Neural tube development depends on notochord-derived Sonic hedgehog released into the sclerotome

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Summary statement: Shh that transits through the sclerotome is presented to the neuroepithelium from its basal aspect to affect motoneuron development.
Abstract

Sonic hedgehog (Shh), produced in notochord and floor plate, is necessary both for neural and mesodermal development. To reach the myotome, Shh has to traverse the sclerotome and a reduction of sclerotomal Shh affects myotome differentiation. By loss and gain of Shh function, and floor plate deletions, we presently report that sclerotomal Shh is also necessary for neural tube development. Reducing the amount of Shh in sclerotome by membrane-tethered hedgehog-interacting protein or by Patched1, but not by dominant active Patched, decreased the number of Olig2+ motoneuron progenitors and of Hb9+ motoneurons without a significant effect on either cell survival or proliferation. These effects were a specific and direct consequence of reducing Shh in mesoderm. In addition, grafting notochords in a basal, but not apical location vis-a-vis the tube, profoundly affected motoneuron development, suggesting that initial ligand presentation occurs at the basal side of epithelia corresponding to the sclerotome-neural tube interface.

Collectively, our results reveal that the sclerotome is a potential site of a Shh gradient that coordinates development of mesodermal and neural progenitors.

Key words: BMP, dermomyotome, dorso-ventral patterning, Hb9, motoneurons, myotome, neural tube, Nkx, paraxial mesoderm, Olig2, Pax7, retinoic acid, somite.
Introduction

Sonic hedgehog (Shh) plays fundamental roles in the development of both neural tube (NT) and somites (Borycki et al., 1998; Briscoe, 2009; Cairns et al., 2008; Ericson et al., 1997a; Ericson et al., 1997b; Gustafsson et al., 2002). Its signaling is initiated by binding of the ligand to the transmembrane receptor Patched (Ptc), that represses the pathway in its absence (Goodrich et al., 1997; Hidalgo and Ingham, 1990; Ingham et al., 1991; Johnson et al., 1996). Ligand binding to Ptc abrogates its repressive effect on Smoothened, a key effector essential for Hedgehog signal transduction (van den Heuvel and Ingham, 1996). The repressive role of Ptc correlates with its localization in the apical cilia, that function as a signal transduction compartment (Caspary et al., 2007; Rohatgi et al., 2007). Binding of Shh to Ptc removes the latter from the cilium, thereby allowing Smoothened to enter and propagate the signal further downstream (Milenkovic et al., 2009; Rohatgi et al., 2009) to regulate Gli activity (Briscoe and Therond, 2013; Ribes and Briscoe, 2009).

Shh signaling is highly regulated by negative and positive modulators. Ptc1, Hedgehog interacting protein (Hhip) and Gli1 are direct targets of Shh and the former two also inhibit its activity (Chuang and McMahon, 1999; Ingham and McMahon, 2001). Sulfatase1 (Dhoot et al., 2001), Boc, Gas and Cdo (Allen et al., 2011; Izzi et al., 2011) enhance ligand activities and are expressed in NT and/or developing mesoderm (Kahane et al., 2013).

Following neurulation, Shh secreted by the notochord (No) induces distinct ventral cell identities in the overlying NT by a mechanism that depends on relative concentrations and duration of exposure (Briscoe and Small, 2015; Dessaud et al., 2010; Stamataki et
Moreover, its activity continues beyond this stage to regulate cell proliferation, survival and differentiation (Cayuso et al., 2006; Charrier et al., 2001). No-derived Shh is also involved in mesoderm patterning (Borycki et al., 1998; Cairns et al., 2008). A ventro-dorsal activity gradient of Shh/Gli signaling in sclerotome was directly visualized using an in vivo reporter in mice (Kahane et al., 2013). In addition, in chick embryos, Shh spreads from the midline through the sclerotome to reach the dermomyotome (DM). There it promotes terminal myogenic differentiation of DM-derived progenitors and maintains epitheliality of DM cells (Kahane et al., 2013). Notably, in both floor plate (FP) and myotome, the activities of Shh are transient, a mechanism that allows dynamic phase transitions to take place (Cruz et al., 2010; Kahane et al., 2013).

Because Shh is important for development of both NT and mesoderm, two functionally interconnected systems, the question arises whether the effects of Shh on either tissue are independent of each other or interrelated. Furthermore, does the NT receive Shh directly from the producing sources (No and FP), or given the ligand is released into the mesoderm, can the latter serve as an “en passant” pathway from which Shh affects aspects of both NT and mesoderm development? Answering these questions is of utmost significance both for better understanding the mechanism of Shh activity and for achieving an integrated molecular view of regional development.

Here we report that, in addition to affecting muscle development, reducing the amount of Shh in sclerotome by Hhip1 or a membrane-tethered Hhip1 (Hhip:CD4), significantly reduces motoneuron numbers. The observed phenotypes are a specific and direct consequence of Shh depletion as they are rescued by excess Shh.
targets are reduced, and its effects are not mediated through other signaling pathways. Notably, the effects of Hhip:CD4 are phenocopied by the transmembrane receptor PtcH1 but not by PTC\textsuperscript{Δloop2} which does not recognize the ligand. In addition, by gain and loss of Shh function, and by FP deletions, we show that the sclerotome constitutes a dynamic substrate of No-derived Shh that acts both on motoneuron as well as on myotome development. Furthermore, grafting No fragments adjacent to the basal, sclerotomal side of the NT profoundly affects its development when compared to apical grafts. A similar basal grafting with respect to the DM significantly enhances myotome formation, suggesting a general need for initial ligand presentation at the basal side of epithelia. Together, our results uncover the sclerotome as a novel pathway through which No-derived Shh disperses to promote aspects of neural development.

**Results**

**Reduction of Shh in sclerotome by Hhip1 affects both myotome and motoneuron differentiation**

To investigate possible interactions between neural and mesodermal progenitors mediated by Shh, electroporations were performed in embryos aged 23-25 somite pairs at the level of epithelial somites. This is the earliest time point at which the prospective sclerotome can be faithfully attained by focal electroporation. In this region, the NT is composed of proliferative cells (Kahane and Kalcheim, 1998) and neural patterning is already apparent and ongoing, as evidenced by expression of \textit{Nkx2.2}, \textit{Olig2}, \textit{Nkx6.2} and \textit{Nkx6.1} (Suppl. Fig.S1, A-D). However, differentiation into Hb9-expressing motoneurons has not yet occurred at this stage (Suppl. Fig. S1, E) and only starts
about 10 hr later at the level of somites 11-12 rostral to the last segmented somites (Supplem. Fig.S1, F, arrows). Hence, the timing of manipulations corresponds to the transition of proliferative progenitors undergoing specification into differentiated motoneurons (Ericson et al., 1996).

Previously, we reported that Shh traversing the sclerotome is necessary for myotome differentiation, as misexpression of the high affinity Shh antagonist Hhip1 in sclerotome resulted in smaller myotomes expressing desmin accompanied by a corresponding accumulation of Pax7+ progenitors [(Kahane et al., 2013), Fig. 1, A,B]. Here, we report that the hemi-NT facing the transfected mesoderm also exhibited a 40% reduction in the number of Hb9+ motoneurons compared to control GFP (asterisks in Fig. 1A’,B’, C, p<0.001, N= 12/ treatment). This is a significant effect given that electroporation is a mosaic technique that attains only a fraction of cells, thus causing ligand reduction rather than a total depletion. Moreover, the ventral boundary of Pax7 expression was frequently shifted ventrally (arrows in Fig. 1A,B), likely due to a reduced size of the ventral extent of the transfected hemi-NT (see Fig.2).

