Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction

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Summary

Sonic hedgehog (Shh) signaling is essential for sclerotome development in the mouse. Gli2 and Gli3 are thought to be the primary transcriptional mediators of Shh signaling; however, their roles in Shh induction of sclerotomal genes have not been investigated. Using a combination of mutant analysis and in vitro explant assays, we demonstrate that *Gli2* and *Gli3* are required for Shh-dependent sclerotome induction. *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos exhibit a severe loss of sclerotomal gene expression, and somitic mesoderm from these embryos cannot activate sclerotomal genes in response to exogenous Shh. We find that one copy of either *Gli2* or *Gli3* is required to mediate Shh induction of sclerotomal markers *Pax1* and *Pax9* in vivo and in vitro. Although *Gli2* is generally considered an activator and *Gli3* a repressor, our results also reveal a repressor function for *Gli2* and an activator function for *Gli3* in the developing somite. To further dissect the function of each Gli, we used adenovirus to overexpress Gli1, Gli2 and Gli3 in presomitic mesoderm explants. We find that each Gli preferentially activates a distinct set of Shh target genes, suggesting that the functions of Shh in patterning, growth and negative feedback are divided preferentially between different Gli proteins in the somite.

Key words: Sonic hedgehog, Somite, Mouse, Patterning, Gli

Supplemental data available online

Introduction

The axial musculoskeletal system of vertebrates derives from somites, segmented blocks of mesoderm flanking each side of the neural tube. Somites form from an unsegmented precursor tissue called the presomitic mesoderm (PSM). The anterior PSM forms the newest somite by undergoing a mesenchymal-to-epithelial transition, resulting in an epithelial sphere with a mesenchymal core. As the somite matures, the dorsal region remains epithelial, while the ventral region undergoes a second transition into mesenchyme. The dorsal epithelium forms the dermomyotome, which gives rise to dermis of the back and skeletal muscles, while the ventral somite forms sclerotome, which gives rise to the ribs and vertebrae (reviewed by Brent and Tabin, 2002).

The secreted signaling molecule Sonic hedgehog (Shh), expressed in the notochord and floorplate, is crucial for sclerotome development as *Shh* mutant mice lack vertebral columns, and form only a few rudimentary rib cartilages (Chiang et al., 1996). Consistent with this, Shh induces expression of sclerotomal markers including the paired-box containing transcription factors *Pax1* and *Pax9*, and the HMG-box containing transcription factor *Sox9* in PSM in vitro (Fan and Tessier-Lavigne, 1994; Murtaugh et al., 1999; Zeng et al., 2002). These target genes are essential for sclerotome development as *Pax1/Pax9* double mutants have severe defects in formation of ribs and vertebrae (Peters et al., 1999), and *Sox9* is required for the transcription of collagen α2, an extracellular component necessary for cartilage formation (Bell et al., 1997; Bi et al., 1999). In addition to the sclerotomal markers, Shh induces proliferation of the somitic mesoderm (Fan et al., 1995), possibly by upregulation of G1 cyclins, which are Shh targets in other tissues (Kenney and Rowitch, 2000; Mill et al., 2003). Shh also negatively regulates its own signaling by upregulation of its own binding receptor patched 1 (*Ptch*) and a decoy receptor hedgehog interacting protein (*Hhip*) (Briscoe et al., 2001; Chuang and McMahon, 1999; Goodrich et al., 1996). Both the proliferation and negative feedback induced by Shh may help define the shape and size of sclerotome-derived skeletal components. Thus, the roles of Shh in the somite can be divided into three categories, patterning, proliferation and negative feedback.

Induction of Shh targets in the somite is thought to be carried out through the conserved Hedgehog (Hh) signaling pathway first described in *Drosophila* (reviewed by McMahon, 2000). In this pathway, Hh binds to its receptor Patched (Ptc) and relieves Ptc inhibition of the signaling component Smoothened (Smo). Smo then signals to the transcription factor Cubitus interruptus (Ci) to activate gene expression. Ci acts as a bipotential transcription factor, repressing some of the same target genes in the absence of Hh (Methot and Basler, 2001; Muller and Basler, 2000). In the mouse, there are three Ci homologs, Gli1, Gli2 and Gli3 (Hui et al., 1994). Gli1 and Gli2 are thought to act primarily as activators, while Gli3 acts

primarily as a repressor (Bai et al., 2002; Lee et al., 1997; Ruiz i Altaba, 1998; Sasaki et al., 1997; Sasaki et al., 1999; Shin et al., 1999). Bipotential functions of Gli2 and Gli3 on reporter genes in cultured cells has been demonstrated (Sasaki et al., 1999), but in vivo evidence of bipotential Gli2 activity is lacking.

Genetic data supports a crucial role for *Gli2* and *Gli3* in formation of the axial skeleton as *Gli2* and *Gli3* mutants exhibit distinct vertebral and rib defects late in development, whereas *Gli1* mutant mice exhibit no developmental defects. *Gli2*$^{-/-}$*Gli3*$^{+/-}$ mice exhibit more severe defects than either single mutant, indicating some overlapping functions in skeletogenesis (Mo et al., 1997). These mice exhibit defects similar to *Pax1/Pax9* double mutants, suggestive of defects early in sclerotome induction. However, the molecular basis for *Gli2* and *Gli3* mutant phenotypes and the role of each Gli in sclerotome development have not been investigated.

To determine whether *Gli2* and *Gli3* are required for Shh-dependent sclerotome induction, we examined Shh target gene expression in *Gli2/Gli3* compound mutants. We find that sclerotomal gene expression is severely reduced in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mice and that at least one copy of either *Gli2* or *Gli3* is required in the somitic mesoderm to confer Shh-responsiveness. We also investigated the specific role of each Gli in activating Shh target genes by overexpression in the PSM in vitro. We find that each Gli displays preferential activation of different sclerotomal targets involved in Shh-directed patterning, proliferation and negative feedback.

