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 Ptc1) (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). Smo 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 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 smoA1640/+ zebrafish were obtained from the Zebrafish International Resource Center (ZIRC), gli2a206/+ and gli2b2017/+ animals were provided by R. Karlstrom (Karlstrom et al., 1999; Karlstrom et al., 2003), and MZsmoA1640/+ 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, cycloamine (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 stage or sphere stage with similar effects), and/or all-trans-retinoic acid (RA; Sigma; 10-100 nM at the one-cell or sphere stage with equal results for each time).

Oligonucleotide injections
shba 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, GTGGGCAATCATGTCTCCGACC (3 ng/embryo) (Ciruna et al., 2002); gli1, CCGACACACCCGCTACCCACAGT (4 ng/embryo) (Karlstrom et al., 2003); gli2a, GGATGATGTAAAAG-CACTAATCC (3 ng/embryo) (Mich et al., 2009); gli2b, AGCTGGAAACCGGCTCCATTCT (8 ng/embryo) (Devine et al., 2009); gli3, ACAACTGGGCTATCCATG (3 ng/embryo) (Huang and Schier, 2009); 5p3, GCCGCCATTGTTGGCGAATAAGT (8 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.
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); nkc2.2 (Woods and Talbot, 2005); ptc1 (Woods and Talbot, 2005); smo (Chen et al., 2001); foxb8, amplified with primers (5′ to 3′) ATGG-ACTGCTCTCACAGGG and TCCACACAGGAAAGATGTCAT; gli1, ATGGCAATGGAATGACG and GATGTGCTCATATTATGATGTGATC; gli2a, ATGGAGACCAACAAGTTCCACAC and CCACC-CATGCCACACAAACT; gli2b, TCCAGCTCCGCAGAGAG and TTGAGACAGGCTCGAACG; gli3, TCTGAGAAATGCGATTTGC and CTCCTCCTATGGCTCTTCT; hoxb1a, TGGCCATGATTTTCGCTGTTGTTTGGCGAAGATGAATTAG and TCCAGCTCCGCAGAGAG; and ptc1, TGCCATTGAAATGGCACCAG and GGTGCAGAAGATGACG.

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 smoH11032 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 adenhoypheseal 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 (nkp2.2a and ptc1). Exogenous shha expanded nkp2.2 and ptc1 expression in wild-type embryos (Fig. 1J,M), and smo mutants responded weakly, but detectably (Fig. 1K,N). 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, gli1a269 mutants [which lack Gli1 activity (Karlstrom et al., 2003)] and gli2a271 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...
Gli2a mutants act as repressors when Smo is inhibited. By contrast, approximately one-quarter of cyclopamine-exposed progeny from *gli1ts269/+* 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 *gli1ts269* and *gli2aty17* 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 *gli2aty17/+* incrossed progeny (Fig. 3J,K,M), but strongly depleted PMNs in *gli1ts269/+* incrossed progeny (Fig. 3L,M). Cyclopamine-treated *gli1ts269/+* 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 *gli1ts269/+* 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 number.
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 amnions 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).

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**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.
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 MZs mo 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 MZs mo 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 MZs mo 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 MZs mo 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. 4H,I,L,M). Exogenous RA increased gli2b

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**Fig. 3. Gli-dependent patterning in the absence of Smo.** (A-L) Zebrafish embryos obtained from wild-type, gli2aty17/ty17 or gli1ts269/t or gli1ts269/zebrafish after treatment with either ethanol or 100 μM cyclopamine. 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 gli2aty17/ty17 and gli1ts269/zebrafish after treatment with either ethanol or 100 μM cyclopamine. Homozygous gli2aty17 and gli1ts269 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. (M) Quantification of PMNs in whole-embryo measurements (supplementary material Figs S11 and S12).

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

\textbf{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 \textit{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.

\textbf{Conclusions}

Surprisingly, zebrafish that completely lack \textit{s\textit{mo}} and therefore Hh signaling retain motoneuronic 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 \textit{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.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4.png}
\caption{Retinoic acid signaling promotes motoneurogenesis and basal Gli activity.}
\end{figure}

(A) Quantification of \textit{isl2a}-positive PMNs at 18 hpf after treatment with DEAB and/or RA. Error bars represent s.e.m. ***, \textit{P}<0.001. (B-G) Wild-type and MZs\textit{mo} zebrafish embryos (14 hpf) were treated with DMSO, 10 \textmu M DEAB or 100 nM RA and stained for \textit{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 \textit{gli2b} expression in the neural tubes of wild-type or MZs\textit{mo} embryos treated with DMSO, 10 \textmu M DEAB or 100 nM RA and then stained for \textit{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 \textmu m.