These results can be explained by the sclerotome constituting a substrate through which Shh disperses and from which the ligand is provided to both mesodermal and neural progenitors. Alternatively, they might result from Hhip1 moving from the transfected mesoderm towards the NT. Indeed, in spite of initial findings that Hhip1 is a transmembrane glycoprotein with cell autonomous functions (Bishop et al., 2009; Chuang and McMahon, 1999), it was reported that Hhip1 is secreted and able to exert long range effects on Shh signaling (Holtz et al., 2015; Kwong et al., 2014).
Differential behavior of secreted Hhip1 compared to membrane-tethered Hhip1:CD4 vis-à-vis Shh

To distinguish between the above possibilities, we produced a Hhip:CD4 plasmid encoding membrane-tethered Hhip1, unable to undergo secretion (Holtz et al., 2015; Kwong et al., 2014). First, we asked whether NT or sclerotomal cells misexpressing either Hhip1 or Hhip:CD4 can sequester Shh. To this end, we implemented a plasmid encoding the N-terminus of Shh fused to YFP (ShhN:YFP). ShhN:YFP is able to undergo palmitoylation but not addition of cholesterol, a property that enables free movement of the mutant protein when compared to native Shh (Beug et al., 2011). When electroporated to sclerotome, secreted ShhN:YFP was apparent along the basement membrane of the NT where it co-localized with laminin, yet no fluorescent signal was detected in neuroepithelial cells (Supplem. Fig. S2, A-C, arrows).

When double electroporations of ShhN:YFP to the NT and Hhip:CD4 to sclerotome or vice-versa were performed, the Hhip:CD4-transfected sclerotome or NT progenitors, respectively, were decorated with ShhN:YFP, demonstrating that Hhip:CD4 binds and immobilizes the ligand on the surface of the expressing cells (Fig. 1D-D”, F-F”). In contrast, no such co-localization could be observed in either sclerotome or NT upon electroporation of ShhN:YFP and Hhip1 (Fig. 1, E-E”, G-G”) consistent with the notion that native Hhip1 is a secreted protein.

In addition, we monitored the expression of endogenous Shh protein upon transfection of Hhip1 or Hhip:CD4 to sclerotome. Shh protein was evident both intracellularly as well as associated with the cell membranes of the FP and No likely exposed to their external surface (Supplem. Fig. S2, D-F”). Electroporation of control GFP and of Hhip:CD4 had no effect on Shh immunoreactive protein in either the No or FP
(Supplem. Fig.S2, D-E’). In contrast, misexpression of Hhip1 markedly reduced Shh levels in both structures unilaterally adjacent to the transfected cells; this reduction was mainly apparent at their basal sides closer to the transfected Hhip1 (Supplem. Fig.S2, arrows in F’). This effect may result from Hhip1 masking antibody binding to the ligand as the 5E1 Shh antibody and Hhip1 bind to the same pseudo-active site on the Shh molecule (Bishop et al., 2009; Maun et al., 2010). Together, these data confirm that both Hhip as well as Hhip:CD4 bind Shh, but, in contrast to Hhip:CD4, Hhip1 is secreted to adsorb Shh at a distance.

The effects of Hhip:CD4 in the NT resemble those observed with other Shh inhibitors

Next, we employed the NT to examine the specificity of Hhip:CD4 relative to other, known inhibitors of Shh. Electroporation of Hhip:CD4, like that of Hhip1, Ptc1 or PTCΔloop2 to hemi-NTs, significantly reduced the number of Hb9+ motoneurons and that of pH3+ mitotic nuclei while enhancing cellular apoptosis. Furthermore, the total area of the transfected hemi-NTs, that reflects overall changes in cell number (see Methods), was significantly smaller in all treatments when compared to controls (Supplem. Fig. S3, N=4/treatment, *p<0.05, **p<0.03, ***p<0.01). Thus, these effects on motoneurons could result from reduced cell differentiation or, indirectly, from effects on progenitor proliferation or survival. These data confirm that Shh acts both as a mitogen and survival factor (Cayuso et al., 2006; Charrier et al., 2001). Most importantly, they demonstrate that Hhip:CD4, by acting like Hhip1, Ptc1 or PTCΔloop2, is a specific tool to abrogate Shh activity.
Local depletion of Shh activity by Hhip:CD4 or Ptc1 in sclerotome inhibits motoneuron specification and/or differentiation in the NT yet has no significant effect on cell proliferation or survival.

Next, we asked whether the effects originally observed across tissues (e.g. between NT and mesoderm) with secreted Hhip1 (Fig.1), can be mimicked by misexpression of two different Shh inhibitors, Hhip:CD4 and the Shh receptor Ptc1, both membrane associated molecules.

First, we confirmed that electroporation of Hhip1 to sclerotome caused significant effects in the NT, as expected from a secreted molecule. These included a reduction in the number of Hb9+ motoneurons (Fig. 2B,B’, U, N=8, p<0.01), a decreased number of pH3+ mitotic nuclei when measured in the entire hemi-NT (Fig.2Q, V, N=4, p<0.03) but not when the ventral domain containing motoneurons was considered (94.2±4% vs. 98.4±6% in Hhip1 and GFP, respectively), enhanced apoptosis (Fig.2, L, N=4) and an overall decrease in the area of the respective hemi-NT (Fig. 2V, N=4, p<0.03) compared to controls (N=5, Fig.2 A,A’,K,P,V).

Transfection of both Hhip:CD4 and Ptc1 also significantly affected motoneuron numbers (N=38, p<0.01 and N=7, p<0.01, respectively, Fig. 2C,C’D,D’,U), yet had no significant effect on overall cell proliferation (Fig. 2V, N=4 and 7), on the proliferation of ventral progenitors (113±10%, 110±14% vs. 98.4±6% for Hhip:CD4, Ptc1 and GFP), or total hemi-NT area (N=4 and 6, respectively) opposite the treated sclerotomes (Fig.2,R,S,V).

Likewise, cell death, as measured by Tunel staining was not qualitatively affected (Fig.2M,N, N=4 and 6). The number of apoptotic nuclei was quantified using caspase 3 immunostaining upon control GFP or Hhip:CD4 electroporation to the sclerotome. A very low number of positive cells and no measurable difference in the ratio of caspase
3+ cells was detected between both treatments (control GFP, 2.3±0.47 and 2.3±0.7 cells/section; and for Hhip:CD4, 1.7±0.5 and 1.2±0.5 cells/section opposite the treated and contralateral sides, respectively; N=4/treatment, Fig. 3A-F, arrowheads). To note, most caspase 3+ apoptotic nuclei were located in the dorsal half of the NT and the amount of caspase 3+ nuclei in the Hb9+ domain was negligible. In contrast, a clear reduction in Hb9+ MNs was observed in the same sections upon Hhip:CD4 electroporation to sclerotome (Fig. 3D-F, arrow).

Thus, reduction of Shh activity in mesoderm by either Hhip:CD4 or Ptc1 mainly affects motoneuron differentiation, contrasting with Shh abrogation in the NT where all parameters were considerably compromised (Supplem. Fig.S3). This suggests that motoneuron differentiation might be more sensitive to a reduced amount of ligand, and further indicates that progenitor proliferation, survival and motoneuron differentiation might be separable processes that depend upon different Shh concentrations.

As a control for Ptc1 activity, we electroporated PTCΔloop2 that is unable to bind circulating Shh and acts cell autonomously to inhibit its signaling. PTCΔloop2, like Hhip1, Hhip:CD4 and Ptc1 adversely affected the size of the electroporated sclerotomes when compared to the intact contralateral ones (Fig.2F-J,W, N=5, p<0.01), altogether demonstrating that Shh traversing the sclerotome is necessary for proliferation and/or survival of these mesodermal progenitors. In contrast, PTCΔloop2 had no significant effect on either proliferation, survival or total area of adjacent NT cells (Fig.2O,T,V), neither did it affect proliferation of motoneuron precursors (111±13% vs 98.4±6% for PTCΔloop2 compared to GFP). As expected, unlike Ptc1, PTCΔloop2 had no effect on motoneurons (Fig.2E,E’,U) suggesting that reduced sclerotomal size is not sufficient to account for the observed loss of motoneurons.

It is worth mentioning that Hhip1, Hhip:CD4 and Ptc1 also caused a slight
ventralization of the ventral boundary of Pax7 expression (arrows in B-D) which was not apparent upon treatment with PTCΔloop2 (Fig. 2E).