Materials and methods

Mice

Gli2$^{+/-}$ mice in a CD1 background (Mo et al., 1997) and *Gli3*$^{XtJ+/-}$ mice in a C3H background (Jackson Laboratory) were crossed to obtain *Gli2*$^{+/-}$*Gli3*$^{+/-}$ mice, which were then mated to obtain various mutant combinations. Genotyping using yolk sac DNA was performed by the polymerase chain reaction (PCR) using primers described elsewhere (Mo et al., 1997; Maynard et al., 2002).

Radioactive section in situ hybridization

Mice at embryonic day 9.5 (E9.5) were fixed overnight in 4% paraformaldehyde and cryosectioned at 12 μm. Riboprobes were labeled with [α-^{35}S]UTP (Amersham Pharmacia) using T3 or T7 RNA Polymerase (Promega). Radioactive section in situ hybridization was performed as described (Frohman et al., 1990). Slides were exposed to NBT emulsion (Kodak), developed, stained by Hematoxylin, and mounted in Permount (VWR). Images were photographed under dark field illumination to visualize silver granules and presented with the corresponding bright-field images.

Whole-mount in situ hybridization

Whole-mount in situ hybridization using digoxigenin (DIG)-UTP (Roche) labeled riboprobes was performed as described (Buttitta et al., 2003). Probes for *Pax1*, *Sim1* and *Pax3* have been described previously (Fan and Tessier-Lavigne, 1994). *Myf5* probe was a gift from Dr M. Buckingham. *Pax9* and *Sox9* probes were generated by reverse transcription coupled with PCR (RT-PCR) of 0.45kb and 1kb regions of the respective transcripts. Primers used for RT-PCR are available upon request. Embryos hybridized to *Pax9*, *Shh* and *Sox9* probes exhibited higher background and were destained in methanol resulting in a bluish signal. Embryos were photographed using an Axiocam camera. Selected embryos were cryosectioned at 20 μm and mounted in Crystal Mount (Biomeda).

Explant induction assays

E9.5 mouse PSM explants from CD1 mice, or *Gli2/Gli3* progeny, were cultured in collagen gels as described (Fan and Tessier-Lavigne, 1994). For inductions in the presence of cycloheximide, PSM was cultured for 10 hours prior to exposure to Shh-N conditioned media, 1 μg/ml cycloheximide (Sigma), or the combination for 8 hours. Shh-N and control conditioned media were collected from COS cells as described (Fan et al., 1995) and used at 500 μg/ml Shh-N. RNA was isolated from explants by RNAsol and used for RT-PCR with 30 cycles of amplification. Primer sequences are available at http://www.ciwemb.edu/labs/fan/index.html. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. Images of gels were captured using a UVP 7500 Gel Documentation System and quantified using Image-Quant v.1.2 (Amersham Pharmacia). All RT-PCR assays were performed within the linear range of amplification for each product as determined by quantitative real-time PCR.

Adenovirus production and explant infection

Adenoviruses carrying full-length mouse Gli1, mouse Gli2, human Gli3 or N-terminally truncated Gli2 as C-terminal EGFP (Clontech) fusion proteins; wild-type Smoothened (Smo); an activated form of Smo (Smo-M2 designated here as Smo*) (Xie et al., 1998); or EGFP driven by a CMV promoter were constructed using the AdenoX system (Clontech). Production of fusion proteins was confirmed by western blot using anti-EGFP (Molecular probes) or anti-Gli1, Gli3 and Smo antibodies (Santa Cruz). For infection, explants were cultured in the presence of 0.5-1.0×10^8 plaque-forming units (pfu) of adenovirus for ~100% infection. Protein expression levels appeared similar as assessed visually by GFP fluorescence. Infected explants were used for RT-PCR or fixed and processed for immunofluorescence as described (Lee et al., 2001).

Results

Gli gene expression in the early somites

Despite the importance of Gli genes in axial skeletal development, a thorough examination of their expression in the early mouse somites has not been reported. To determine which Gli genes might play a role in sclerotome induction, we performed radioactive in situ hybridization on posterior regions of mice at E9.5 to investigate *Gli* expression within the early somite.

Expression of *Gli1* is absent throughout the PSM. Upon formation of the somites, *Gli1* is expressed in the ventral domain (Fig. 1A,A′). Transverse sections through the early somites reveal expression in the most ventromedial domain of the sclerotome (Fig. 1D,D′). By comparison, *Gli2* is more widely expressed. *Gli2* is weak in the posterior two-thirds of the PSM, but becomes stronger in the anterior domain (Fig. 1B,B′). *Gli2* expression is absent from the dermomyotome but found in the sclerotome, expanding more dorsally and laterally than *Gli1* (Fig. 1E,E′). *Gli3* exhibits the broadest expression of all three genes. *Gli3* is expressed throughout the anterior two-thirds of the PSM as well as both dorsal and ventral domains of the early somites (Fig. 1C,C′). Transverse sections reveal that *Gli3* is expressed throughout the sclerotome, extending laterally like *Gli2*, but has stronger expression in the dermomyotome (Fig. 1F,F′). Thus, all three Gli genes are expressed in the ventral domain of the early somites. As *Gli2* and *Gli3* are expressed in the anterior PSM, they may act earliest in sclerotome patterning.

