Novel Mechanisms and Functions for Hedgehog Signaling in Development and Disease
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Summary
The Hedgehog (Hh) pathway is required for many developmental processes as well as for adult homeostasis. Although all known effects of Hh signaling affecting patterning and differentiation are mediated by members of the Gli family of zinc finger transcription factors, we demonstrate that the Hh-dependent formation of neurites from motorneurons, like migration of fibroblasts, requires leukotriene synthesis and is different from the Gli-mediated Hh response. Smo activity is required for the utilization of the leukotriene metabolism and inversely, the leukotriene metabolism is required for mediating the Hh effects on neurite projection. These data establish a function for the previously described arachidonic acid-dependent Hh pathway in a developmentally relevant model system.

Introduction
Hh signaling is involved in a large number of developmental processes as well as in adult pathophysiology (Bijlsma et al., 2004), however its role in inducing ventral neurons was one of the first and best characterized functions to be found for Sonic hedgehog (Shh) signaling (Roelink et al., 1994). Activation of the Hh pathway involves a particularly complicated sequence of events. The Hh ligands for the pathway are autocatalytically cleaved (Lee et al., 1994), sterolated (Porter et al., 1996), palmitoylated (Pepinsky et al., 1998), and despite its apparent hydrophobicity, Hh mediates long-range signaling. Hh binds to one of the two receptors of the pathway, Patched1 (Ptc1) (Marigo et al., 1996), which results in the internalization of the Ptc1/Hh complex (Incardona et al., 2000). The 12-pass transmembrane protein Ptc1 probably inhibits the activity of the 7-pass transmembrane protein Smoothened (Smo) via the re-distribution of (pro-)vitamin D, or similar molecules (Bijlsma et al., 2006). Smo accompanies the Ptc1/Hh complex into the cell after which it segregates away from the complex thereby removing itself from the inhibitory action of Ptc1 and becoming active (Incardona et al., 2002). This activity is relayed to the pathway’s transcription factors (the family of zinc finger containing glioma-associated oncogene (Gli) proteins) through an intracellular complex of pathway components, the exact composition and function of which is not yet clear and appears to be different in distinct phyla (Varjosalo et al., 2006). In the absence of a signal from Smo, the pathway’s transcription factors are either kept inactive by sequestration or processing into repressor forms by this complex (Ruiz i Altaba, 1999).

There were several reasons to postulate a Hh response independent from the Gli-mediated activation or suppression of transcription. For instance, Shh has been shown to be an important chemotactic factor with respect to axonal guidance during development of the central nervous system (Charron et al., 2003), an action that fits poorly with Shh acting through Gli-dependent transcription as these actions take place outside the cell body and on a timescale seemingly incompatible with transcription/translation. We recently showed that besides the traditional Hh pathway, an arachidonic acid-dependent but Gli-independent pathway exists (Bijlsma et al., 2007). This pathway was shown to function in mediating fibroblast motility independently of transcriptional
activity and to require a functional arachidonate metabolism. We set out to investigate the existence and function of the leukotriene-dependent Hh pathway in a neuronal model for Shh-mediated differentiation.

By virtue of their pluripotency and sensitivity to differentiation cues, embryonic stem (ES) cells provide a good in vitro model for neural differentiation (Wichterle et al., 2002). In neuralized ES cells, the response to Shh results in expression of HB9, a marker of motorneuron differentiation (Pfaff et al., 1996). By using ES cells derived from an embryo transgenic for an HB9 promoter-driven green fluorescent protein (GFP) (Wichterle et al., 2002), we were thus able to assess motorneuron differentiation in neuralized embryoid bodies (EBs) derived from these ES cells. By staining for class III β-tubulin (a neuron specific cytoskeletal protein), we could simultaneously visualize neurites of neurons induced in neuralized EBs (Ferreira and Caceres, 1992), allowing us to assess the effect of Hh signaling on neuronal differentiation and neurite formation independently.

To address the role of leukotrienes in neurite formation, we focused on a key enzyme involved in their synthesis, 5-lipoxygenase (5-LOX), which catalyzes the formation of the leukotriene precursor 5-hydroperoxyicosatetraenoic acid (5-HPETE) from arachidonic acid. Due to its function in generating leukotrienes, the role of 5-LOX in immunology is clear and well established, and very specific inhibitors have been developed for pharmaceutical use. We showed that one of these inhibitors, MK-886, inhibited neurite outgrowth in of Shh induced motorneurons, while not affecting the induction of motorneuron specific gene expression. We further showed that Smo function is required for MK-886 to inhibit neurite growth. These results indicate the presence of two independent, Smo-dependent mechanisms to relay the Hh response, the classical pathway, resulting in the induction of motorneurons, as well as a 5-LOX dependent response affecting neurite outgrowth.

