obtained from five embryos \pm s.d. The overall average is an average of the results for all five ventral rows.

[†]The overall averages for somites 6 + 10 are not significantly different ($P=0.58$, Student's *t*-test).

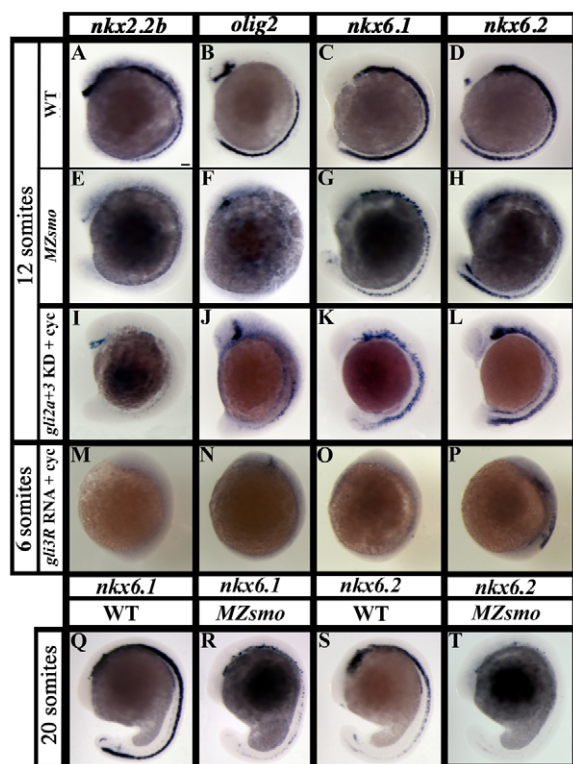


Fig. 8. Progenitor domain expression at somitogenesis stages.

(A-T) Lateral views of WT zebrafish embryos (A-D, Q, S), *MZsmo* mutants (E-H, R, T) and embryos treated with cyclopamine (cyc) and injected with *gli2a*, *gli3* and *p53* MOs (*gli2a* + 3 KD; I-L) or *gli3R* RNA (M-P). When *gli2a* and *gli3* were knocked down, *nkx2.2b* was absent ($n=23$; I), a small amount of *olig2* expression was recovered ($n=25$; J), and *nkx6.1* ($n=22$; K) and *nkx6.2* ($n=19$; L) expression was much stronger in all injected embryos. When *gli3R* RNA was injected, all embryos completely lacked *nkx2.2b* ($n=21$; M) and *olig2* ($n=45$; N) expression. *nkx6.1* expression was lost in 54% of embryos; the remaining embryos had strongly reduced staining ($n=28$; O). *nkx6.2* expression was lost in 8%, strongly reduced in 81%, and less reduced in 11% of injected embryos ($n=26$; P). Scale bar: 50 μ m.

studies on amniotes as the analysis was conducted on spinal cord transverse sections (Litingtung and Chiang, 2000; Wijgerde et al., 2002), whereas we examined the whole spinal cord in lateral view. Alternatively, it is possible that Gli repressor activity inhibits this Hh-independent specification in amniotes, but is not present at early enough stages to do this in zebrafish.

When we knocked down Gli1 activity in embryos with reduced Hh signalling, we found that almost all MNs and V3 domain cells were lost (Fig. 9B,E,K, Fig. 10A-C). The remaining *tal2*-expressing cells are probably cells that normally form more dorsally. We are confident that these results reflect a Hh-independent role for Gli1, rather than a non-specific effect from either the cyclopamine treatment or the *gli1* MO, for three main reasons. First, we observe a similar effect on MNs when we treat *gli1* (*detour*) mutants with cyclopamine (Fig. 9H). Second, we do not observe any reduction of V2a, V1 or V0v cells in our cyclopamine- and *gli1* MO-treated embryos (Fig. 10D; data not shown), arguing against any non-specific toxic effect. Third, our results agree with those of Mich and Chen (Mich and Chen, 2011), who also observe a Hh-independent role for Gli1 in specifying