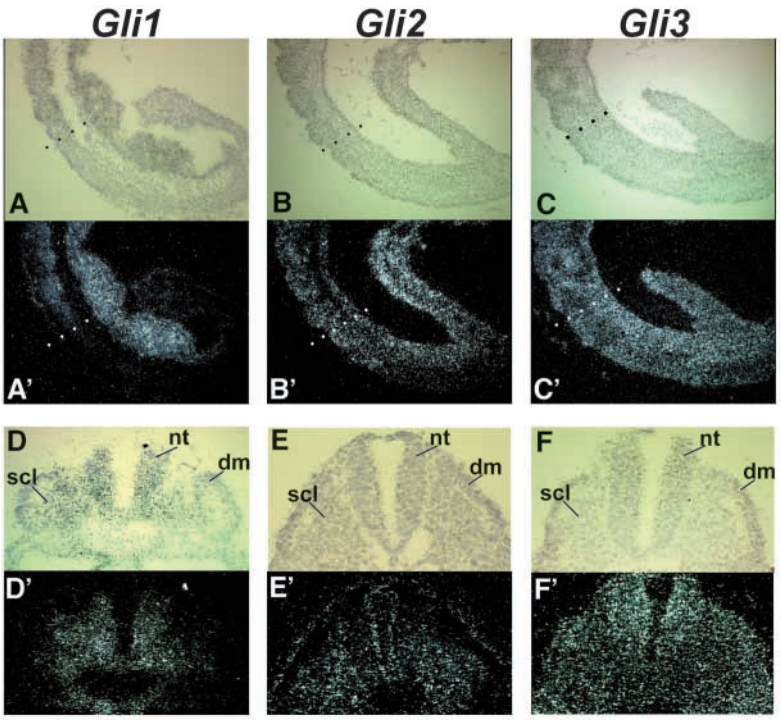

Fig. 1. All three Gli genes are expressed in the ventral somite. Gene expression within the PSM and early somites was examined by radioactive section in situ hybridization on sagittal (A-C′) and transverse (D-F′) sections through E9.5 embryos. Bright-field images (A-F) are shown with corresponding dark field images (A′-F′). Radioactive section in situ hybridization signal corresponds to silver granules highlighted by dark-field illumination. Dotted lines indicate the boundary between the PSM and first fully formed somite (A-C′). *Gli1* is expressed upon somite formation (A,A′) in the sclerotome (D,D′). *Gli2* is expressed in the anterior PSM and the somites (B,B′,E,E′). *Gli3* is expressed in the PSM (C,C′) and the somites (F,F′). nt, neural tube; dm, dermomyotome; scl, sclerotome.

Skeletal defects in *Gli2*$^{-/-}$, *Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ mice are not due to an initial defect in sclerotome induction

The skeletal defects observed in *Gli2* and *Gli3* mutants are suggestive of defects in sclerotome gene induction (Mo et al., 1997). We therefore examined the expression of the sclerotomal markers *Pax1*, *Pax9* and *Sox9* in *Gli2*, *Gli3* and *Gli2/Gli3* mutant mice by whole-mount in situ hybridization. Surprisingly, *Pax1* and *Pax9* expression appeared normal in *Gli2*$^{-/-}$, *Gli3*$^{-/-}$, *Gli2*$^{-/-}$*Gli3*$^{+/-}$ and *Gli2*$^{+/-}$*Gli3*$^{-/-}$ embryos at E9.5 (Fig. 2A-D,A′-D′; see Fig. S1A at http://dev.biologists.org/supplemental/) and E10.5 (data not shown). *Sox9* expression also appeared normal in the early somites of *Gli2*$^{-/-}$, *Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos, but we did observe a loss of *Sox9* specifically in the mature anterior somites at E9.5 (Fig. 2, inset A″-D″) and E10.5 (data not shown). *Sox9* expression is normally found in both ventral and dorsal domains of the sclerotome in anterior somites (arrowheads in inset Fig. 2A″). Although we observed the most severe loss of *Sox9* in *Gli3*$^{-/-}$ embryos to be specific to the dorsal sclerotome domain, *Gli2*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos exhibited a reduction of *Sox9* expression in both ventral and dorsal sclerotome domains (arrowheads in inset Fig. 2B″,D″; see Fig. S1B at http://dev.biologists.org/supplemental/). This is consistent with the finding that *Gli2*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos exhibit more severe skeletal defects than *Gli3*$^{-/-}$ embryos (Mo et al., 1997), and suggests that the skeletal defects observed in these mice are due to an inability to maintain high levels of *Sox9* expression in maturing somites.

Gli2$^{-/-}$*Gli3*$^{-/-}$ mice have severely reduced sclerotomal gene expression

In contrast to the other allelic combinations, *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos display dramatically reduced *Pax1* and *Pax9* expression and undetectable *Sox9* expression in the somites at E9.5 (Fig. 2E-E″). Both the initiation and maintenance of *Pax1* and *Pax9* were affected, as only a low level of expression is restricted to a small number of interlimb somites (arrowheads in Fig. 2E,E′). This demonstrates that loss of both *Gli2* and *Gli3* leads to a severe defect in sclerotomal gene expression and that one copy of either *Gli2* or *Gli3* is sufficient to substantially restore *Pax1* and *Pax9* expression in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ and *Gli2*$^{+/-}$*Gli3*$^{-/-}$ embryos.

Somite morphology is abnormal in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos

The severe loss of sclerotomal gene expression in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos prompted us to investigate the somite morphology in these embryos. In wild-type, *Gli2*$^{+/-}$*Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos, the dermomyotome (dm), myotome (my) and sclerotome (scl) are visible and organized (Fig. 3A, see Fig. S1C at http://dev.biologists.org/supplemental/). By contrast, in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites, the dermomyotome had an abnormal upside down U-shape, owing to ectopic epithelium that extends ventromedially adjacent to the neural tube (Fig. 3B, open arrowheads). We also frequently observed a closed sphere of epithelium in the trunk somites of these embryos (indicated by broken lines in Fig. 3H,J,L,P). In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, mesenchyme resembling the sclerotome was present but reduced in size, and the myotome was not clearly distinguishable.

