Development 138, 5113-5119 (2011) doi:10.1242/dev.066225

Hedgehog and retinoic acid signaling cooperate to promote motoneurogenesis in zebrafish

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SUMMARY

The precise requirements of Hedgehog (Hh) pathway activity in vertebrate central nervous system development remain unclear, particularly in organisms with both maternally and zygotically derived signaling. Here we describe the motoneural phenotype of zebrafish that lack maternal and zygotic contributions of the Hh signaling transducer Smoothened (*MZsmo* mutants) and therefore are completely devoid of ligand-dependent pathway activation. Some functional primary motoneurons (PMNs) persist in the absence of Hh signaling, and we find that their induction requires both basal Gli transcription factor activity and retinoic acid (RA) signaling. We also provide evidence that RA pathway activation can modulate Gli function in a Hh ligand-independent manner. These findings support a model in which Hh and RA signaling cooperate to promote PMN cell fates in zebrafish.

KEY WORDS: Hedgehog, Motoneuron, Retinoic acid, Zebrafish

INTRODUCTION

Hedgehog (Hh) pathway activation is essential for vertebrate development (Ingham and McMahon, 2001) and can lead to oncogenesis (Teglund and Toftgard, 2010). Hh ligands (Echelard et al., 1993) bind to the transmembrane receptor Patched 1 (Ptc1, or Ptch1) (Stone et al., 1996), thereby alleviating Ptc1-mediated inhibition of Smoothened (Smo), a G protein-coupled receptor-like protein (Zhang et al., 2001). Smo in turn regulates Gli transcription factors, which exist as full-length transcriptional activators and N-terminal repressors (Wang et al., 2000; Bai et al., 2002).

In mammals, sonic hedgehog (Shh) is expressed in the notochord and floor plate of the developing spinal cord, establishing a dorsoventral gradient of Hh activity that is required for ventral neural cell fates including motoneurons (Ericson et al., 1995; Roelink et al., 1995; Chiang et al., 1996). The role of Hh signaling in teleost motoneuron development, however, is less clear. Zebrafish have primary and secondary motoneurons (PMNs and SMNs), which differ in their time of appearance during development (Kimmel and Westerfield, 1990). Elevated Hh signaling induces extra PMNs and SMNs (Chandrasekhar et al., 1998), and Hh signaling is necessary for SMN specification (Varga et al., 2001). Although zebrafish lacking zygotic Smo retain significant numbers of PMNs (Chen et al., 2001; Varga et al., 2001), it has been reported that nearly all PMNs can be eliminated in embryos treated with the Smo antagonist cyclopamine (Chen et al., 2001) or injected with morpholinos (MOs) targeting Hh ligands (Lewis and Eisen, 2001). These observations have led to the hypothesis that PMNs remaining in zygotic *smo* mutants are specified by maternal *smo* activity (Chen et al., 2001; Varga et al., 2001).

Alternatively, the depletion of PMNs in zebrafish treated with cyclopamine or Hh ligand-targeting MOs could be due to off-target effects of these reagents or associated impurities, and other pathways might induce PMNs in the absence of Hh signaling. One candidate is retinoic acid (RA) signaling, which can promote mammalian motoneuron specification (Pierani et al., 1999; Novitch et al., 2003) and is localized to the anterior zebrafish spinal cord (Perz-Edwards et al., 2001), where PMNs persist in zygotic *smo* mutants. Basal Gli activity could also enable PMN induction, as zebrafish Gli function is not fully dependent on Hh signaling (Huang and Schier, 2009).

Here we report that zebrafish lacking both maternal and zygotic *smo* function (*MZsmo* mutants) exhibit a complete loss of Hh signaling yet still form PMNs. We find that both RA signaling and Smo-independent Gli activity promote PMN genesis in *MZsmo* embryos, in part by fostering PMN progenitor formation and neuronal proliferation. Furthermore, RA signaling can increase basal Gli activity. These results suggest that Hh and RA signaling act in concert to induce PMNs during neural tube development.

MATERIALS AND METHODS

Zebrafish husbandry

Wild-type AB and $smo^{hi1640/+}$ zebrafish were obtained from the Zebrafish International Resource Center (ZIRC), $gli1^{ts269/+}$ and $gli2a^{ty17/+}$ animals were provided by R. Karlstrom (Karlstrom et al., 1999; Karlstrom et al., 2003), and $MZsmo^{hi1640}$ zebrafish were created as described (Mich et al., 2009). Naturally fertilized embryos were cultured in E3 medium at 28.5°C with a vehicle control, cyclopamine (a gift from Infinity Pharmaceuticals; 100 μM at the one-cell stage), SANT75 [a gift from S. Lin (Yang et al., 2009); 100 μM at the one-cell stage], diethylaminobenzaldehyde (DEAB; Sigma; 10 μM at the one-cell or sphere stage with similar effects), and/or all-trans-retinoic acid (RA; Sigma; 10 or 100 nM at the one-cell or sphere stage with equal results for each time).

Oligonucleotide injections

shha mRNA was synthesized with the mMessage mMachine Kit (Applied Biosystems) and injected at 100 pg/embryo. MOs (Gene Tools) were (5′ to 3′): *dead end*, GCTGGGCATCCATGTCTCCGACCAT (3 ng/embryo) (Ciruna et al., 2002); *gli1*, CCGACACACCCGCTACACCCACAGT (4 ng/embryo) (Karlstrom et al., 2003); *gli2a*, GGATGATGTAAA-GTTCGTCAGTTGC (8 ng/embryo) (Karlstrom et al., 2003); *gli2b*, AGCTGGAACACCGGCCTCCATTCTG (8 ng/embryo) (Devine et al., 2009); *gli3*, ACAACTGGGCATTCCTCAGAGCATC (8 ng/embryo) (Tyurina et al., 2005); and *tp53*, GCGCCATTGCTTTGCAAGAATTG (4 ng/embryo) (Robu et al., 2007). Gli MOs were co-injected with *tp53* MO, and *gli2a*/*gli3* and *gli2a*/*gli2b*/*gli3* MO combinations were co-injected in equimolar amounts for a total of 8 ng plus 4 ng *tp53* MO.