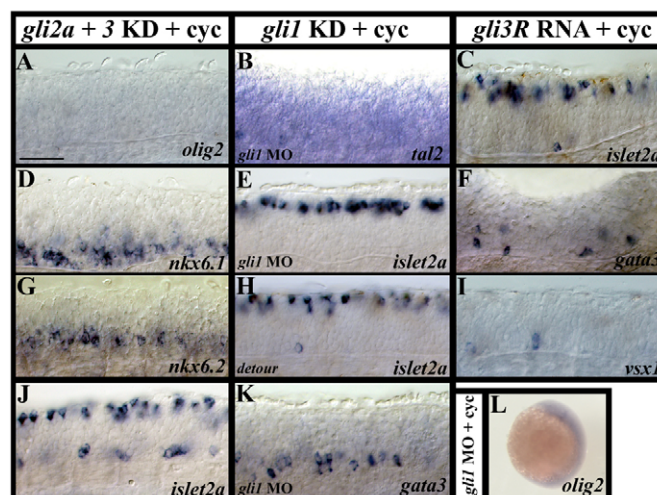


Fig. 9. Manipulating Gli activity in embryos with abrogated Hh signalling.

(A-L) Lateral views of spinal cord expression in 24-hpf zebrafish embryos treated with cyclopamine (cyc) and injected with either *gli2a*, *gli3* and *p53* MOs (A,D,G,I), *gli1* and *p53* MOs (B,E,K) or *gli3R* RNA (C,F,I), a 24-hpf *gli1* mutant (*detour*) embryo treated with cyclopamine (H) and a 6-somite (12-hpf) embryo treated with cyclopamine and injected with *gli1* and *p53* MOs (L). For L, 28/29 injected embryos had a complete lack of spinal cord *olig2* expression; the remaining embryo had a very small amount of spinal cord staining. Scale bar: 50 μ m in A-K; 250 μ m in L.

MNs. These results strongly suggest that Gli1 acts in a Hh-independent manner to specify at least most of the remaining MNs and V3 cells in *MZsmo* mutants. By contrast, there was no change in the number of cells with more dorsal cell fates, suggesting that Gli1 activity is not required for V2, V1 and V0v cells to form, even in the absence of Hh signalling.

RA signalling is required for correct cell proliferation and cell number in the zebrafish spinal cord

Previous studies suggested that RA signalling was a good candidate for specifying at least MNs, V0v cells and V1 cells in the absence of Hh signalling (Diez del Corral et al., 2003; Novitsch et al., 2003; Pierani et al., 1999; Wilson et al., 2004). Our results show that, in zebrafish spinal cord, RA signalling is required for the correct number of MNs and V0v, V1, V2 and V3 domain cells to form (Fig. 11A-F', Fig. 12A,B). In addition, the number of cells expressing ventral or dorsal progenitor domain markers are also reduced when RA signalling is abrogated (Fig. 11G-K', Fig. 12C). The spinal cord phenotypes obtained from loss of Hh and RA signalling appear to be additive, suggesting that RA is required independently of Shh for the formation of correct numbers of both progenitor and postmitotic cells. This is probably at least partly due to RA signalling regulating cell proliferation, as we see fewer phospho-Histone H3-positive cells in embryos in which RA signalling has been inhibited (Fig. 13A-E).

Our data do not exclude the possibility that RA also has other roles in spinal cord development, such as in directly upregulating Gli activator or downregulating Gli repressor activity (e.g. Mich and Chen, 2011). Consistent with the idea that RA has more than one function in spinal cord development, although inhibiting RA signalling has widespread effects in the spinal cord, there do