Dermomyotomal and myotomal gene expression is abnormal in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos

The abnormal epithelium in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos suggested defects in dermomyotome patterning. We therefore investigated the expression of the dermomyotomal marker *Pax3* in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos by whole-mount in situ hybridization. Expression of *Pax3* in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos appeared largely normal by wholemount at E9.5 (Fig. 3C,D). However transverse sections through the trunk revealed weak

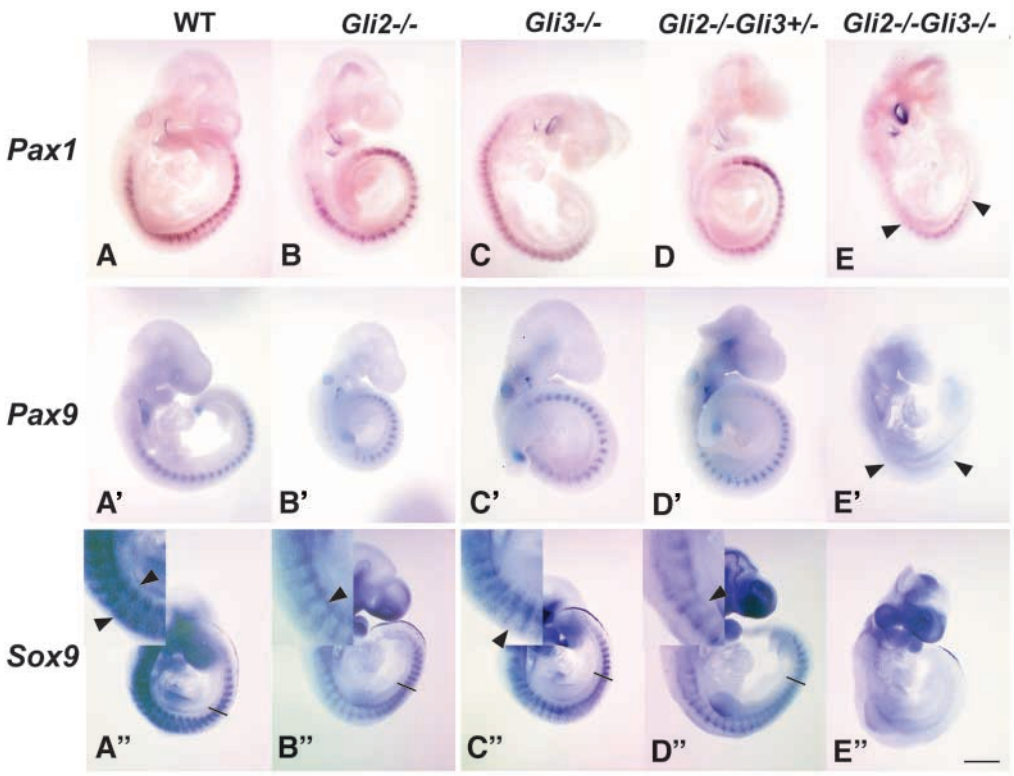

Fig. 2. *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mutant mice exhibit a severe loss of sclerotomal gene expression. Expression of *Pax1*, *Pax9* and *Sox9* was assessed by whole-mount in situ hybridization in wild type (A,A′,A″), *Gli2*$^{-/-}$ (B,B′,B″), *Gli3*$^{-/-}$ (C,C′,C″), *Gli2*$^{-/-}$*Gli3*$^{+/-}$ (D,D′,D″) and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ (E,E′,E″) E9.5 embryos. *Pax1* and *Pax9* are expressed normally in all allelic combinations (A-D,A′-D′) except for *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, which exhibit a severe reduction and delay in *Pax1* and *Pax9* (between arrowheads in E and E′) expression. In *Gli3*$^{-/-}$ embryos *Sox9* expression is reduced in the anterior somites, with the dorsal region most affected (compare arrowheads in inset of A″ and C″). *Gli2*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos exhibit reduced *Sox9* in both ventral and dorsal domains of anterior somites (arrowheads in B″ and D″). *Sox9* appears normal in the posterior somites of these embryos (indicated by the black line A″-D″). *Sox9* is undetectable in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos. Scale bar: 0.5 mm.

Pax3 expression in the ectopic ventromedial epithelium (Fig. 3H).

We next examined the expression of the myotomal marker and *Gli* target gene *Myf5* (Gustafsson et al., 2002) by whole-mount in situ hybridization. *Myf5* expression is normally restricted to the dorsomedial lip of the dermomyotome and the developing myotome. In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, *Myf5* expression appeared diffuse and laterally expanded throughout the somites (arrowheads in Fig. 3E,F). In contrast to *Pax3* and *Myf5*, expression of the lateral somite marker *Sim1* appeared normal in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (arrowheads in Fig. 3I,J).

Somites of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos exhibit more severe defects than somites of *Shh*$^{-/-}$*Gli3*$^{-/-}$ embryos

The somite defects we observed in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mutants appear less severe than those described for *Shh* mutants (Chiang et al., 1996). Loss of *Gli3* in a *Shh* mutant background rescues specific aspects of neural tube patterning due to the removal of *Gli3* repressor function (Litingtung and Chiang, 2000), but whether *Gli3* also acts as a repressor in the somites has not been examined. To test whether *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos more closely resemble those in which both the Shh signal and Gli3 repressor activity is lost, we compared the expression of *Pax1* and *Myf5* in *Gli2*$^{-/-}$*Gli3*$^{-/-}$, *Shh*$^{-/-}$ and *Shh*$^{-/-}$*Gli3*$^{-/-}$ embryos.