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Accepted 3 October 2011

Whole-mount in situ hybridization

Protocols were as described (Jowett and Lettice, 1994) using the following probes: *isl1* (Lewis and Eisen, 2001); *isl2a* (Lewis and Eisen, 2001); *nkx2.2a* (Woods and Talbot, 2005); *ptc1* (Woods and Talbot, 2005); *smo* (Chen et al., 2001); *fzd8b*, amplified with primers (5′ to 3′) ATGGACTCGCCTACACAGGG and TCACACACGAGAAAGTGGCATT; *gli1*, ATGCCAGTGGATATGCAGC and GATGTGCTCATTATTGATGTGATGC; *gli2a*, ATGGAGACCACAAGTCCCACC and CCACCCATCGACACACAAACT; *gli2b*, TCCAGCTCCCGCAGAGAAG and TTGAGCAGCCCTCGAACG; *gli3*, TCTGAGGAATGCCCAGTTGTC and CCTCCTCTTTATCGCCCTCTT; *hoxb4a*, TGGCCATGAGTTCCTATTTGAT and CTATAGACTTGGCGGAGGTCC; *olig2*, ATGGACTCTGACACGAGCCG and GGCTGAGGAAGGTTTGCCA; and *pax6a*, ATGCCTCAAAAAGAATACTATAACCG and TCACTGTAGTCTGGGCCAGTATTG. For PMN quantification, at least 40 embryos were analyzed per condition using *isl2a* staining of 18-somite [18 hours post-fertilization (hpf)] embryos, except for the *smo* mutant data in Fig. 2O (at least five embryos per condition in this case).

gli2b transcript levels were quantified by acquiring color images of *gli2b*-stained flat-mounted embryos using a Leica M205FA stereomicroscope. The images were cropped to the neural plate region using Photoshop (Adobe) and converted into monochromatic images with inverted pixel intensities to correlate *gli2b* staining and pixel intensity. Raw pixel intensity files were then generated by ImageJ (NIH) and imported into R software for data analysis. Five to seven embryos were imaged for each experimental condition.

Whole-mount immunostaining

Protocols were as described (Macdonald, 1999) using the following antibodies: slow muscle myosin [F59, Developmental Studies Hybridoma Bank (DSHB), 1:5 dilution]; fast muscle myosin (F310, DSHB, 1:5); synaptic vesicles (SV2, DSHB, 1:10); Mnx1 (81.5C10, DSHB, 1:20); motoneurons (zn-1, ZIRC, 1:50); synaptotagmin 2 (znp-1, ZIRC, 1:10); lens fiber cells (zl-1, ZIRC, 1:500); Isl1/2 (39.4D5, DSHB, 1:10); phosphohistone H3 (3465S, Cell Signaling, 1:750); and Alexa Fluor 594-conjugated α-bungarotoxin (Invitrogen, 1:250). Alexa Fluor 488- and 594-conjugated secondary antibodies (Invitrogen) were diluted 1:500.

Twitching assays

For stimulus-induced twitching, 24-hpf embryos were prodded on their yolk, and their responses were tabulated (n>250). For spontaneous twitching, a field of 24-hpf embryos was videorecorded at room temperature for 5 minutes (8× magnification, 100 mseconds/frame) and scored (n>150). For voltage-induced twitching, a square-wave (10-msecond pulse of 5 V) was applied to 24-hpf embryos (n=25) using an electroporation apparatus (Harvard Apparatus BTX ECM 830), and responses were videorecorded.

Quantitative (q) RT-PCR

Fifty embryos were lysed in 1 ml TRIzol (Invitrogen) to obtain crude RNA. Then, 10 μg crude RNA was treated with 2 units of DNase TURBO (Applied Biosystems) at 37°C for 30 minutes, purified by phenol/chloroform extraction, and reverse transcribed using the Superscript III Kit (Invitrogen). qPCR was performed using a Roche 480 LightCycler, SYBR Green I Master Kit and the following primers (5′ to 3′): β-*actin*, TCACCTCTCTTGCTCCTTCC and GGGCCAGACTCATCGTACTC; *ef1a*, AACCCCAAGGCTCTCAAATC and TCTCAACGCTCTTGATGACG; *gli1*, GTTTGCCACTGGAAGGACTG and GATGCGTCTTCAGGTTCTCC; *hhip*, GGTTTGGCGAAGATGAATTAG and CACTCCTTTGGAACTTGTGGT; *nkx2.2a*, TACTCATTACACGGCCTGTCC and CTTGGAAAAGAGCACCCTCC; *ptc1*, TGCCATTGAAACGGACATAG and TTCCTTCCTGTTTGGGTGTC; and *ptc2*, GCAGGCTGGTAGGATCACTC and GCGTTTGGGTTGATTATTCC.

RESULTS AND DISCUSSION

Zebrafish maternal *smo* persists through gastrulation and mediates patterning

As described previously (Mich et al., 2009), we generated *MZsmo* zebrafish, which exhibit Hh signaling-deficient phenotypes of partial cyclopia, ventral body curvature, circulation defects, and U-shaped somites (Fig. 1A-C). The smo^{hi1640} allele used in these studies strongly disrupts *smo* transcription (Chen et al., 2001), allowing observation of maternal *smo* by comparing *smo* transcript levels in *MZsmo* and *smo* mutants. Maternal *smo* persisted until the bud stage (10 hpf) (Fig. 1D-F), at which point it was primarily restricted to the anterior neural plate (Fig. 1E).

In comparison to wild-type zebrafish and zygotic *smo* mutants, *MZsmo* embryos exhibit more severe mispatterning of the adenohypophyseal placode (supplementary material Fig. S1) and the zona limitans intrathalamica (Fig. 1G-I). Maternal *smo* therefore promotes some aspects of zebrafish embryogenesis, presumably by transducing Hh signals.