The possibility that Hhip:CD4 or Hhip1 affect specification of NT cells was further tested by examining expression of Olig2, a marker of motoneuron progenitors and of Nkx6.1 which also extends more ventrally. Transfections were performed either at 12 ss (early) or at 25ss (late) and embryos were fixed 14 hours later. Expression of Olig2 and Nkx6.1 was significantly reduced adjacent to the electroporated sclerotomes of both Hhip1 and Hip:CD4-treated embryos. Moreover, no difference in the extent of the effects was monitored in early vs. late electroporations, yet Hhip1 exhibited a stronger phenotype than Hhip:CD4 at either stage (Fig. 3, N= 5/treatment), consistent with the former being soluble and therefore, more efficient in ligand sequestration. This suggests that depletion of Shh in sclerotome also affects ongoing specification of motoneuron progenitors, a process that already begun before the time electroporations were performed (Supplem. Fig. S1).

Next, we further assessed the specificity of Hhip:CD4. While sclerotomal misexpression of Hhip:CD4 significantly affected the number of Hb9+ cells, co-treatment of Hhip:CD4 with Shh rescued the effect back to control levels, further suggesting that Hhip:CD4 abrogates Shh activity. Moreover, Shh alone significantly enhanced motoneuron differentiation (Fig. 4, N= 12, 26, 8 and 16, for control GFP, Hhip:CD4, Hhip:CD4+Shh and Shh alone, respectively, p<0.001).
Local depletion of Shh activity by Hhip:CD4 or Ptc1 in NT inhibits myotome differentiation in mesoderm

Next, we examined whether reduction of Shh in the NT influences myotomal size, measured as the area of desmin+ immunoreactivity. Electroporation of control GFP had no effect of myotome size (N=14) whereas Hhip1 and Hhip:CD4 caused a significant decrease in myotomal size adjacent to the transfected side (N=3,10, respectively, p<0.001, Fig. 5, A-C, F). Electroporation of Ptc1 exhibited a similar effect (N=16, p<0.001); in contrast, PTCΔloop2 revealed no reduction (N=6, Fig. 5D,E,F).

Together, our data suggest that reducing the amount of Shh circulating through the sclerotome promotes a concomitant loss of effective Shh in the NT and vice-versa. Since Hhip:CD4 and Ptc1 are membrane-bound and not secreted, the present results could be explained by the sclerotome, which is a substrate for Shh dispersal, representing a common pool that supplies the ligand to both tissues. Consistent with this notion, sclerotomal Shh is not likely to act by affecting ligand levels in the producing cells because inhibition of Shh by Hhip:CD4 in sclerotome had no effect on Shh expression in either FP or No (Supplem. Fig. 2, E,E’).

The effects of Hhip:CD4 are a direct consequence of Shh depletion

The similarity between the effects of Hhip:CD4 and Ptc1 (Fig. 2) and the rescue of motoneurons by co-electroporation of Shh and Hhip:CD4 (Fig. 4) suggests that the effects of Hhip1 and Hhip:CD4 are specifically mediated by ligand depletion. To further investigate the possibility of direct versus indirect effects, we examined the expression of Gli1, Hhip1 and Ptc2, transcriptional targets of Shh and compared it to Gli3 mRNA expression, which is not directly regulated by Shh (Sasaki et al., 1999). Electroporation of control GFP, Hhip1 or Hhip:CD4 to sclerotome had no effect on
Gli3 mRNA in either the NT or sclerotome (Fig. 7, A-C). In contrast, transfections of Hhip1 or Hhip:CD4 to sclerotome reduced Gli1, Hhip1 and Ptc2 mRNAs in the adjacent hemi-NT, as well as in the transfected sclerotomes, compared to contralateral sides and to control GFP (Fig. 7, D-O, arrowheads). Ptc2 expression was further quantified in embryos electroporated at either 25ss (late) or 12ss (early); both revealed a significant reduction when compared to control GFP, but no difference between the stages was monitored (Fig. 7J-P, N=5, *p<0.001, **p<0.001). Notably, although both plasmids resulted in visible reductions, the phenotypes obtained with soluble Hhip1 were more striking than those with Hhip:CD4, consistent with a stronger effect of the former on motoneuron numbers and on Olig2 expression (Figs. 1C, 2V, Fig. 3). Thus, the observed effects specifically and directly result from ligand reduction.

Next, we examined the possibility of secondary effects across tissues mediated by Shh depletion. Two candidates are BMP which acts antagonistically to Shh (Dessaud et al., 2007; Ericson et al., 1997a; McMahon et al., 1998) and retinoic acid from the somite that affects NT development (Diez del Corral et al., 2003; Wilson et al., 2003). To this end, control GFP or Hhip:CD4 were electroporated into sclerotome. If the effects on the NT of Shh deprivation in sclerotome are mediated by BMP, it is predicted that the dorsal extent of BMP signaling is expanded and or its intensity increased. No changes in the area or intensity of pSmad 1,5,8 immunoreactivity, a readout of BMP activity restricted to the dorsal NT, were measured upon Hhip:CD4 treatment (Suppl. Fig.S4, N=4 per treatment). As an internal control, we observed a reduced number of Hb9+ motoneurons in the ventral NT adjacent to the transfected side (Suppl. Fig.S4B, arrow).

In addition, control GFP or Hhip:CD4 were electroporated into sclerotome and RARE-AP, a specific reporter of retinoic acid activity was co-electroporated into the NT along
with GFP to monitor electroporation efficiency. The specificity of RARE-AP was first tested by co-electroporating it with a dominant negative receptor plasmid, that abolished RARE-AP signal (Supplem. Fig. S5A-B’). No measurable effect in the relative intensity of RARE-AP compared to GFP was observed in the NT upon reduction of Shh in the neighboring sclerotome when compared to control (N= 9/treatment, Supplem. Fig. S5, C-E).

Together, these results show that the observed effects are a direct consequence of Shh depletion. Thus, sequestering Shh in the sclerotome by Hhip1, Hip:CD4 or Ptc1 results in a corresponding reduction of Shh ligand in the NT and vice-versa.

Gain of Shh function in sclerotome enhances motoneuron specification and/or differentiation but Shh misexpression in NT has no effect on myotome

Our loss of function results would be consistent with the possibility that Shh can translocate bidirectionally between mesoderm and NT. To gain additional insight into the directionality of Shh effects across NT and mesoderm, we adopted a complementary gain of function approach. Control GFP or full length Shh were electroporated into sclerotome. A day later, an increase of 30-40% in the number of Hb9+ motoneurons was monitored in the NT of Shh-treated embryos ipsilateral to the treated side compared to controls (p<0.001, N=9/treatment, Fig. 6A-C, see also Fig. 4E).

Reciprocally, control GFP or full length Shh were electroporated into hemi-NTs and the size of adjacent desmin+ myotomes was examined. Although the overall size of the transfected hemi-NT increased, no significant change in myotomal size was measured
and no apparent effects on DM or sclerotome were observed (N=6 and 5 in control and treated embryos, respectively, Fig. 6D-F).

Hence, our gain of function data are inconsistent with the simpler possibility that Shh moves bidirectionally between mesoderm and NT, that is based solely on data from loss of Shh activity. While excess Shh in mesoderm profoundly affects NT development, the observation that gain of Shh in NT has no effect on myotome development is in line with Shh being transported into the neuroepithelium but not outside into mesoderm. In this regard, our finding that Shh depletion in the NT inhibits myotome differentiation (Fig. 5) is consistent with this procedure causing a corresponding lower effective concentration in mesoderm. This could be accounted for by enhanced uptake of the ligand into NT cells via directional baso-apical transport from the sclerotome. This suggests that the sclerotome constitutes a common substrate of No-derived Shh that serves both tissues.