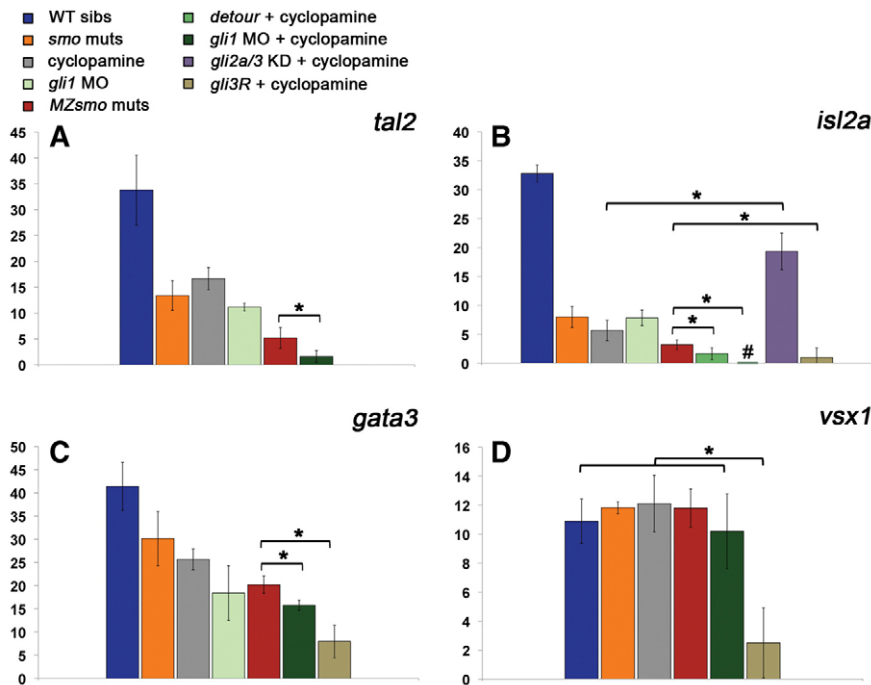


Fig. 10. Effect of manipulating Gli activity in embryos that lack Hh signalling.

(A-D) Average number ($n \geq 5$ embryos) of cells in spinal cord adjacent to somites 6-10 expressing the indicated markers. Note that treating WT with cyclopamine does not produce a phenotype as severe as that of *MZsmo* mutants (supplementary material Fig. S1). There were no *islet2a*-expressing cells in *gli1* MO-injected embryos treated with cyclopamine (# in B). The most relevant statistically significant reductions are indicated (*, $P < 0.05$, Student's *t*-test). With the exception of *vsx1*, where the only result that is significantly different is *Gli3R* + cyclopamine, most pairwise comparisons for the other genes are significantly different [the exceptions are: *smo* versus cyclopamine (*tal2*, *gata3*); *smo* versus *gli1* MO (*tal2*, *islet2a*); *detour* + cyclopamine and *gli1* MO + cyclopamine versus *gli3R* + cyclopamine (*islet2a*); *MZsmo* and *gli1* MO + cyclopamine versus *gli1* MO (*gata3*)]. Error bars denote s.d.

appear to be some cell type-specific or domain-specific differences in the phenotype. For example, at 24 hpf *olig2* expression appears to be reduced to a greater extent than other

progenitor domain markers, and *nkx6.1* expression is only subtly reduced (Fig. 12C). Very similar differences have been observed in amniotes (Diez del Corral et al., 2003; Novitsch et al., 2003),