MZsmo zebrafish lack detectable Hh signaling

To assay Hh responsiveness in wild-type and mutant embryos, we injected zygotes with *shha* mRNA and assessed Hh target gene transcription (*nkx2.2a* and *ptc1*). Exogenous *shha* expanded *nkx2.2* and *ptc1* expression in wild-type embryos (Fig. 1J,M), and *smo* mutants responded weakly, but detectably (Fig. 1K,N), as previously reported (Varga et al., 2001). However, *MZsmo* embryos did not respond to exogenous Hh ligand (Fig. 1L,O). qRT-PCR analysis confirmed that *shha* mRNA-injected *MZsmo* embryos lack Hh pathway activation (Fig. 1P).

MZsmo embryos have motor defects yet retain anterior PMNs

MZsmo zebrafish exhibit more severe paralysis than *smo* mutants in response to stimulus-induced and spontaneous twitching but not voltage-induced twitching (Fig. 2A). The muscle is not more severely mispatterned in *MZsmo* embryos (supplementary material Fig. S2) and Rohon-Beard (RB) sensory neurons were retained (Fig. 2E,N). These data suggest that the greater paralysis of *MZsmo* embryos is due to a defective motoneural apparatus.

To investigate the basis of these motoneural defects, we examined PMN genesis in *MZsmo* embryos. We observed fewer and more anteriorly restricted PMNs (Fig. 2C-N), which retained native identity and activity as assessed by a battery of PMN markers (supplementary material Fig. S3). The *MZsmo* motor defect is therefore likely to be due to decreased numbers of PMNs rather than to attenuated intrinsic activity of the remaining neurons. Yet, contrary to earlier predictions (Chen et al., 2001; Varga et al., 2001), all *MZsmo* mutants retained PMNs, indicating that Hh signaling is not required for their formation.

Basal Gli activity promotes motoneurogenesis

Since PMNs can form in *MZsmo* zebrafish, one possibility is that basal Gli activity is sufficient to induce PMN development. In addition to a single *smo* gene, zebrafish have four Gli homologs – the constitutively active *gli1* and *gli2a*, *gli2b* and *gli3* – which have full-length activator and C-terminally truncated repressor states. To assess basal Gli activities, we treated wild-type zebrafish embryos, $gli1^{ts269}$ mutants [which lack Gli1 function (Karlstrom et al., 2003)] and $gli2a^{ty17}$ mutants [which express a repressor form of Gli2a (Karlstrom et al., 1999)] with cyclopamine and assessed *ptc1* expression (Fig. 3A-F). Cyclopamine treatment (100 μM) reduced *ptc1* transcription in wild-type embryos to levels similar to that in *MZsmo* mutants (Fig. 3D; supplementary material Fig. S4); homozygous *gli2a* mutant embryos were indistinguishable from their wild-type and heterozygous siblings after cyclopamine treatment (Fig. 3E), which is likely to be because both wild-type Gli2a and truncated