Sclerotome, but not FP-derived Shh is necessary for motoneuron specification and/or differentiation

The accepted view sustains that development of various NT cell types depends upon a local ventrodorsal gradient of Shh directly emanating from the No and/or FP (Smith, 1993; Trousse et al., 1995). Our present results, show that the sclerotomal pool of Shh, originally released from No/FP, is also necessary for motoneuron development (Figs. 5,6). Hence, we examined the relative contribution of the FP compared to sclerotomal Shh to development of Hb9+ neurons.
Control GFP or Hhip:CD4 were electroporated dorsoventrally to attain the FP. Control GFP had no effect on FP integrity or on Shh immunoreactivity (N= 7, Fig.8A). Surprisingly, electroporation of Hhip:CD4 caused by 24 hours, the total absence of the FP and consequent loss of FP-derived Shh (N= 10, Fig. 8, D,E, asterisks). Notably, already by 10 hours post-electroporation, the beginning of FP disintegration, reflected by the presence of pyknotic nuclei, loss of Shh and punctate appearance of transfected Hhip:CD4-GFP, were observed (Fig. 8G-G”, arrows and arrowheads, N=5). This early loss of FP enabled us to accurately monitor its contribution to motoneuron development. Because the effect is bilateral, in order to obtain a reliable measurement, the proportion of Hb9+ neurons was measured as the ratio between neurons in flank lacking a FP to intact neck of the same embryos. In spite of FP disappearance, the proportion of flank-level motoneurons was unaltered when compared to control embryos that received GFP (N=4/ treatment, Fig. 8, B,E,I). To further examine the need for the FP, control GFP and Hhip:CD4-treated embryos were fixed 40 hours following transfection to the brachial level. As shown in Supplem. Fig. S6, no FP remained after electroporation with Hhip:CD4, revealed by lack of GFP signal (A, *in E), disappearance of Shh mRNA, the latter still apparent in the No (J,L), and by nuclear Hoechst (D,D’,H, H’, K,K’,M,M’). Nevertheless, no apparent reduction in motoneurons was detected in treated vs. control GFP cases (A-C, E-G), and the proportion of Hb9+ motoneurons compared between caudal brachial (electroporated) to rostral brachial (intact) levels did not significantly change (N=5/treatment, A-I). Likewise, expression of Olig2 mRNA, that was visible dorsal to the strong Shh mRNA signal in the FP of controls (J,K), remained similar in Hhip:CD4-treated embryos that lacked a FP (N=6/ treatment). Notably, as shown in Supplem. Fig. S6 (L,M, M’), sometimes cells filled the gap at the ventral midline into which Olig2 expression
extended. In such cases, nuclear organization was diffuse, compared to the ordered basal location of nuclei in FP-containing NTs (compare M’ to K’ and D’).

Similar to what was observed with Hhip:CD4, electroporation of Ptc1 also compromised FP integrity while having no apparent effect on motoneurons (N=5, Fig.8H). In contrast, inhibition of Shh in sclerotome by Hhip:CD4 exhibited a visible reduction in motoneurons (Fig. 8, C,F arrow, and Figs. 2 and 3). Thus, we conclude that Shh traversing the sclerotome plays a significant part in motoneuron differentiation whereas direct supply of ligand by FP has no apparent contribution at least at the stages here examined.

A basal, but not apical, presentation of Shh is required for ligand activity on both NT and DM/myotome

Since Shh transiting through the sclerotome is needed for NT development, we predicted that the NT would be more sensitive to Shh presented from its basal side abutting the sclerotome than from its apical side. To examine this hypothesis, fragments of No were grafted into either the lumen of the NT (apical) or between somites and NT (basal grafts). A day later, the there was no apparent reduction in the extent of Pax7 expression in the luminal grafts; and only a mild reduction in the intensity of Pax7 was monitored (Fig. 9A, B). In contrast, Pax7 was dorsally restricted when facing the basal grafts (Fig. 7B). The latter also caused a characteristic bending of the NT, previously reported to represent an ectopic FP-like structure (van Straaten et al., 1988)(N= 6 and 6 for apical vs. basal grafts, Fig. 9, B,D). In addition, the amount of Hb9+ motoneurons was unchanged by the apical grafts, yet was markedly increased in the basal grafts in which the No’s were similarly localized in a dorsal position vis-a-vis the NT (N= 6 and
Likewise, implantation of No fragments at epithelial somite levels and at an apical position with respect to the DM had only a mild effect on the subsequent development of desmin-positive myotomes with no apparent alteration in Pax7 expression in the DM. In striking contrast, equivalent grafts performed basal to the prospective DM, produced large myotomes expressing desmin and a radical \textit{in situ} differentiation of the DM into muscle at the expense of Pax7-positive progenitors (N= 5 and 7 for apical vs. basal grafts, Fig. 9, E,F). Therefore, an initial basal presentation of the ligand \textit{vis-à-vis} the target epithelium is required for the activity of Shh. This is consistent with our results showing that Shh emanating from the sclerotome and reaching the NT from its basal side, is necessary and sufficient for aspects of NT differentiation.

\textbf{Discussion}

We uncover a previously unknown domain, the sclerotome, as being an important “en passant” substrate of Shh that influences not only DM and myotome development, as previously shown (Kahane et al., 2013) but also aspects of NT development such as specification and differentiation of motoneurons (Fig. 9G). Loss of function data show that local depletion of Shh in either NT or sclerotome results in major defects across tissues such as reduced myotomal size and less motoneurons. Reciprocally, only gain of Shh function in the sclerotome enhances motoneuron differentiation while misexpression of the ligand in NT has no effect on myotomal size. Together, these results suggest that Shh specifically secreted into sclerotome constitutes a common pool that supplies both tissues.
In contrast to reduced motoneurons observed upon inhibition of Shh in sclerotome, ablation of the FP has no short-term effects on motoneurons. In addition, embryos without a FP, fixed two days following electroporation, did not exhibit a significant reduction in Olig2 or Hb9. This is consistent with the development of a normal ventral pattern in Gli2 mutants lacking a FP (Chamberlain et al., 2008; Matise et al., 1998a). Likewise, loss of Shh in FP did not dramatically alter ventral neural patterning, yet altered later gliogenesis (Yu et al., 2013). Furthermore, abrogating Shh in FP under the regulation of Brn4, revealed a normal short-term expression of Nkx2.2 and Olig2, but a reduction at later stages (Dessaud et al., 2010). Although this initial phenotype is consistent with our results, we did not observe a later effect in avian embryos when electroporation was performed at the epithelial somite stage. Perhaps, an even earlier deletion of the FP, technically challenging in the avian embryo, would have resulted in effects similar than those obtained in mice. The above results indicate that Shh required at this stage for motoneuron development does not have to emanate by direct contact with the FP which is an integral part of the neuroepithelium, and any basal source of ligand is sufficient. It is therefore possible that in the absence of a FP, the No compensates for the loss of FP-derived Shh as suggested for Gli2 mutants (Danesin and Soula, 2017). Hence, No-derived Shh would follow a basal pathway. Consistently, abrogation of Shh in mesoderm, performed at the same stage and for a similar duration, revealed a significant NT phenotype even in the presence of both Shh-producing axial structures. Altogether, we propose that at least a significant fraction of Shh operating on the neuroepithelium stems from the No via a sclerotomal pathway which, at the stages examined, seems more active than the FP in promoting motoneuron development.
It is noteworthy that different procedures that perturb the production of Shh or its signaling have different effects on FP integrity. Whereas Gli2 mutants lack a FP (Matise et al., 1998b), deletion of Shh in FP does not affect its maintenance (Dessaud et al., 2010). In both chick and mouse, Shh was suggested to be necessary for initial FP induction but later on the FP becomes refractory to the ligand (Ribes et al., 2010). In our experiments, we implemented a membrane tethered version of the high affinity Shh inhibitor Hhip and also the Shh receptor Ptc1, both resulting in the death of FP cells. This might be accounted for by a combination of ligand depletion with accumulation of Shh-Hhip or Shh-Ptc1 complexes at the cell membrane altogether compromising the epithelial integrity.