Transverse sections through the trunk reveal that the weak *Pax1* expression observed in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos is restricted to a group of medially located mesenchymal cells within an epithelial sphere (Fig. 3L). In *Shh*$^{-/-}$ embryos *Pax1* expression in the trunk was undetectable in the ventral mesenchyme (Fig. 3M). Strikingly, strong *Pax1* expression was restored in *Shh*$^{-/-}$*Gli3*$^{-/-}$ embryos (Fig. 3N). In wild-type embryos, *Myf5* expression is restricted to the developing myotome (Fig. 3O). In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, *Myf5* expression was observed throughout mesenchymal cells within the epithelial spheres without forming a distinct layer (Fig. 3P). Expression of *Myf5* in *Shh*$^{-/-}$ embryos was not as tightly organized as in the wild-type and dorsomedial expression was reduced (arrowhead in Fig. 3Q). By contrast, in *Shh*$^{-/-}$*Gli3*$^{-/-}$ embryos strong dorsomedial expression of *Myf5* was restored, but remained less organized than in the wild type (Fig. 3R). These findings demonstrate that *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos exhibit more severe sclerotomal and myotomal phenotypes than *Shh*$^{-/-}$*Gli3*$^{-/-}$ embryos, and that similar to the developing neural tube, loss of *Gli3* function ameliorates the sclerotomal phenotype of *Shh* mutants.

At least one copy of either *Gli2* or *Gli3* is required for Shh-dependent sclerotome induction tissue autonomously