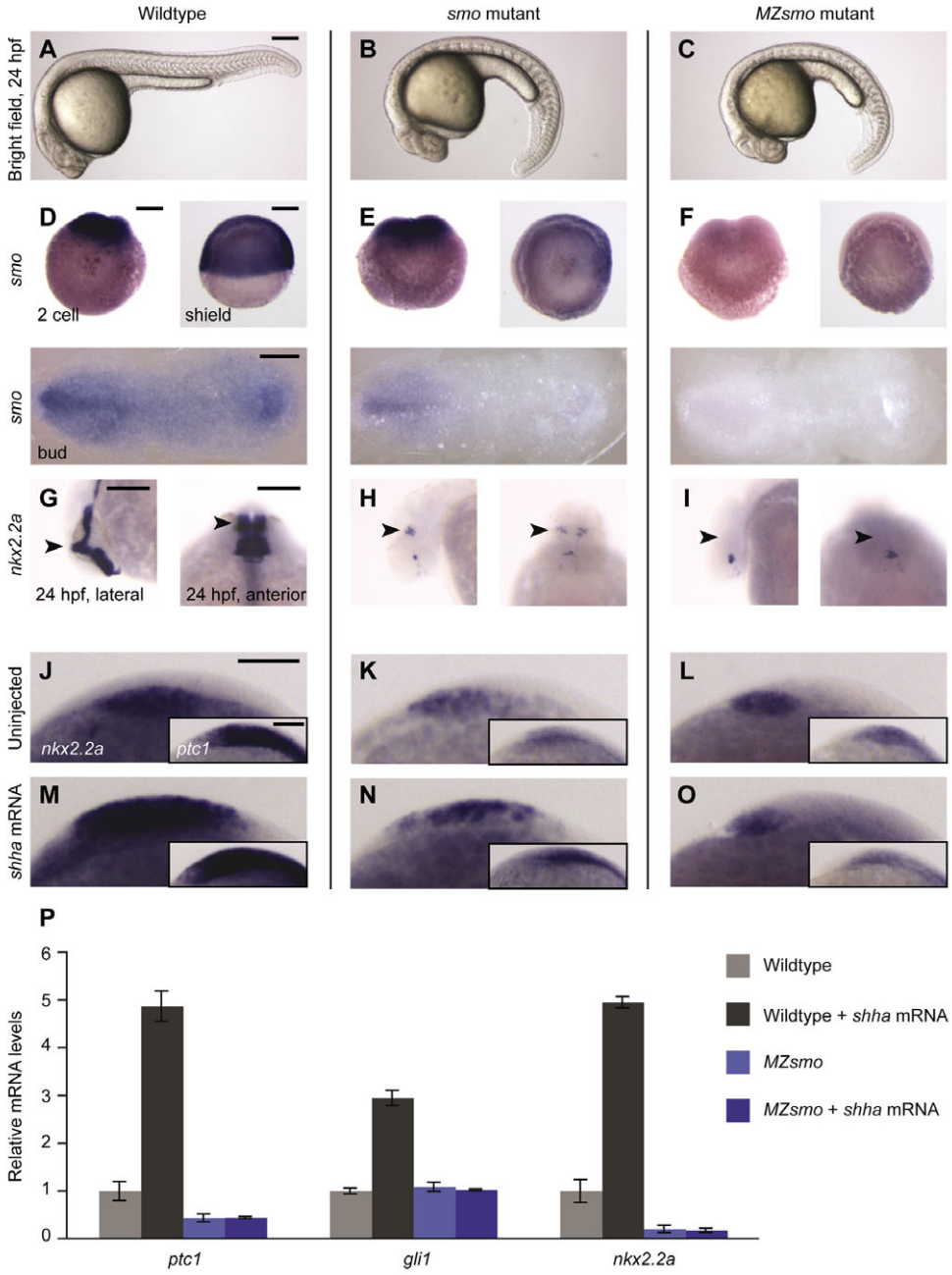

Fig. 1. *MZsmo* zebrafish embryos have patterning defects and lack detectable Hh signaling. (**A-C**) Wild-type, *smo* and *MZsmo* embryos (24 hpf). (**D-F**) Maternal *smo* mRNA is present in zygotic *smo* mutants but depleted in *MZsmo* embryos. Homozygous smo^{hi1640} embryos could be distinguished from siblings at the shield and bud stages but not at the two-cell stage. Two-cell and shield embryos are viewed laterally; bud-stage embryos are flat-mounted and viewed dorsally, anterior to left. (**G-I**) Maternal *smo*-dependent *nkx2.2a* expression in the zona limitans intrathalamica (arrowheads). (**J-O**) Wild-type, *smo* and *MZsmo* embryos (10 hpf) were either uninjected or injected with *shha* mRNA and stained for *nkx2.2a* (primary micrographs) or *ptc1* (insets) transcripts. Images are lateral views of the anterior neural plate, anterior to the left. (**P**) Wild-type and *MZsmo* embryos were treated as in J-O and analyzed by qRT-PCR. Transcript levels are normalized to the wild-type control; error bars represent s.e.m. Scale bars: 200 μm in A-I; 100 μm in J-O.

Gli2a mutants act as repressors when Smo is inhibited. By contrast, approximately one-quarter of cyclopamine-exposed progeny from $gli1^{ts269/+}$ incrosses exhibited a near complete loss of *ptc1* expression (Fig. 3F), although the embryos did not segregate into two distinct groups (see below). *MZsmo* embryos injected with *gli1* MO had significantly reduced Hh target gene expression as gauged by qRT-PCR (supplementary material Fig. S5).

Next, we tested whether Gli1 promotes Smo-independent motoneurogenesis. Identifiable by their missing hindbrain cranial motoneurons (Chandrasekhar et al., 1999), homozygous $gli1^{ts269}$ and $gli2a^{ty17}$ mutants have disorganized PMNs (Zeller et al., 2002), which are fewer in number than those in wild-type embryos (Fig. 3G-I). Cyclopamine decreased PMN numbers to a similar extent in wild-type and $gli2a^{ty17/+}$ incrossed progeny (Fig. 3J,K,M), but strongly depleted PMNs in $gli1^{ts269/+}$ incrossed progeny (Fig. 3L,M). Cyclopamine-treated $gli1^{ts269/+}$ incrosses did not segregate clearly into a 3:1 phenotypic ratio (Fig. 3M), possibly due to *gli1* haploinsufficiency (Karlstrom et al., 2003), although likely homozygous *gli1* mutants could be identified (Fig. 3F,L). The effect of cyclopamine on $gli1^{ts269/+}$ progeny was confirmed in *MZsmo* mutants injected with *gli1* MO (Fig. 3N), supporting a model in which Hh signaling-independent Gli1 activity promotes PMN formation.

To study the roles of other zebrafish Gli proteins in PMN development, we quantified these neurons in *MZsmo* embryos injected with MOs targeting *gli2a*, *gli2b* or *gli3*. In comparison to uninjected *MZsmo* embryos, *gli2b* knockdown significantly depleted PMNs (Fig. 3N). Knockdown of *gli2a* increased PMN number in *MZsmo* zebrafish, and, although loss of *gli3* function did not significantly perturb PMN formation, tandem *gli2a/gli3* or *gli2a/gli2b/gli3* knockdowns led to even greater increases in PMN