Results

Motorneuron Neurite Projection in Neuralized Embryoid Bodies Is Dependent on 5-Lipoxygenase Activity

To study the possible involvement of the arachidonic acid-dependent Hh pathway in a model for neuronal development, we investigated motorneuron differentiation in HB9::GFP EBs. motorneuron differentiation is known to be critically dependent on Gli transcription factor activity (Bai et al., 2004; Wichterle et al., 2002), and this differentiation thus represents the classical Hh signaling pathway in our assay. Following neuralization by retinoic acid treatment and ventralization by a pharmacological Hh agonist, all EBs contained motorneurons as shown by GFP expression (Figure 1E). The Hh agonist used is similar to the previously described HhAg1.3 (Frank-Kamenetsky et al., 2002; Wichterle et al., 2002), and specifically binds and activates Smo.

In addition to assessing proper motorneuron differentiation, staining for Tuj-1 enabled us to visualize the projection of class III β-Tubulin positive neurites in these EBs. In response to Smo activation, a dense network of neurite projections was formed. As we used conventional epifluorescence microscopy, we predominantly visualized motorneuron projections on the outside of the EBs (Figure 1B). In the absence of Hh agonist, this fine neurite network was largely absent and only a few projections could be seen per optical field (Figure 1A). When the EBs were stimulated with Hh agonist as well as with the specific inhibitor of 5-lipoxygenase (5-LOX), MK-886, the number of neurites was significantly diminished (Figure 1C), while motorneuron differentiation as measured by GFP expression was unaffected (Figure 1F).

To quantify the density or reticularity of this network, we measured neurite length in between crossing neurites (nodes). This quantification showed two distinct classes of neurite network density evident from by the frequency distribution analysis of the measured lengths (Figure 1G). In the dense network that formed in the presence of Hh agonist the distances between crossing neurites were small, while longer distances between these nodes characterized a less dense network. The latter EBs were referred to as class I, while EBs containing a dense network were referred to as class II. Activation of the Hh response by Hh agonist caused the formation of class II EBs, while in the presence of Hh agonist as well as MK-886, the neurite network became significantly less dense and these EBs were classified class I. All subsequent analyses of proper neurite projection were performed using this classification.

Quantification of the percentage of class II EBs demonstrated that increasing concentrations of MK-886 decreased the induction of class II EBs by Hh agonist (Figure 1H). Conversely, quantification of the percentage of GFP positive EBs showed that motorneuron differentiation was not affected by 5-LOX inhibition (Figure 1I). Thus, although MK-886 left the developmental program leading to motorneurons unaltered, it efficiently disturbed the formation of neurite projections from these motorneurons. This suggests that neurite extension, but not the differentiation of motorneurons relies on leukotriene synthesis.

Using a more general leukotriene inhibitor, nordihydroguaiaretic acid (NDGA), we found a similar response on class II EB induction (Figure 1J), although NDGA also caused some inhibition of GFP expression (Figure 1K), probably due
FIGURE 1. HH agonist treatment induces motorneurons and a dense network of TuJ1-positive neurite projections in neuralized EBs.

(A-F) As indicated by HB9 promoter-driven GFP expression in neuralized EBs, HH agonist induced differentiation to motorneurons (GFP expression, lower row of panels). This differentiation was not abrogated by treatment with 5 μM MK-886. In the presence of HH agonist, a dense network of class III β-tubulin positive neurite projections (stained by α-Tuj1, upper row of panels) could be seen. This network was, however, disturbed by inhibition of 5-LOX with 5 μM MK-886, a concentration that did not affect GFP expression. EBs were stimulated with 500 nM HH agonist and 1 μM retinoic acid for 3d after 2d of allowing aggregation in the absence of stimulus. MK-886 was added simultaneously with HH agonist.

(G) Frequency distribution analysis of neurite length (in pixels) in between crossing neurites as measure of network reticularity. Two distinct classes of neurite network density could be seen. All conditions included RA treatment. n=40

(H) Quantification of percentage of class II EBs showed strong inhibition by MK-886 treatment (as in C), whereas the population of GFP positive (motorneuron) EBs was hardly disturbed (I). Approximately 50 EBs were quantified in at least 3 individual experiments. Shown is the mean ± SEM.