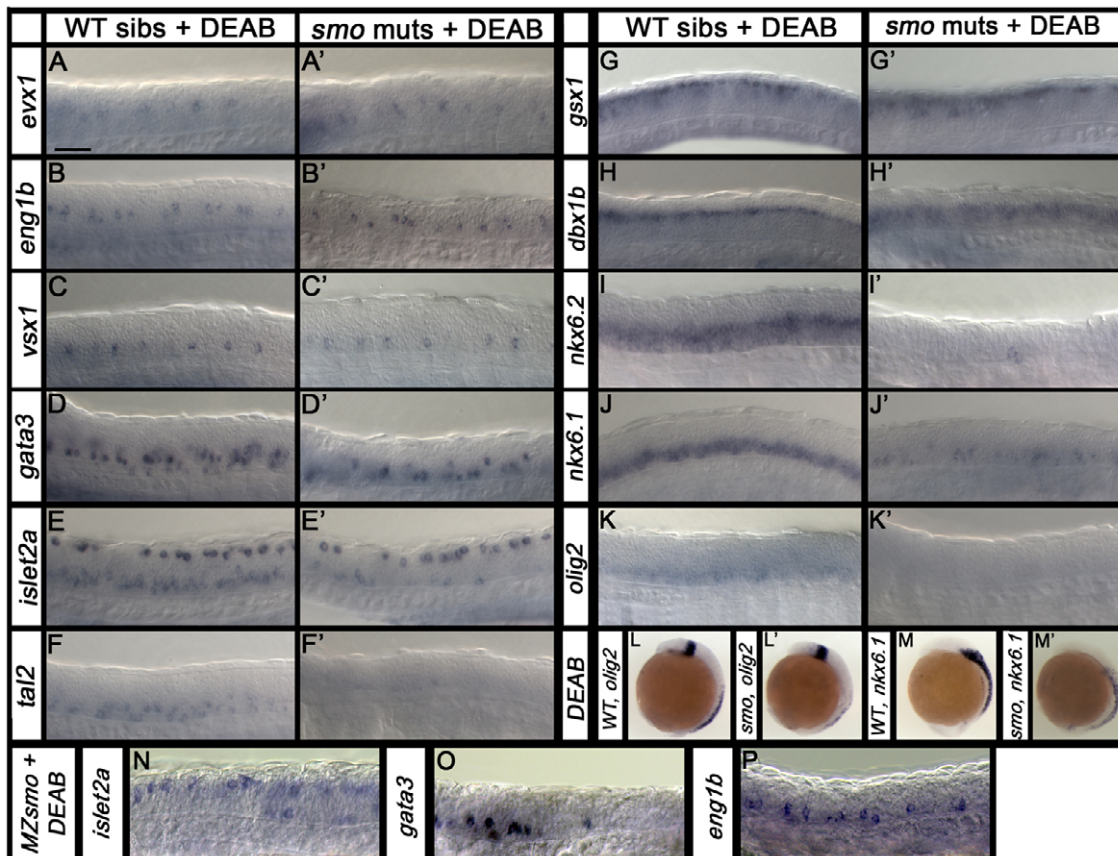


Fig. 11. Postmitotic and progenitor domain markers are reduced in the absence of RA signalling. Lateral views of WT sibling, *smo* mutant and *MZsmo* mutant 24-hpf spinal cords (A-K', N-P) and WT sibling and *smo* mutant 6-somite (12-hpf) embryos (L-M') treated with DEAB. Scale bar: 50 μ m in A-K', N-P; 250 μ m in L-M'.

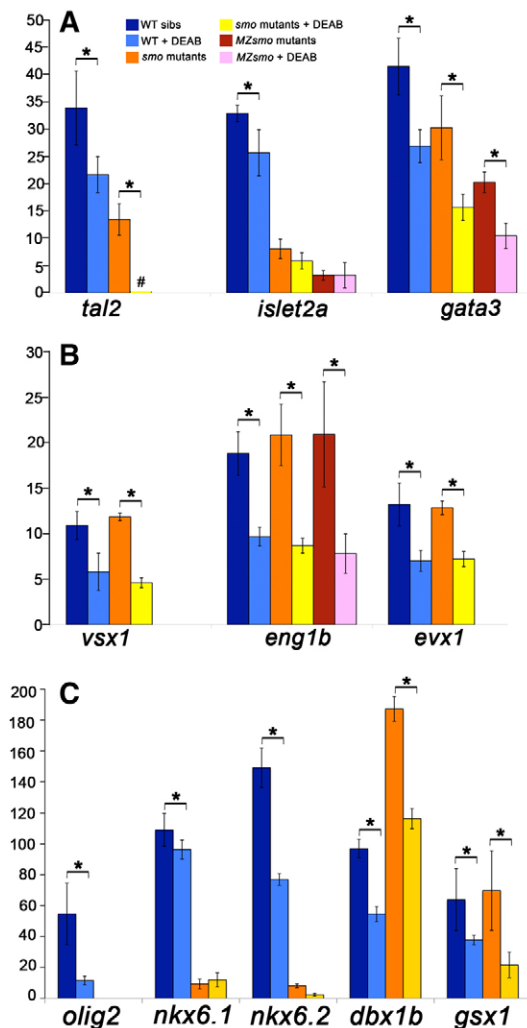


Fig. 12. Loss of RA signalling results in fewer spinal cord cells expressing postmitotic and progenitor domain transcription factors. (A-C) Average numbers ($n \geq 5$ embryos) of cells expressing the indicated markers in WT siblings, *smo* mutants and *MZsmo* mutants treated with DEAB or DMSO (control) in spinal cord adjacent to somites 6-10. There were no *tal2*-expressing cells in *smo* mutants treated with DEAB (# in A). Statistically significant differences between embryos of the same genotype are indicated (*, $P < 0.05$, Student's *t*-test). There is no significant difference between the number of *islet2a*-expressing cells in *smo* and *MZsmo* mutants with and without DEAB (A). This might reflect the fact that most of the remaining *islet2a*-expressing cells in these mutants are found anterior to somite 6 (see Mich and Chen, 2011). Error bars denote s.d.

where it has also been suggested that RA signalling has multiple roles at different times in many, but perhaps not all, spinal cord cells, including the regulation of progenitor domain transcription factor expression and the control of neuronal differentiation. Intriguingly, a recent paper (Lee et al., 2009) suggests that RA signalling helps to establish transcriptionally active chromatin in MN domain cells, suggesting a possible mechanism for some of these functions.