Furthermore, if Shh from sclerotome is important for neuroepithelial development, is it possible to directly show its presence in this domain? Shh in the synthesizing cells of the FP and No is intracellular and membrane-bound [(Chamberlain et al., 2008) and Supplem. Fig. S2)] whereas in the sclerotome it is expected to be extracellular and/or included in organelles (e.g, exosomes, etc). Most protocols used for tissue processing are likely to keep only the former type of immunoreactive protein and wash away the extracellular ligand in sclerotome. Notably, when using a method that allowed proteoglycan/glycosaminoglycan preservation and/or perhaps also a different antibody, a previous study showed a sclerotomal localization of Shh protein (Gritli-Linde et al., 2001).

One open question is how is Shh transported through the sclerotome. Possible models could be packaging of the ligand in No-derived exosomes (Vyas et al., 2014), diffusion of Shh released by matrix metalloproteinases in a lipid-free form (Dierker et al., 2009); secretion as multimeric complexes (Chen et al., 2004) and/or via carrier-mediated
transport through the extracellular space (Parchure et al., 2018). The precise mechanism responsible for Shh transport in the present context remains to be unraveled.

If provided to the NT from the sclerotome, it is inferred that neurepithelial cells sense Shh from their basal pole that faces the mesoderm. Consistently, grafting No fragments in a basal, but not apical position with respect to the NT, profoundly affects NT shape, motoneuron differentiation and Pax7 expression. Indeed, during normal development, the No underlies the basal domain of the NT, further supporting the idea that No-derived Shh can only reach the NT via a basal route. In line with the above, it was reported that lipidated Shh enters cells of the imaginal disc in Drosophila only through its basolateral surface (Callejo et al., 2006). A similar phenomenon was observed in high density human gastruloids, that self organize into an epithelium. In these cultures, cells were responsive to BMP4 or Activin ligands only when presented from the basal side (Etoc et al., 2016), suggesting that cell polarization controls ligand response.

The above findings are interesting in light of the proposed concept that apically-localized cilia serve as antennae to sense and transduce a Shh signal (Corbit et al., 2005; Singla and Reiter, 2006). We suggest that, initially, Shh is presented from the basal side of epithelial cells from which it may be transported to the apical domain where cilia are localized. Since an apical presentation of the No and associated Shh was without a significant effect in our implant experiments, we propose that cilia act as transducers of a Shh signal, but not as the primary antennae sensing the ligand. This further suggests that basal reception followed by baso-apical transport might be necessary for the activity of Shh arriving at the cilia. Growing evidence suggests cilia-independent Shh reception that occurs through basally localized cytonemes [(Gradilla et al., 2018) and refs. therein]. Likewise, in the retina neuroepithelium, Shh and its coreceptor Cdo
colocalize at the basal side of the cells where filopodia-like structures are present (Cardozo et al., 2014). An extreme example is provided by some cell types where Shh signaling takes place even in the absence of cilia [(Gradilla et al., 2018) and refs. therein].

In the NT, direct visualization of labeled Shh, revealed that the ligand concentrates in association with the apically localized basal bodies from which cilia stem, while forming a dynamic gradient in the ventral NT (Chamberlain et al., 2008). In light of the present results, the above observed graded distribution of ligand could be explained as being the end point of a transport process that begins at the No, travels through the sclerotome forming there a ventro-dorsal gradient, then reaches the neuroepithelium through its basal side finally concentrating in the apical cilia (Fig. 9G). Possible mechanisms mediating such a baso-apical transport associated with possible biochemical changes of the protein to make it available to cilia for signaling remain to be unraveled. A microtubular network spanning the extent of neuroepithelial cells could be involved in this process (Chamberlain et al., 2008).

An initial presentation of Shh from the basal side of an epithelium seems to be of general significance as basal grafting of a No also elicited robust in situ differentiation of DM progenitors into myocytes when compared to an apical implant that exhibited only a mild phenotype. How can this differential effect be explained given that the endogenous ligand apparently arrives from the apical sclerotomal direction? Initially, pioneer myotomal cells in the epithelial somite face the No from their basal aspect (Kahane et al., 1998). Upon sclerotome dissociation, the epithelial DM is consolidated and becomes composed of a central sheet and four inwardly curved lips pointing towards the sclerotome. We and others showed that the four DM lips are the main sources of myotomal cells (Kalcheim et al., 1999) and it is their basal domain that points
towards the sclerotome from which Shh arrives. Next, myotomal progenitors enter the nascent myotome and a basement membrane begins assembling between myotome and sclerotome (Borycki, 2013). At this time, the central DM sheet also contributes myotomal progenitors by direct translocation (Ben-Yair et al., 2011), which then differentiate in a Shh-dependent manner (Kahane et al., 2013). These precursors exhibit an inverse apicobasal polarity when compared to central DM cells (Ben-Yair et al., 2011). Hence, both the DM lips and these translocating progenitors point with their basal surfaces towards the source of endogenous Shh, likely being the main targets for its activity and also accounting for the partial effect of the apical grafts.

As discussed above, the basal domain of the DM/myotome and NT epithelia are characterized by the presence of a surrounding basement membrane. An association between Shh and the basal lamina has been shown. In cerebellar granule precursors, the laminin-containing basement membrane binds and enhances Shh signaling (Blaess et al., 2004). Similarly, Shh induces activation of Myf5 in mouse DM. Myf5+ cells then translocate to the myotome and upregulate α6β1 integrin and dystroglycan, allowing a myotomal basement membrane to be assembled (Borycki, 2013). This is further confirmed in Shh-deficient mice, which fail to form a myotomal basement membrane, and in which myotomal differentiation is delayed (Anderson et al., 2009; Borycki et al., 1999). Additionally, Shh immunoreactive protein was found to localize in the basement membrane surrounding the NT (Gritli-Linde et al., 2001). Taken together, these results suggest that in both NT and DM/myotome, a feedforward mechanism may exist whereby Shh controls laminin expression and basement membrane assembly. This could allow a local concentration of the ligand and/or signal stabilization. However, the basement membrane alone is unlikely to serve as the common pool, as sequestering the ligand specifically in sclerotome by membrane-associated Hhip:CD4 was highly
effective. In addition, others have shown that the ligand is present in the mesoderm itself (Gritli-Linde et al., 2001).

Our present findings support the importance of NT-somite interactions which are pivotal for the normal patterning of trunk components. For instance, opposite gradients of retinoic acid and Fgf8 in mesoderm are required for NT development (Diez del Corral et al., 2003). In addition, the nascent DM controls the timing of neural crest delamination by modulating noggin mRNA and BMP activity in the NT (Sela-Donenfeld and Kalcheim, 2000). Reciprocally, Bmp4 and/or Wnt1 from the NT pattern somite-derived myogenesis (Abu-Elmagd et al., 2010; Marcelle et al., 1997). Formation of the dorsal dermis is influenced by NT-derived Wnt1 and by neurotrophin-3 (Brill et al., 1995; Marcelle et al., 1997; Sela-Donenfeld and Kalcheim, 2002). Furthermore, interactions between neural and somitic cells control neural crest migration and segmentation of peripheral ganglia and nerves as well as specific aspects of myogenesis (Kalcheim, 2011; Kalcheim and Goldstein, 1991). The present results raise the intriguing possibility that the dual activity on both motoneurons and myotome of Shh released into sclerotome, serves to couple and coordinate development of the neuromuscular system.
Materials and Methods

Embryos

Chick (Gallus gallus) and quail (Coturnix japonica) eggs were from commercial sources (Moshav Orot and Moshav Mata, respectively). All experiments were performed using quails except for those involving in situ hybridizations which were done in chick embryos.

Expression vectors and electroporation

Expression vectors were: pCAGGS-GFP, pCAGGS-RFP (Krispin et al., 2010), Ptc1 (Briscoe et al., 2001), PTCΔloop2 (Briscoe et al., 2001; Kahane et al., 2013), a retinoic acid reporter fused to alkaline phosphatase (pRARE-AP, from J. Sen) (Gupta and Sen, 2015), a dominant negative pan-retinoic acid receptor (RAR403dn-IRES-GFP, from S. Sockanathan) that abrogates retinoic acid signaling (Novitch et al., 2003), full length Shh (Kahane et al., 2013), cholesterol deficient Shh (mShh-N-YFP, from V. Wallace) (Beug et al., 2011) that was subcloned into pCAGGS, and mHhip1 (Kahane et al., 2013). To produce Hhip1:CD4, a membrane-tethered version of Hhip1, the transmembrane and intracellular domains of mouse CD4 were fused to the C-terminal domain of Hhip1 lacking amino acids A679-V700, as previously described (Holtz et al., 2015; Kwong et al., 2014) and further subcloned into pCAGGS for electroporation.