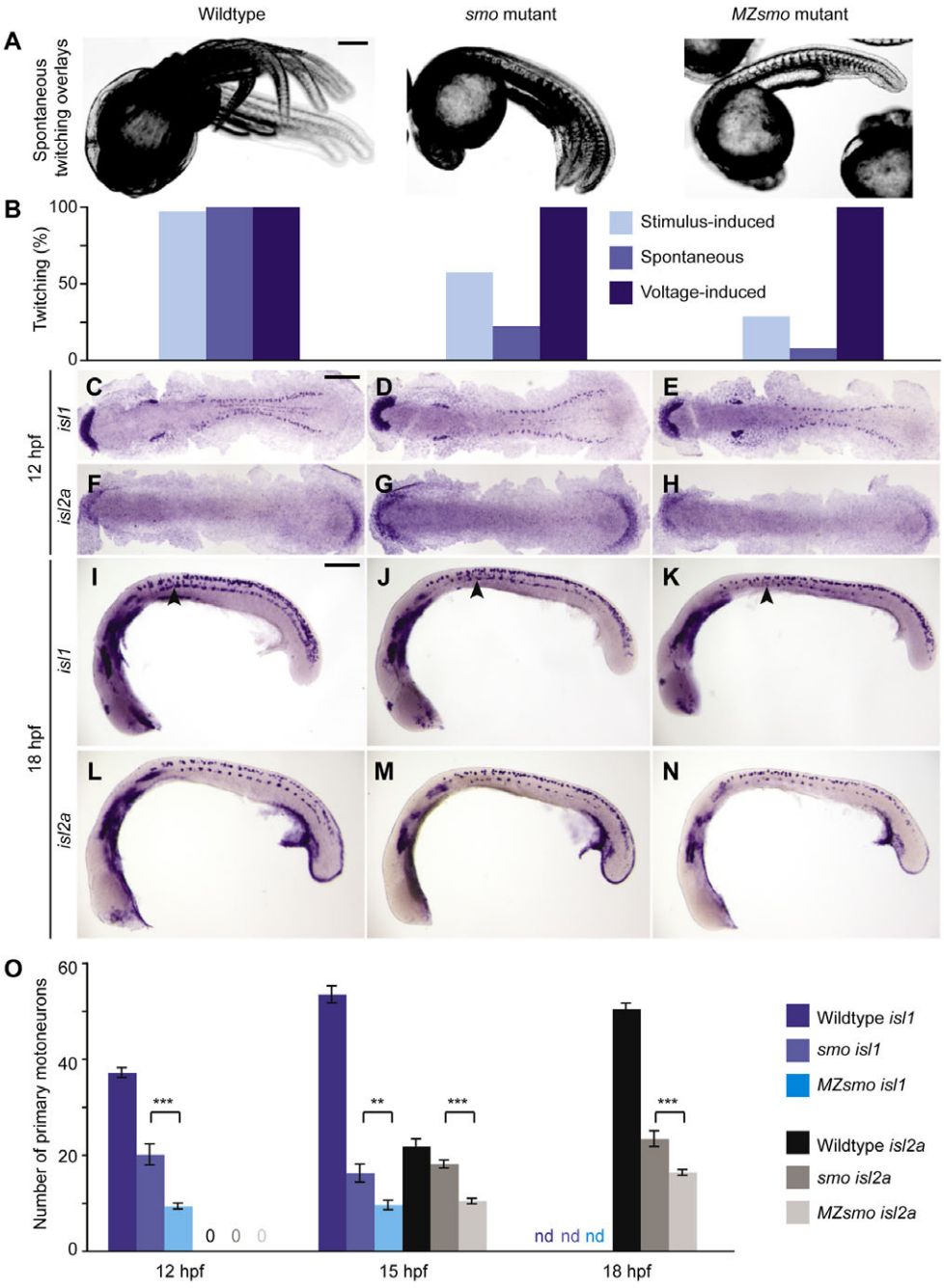

Fig. 2. Maternal *smo* promotes motor activity and motoneurogenesis. (**A**) Wild-type, *smo* and *MZsmo* zebrafish embryos were imaged at 24 hpf during spontaneous twitching. Ten overlaid images taken at 100-msecond intervals are shown. (**B**) Embryos at 24 hpf were observed for the ability to twitch in response to physical stimulus, spontaneously, or in response to applied voltage. (**C-N**) Wild-type, *smo* and *MZsmo* embryos at the 6-somite (12 hpf, dorsal view) and 18-somite (18 hpf, lateral view) stages were stained for *isl1* or *isl2a* transcripts, which mark ventral PMNs and dorsal RB neurons. Anterior is to the left. Arrowheads in I-K indicate non-PMN *isl1*-labeled interneurons that preclude accurate counting of *isl1*-positive PMNs at this developmental stage. (**O**) Average number of *isl1*-positive or *isl2a*-positive PMNs per embryo for each genotype and developmental stage. For each condition, 100-200 PMNs were counted. nd, not determined owing to technical challenges in quantifying PMNs at this stage. Error bars represent s.e.m. **, $P<0.01$; ***, $P<0.001$. Scale bars: 200 μm.

number. These manipulations did not affect RB neurons but did alter *ptc1* transcript levels (supplementary material Fig. S6), confirming the specificity of these reagents. Analogous perturbations do not influence more dorsal interneuron types (England et al., 2011). Thus, in the absence of Hh signaling, *gli1* and *gli2b* can promote PMN formation, whereas *gli2a*, and to a lesser extent *gli3*, inhibit this process.

RA signaling promotes PMN induction in the absence of Hh signaling

Since RA signaling can promote motoneuron specification in amniotes and is localized to the anterior region where PMNs persist in *MZsmo* embryos, we investigated whether RA pathway activity specifies PMNs in these mutants. We treated wild-type and *MZsmo* zebrafish with either exogenous RA or the aldehyde dehydrogenase 1a2 (Aldh1a2) inhibitor DEAB (supplementary material Fig. S7) (Grandel et al., 2002; Begemann et al., 2004), and quantified PMN specification. DEAB inhibited PMN formation slightly in wild-type embryos but more potently in *MZsmo* mutants (Fig. 3N, Fig. 4A), with the few PMNs remaining in DEAB-treated *MZsmo* zebrafish being largely restricted to rostral regions (supplementary material Fig. S8). This effect was rescued by exogenous RA dosed at physiological levels (10 nM), and higher concentrations (100 nM) induced supernumerary PMNs in both wild-type and *MZsmo* embryos (Fig. 4A; supplementary material Fig. S8). These observations suggest that the PMNs remaining in *MZsmo* zebrafish are specified, at least in part, through RA signaling (supplementary material Fig. S7D). RA also induced greater numbers of RB sensory neurons (supplementary material Fig. S9), consistent with findings in *Xenopus* (Franco et al., 1999).