Motoyama et al. (Motoyama et al., 2003) have demonstrated

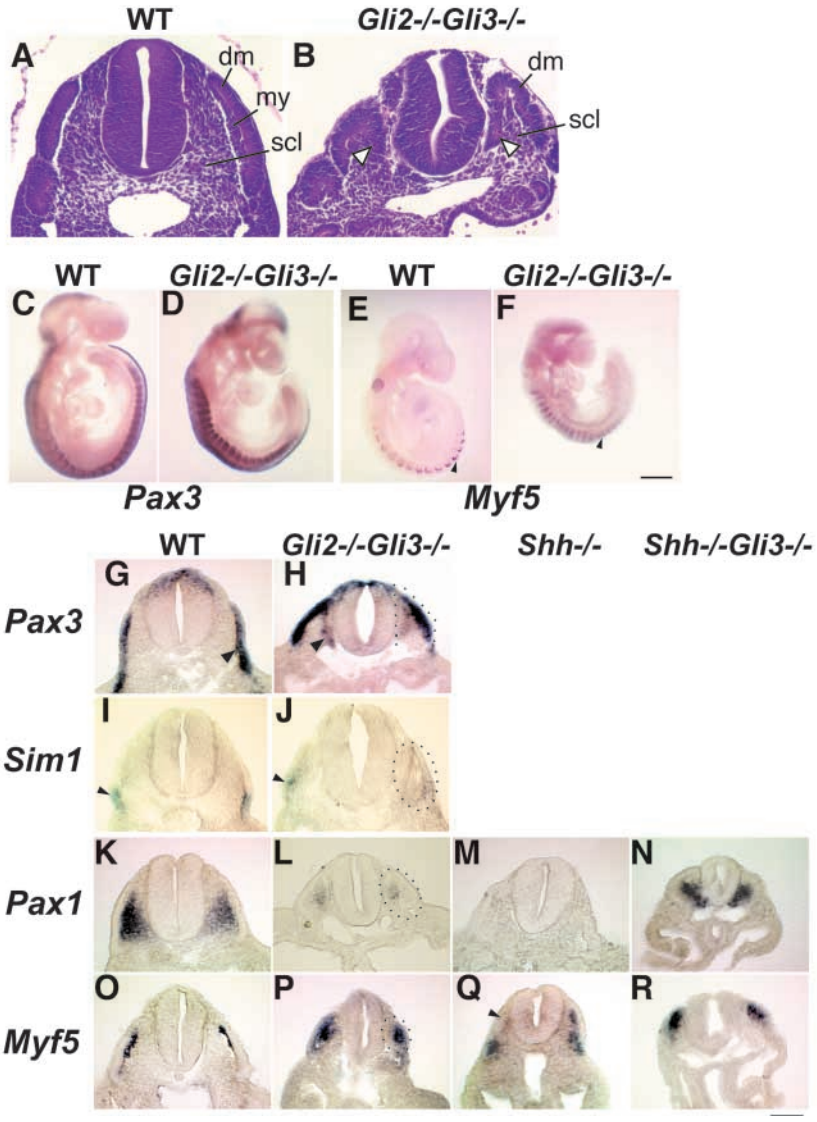

Fig. 3. Dorsoventral patterning of the somite is abnormal in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos. Transverse sections through the trunk of wild-type (A), and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ (B) embryos at E10.5 were stained to reveal tissue morphology. In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, ectopic epithelium extends ventromedially (open arrowheads), the sclerotome is reduced and myotome is not distinguishable. dm dermomyotome, scl sclerotome, my myotome. Expression of *Pax3* and *Myf5* were assessed by whole-mount in situ hybridization in wild-type (C,E) and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ (D,F) E9.5 embryos. *Pax3* in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos appeared normal in wholemount. *Myf5* expression in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites was diffuse and expanded laterally. Additionally, dorsomedial-specific expression (arrowhead in E) was absent (arrowhead in F). Scale bar: 0.5 mm. (G-R) Expression of *Pax3*, *Sim1*, *Pax1* and *Myf5*, were assessed by whole-mount in situ hybridization and sectioned. Transverse sections though the trunks of E.9.5 wild-type (G,I,K,O), *Gli2*$^{-/-}$*Gli3*$^{-/-}$ (H,J,L,P), *Shh*$^{-/-}$ (M,Q) and *Shh*$^{-/-}$*Gli3*$^{-/-}$ (N,R) embryos were collected. Broken lines outline ectopic epithelium in H,J,L,P. In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos *Pax3* is expressed in the ectopic ventromedial epithelium of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites (arrowhead in H). *Sim1* expression is normal in wild-type (arrowhead in I) and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mutants (arrowhead in J). *Pax1* expression is weak in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (L), undetectable in the trunk of *Shh*$^{-/-}$ embryos (M), but restored in *Shh*$^{-/-}$*Gli3*$^{-/-}$ embryos (N). In wild type, *Myf5* is restricted to the layer of myotome (O), whereas in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites, *Myf5* is expressed throughout the mesenchymal cells within the epithelial spheres (P). In *Shh*$^{-/-}$ somites, dorsomedial expression of *Myf5* is reduced (arrowhead in Q), whereas in *Shh*$^{-/-}$*Gli3*$^{-/-}$ somites *Myf5* is restored in the dorsomedial lip (R). Scale bar: 50 μm.

that *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos have severely reduced expression of *Shh* in the brain and brachial regions. Thus, the reduced sclerotomal gene expression in these embryos might be due to a loss of *Shh* expression. As shown in Fig. 4A, *Shh* expression in the notochord and floorplate of double mutants is significantly weaker than in wild type. This finding questions whether the reduction in sclerotome gene expression in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos is due to a reduction of Shh, or an inability to transcriptionally activate Shh targets in the somites. To distinguish between these possibilities, we tested whether PSM isolated from *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos can activate sclerotomal genes in response to exogenously provided Shh. If loss of Shh is the cause of the sclerotomal defects, PSM from *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos will be able respond to exogenous Shh. If an inability to transcriptionally activate Shh target genes is the cause of the sclerotomal defects, exogenous Shh will not be able to induce Shh target genes in the PSM from *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos.

E9.5 PSM isolated from various *Gli2/Gli3* allelic combinations was cultured either in the absence or presence of 500 ng/ml Shh-N for 24 hours, and Shh target gene induction was assessed by RT-PCR. β-actin expression was assessed as a control for normalization and the Shh target genes tested include *Ptch*, cyclin D2 (*Ccnd2*), *Hhip*, *Pax1* and *Pax9*. As shown in Fig. 4B, wild-type PSM responded normally to Shh-N by inducing all target genes tested. PSM from *Gli3*$^{-/-}$ embryos also responded to Shh-N by inducing target genes. However, a 2.3-fold increase in the level of *Pax9* expression in the absence of Shh was observed. *Gli2*$^{-/-}$ PSM showed an intermediate response to Shh-N, with normal induction of *Ccnd2*, *Pax1* and *Gli1* expression, but a 3.0-fold reduction in *Pax9* induction. Interestingly, *Ptch* expression was not induced by Shh in the *Gli2*$^{-/-}$ PSM. By contrast, *Gli2*$^{-/-}$*Gli3*$^{+/-}$ PSM and *Gli2*$^{+/-}$*Gli3*$^{-/-}$ PSM (see Fig. S1D at http://dev.biologists.org/supplemental/) exhibited normal Shh-induction of *Ptch*, *Pax1* and *Gli1* but *Gli2*$^{-/-}$*Gli3*$^{+/-}$ PSM still exhibited a 2.1-fold reduction in *Pax9* induction. Consistent with the expression data in the mutants, this result suggests that one copy of either *Gli2* or *Gli3* is sufficient to activate Shh target genes. Last, *Gli2*$^{-/-}$*Gli3*$^{-/-}$ PSM did not exhibit any responsiveness to exogenous Shh-N, as the expression of all target genes was unchanged in the presence of Shh-N,