(J) NDGA treatment in abovementioned experimental setup and quantification shows a similar response but slightly more pronounced effect on motorneuron differentiation as indicated by GFP expression (K). Statistics as (H).
to the relative low specificity of NDGA as compared to MK-886 in inhibiting LOX enzymes.

**Neurite Projection from Ventral Neural Explants is Dependent on 5-Lipoxygenase Activity**

To be able to quantitatively study single neurite dynamics under leukotriene inhibition and confirm the observations from the EB model, we used a ventral quail neural explant model (Yamada et al., 1993) in which we observed neurite projections, likely from motor neurons, exiting the explant and adhering to the substrate in the presence of Hh agonist (Figure 2A). In this experimental setup, we observed that MK-866 caused shortening of the neurites outside the explant in a dose dependent manner (Figure 2B). Trypan Blue exclusion revealed that this was not due to cytotoxicity (data not shown). Quantification of the effect of MK-866 on the length of neurite outgrowth is shown in Figure 2C. Although these explant data do not allow us to precisely separate the effects of Hh signaling on differentiation and neurite extension, they do emphasize that 5-lipoxygenase activity is required for neurite outgrowth from ventral neural explants.

**Specificity of Leukotriene Inhibition for Class II EB Induction**

Arachidonate is generated from membrane phospholipids by phospholipase A2 (PLA2, see Figure 3A for overview). Leukotrienes (LTs) are subsequently synthesized from arachidonate by the lipoxygenases (LOX), through hydroperoxyeicosatetraenoic acid (HPETE) intermediates whereas the cyclooxygenases (COX) mediate synthesis of prostaglandins (PGs). The group of cysteinyl leukotrienes (cysLT) includes the leukotrienes that are indirectly synthesized by 5-lipoxygenase and consists of leukotrienes C4, -D4 and -E4. These leukotrienes are the focus of this study.

We determined the IC<sub>50</sub> for inhibition of class II EB formation as well as the IC<sub>50</sub> for inhibition of GFP expression by compounds inhibiting lipoxygenases. 5-LOX inhibitors MK-886 and NDGA as well as the 12-LOX inhibitors baicalein and cinnamyl 3,4-dihydroxy-(alpha)-cyanocinnamate (CDC) and cyclooxygenases inhibitor indomethacin were tested. Inhibition of cyclooxygenases was approximately 25 fold less efficient in inhibiting class II EB formation, whereas its effect on GFP expression was comparable to that of MK-886 (Figure 3B). These data suggest a specific role for leukotrienes as the primary arachidonate metabolites required for the projection of neurites from motorneurons.

To confirm that inhibition of leukotriene synthesis does not inhibit motorneuron differentiation in EB development, but rather affects the projection of neurites after completed motorneuron differentiation, EBs were stimulated for 3 days with Hh agonist and during the last 24h, MK-886 was added (Figure 4A). After 2 days, we observed widespread motorneuron differentiation. Subsequent addition of MK-886 affected only processes in differentiated motorneurons and resulted in significant inhibition of class II EB induction (Figure 4B). The magnitude of this inhibition was similar to that found in Figure 1H. To formally exclude a detrimental effect of the inhibitors used on cell viability in general, we assayed cell viability in EBs treated with various concentrations of MK-886 and NDGA by Trypan Blue exclusion. NDGA and MK-886 were only toxic to cells at concentrations 10-fold
higher (50 μM) than those affecting neurite outgrowth (Figure 4B). At lower concentrations, these compound appeared to slightly enhance cell viability.

To verify that the effects of MK-886 on the induction of class I EBs are dependent on Hh signaling, we assessed the effects of 5-lipoxygenase inhibition on neurite projection require an active Shh response. To confirm this, we included the Shh inhibiting antibody 5E1 in the culture medium, or used EBs derived from Smo⁻/⁻ ES cells, which are unable to activate a Hh response. The presence of the Shh blocking antibody 5E1 even resulted in a slight reduction of neurite outgrowth indicating some low level of Shh expression in the EBs (Figure 5A). The inhibition of basal class II EB induction by 5E1 also tells us that this basal level is not due to any non-Hh related artifacts such as the induction of PLA₂ by retinoic acid (Antony et al., 2001). Inclusion of MK-886 in the presence of 5E1 had little effect, which might indicate that those neurites that form in the absence of Hh agonist are not sensitive to this compound. Similarly, Smo⁻/⁻ EBs, which are insensitive to Hh agonist, were not affected by MK-886, although at 5 μM MK-886, no EBs remained, probably due to non-specific toxicity to Smo⁻/⁻ ES cells (Figure 5B). Since very few neurites formed under these conditions in Smo⁻/⁻ EBs in the first place, we wanted to test if the neurite extensions that appear in the neutralized EBs in the absence of Hh pathway activation are MK-886 sensitive. Note that the cells from which these neurites extended were probably Lim1/2 interneurons, representing the dorsal and intermediate population of neurons, as formed in neutralized tissue in the absence of Shh.