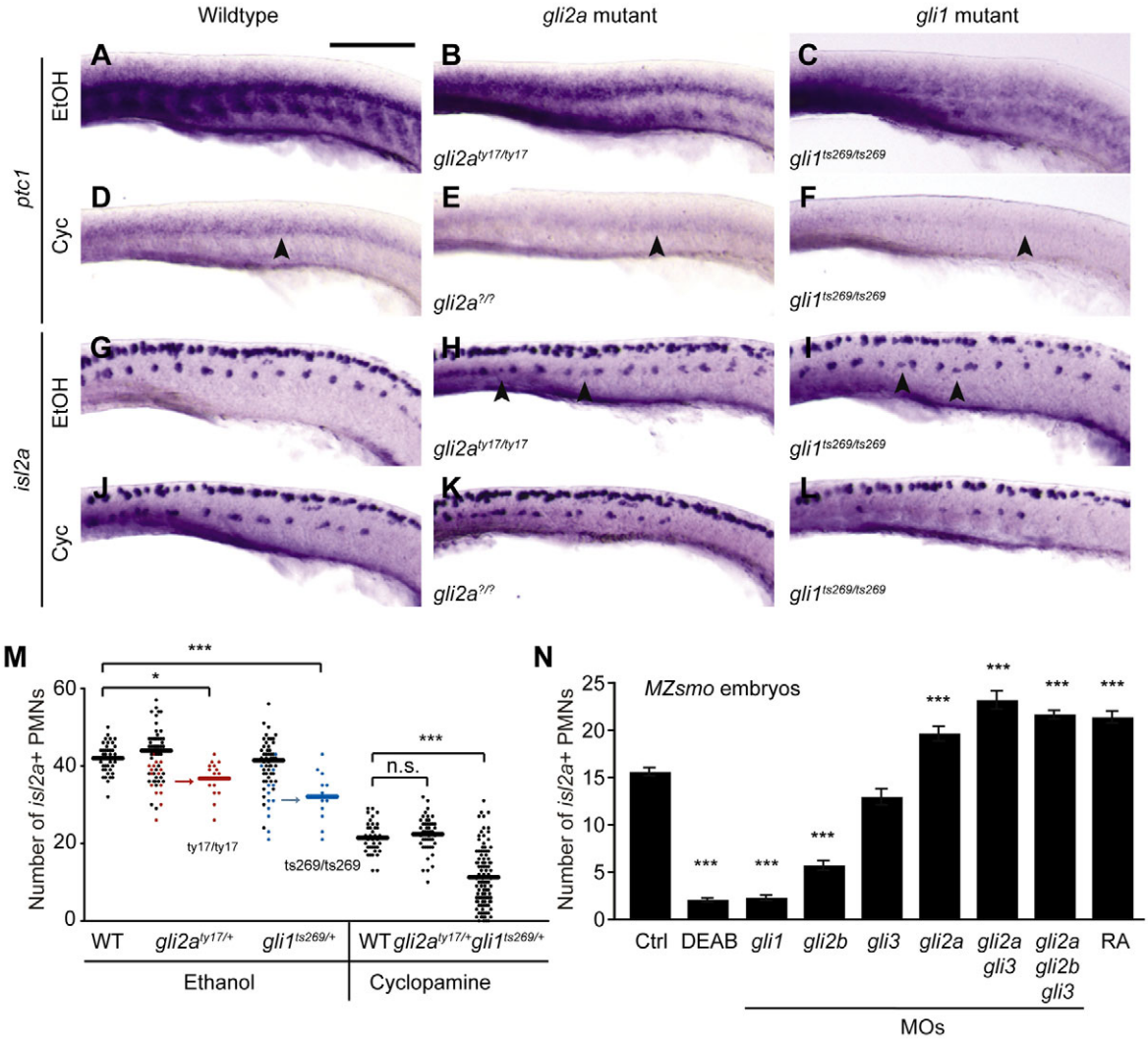

Fig. 3. Gli-dependent patterning in the absence of Smo. (**A-L**) Zebrafish embryos obtained from wild-type, *gli2a*$^{ty17/+}$ or *gli1*$^{ts269/+}$ incrosses (18 hpf) were treated with either ethanol (EtOH) or 100 μM cyclopamine and stained for *ptc1* or *isl2a* transcripts. The trunk region between approximately the third and twelfth somites is shown laterally, anterior to the left. Arrowheads in D-F show basal *ptc1* expression in the neural tube; arrowheads in H,I show disorganized PMNs in *gli2a*$^{ty17/ty17}$ and *gli1*$^{ts269/ts269}$ embryos. (**M**) Quantification of PMNs in wild-type embryos and those resulting from incrosses of *gli2a*$^{ty17/+}$ or *gli1*$^{ts269/+}$ zebrafish after treatment with either ethanol or 100 μM cyclopamine. Homozygous *gli2a*^{ty17} and *gli1*^{ts269} mutants were identified by the absence of hindbrain cranial motoneurons, which cannot be assessed after cyclopamine treatment. *, $P<0.05$; ***, $P<0.001$; n.s., not significant. Bar indicates the mean. (**N**) Quantification of PMNs in *MZsmo* embryos treated as indicated. Error bars represent s.e.m. ***, $P<0.001$, compared with control *MZsmo* embryos. Scale bar: 100 μm.

RA signaling can promote basal Gli activity

To determine whether basal Gli activity and RA signaling act independently to promote PMN specification, we analyzed the effects of RA signaling perturbations on the Hh pathway state in wild-type and *MZsmo* embryos. Altered RA signaling did not significantly change *ptc1* expression within the neural tube of wild-type embryos (Fig. 4B,D,F), although transcription of the RA target *hoxb4a* was affected (supplementary material Fig. S10). Nor could significant RA signaling-dependent changes in *ptc1* transcription be detected by qRT-PCR analyses of these embryos (supplementary material Fig. S11). However, *ptc1* within the neural tube of *MZsmo* embryos was moderately inhibited by DEAB (Fig. 4C,E; supplementary material Fig. S10), and exogenous RA increased *ptc1* transcription in the posterior neural tube (Fig. 4G). qRT-PCR analysis of *MZsmo* zebrafish also showed that elevated RA signaling can promote expression of the Hh targets *gli1*, *hedgehog-interacting protein* (*hhip*) and *ptc2*, although the neural tube-localized increase in *ptc1* staining was less apparent in these whole-embryo measurements (supplementary material Figs S11 and S12).

Intrigued by the effects of exogenous RA on global *gli1* transcript levels, we investigated whether RA signaling can regulate *gli1* activity in the zebrafish neural tube. RA or DEAB treatment did not detectably affect *gli1* expression within the developing spinal cords of wild-type or *MZsmo* embryos (supplementary material Fig. S13), suggesting that RA signaling does not modulate this factor in this tissue. Nor did RA or DEAB treatment perturb *gli2a* or *gli3* expression (data not shown). However, *gli2b* is expressed in an anteroposterior gradient within the neural tube (Fig. 4H-K), and DEAB treatment reduced *gli2b* transcript levels (Fig. 4,H,I,L,M). Exogenous RA increased *gli2b*

transcription posteriorly (Fig. 4,H,I,N,O). The correlation between RA signaling-dependent *gli2b* expression and *MZsmo* PMN induction, along with the observation that the *gli2b* MO depletes these PMNs, suggest that modulation of *gli2b* expression and thereby its basal activity is at least one component of RA-dependent motoneurogenesis. England et al. have similarly proposed that RA is required independently of Shh signaling to pattern the ventral spinal cord (England et al., 2011).