For electroporations, DNA (1-4 µg/µl) was microinjected into the center of flank-level epithelial somites (somites 20-25) of 23-25 somite-stage embryos. Electroporations were performed to the ventral half of epithelial somites (prospective sclerotome). To this end, the positive tungsten electrode was placed under the blastoderm in a location
corresponding to the ventro-medial portion of epithelial somites on a length of about 7 segments, and the negative electrode was placed in a superficial, dorso-lateral position with respect to the same somites. (Ben-Yair et al., 2011; Halperin-Barlev and Kalcheim, 2011; Kahane et al., 2007; Kahane et al., 2013).

For hemi-NT electroporations, DNA was microinjected into the lumen of the NT. One tungsten electrode was placed underneath the blastoderm on one side of the embryo and the other electrode was placed in a superficial position at the contralateral side. For FP electroporations, the positive electrode was inserted under the blastoderm near the midline and the negative electrode was placed over the dorsal NT. In some cases double electroporations to the hemi NT and sclerotome were performed sequentially. A square wave electroporator (ECM 830, BTX, Inc.) was used. One pulse x12V for 5msec was applied.

No grafts

No fragments comprising a length of 7-8 segments were enzymatically excised from donor embryos aged 25 somite pairs as previously described (Charrier et al., 2001) and kept in cold phosphate buffered saline until grafting. For grafting at the apical side of the NT, the ectoderm and dorsal NT of host embryos were cut and the No piece placed in the NT lumen. A day following implantation, the grafts were usually found in the dorsal portion of the NT facing its cavity. For basal grafting with respect to the NT, a slit was performed between somites and NT and the No fragments were inserted. A similar, but more profound slit, was performed to reach the ventral sclerotome abutting the apical side of the DM. To reach the basal domain of the DM, the ectoderm was cut to precisely accommodate the length of the No fragment. Following microsurgery, embryos were reincubated for additional 24 hours.
**Immunohistochemistry**

Embryos were fixed overnight at 4°C with 4% formaldehyde in phosphate-buffered saline (PBS) (pH 7.4) followed by washings in PBS. Most immunostainings except for desmin were performed on whole embryo fragments. Immunolabeling for desmin was performed on tissue sections, as described (Burstyn-Cohen and Kalcheim, 2002; Kahane et al., 2001).

For wholmount immunostaining, antibodies were diluted in PBS containing 1% Triton X-100 and 5% newborn calf serum and tissues were incubated overnight at 4°C on a rotatory shaker. Next, they were washed twice in a large volume of PBS/1% Triton X-100 first for 10 min and then for 2 hours at room temperature. Secondary antibodies were similarly diluted in PBS/ 1% Triton X-100/ 5% newborn calf serum and incubated overnight followed by repetitive washings. Embryo fragments were dehydrated in increasing ethanol solutions (30%, 70%, 90% and 100%, 10 minutes each) followed by toluene (2 times, 10 minutes each), then embedded in paraffin wax and sectioned at 8μm. Paraffin was removed in Xylene and slides were rehydrated in decreasing ethanol solutions.

The following antibodies were used: rabbit anti GFP (1:2000, Invitrogen, Thermo Fisher Scientific, A6455) and mouse anti-desmin (1:200, Molecular Probes, 10519). Monoclonal antibodies against Pax7 (PAX7-s, 1:20), Shh (5E1, 1:20) and Hb9 (1:200) were from DSHB, University of Iowa). Phosphorylated Smad 1-5-8 (PSmad, 1:1000) was a gift from Ed Laufer. Anti-Histone H3 (phospho S10) was from Abcam (mAbcam 14955, 1:500), and anti-caspase 3 (Cell Signaling, 1:200). Detection of DNA fragmentation was done by TUNEL (ab66110, Abcam) according to manufacturer’s instructions. Nuclei were visualized with Hoechst.
In situ hybridization

Embryos were fixed in Fornoy (60% ethanol, 30% formaldehyde, 10% acetic acid), then dehydrated in ethanol/toluene, processed for paraffin wax embedding and sectioned at 10 μm. Slides were rehydrated in toluene/ethanol/PBS, treated with proteinase K (1µg/ml, Sigma Aldrich P2308) at 37°C for 7 minutes, and then fixed in 4% formaldehyde at room temperature for 20 minutes. Next, slides were washed in PBS followed by 2X SSC and hybridized in hybridization buffer (1X salt solution composed of 2M NaCl, 0.12M Tris, 0.04M NaH₂PO₄·2H₂O, 0.05M Na₂HPO₄, 0.05M EDTA, pH7.5], 50% formamide, 10% dextran sulfate, 1mg/ml Yeast RNA, 1X Denhardt solution) containing 1µg/ml DIG labeled RNA probes (prepared with a DIG RNA labeling mix, Roche, 11277073910) for overnight at 65°C in a humid chamber. Post-hybridization, slides were rinsed in a rotating incubator with 50% formamide, 1X SSC, 0.1% Tween 20, until coverslips dropped and then an additional wash for 1 hour followed by 2 washes in MABT (10% Maleic acid 1M pH 7.5, 3% NaCl 5M, 0.1% Tween 20) and preincubation in MABT/ 2.5% FCS. Anti-DIG-AP antibody (1/1000, Roche 11093274910) diluted in MABT+2% BBR+20% FCS was then added for overnight at room temperature. This was followed by rinsing in MABT and then in NTMT (2% NaCl 5M, 10% Tris HCl 1M pH9.5, 5% MgCl₂ 1M, 0.1% Tween20), and then incubation in NTMT + 1:200 NBT/BCIP Stock Solution (Sigma-Aldrich, 11681451001) at 37°C until the AP reaction was completed.

The following probes were employed: Hhip1 (from J. Briscoe), Nkx2.2, Nkx 6.1, Nkx 6.2, Olig2, Ptc2 (from J. Ericson), and Gli1, Gli3 from A.G. Borycki.
Data analysis and statistics

Four to 26 embryos were analyzed per experimental treatment. Each experiment was repeated at least 3-5 times. The number of Hb9-positive motoneurons was counted in 5-10 alternate sections per embryo. The average number of Hb9 cells counted per embryo was 650, ranging between 200 to 1400 cells. The number of phospho-histone H3 (pH3) or caspase 3-positive nuclei was monitored in 7-10 sections per embryo in 4-8 embryos per treatment. The average number of pH3 cells counted per embryo was 150, ranging from 53-240 cells, respectively. Caspase quantifications are in text.

Myotomes were defined by desmin staining. The area occupied by desmin+ myotomes was measured in alternate sections of 3 to 16 embryos per experimental treatment. Sclerotomes of 4 to 6 embryos were defined in the mediolateral aspect as the tissue between myotome and NT, and in the dorsoventral extent as the mesenchyme between the dorsomedial lip of the DM up to the dorsal border of the cardinal vein and aorta. The surface area of hemi-NTs was monitored in 4 sections per embryo. Myotomal, sclerotomal and hemi-NT areas as well as the area and intensity of cells expressing the p-Smad 1,5,8 or pRARE-AP were measured using Image J software (NIH). All results are expressed as the mean proportion of positive cells or area in treated compared to control contralateral sides, or to control GFP ±SEM.

In a control experiment, we tested whether area measurements are a faithful representation of cell number. To this end, we compared the number of Hoechst+ nuclei in hemi-NTs electroporated with Hhip:CD4/contralateral side vs. control GFP/contralateral side, and found them to be not significantly different from the equivalent ratio of surface area. Consequently, the calculated ratio of area to cell number was similar (1.03±0.032, N=6 embryos sectioned at 5μM, 5 sections/embryo).
Thus, surface area was validated as a reliable measure of overall cell number.