When Smo⁻/⁻ EBs were cultured for an additional 3 days, we could observe the induction of class II EBs (Figure 5C-E, Figure 4F). The appearance of these EBs was insensitive to MK-886 (Figure 5D-F), demonstrating that Smo-independent formation of neurite projections does not rely on leukotriene synthesis. This implies that the observed effect of 5-LOX inhibition on motorneuron projections is Smo-specific and -dependent.

**Gli transcription factors do not influence leukotriene synthesis**

To consolidate the Gli-independence of neurite projections from motorneurons, it is important to uncouple leukotriene synthesis from transcriptional activity of the Hh pathway through the Gli proteins. To formally exclude the family of Gli transcription factors to be responsible for modulation of leukotriene synthesis, we investigated whether fibroblasts from mice knockout for these transcription factors had an inhibited or enhanced leukotriene synthesis. We used cells from wild type, Gli1⁻/⁻ Gli2⁻/⁻, and Gli2⁻/⁻ Gli3⁻/⁻ double knockout mice. Although none of the cells used were knockout for all 3 Gli proteins, Gli3 is a potent inhibitor of the Hh response. Consequently, the Gli1⁻/⁻ Gli2⁻/⁻ knockout fibroblasts lack all activating functions of the Gli transcription factors (see also (Lipinski et al., 2006)).

Cells were treated with 30 μM arachidonate for 5 minutes and leukotriene content was determined using a cysteinyl leukotriene EIA. The excess of arachidonate (not recognized by the EIA) served as a positive control for a functional leukotriene synthesis machinery (Peppelenbosch et al., 1993). Intracellular leukotriene content was not different between the different cell lines under both basal and stimulated (arachidonate) conditions, meaning that the presence or absence of Gli transcription factors does not influence leukotriene synthesis, and suggesting that the requirement for leukotriene synthesis for motorneuron neurite projection is Gli-independent as well.
**EXOGENOUS ARACHIDONATE BYPASSES THE REQUIREMENT FOR HH AGONIST FOR NEURITE PROJECTION**

We reasoned that if HH activates and requires the arachidonic metabolism for cytoskeletal rearrangement in motorneurons as it does in mesenchymal fibroblasts, enhancing the production of leukotriene synthesis via the addition of substrate (arachidonate) while Smo activity is low should mimic the HH agonist effect on neurite outgrowth. Indeed, we observed that the addition of 5 μM arachidonate was sufficient to induce class II EBs in the absence of HH agonist (Figure 6A). Under these conditions, motorneuron differentiation did not occur, but the low Smo activity was adequate to utilize the exogenously added arachidonate to increase neurite outgrowth. In other words; by adding arachidonate in excess we augment the non-Gli HH pathway and this is shown by the presence of neurites but absence of GFP expression. In the presence of HH agonist, arachidonate was not able to further increase the number of neurite rich EBs, which has apparently reached the highest possible level (Figure 6A).

Although pathway activation by HH agonist was not required for arachidonate-induced class II EB formation, basal Smo activity was required; in Smo−/− EBs the increase in class II EBs after arachidonate stimulation was non-significant. This is important, as it confirms that Smo signals to the leukotriene dependent HH pathway. This is consistent with the notion that Smo mediates two distinct activities, one that signals to the Gli transcription factors and which mediates patterning, and another that utilizes leukotrienes, and is involved in cytoskeletal rearrangement (Bijlsma et al., 2007).

**DISCUSSION**

In this study, we describe the requirement of arachidonic acid for HH action in an in vitro model for neurite projection. A major advantage of deriving neurons from embryonic stem cells in vitro is that this allows us to use small molecule activators and inhibitors in a controlled manner, in contrast to in vivo systems, where pharmacokinetics and side effects greatly complicate interpretation of results. Another advantage is that we can use stem cell lines with specific mutations that would be lethal in experimental animals. For example, they allow us to easily visualize neurons in cell lines mutant for Smo, which are thus completely insensitive to HH, inherently superior to the use of Smo inhibitors such as cyclopamine.