RA signaling and basal Gli activity modulate PMN progenitor cell formation and neural proliferation

To investigate how RA signaling and basal Gli activity might cooperate to induce motoneurogenesis, we investigated their contributions to the formation of *olig2*-expressing motoneuron progenitors and cell proliferation within the neural tube. Motoneuron progenitors that persist after Smo inhibition by cyclopamine or SANT75, a structurally distinct Smo antagonist (Yang et al., 2009), require both RA signaling and basal Gli activity for their formation (supplementary material Fig. S14) [for analyses of other progenitor domains, see England et al. (England et al., 2011)]. We also found that ablation of Smo function diminished neural proliferation, which could be further reduced by DEAB treatment (supplementary material Fig. S15). Loss of basal Gli activity decreased the number of mitotic cells as well, although to a lesser extent. These results suggest that both RA signaling and basal Gli activity promote motoneurogenesis at least in part by inducing progenitor cell formation and neural proliferation.

Conclusions

Surprisingly, zebrafish that completely lack *smo* and therefore Hh signaling retain motoneurogenic capabilities, particularly within the rostral regions of the developing spinal cord. PMN formation in these embryos is promoted by basal, Hh ligand-independent Gli activity and RA signaling, with the latter acting in part by inducing *gli2b* transcription and potentiating Hh target gene expression. Although we cannot rule out the possibility that other mechanisms enable PMN development in the absence of Hh signaling, our findings demonstrate the functional significance of basal Gli activity and Hh signaling-independent modes of Gli control, and they reconcile the apparent discrepancies between Hh pathway-dependent ventral neural tube patterning in teleosts (Chen et al., 2001; Varga et al., 2001) and in mammals (Chiang et al., 1996). Similar Hh ligand-independent mechanisms of Gli regulation might contribute to other cell fate decisions in developmental and oncogenic contexts.

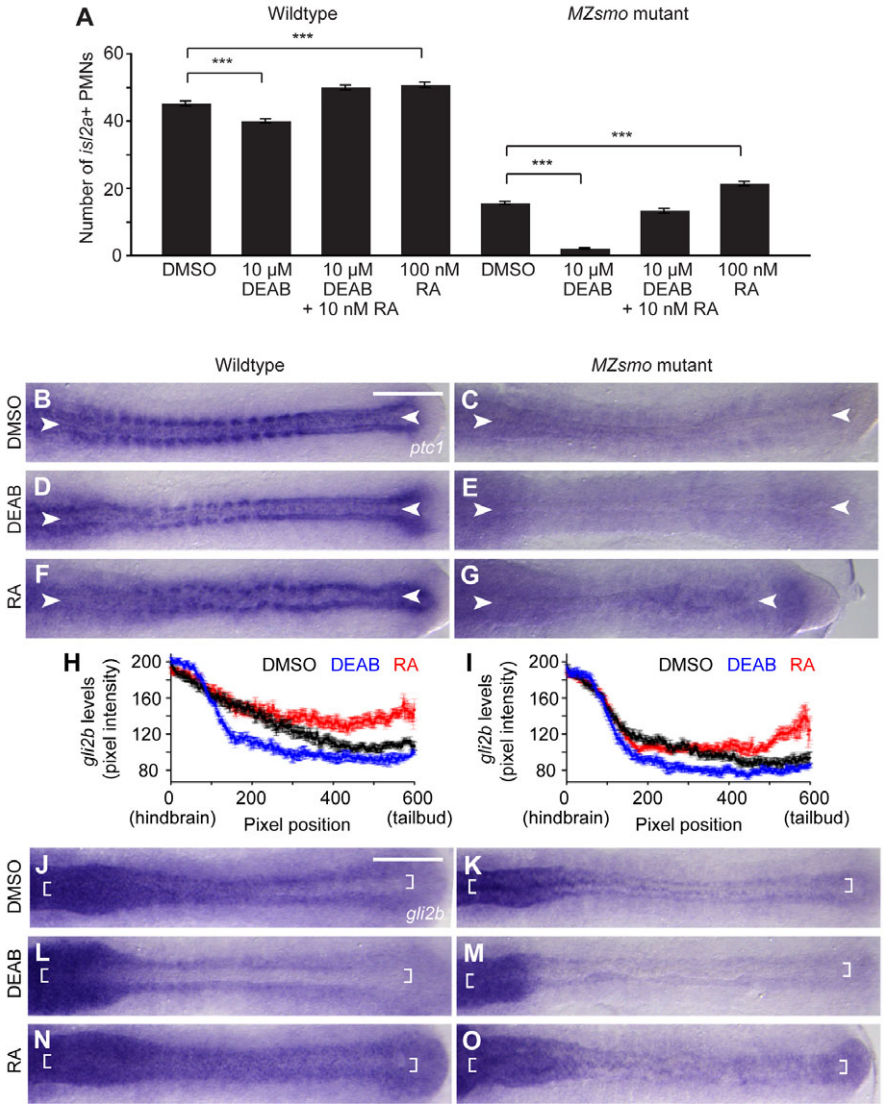

Fig. 4. Retinoic acid signaling promotes motoneurogenesis and basal Gli activity. (**A**) Quantification of *isl2a*-positive PMNs at 18 hpf after treatment with DEAB and/or RA. Error bars represent s.e.m. ***, $P<0.001$. (**B-G**) Wild-type and *MZsmo* zebrafish embryos (14 hpf) were treated with DMSO, 10 µM DEAB or 100 nM RA and stained for *ptc1* transcripts to assess the Hh pathway state. Embryos are flat-mounted, anterior to the left and viewed dorsally, with arrowheads demarcating the neural tube region. (**H**,**I**) Quantification of *gli2b* expression in the neural tubes of wild-type or *MZsmo* embryos treated with DMSO, 10 µM DEAB or 100 nM RA and then stained for *gli2b* transcripts. Data are the average of at least five embryos per condition ± s.e.m. (**J-O**) Representative images of embryos treated, stained and quantified as described for H,I, with the analyzed regions highlighted in white brackets. Scale bars: 200 µm.

Acknowledgements
We thank Dr Katharine E. Lewis for helpful discussions and Dr Rolf O. Karlstrom for providing Gli mutant zebrafish.

Funding
This work was supported by an American Cancer Society Research Scholar Grant [RSG-08-041-01-DDC to J.K.C.]; and a California Institute for Regenerative Medicine Scholar Award [T1-0001 to J.K.M.].