In conclusion, our data uncover a major difference between the spinal cord phenotypes of mouse and zebrafish embryos that lack Hh signalling. Notably, in zebrafish embryos, Hh signalling is not required for the early expression of many ventral progenitor

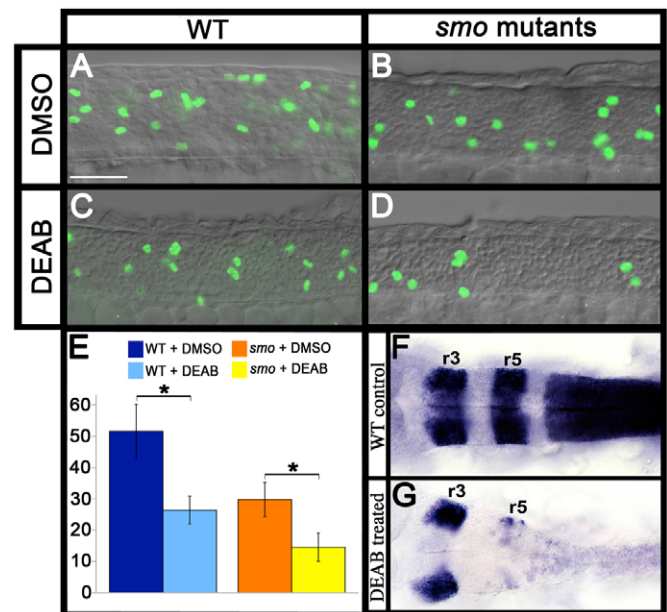


Fig. 13. DEAB treatment causes a reduction in cell proliferation. (A-D) Lateral views of phospho-Histone H3 staining in DMSO-treated control and DEAB-treated spinal cords of WT zebrafish embryos and *smo* mutants at 24 hpf. Scale bar: 50 μ m. (E) Average number ($n = 5$ embryos) of phospho-Histone H3-positive cells in spinal cord adjacent to somites 6-10. Statistically significant differences between embryos of the same genotype are indicated (*, $P < 0.05$, Student's *t*-test). Error bars denote s.d. (F, G) dorsal views of hindbrain showing dramatically reduced *egr2b* expression in rhombomere (r) 5 and the loss of *hoXB4a* expression in the anterior spinal cord of DEAB-treated embryos (G).

domain transcription factors or for the formation of V2, V1 and V0v cells. Our data suggest that this is because Gli repressor activity is not present early enough to repress ventral progenitor domain genes at early somitogenesis stages. The results also suggest that the remaining expression of pMN, p2 and p1 domain genes is sufficient to specify the small numbers of MNs and the normal numbers of V2 and V1 cells that we observe at 24 hpf in *MZsmo* mutants. We also show that the remaining early expression of the pMN domain gene *olig2* and that most of the remaining MNs in *MZsmo* mutants require a Hh-independent Gli1 activity. However, it remains a mystery, in both zebrafish and mouse, what (if anything) induces/specifies the expression of p2 and p1 domain genes in the absence of both Hh signalling and Gli repressor activity. The data that we present in this paper strongly suggest that this 'missing signal' is not RA. RA signalling is required in zebrafish embryos to specify the correct numbers of ventral spinal cord neurons, but our data suggest that this is because in the absence of RA signalling there are reduced levels of cell proliferation in the spinal cord. Although not exhaustive, our preliminary analyses also suggest that the 'missing signal' is not a BMP or Wnt signal because when we knock down these signalling pathways, either individually or in combination with Hh signalling, V2, V1 and V0v cells still form (M.F.B., S.E. and K.E.L., unpublished data).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at

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