Images were photographed using a DP73 (Olympus) cooled CCD digital camera mounted on a BX51 microscope (Olympus) with Uplan FL-N 20x/0.5 and 40x/0.75 dry objectives (Olympus). For quantification, images of control and treated sections were photographed under the same conditions. For figure preparation, images were exported into Photoshop CS6 (Adobe). If necessary, the levels of brightness and contrast were adjusted to the entire image and images were cropped without color correction adjustments or \( \gamma \) adjustments. Final figures were prepared using Photoshop CS6.

Significance of results was determined using the non-parametric Mann–Whitney test. All tests applied were two-tailed, and a \( P \)-value of 0.05 or less was considered statistically significant. Data were analyzed using the IBM SPSS software version 25. The number of embryos analyzed for each treatment (N) is detailed in the Results Section. \( P \)-values can be found both in the Results Section and in the corresponding Legends.

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**Competing interests**

No competing interests declared
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Fig. 1- Reduction of Shh in sclerotome by Hhip1 affects both myotome and motoneuron differentiation.

(A, B) Electroporation of control GFP (A) or Hhip1/GFP (B) to the prospective sclerotome. A day later a reduction in myotome size (blue desmin staining) is apparent...
adjacent to the transfected cells. In addition, note ventral shift of the Pax7+ boundary in B (small arrows).

(A’, B’) Higher magnification of the insets in A,B, respectively, depicting a reduction of Hb9+ motoneurons in B upon Hhip1 treatment compared to the control side and to control GFP (A’). Asterisks (*) denote motoneurons adjacent to electroporated sclerotomes. (C) Quantification of Hb9+ motoneurons (***, p<0.001).

(D-D”) Double electroporation of ShhN:YFP to NT and Hhip1:CD4 to sclerotome. Note that sclerotomal cells misexpressing Hhip:CD4 (red) are decorated by Shh (green) immunolabeling (D’, D” arrows).

(E-E”) Double electroporation of ShhN:YFP to NT and Hhip1 to sclerotome. No labeling of Shh (green) is apparent in sclerotomal cells misexpressing Hhip1 (red). D” and E”, high magnifications of respective insets.

(F-F”) Double electroporation of ShhN:YFP to sclerotome and Hhip1:CD4 to NT. Note labeling of Shh (green) in both the basement membrane as well as along neuroepithelial cells misexpressing Hhip:CD4 (F’, F”). F” is a higher magnification of F/F’ where only ShhN:YFP (green) is shown to highlight that Shh co-localizes with the red, Hhip1:CD4-misexpressing cells, in the neuroepithelium (F).

(G-G”) Double electroporation of ShhN:YFP to sclerotome and Hhip1 to NT. Note labeling of Shh (green) in basement membrane but no co-staining of neuroepithelial cells. G”, high magnifications of inset in G’. Abbreviations, DM, dermomyotome; M, myotome; NT, neural tube; No, notochord; Scl, sclerotome. Bars=50 μM.
Fig. 2 - Electroporation of Hhip:CD4 and Ptc1 into sclerotome inhibit motoneuron development without significantly affecting progenitor proliferation or survival. (A-E’) Electroporation (green) to sclerotome followed a day later by immunostaining for Pax7 and Hb9. A’-E’ represent higher magnifications of the boxed regions in A-E. Note that all plasmids except PTCΔloop2 reduced the number of motoneurons adjacent to the transfected sclerotomes. Arrows in B-D show a slight ventralization of the ventral boundary of Pax7.
(F-J) The same sections as in A-E in which the sclerotomes were delineated by a white lines.

(K-O) Tunel staining (red). Note weak immunostaining of electroporated plasmids (green) because no anti-GFP antibodies were implemented to enable better visualization of apoptotic nuclei. Only Hhip1 caused numerous Tunel+ nuclei adjacent to the transfected sclerotome. Ectodermal staining reflects non-specific reactivity.

(P-T) staining of mitotic nuclei with anti pH3 (red).

(U) Quantification of motoneurons

(V) Quantification of mitotic nuclei and area in the hemi-NT adjacent to the electroporated sclerotome.

(W) Quantification of the relative area of electroporated sclerotomes based on sections such as those shown in F-J. Abbreviations, NT, neural tube; Scl, sclerotome. *p<0.05, **p<0.03, ***p<0.01. Bar=50μM.
Fig. 3- Electroporation of Hhip:CD4 into sclerotome reduces the extent of *Olig2* and *Nkx.6.1* expression in NT without affecting cell survival.

(A-F) Electroporation of control GFP (A-C) or Hhip:CD4 (D-F) (green cells in sclerotome). Note unilateral reduction of Hb9+ motoneurons adjacent to transfected sclerotome (arrow in D). Green cells in NT represent caspase 3+ nuclei (arrowheads). Only few apoptotic nuclei are evident in both control and treated embryos, primarily localized to the dorsal NT but not to the motoneuron area. See text for quantification.

(G-R) Electroporation of control GFP, Hhip1 or Hhip:CD4. G-L represent early electroporations, and M-R late electroporations. Asterisks denote transfected sclerotomes. Arrows mark mRNA expression on the experimental side. The extent and/or intensity of mRNA expression were reduced in Hhip1/Hhip:CD4-treated embryos.
(S,T) Quantification of the relative expression of *Olig2* and *Nkx6.1* upon early vs. late electroporations. **p<0.001, *p<0.01. Abbreviations, NT, neural tube, No, notochord, Scl, sclerotome. Bar=50μM.
Fig. 4- Depletion of Shh activity by Hhip:CD4 in sclerotome inhibits motoneuron development and is rescued by co-treatment with Shh

(A-D) Electroporation of the depicted plasmids to sclerotome (Scl) (green). Hhip:CD4 to Scl reduces motoneuron numbers compared to control GFP, whereas co-transfection with Shh rescues the effect. A’-D’ are higher magnifications of the insets in A-D. (E) Quantification of Hb9+ motoneurons, ***p<0.001. Bar=50 μM.
Fig. 5- Electroporations of Hhip, Hhip:CD4 or Ptc1, but not of PTCΔloop2 into the neural tube, reduce myotome size.

(A-E) Electroporation of the depicted plasmids (green). Hhip1, Hhip:CD4 and Ptc1 reduce the size of adjacent desmin+ myotomes (arrows, red) compared to control GFP yet PTCΔloop2 has no significant effect. (F) Quantification of myotome size. ***p<0.001. Bar=50 μM.
Fig.6- Gain of Shh function in sclerotome enhances motoneuron differentiation but Shh misexpression in NT has no effect on myotome

(A-C) Electroporation of Shh (green) to the sclerotome (Scl) enhances the number of Hb9+ motoneurons compared to control (arrow in B, red). Quantification in C, ***p<0001. (D-F) Electroporation of Shh (green) to the NT has no effect on the size of desmin+ myotomes (blue) or on expression of Pax7 (red) in the DM. Quantification in F. Abbreviations, No, notochord; NT, neural tube, Scl, sclerotome. Bar=50µM.
Fig. 7- The effects of Hhip1 and Hhip:CD4 on NT are a direct consequence of Shh depletion

(A-I) In situ hybridization for Gli3, Gli1 and Hhip1 following electroporation of depicted plasmids. (J-O) In situ hybridization for Ptc2 following late or early electroporations of control GFP, Hhip1 or Hhip:CD4. Asterisks (*) mark the electroporated sites. Control GFP had no effect on bilateral expression of either gene in
NT. In contrast, Hhip1 and Hhip:CD4 transfected to sclerotome reduced \textit{Gli1}, \textit{Hhip1} and \textit{Ptc2} mRNAs unilaterally (D-O, arrowheads), but not \textit{Gli3} mRNA (A-C). (P) Quantification of the relative expression of \textit{Ptc2} in treated/contralateral sides of late and early electroporations (N=5 embryos/treatment,**p<0.001,*p<0.01). Abbreviations; NT, neural tube, No, notochord, Scl, sclerotome. Bar=50 \textmu M.
Fig. 8- Sclerotome, but not FP-derived Shh is necessary for motoneuron development

(A,B) Dorsoventral electroporation of control GFP showing in A, the presence of the labeled floor plate (FP) that co-expresses Shh protein. In B, note Hb9+ motoneurons dorsal to the labeled FP. (C) Hb9+ motoneurons in control GFP electroporation to sclerotome.
(D,E) Loss of FP tissue 24 hours post- Hhip:CD4 electroporation. Asterisks (**) in D and E denote absence of a FP and panel D shows concomitant absence of Shh expression. Hhip:CD4 electroporation has no effect on ventral Hb9+ motoneurons or dorsal Pax7 expression (both in red). (F) In contrast, less motoneurons are apparent adjacent to the sclerotome transfected with Hhip:CD4 (arrow).