We have shown earlier that Shh induces cytoskeletal rearrangement and migration regulated by ara-

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**FIGURE 6B** summarizes the experiments as performed in Figures 1-5. Stimulation of EBs with HH agonist activates Smo and this induces a transcriptional response as well as a Gli-independent, non-transcriptional response. This latter pathway is sensitive to leukotriene inhibition and requires Smo. If Smo activity is low, the addition of excess substrate (arachidonate) is required and sufficient to induce the non-transcriptional response (Figure 6C). Under these conditions, there is no transcriptional HH response as determined by the lack of HB9 promoter-driven GFP expression, separating the effects of the two pathways. If Smo is absent, additional arachidonate has no effect and no class II EBs are formed (Figure 6D). These experiments provide solid evidence for a leukotriene-dependent HH pathway that is Gli-independent, but mediated through Smo.

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**FIGURE 4. THE INHIBITION OF CLASS II EB FORMATION BY MK-886 IS NOT CAUSED BY ABROGATION OF MOTORNEURON DIFFERENTIATION OR DIMINISHED CELL ViABILITY**

(A) EBs were stimulated with retinoic acid and HH agonist for 2d, and during the last 24h, MK-886 was added at the concentrations as indicated. EBs were fixed and stained with α-Tuj1 and classified as for Figure 1H.

(B) EBs were treated as for Figure 1, with increasing concentrations of MK-886 or NDGA and subsequently incubated for 5 min in 0.1% Trypan Blue. EBs were mounted in aqueous medium, photographed and subsequently, intensity of the blue RGB channel was quantified using ImageJ software. Statistics as Figure 1H. * = p<0.05. ** = p<0.01, Student’s t-test.
FIGURE 5. THE INHIBITION OF MOTOR-NEURON NEURITE PROJECTIONS BY MK-886 IS HH PATHWAY-SPECIFIC

(A) EBs were treated with 1 μg/ml Shh blocking antibody (5E1) simultaneously with RA, resulting in a low amount of class II EBs. In these EBs, MK-886 addition showed no effect.

(B) Low baseline levels of class II amongst Smo-/- EBs after 3d of Hh agonist treatment could not be lowered by MK-886 treatment.

(C-E): Following incubation for 6d, Smo-/- EBs form proper (Hh-independent) neurite projections. These were insensitive to treatment with MK-886, quantification in (F).

(G) To exclude Gli action on the leukotriene metabolism, fibroblasts from wild-type, Gli1-/-2-/-, and Gli2-/-3-/- double knock-outs were stimulated with 30 μM arachidonate or solvent control for 5 minutes and intracellular cysteinyl leukotriene (cysLT) content was determined and not found to significantly differ in both baseline and stimulated conditions. n=6.
paired motorneuron functioning, consistent with our finding that leukotriene synthesis is necessary for proper formation of motorneuron neurites.

Another interesting implication of our findings is that motor neurons appear to go through multiple developmental phases where they are dependent on Hh signaling. Initially Hh dependence is via the classical pathway involving Gli activity, but the later phases, involving the establishment of the correct connectivity, rely on the leukotriene-dependent pathway focused on in this study. Since we have shown that neurite extension is independent of Gli-mediated Shh signaling, it is expected that Hh mediated neurite extension can affect many aspects of Hh mediated growth cone guidance. This might be particularly important for the many axons that cross the ventral midline. The morphogen Shh is an axonal chemotactant that collaborates with netrin-1 in midline axon guidance (Charron et al., 2003). This chemotraction is known to require the Hh co-receptor brother of cdo (BOC), and this Shh-mediated pathfinding is a prime candidate to be mediated in a Smo dependent, leukotriene dependent pathway. Not only the axons of dorsal sensory relay neurons cross the midline. Other examples include the axons in the lateral corticospinal tract, and the vast majority of retinal ganglion cells. In particular in these cases, the growth cones are at a significant distance from the transcriptional machinery, making any involvement of Gli mediated signaling unlikely, but instead it would be consistent with our observations that some of the required guidance occurs via arachidonic acid metabolites.