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.066225/-/DC1

References

Bai, C. B., Auerbach, W., Lee, J. S., Stephen, D. and Joyner, A. L. (2002). Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development* **129**, 4753-4761.
Begemann, G., Marx, M., Mebus, K., Meyer, A. and Bastmeyer, M. (2004). Beyond the neckless phenotype: influence of reduced retinoic acid signaling on motor neuron development in the zebrafish hindbrain. *Dev. Biol.* **271**, 119-129.
Chandrasekhar, A., Warren, J. T., Jr, Takahashi, K., Schauerte, H. E., van Eeden, F. J., Haffter, P. and Kuwada, J. Y. (1998). Role of sonic hedgehog in branchiomotor neuron induction in zebrafish. *Mech. Dev.* **76**, 101-115.
Chandrasekhar, A., Schauerte, H. E., Haffter, P. and Kuwada, J. Y. (1999). The zebrafish detour gene is essential for cranial but not spinal motor neuron induction. *Development* **126**, 2727-2737.
Chen, W., Burgess, S. and Hopkins, N. (2001). Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. *Development* **128**, 2385-2396.
Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.
Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E. and Schier, A. F. (2002). Production of maternal-zygotic mutant zebrafish by germ-line replacement. *Proc. Natl. Acad. Sci. USA* **99**, 14919-14924.
Devine, C. A., Sbrogna, J. L., Guner, B., Osgood, M., Shen, M. C. and Karlstrom, R. O. (2009). A dynamic Gli code interprets Hh signals to regulate induction, patterning, and endocrine cell specification in the zebrafish pituitary. *Dev. Biol.* **326**, 143-154.
Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.
England, S., Batista, M. F., Mich, J. K., Chen, J. K. and Lewis, K. E. (2011). Roles of Hedgehog pathway components and retinoic acid signalling in specifying zebrafish ventral spinal cord neurons. *Development* **138**, 5121-5134.
Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81**, 747-756.
Franco, P. G., Paganelli, A. R., Lopez, S. L. and Carrasco, A. E. (1999). Functional association of retinoic acid and hedgehog signaling in Xenopus primary neurogenesis. *Development* **126**, 4257-4265.
Grandel, H., Lun, K., Rauch, G. J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Kuchler, A. M., Schulte-Merker, S., Geisler, R. et al. (2002). Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. *Development* **129**, 2851-2865.
Huang, P. and Schier, A. F. (2009). Dampened Hedgehog signaling but normal Wnt signaling in zebrafish without cilia. *Development* **136**, 3089-3098.
Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
Jowett, T. and Lettice, L. (1994). Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. *Trends Genet.* **10**, 73-74.
Karlstrom, R. O., Talbot, W. S. and Schier, A. F. (1999). Comparative synteny cloning of zebrafish you-too: mutations in the Hedgehog target gli2 affect ventral forebrain patterning. *Genes Dev.* **13**, 388-393.
Karlstrom, R. O., Tyurina, O. V., Kawakami, A., Nishioka, N., Talbot, W. S., Sasaki, H. and Schier, A. F. (2003). Genetic analysis of zebrafish gli1 and gli2 reveals divergent requirements for gli genes in vertebrate development. *Development* **130**, 1549-1564.
Kimmel, C. B. and Westerfield, M. (1990). Primary neurons of the zebrafish. In *Signals and Sense: Local and Global Order in Perceptual Maps* (ed. G. M. Edelman, W. E. Gall and W. M. Cowan), pp. 561-588. New York: Wiley-Liss.
Lewis, K. E. and Eisen, J. S. (2001). Hedgehog signaling is required for primary motoneuron induction in zebrafish. *Development* **128**, 3485-3495.
Macdonald, R. (1999). Zebrafish immunohistochemistry. *Methods Mol. Biol.* **127**, 77-88.
Mich, J. K., Blaser, H., Thomas, N. A., Firestone, A. J., Yelon, D., Raz, E. and Chen, J. K. (2009). Germ cell migration in zebrafish is cyclopamine-sensitive but Smoothened-independent. *Dev. Biol.* **328**, 342-354.
Novitch, B. G., Wichterle, H., Jessell, T. M. and Sockanathan, S. (2003). A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* **40**, 81-95.
Perz-Edwards, A., Hardison, N. L. and Linney, E. (2001). Retinoic acid-mediated gene expression in transgenic reporter zebrafish. *Dev. Biol.* **229**, 89-101.
Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.
Robu, M. E., Larson, J. D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S. A. and Ekker, S. C. (2007). p53 activation by knockdown technologies. *PLoS Genet.* **3**, e78.
Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.
Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H. et al. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**, 129-134.
Teglund, S. and Toftgard, R. (2010). Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim. Biophys. Acta* **1805**, 181-208.
Tyurina, O. V., Guner, B., Popova, E., Feng, J., Schier, A. F., Kohtz, J. D. and Karlstrom, R. O. (2005). Zebrafish Gli3 functions as both an activator and a repressor in Hedgehog signaling. *Dev. Biol.* **277**, 537-556.
Varga, Z. M., Amores, A., Lewis, K. E., Yan, Y. L., Postlethwait, J. H., Eisen, J. S. and Westerfield, M. (2001). Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497-3509.
Wang, B., Fallon, J. F. and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423-434.
Woods, I. G. and Talbot, W. S. (2005). The you gene encodes an EGF-CUB protein essential for Hedgehog signaling in zebrafish. *PLoS Biol.* **3**, e66.
Yang, H., Xiang, J., Wang, N., Zhao, Y., Hyman, J., Li, S., Jiang, J., Chen, J., Yang, Z. and Lin, S. (2009). Converse conformational control of smoothened activity by structurally related small molecules. *J. Biol. Chem.* **284**, 20876-20884.
Zeller, J., Schneider, V., Malayaman, S., Higashijima, S., Okamoto, H., Gui, J., Lin, S. and Granato, M. (2002). Migration of zebrafish spinal motor nerves into the periphery requires multiple myotome-derived cues. *Dev. Biol.* **252**, 241-256.
Zhang, X. M., Ramalho-Santos, M. and McMahon, A. P. (2001). Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. *Cell* **105**, 781-792.