(G-G”) Disintegration of the FP 10 hours following Hhip:CD4 transfection. Note in G and G’ the almost complete absence of GFP signal in the FP domain (with only one GFP+ cell remaining) as well as the punctate expression of GFP (arrows) corresponding to disorganized and pyknotic nuclei (arrowheads in G”).

(H) Loss of the FP upon electroporation of Ptc1 (**) shows no apparent effect on motoneurons. (I) Quantification of the proportion of Hb9+ motoneurons in flank (electroporated)/neck (intact region). Abbreviations, No, notochord; NT, neural tube. Bar=50 µM.
Fig. 9- A basal, but not apical, presentation of Shh is required for ligand activity on both NT and DM/myotome. A model accounting for the effects of Shh traversing the sclerotome on both NT and myotome development.

Implantation of No fragments (dotted circles) in apical (A,C,E) or basal (B,D,F) positions vis-à-vis the dorsal NT, or the dermomyotome (DM) (E,F).

(A,C) A No piece was grafted inside the NT facing its luminal side. Note that a day later it localizes in the dorsal NT. No significant effect on Pax7 (arrows) or Hb9+ motoneurons (arrowheads) is apparent.
(B,D) A No piece was grafted in a basal position vis-à-vis the dorsal NT. Note a dorsal restriction of Pax7 (arrows in B) as well as a dorsalward expansion of Hb9+ motoneurons (arrowheads in D).

(E,F) A No fragment localized apical to the DM causes a mild change in the adjacent desmin+ myotome (green, E) whereas the basally located No strongly increases myotomal size and promotes an in situ differentiation of the DM into myotome with concomitant loss of Pax7 expression.

(G) A proposed model for the activity of No-derived Shh. The No secretes Shh that acts on the ventral NT and also traverses the sclerotome (Scl) which is both a pathway for ligand movement and also a target of its activity. We propose that a ventral to dorsal gradient of ligand is created in Scl and plays a pivotal role both on myotome as well as on motoneuron development. Shh is thus presented to the target epithelial cells via its basal domain, probably by initial association with the laminin-containing basement membrane. See text for details. Bar=50 µM.
Fig. S1- Expression of neural tube markers by the time of in ovo electroporation

(A-D) In situ hybridization of embryos aged 25 somite pairs showing expression of ventral neural tube (NT) markers at the epithelial somite (ES) stage.

(E,F) Co-immunolabeling of Pax7 and Hb9 antibodies. Note in E that only Pax7 is expressed in both dorsal NT and dorsal aspect of the ES, whereas the first Hb9+ ventral motoneurons appear at a later stage following somite dissociation (arrows in F). Both dorsal Pax7 and ventral Hb9 immunostainings are in red as the respective antibodies are monoclonal of the same subtype. Abbreviations, No, notochord. Bar=50µM.
**Fig. S2**- Differential behavior of secreted Hhip1 compared to membrane-tethered Hhip:CD4

(A-C) ShhN:YFP, electroporated into sclerotome (Scl, green), colocalizes with laminin (red) in the basement membrane around the neural tube (NT) (arrows).

(D-F’) Immunolabeling with Shh and Hb9 antibodies of embryos electroporated with control GFP (D,D’), Hhip:CD4 (E,E’) or Hhip1 (F,F’). Note that Hhip1 caused a reduction in ipsilateral Shh in both notochord (No) and FP (FP) (arrows in F’) whereas GFP or Hhip:CD4 were without effect. Bars=50 µM.
**Fig. S3- Depletion of Shh in the NT by Hhip:CD4 phenocopy those exerted by Hhip, Ptc1 and PTCΔloop2**

(A-E’) Electroporation of depicted plasmids (green) to hemi-NTs followed a day later by immunostaining for Pax7 and Hb9. A’-E’ represent higher magnifications of the boxed regions in A-E.

(F-J) Staining of mitotic nuclei with anti pH3 (red) following electroporation with depicted plasmids.

(K-O) Tunel staining (red). Enhanced apoptosis is seen in the hemi-NTs adjacent to all electroporations when compared to the respective contralateral sides or to control GFP. Note weak immunostaining of electroporated plasmids (green) because no anti-GFP antibodies were implemented to enable better visualization of apoptotic nuclei.

(P) Quantification of motoneurons, mitotic nuclei and area. *p<0.05, **p<0.03, ***p<0.01. Bar=50µM.
Fig. S4- Electroporation of Hhip:CD4 to sclerotome has no effect on BMP signaling in dorsal NT.

(A,B) Misexpression of Hhip:CD4 in sclerotome (green) had no effect on expression of phospho-Smad 1,5,8 in the dorsal NT compared to control GFP (arrowheads), yet reduced the number of Hb9+ motoneurons (arrow in B). (C) Quantification of the area and intensity of phospho-Smad 1,5,8 expression. Bar=50 µM.
Fig. S5- Inhibition of Shh activity in sclerotome has no effect on retinoic acid signaling in the neural tube.

(A-B””) Specificity of the retinoic acid reporter (pRARE-AP). Note in A-A” the expression of pRARE-AP in control GFP-electroporated NT. In contrast, no signal is apparent when retinoic acid activity is abolished by a dnRAR plasmid (B-B”). A” and B” are overlays of the precedent panels, respectively.

(C-D’) Double electroporations of control GFP or Hhip:CD4 to the sclerotome (C and D, respectively) and co-electroporation of RARE-AP together with GFP to the NT of the same embryos (C,C’, D,D’).

(E) Data quantification. Misexpression of Hhip:CD4 in sclerotome had no effect on RARE-AP/GFP activity in NT. Results represent mean values of RARE-AP/GFP±SEM. Bars=50 µM.
Figure S6- Deletion of the FP has no significant effect on Hb9+ motoneurons or Olig2+ precursors

(A-D’) Dorsoventral electroporation of control GFP showing in A, the presence of the labeled floor plate (FP). In B, note Hb9+ motoneurons dorsal to the labeled FP. (C) Overlay of A and B. (D, D’) Hoechst nuclear stain. Note in the higher magnification (D’) the basal localization of nuclei (arrows).

(E-H’) Loss of FP tissue 40 hours post-Hhip:CD4 electroporation as marked by absence of GFP. Asterisks (**) denote absence of a FP. Arrowheads in H’ mark an open ventral NT lacking FP cells. (I) Quantification showing no effect of FP deletion on the proportion of Hb9+ motoneurons in caudal brachial (electroporated)/rostral brachial (intact region).
(J-M') Double in situ hybridization for Olig2 (light blue) and Shh (dark blue) of control GFP (J,K,K') or Hhip:CD4 electroporated embryos (L, M,M'). Note that in controls, Olig2 (light blue) is dorsal to Shh mRNA expression (intense blue in FP and also seen in No). Between them, a characteristic gap corresponding to ventral interneurons is apparent. In contrast, upon electroporation of Hhip:CD4, the FP, with its typical basal nuclei is lost (arrows in D’,K’ compared to arrowheads in M’), and a concomitant disappearance of Shh mRNA signal is apparent. In spite of that, Olig2 expression seems unaffected (L). Abbreviations, No, notochord; NT, neural tube. Bars=50 µM.