Given the analogy between neurite formation and membrane ruffling, it is possible that this Hh dependent process also plays a role in tumor metastasis. Not only is the activation of the Shh pathway the cause of many tumors, increased Hedgehog signaling is also known to enhance metastasis of some tumors (Feldmann et al., 2007). Inhibitors of leukotriene synthesis are commonly used as anti-allergens, and such compounds could be anticipated to have a role in the inhibition of Hedgehog-induced metastases. It has been
shown that inhibition of the arachidonic acid metabolism has an inhibitory effect on pancreatic tumors (Schuller et al., 2002), which often are induced in response to Shh and it could be argued that the novel Hh pathway is responsible for the observed beneficiary effects (Thayer et al., 2003). In addition, the use of leukotriene-inhibiting compounds might need more scrutiny in regard of their use during pregnancy. Conversely, the widespread use of these leukotriene synthesis inhibitors might help in determining the in vivo importance of this pathway in Hedgehog-dependent cellular migration, neuron guidance, and other biological phenomena.

**MATERIALS AND METHODS**

**ES CELL CULTURE**

HB9::GFP or Semo/- mouse-derived ES cells were maintained in ES medium DMEM with 4.5 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate (Invitrogen, Carlsbad, CA) and 3.7 g/L Na-bicarbonate (Mallinkrodt Baker, Phillipsburg, NJ), supplemented with 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 15% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), 1% Penicillin-Streptomycin-Glutamine, 1% Non-Essential Amino Acids (all Invitrogen), 1% ES cell Nucleosides and 1000 Units/ml recombinant murine leukemia inhibitory factor (LIF, both from Chemicon-Millipore, Billerica, MA). For EB differentiation cells were trypsinized, washed and diluted to a concentration of 50,000 cells/ml in DFNB (25% DMEM with 4.5 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate (Invitrogen) and 3.7 g/L Na-bicarbonate (Mallinkrodt Baker), 48% Neurobasal, 25% Ham’s F12 (both Invitrogen), supplemented with 80 mM FM2-mercaptoethanol (Sigma), 1% Penicillin-Streptomycin-Glutamine, and 1% B-27 Supplement (all from Invitrogen). To induce motor neurons, the cells were grown in non-adherent bacterial grade Petri dishes for 2 days to allow aggregation into EBs. On day 2, medium was changed to DFNB supplemented with appropriate combinations of 1 μM retinoic acid (RA) (Sigma), and Hh agonist. Produced EBs were grown for an additional 3 days in this supplemented DFNB with a media change after 2 days. For the experiments described in Fig. 3C, EBs were grown in the supplemented DFNB for 6 days.

**SHH RESPONSE QUANTIFICATION**

After culture, EBs were fixed in 4% paraformaldehyde for 30 min, blocked and permeabilized in phosphate buffered saline (PBS:-Triton X-100 0.1% with 10% normal goat serum (NGS) for 1h and incubated in 1:500 α-Tuj1 primary antibody (Covance, Princeton, NJ) overnight. After overnight incubation with Cy3-conjugated secondary antibody, EBs were mounted in ProLong Gold (Invitrogen) and imaged on a Nikon fluorescence microscope. For classification, EBs were counted on a Zeiss dissection microscope. EBs were simultaneously quantified for proper neurite projection (see text body and Figure 1 for details on this) and GFP expression.

**NEURAL PLATE EXPLANTS**

Ventral neural plate explants were obtained as previously described (Robertson et al., 2004; Yamada et al., 1993), and maintained on growth factor-reduced Matrigel substrate (Invitrogen) in the presence of Hh agonist for 3d. Leukotriene synthesis was inhibited by adding 500 nM or 5 μM MK-886. Explants were processed for microscopy as described above and neurite projection length was quantified using ImageJ (NIH, public domain software). Image colors were shown inverted for better visibility of projections.

**LEUKOTRIENE MEASUREMENT** (Bijlsma et al., 2007)

Cells from wild type, Gli1-/-, and Gli2-/- double knock-out mice(Lipinski et al., 2006) were grown in 145 mm culture dishes, and stimulated with arachidonate or solvent control. Cells were lysed in 2 ml of methanol, after which the methanol was evaporated in a SpeedVac concentrator. After reconstitution in 200 μl of enzyme immunoassay (EIA) buffer, cysteinyl leukotriene content was assayed according to manufacturer’s directions (Cysteinyl Leukotriene ELA Kit, Cayman Chemicals, Ann Arbor, MI). Measured values of all samples were mid-range of the standard curve.

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**REFERENCES**