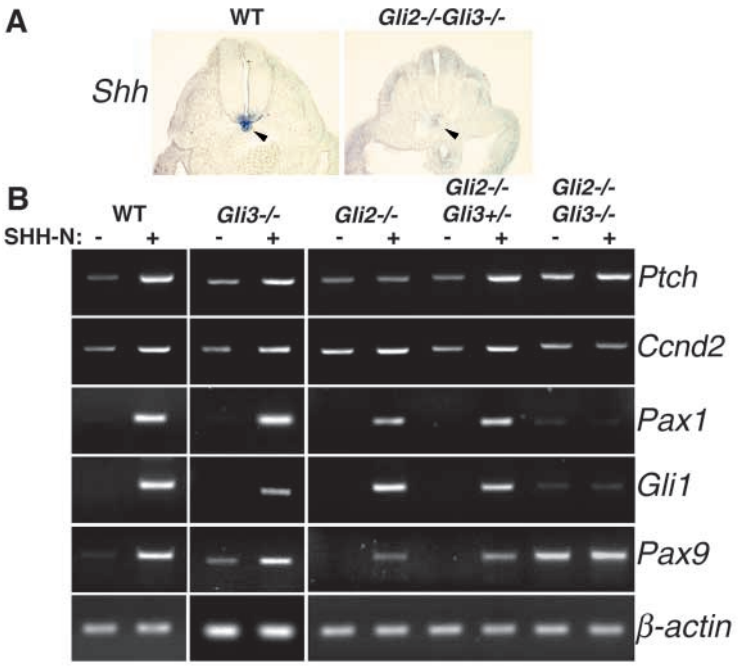

Fig. 4. One copy of either *Gli2* or *Gli3* is required for tissue autonomous Shh responsiveness. (A) *Shh* expression was assessed by whole-mount in situ hybridization at E9.5. Transverse sections through the trunk reveal dramatically reduced *Shh* in the notochord and floorplate of $Gli2^{-/-}Gli3^{-/-}$ embryos (compare arrowheads in A). (B) PSM tissue was isolated from E9.5 wild-type, $Gli3^{-/-}$, $Gli2^{-/-}$, $Gli2^{-/-}Gli3^{+/-}$ and $Gli2^{-/-}Gli3^{-/-}$ embryos and cultured in the presence (+) or absence (–) of Shh-N for 24 hours. Induction of target genes was assessed by RT-PCR. β-Actin serves as a control for normalization. Compared with wild-type PSM, $Gli3^{-/-}$ PSM shows Shh-responsiveness with an increase in *Pax9* expression in the absence of Shh-N (*n*=4). $Gli2^{-/-}$ PSM shows reduced responsiveness in activating *Ptch* and *Pax9*, but normal induction of other targets (*n*=4). In $Gli2^{-/-}Gli3^{+/-}$ PSM, all targets are induced with some reduction in *Pax9* induction (*n*=2). In $Gli2^{-/-}$ $Gli3^{-/-}$ PSM, all targets tested are expressed identically in the presence and absence of Shh-N. There is increased basal expression of *Ptch* and *Pax9* in $Gli2^{-/-}Gli3^{-/-}$ PSM independent of Shh-N (*n*=3).

indicating that *Gli2* and *Gli3* are required for Shh-induction of these genes. Interestingly, the expression of *Ptch*, *Gli1* and *Pax9* in the absence of Shh-N was increased (3.5-, 2.0- and 10-fold respectively) above wild-type control levels suggesting one copy of *Gli3* is required for repression of these genes in the absence of Shh. These findings demonstrate a tissue autonomous requirement in the PSM for one copy of either *Gli2* or *Gli3* to mediate Shh induction of target genes.

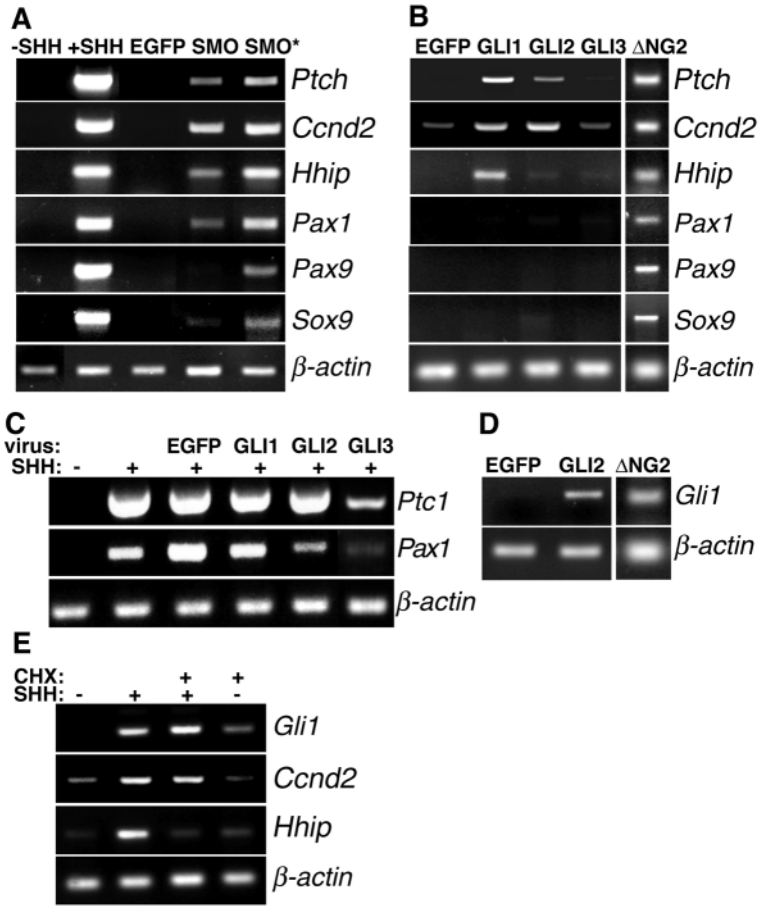

Fig. 5. ΔNG2 can mimic Shh and Smo* induction of targets in presomitic mesoderm. (A) PSM explants were cultured alone (–Shh), with Shh-N (+Shh) or infected with adenoviruses carrying EGFP, Smo or Smo* for 24 hours and analyzed for induction of Shh target genes by RT-PCR. Shh-N induces target genes, while infection with EGFP virus does not. Expression of Smo* induces all Shh targets tested (*n*=8). (B) PSM explants were infected with adenoviruses carrying either EGFP, full-length Gli1, Gli2, Gli3 or ΔNG2 for 24 hours and analyzed for induction of Shh targets by RT-PCR. Gli1 induced *Ptch*, *Hhip* and moderate levels of *Ccnd2*. Gli2 induced *Ccnd2*, and moderate levels of *Ptch*. Gli3 did not induce any Shh targets, while ΔNG2 activated all targets tested (*n*=8). (C) PSM was cultured for 24 hours in the absence (–) or presence (+) of Shh-N and adenovirus as indicated. Expression of *Ptch* and *Pax1*, was assessed by RT-PCR. Infection with EGFP and Gli1 does not affect induction of target genes by Shh, while infection with Gli2 does seem to have a moderate inhibitory affect specifically on *Pax1*. Infection with Gli3 acts to repress Shh induction of both *Ptch* and *Pax1* (*n*=3). (D) PSM infected with Gli2 and ΔNG2 induce *Gli1* at 24 hours (*n*=8). (E) PSM explants cultured alone, with Shh-N, with 1 μg/ml cycloheximide (CHX) or the combination for 9 hours were analyzed for *Gli1*, *Ccnd2* and *Hhip* by RT-PCR. In the presence of CHX, Shh activates only *Ccnd2* and *Gli1*, but not *Hhip* (*n*=2). CHX (1 μg/ml) effectively inhibits over 90% of new protein synthesis under similar culture conditions (Fu et al., 2002).

ΔNG2 mimics Shh and Smo* signaling in the somitic mesoderm

To further dissect the function of each Gli in mediating specific target gene expression, we generated adenoviral vectors for overexpression of the Gli genes in the PSM. Adenoviral vectors contained either full-length Gli1, Gli2, Gli3 or an activated N-terminally truncated form of Gli2 (ΔNG2) (Sasaki et al., 1999), as C-terminal fusions to EGFP driven by a cytomegalovirus (CMV) promoter (see Fig. S2A at http://dev.biologists.org/supplemental/). Additional vectors serving as negative and positive controls contained EGFP alone, wild-type Smo or a constitutively active form of Smo (Smo*) (Xie et al., 1998). Production of the desired protein products upon adenoviral infection was confirmed by western analysis (see Fig. S2B-D at http://dev.biologists.org/supplemental/) and functionality of Gli-EGFPs was tested using a Shh-responsive cell line and a luciferase reporter downstream of eight Gli binding sites (see Fig. S2E at http://dev.biologists.org/supplemental/). Virally expressed Gli genes, Smo and Smo* functioned as predicted from previous studies (Sasaki et al., 1997; Sasaki et al., 1999; Shin et al., 1999; Taipale et al., 2000).

As Shh signaling in the somite is thought to occur via the conserved HH-PTC-Smo signaling pathway (Zhang et al., 2001), we first wanted to establish whether overexpression of Smo* by adenovirus can activate Shh target genes in cultured PSM. To test this, PSM from E9.5 mice was cultured alone, with Shh-N (500 ng/ml), or infected with adenovirus carrying EGFP, Smo or Smo* for 24 hours and analyzed for induction of the Shh target genes *Ptch*, *Ccnd2*, *Hhip*, *Pax1*, *Pax9* and *Sox9*. As shown by RT-PCR in Fig. 5A, treatment with Shh-N induces all Shh target genes tested, while infection with EGFP adenovirus does not affect any Shh targets. Expression of Smo induces *Ptch*, *Ccnd2*, *Hhip* and weak *Pax1*, while expression of Smo* induces all Shh target genes tested. In conclusion, Shh target gene induction in the somite can be recapitulated by overexpression of Smo* in the PSM.

As all three Gli genes are expressed in the sclerotome, we next investigated whether overexpression of Gli genes can mimic Shh transcriptional target gene induction in the PSM. PSM explants were infected with adenovirus carrying EGFP alone or each of the Gli genes, for 24 hours and analyzed for Shh target gene expression. As shown in Fig. 5B, Gli1 and Gli2, each induced a subset of Shh target genes, whereas Gli3 did not induce any targets. Gli1 acted as a strong activator of *Ptch* and *Hhip* expression, but had a moderate effect on *Ccnd2* expression. By contrast, Gli2 was a stronger activator of *Ccnd2* (6.5-fold induction by Gli1 compared to 8.0-fold by Gli2), but had a weaker effect on *Ptch* (34-fold induction by Gli1 compared with 15-fold by Gli2) and no effect on *Hhip*. As none of the full-length Gli genes activated expression of *Pax1*, *Pax9* and *Sox9*, we tested an N-terminally truncated form of Gli2, ΔNG2, previously shown to strongly activate Shh target genes in the neural tube (Sasaki et al., 1999) and skin (Mill et al., 2003). Indeed, infection with ΔNG2 adenovirus for 24 hours activated all Shh target genes tested in the PSM, suggesting that an activated form of Gli2 may mediate Shh signaling in the somite.

We next tested the activity of full-length Gli genes in the presence of Shh-N to determine whether the Gli genes can also exhibit repressive effects when overexpressed. PSM was cultured for 24 hours with each Gli adenovirus in the absence or presence of 500 ng/ml Shh-N, and expression of *Ptch* and *Pax1* was assessed by RT-PCR. As shown in Fig. 5C, infection with EGFP and Gli1 adenoviruses does not affect induction of *Pax1* and *Ptch* genes by Shh, while infection with Gli2 seems to have a moderate inhibitory affect (threefold) specifically on *Pax1*. By contrast, infection with Gli3 strongly represses Shh induction of both *Ptch* and *Pax1*. These results confirm the repressive ability of Gli3, and suggest that although full-length Gli2 can activate some Shh targets, full-length Gli2 expressed at high levels can also repress specific targets.

In other tissues *Gli2* has been shown to be critical for induction of *Gli1* expression (Bai et al., 2002; Ding et al., 1998). Similarly, we found that Gli2 and ΔNG2 can induce the expression of *Gli1* in the PSM, although neither Gli1 nor Gli3 induce *Gli1* expression (Fig. 5D and data not shown), demonstrating that Gli1 and Gli2 can preferentially activate different target genes.

Ptch, *Pax1* and *Pax9* are direct transcriptional targets of Shh signaling in the PSM that can be induced in the absence of protein synthesis (Dockter, 2000) (C.M.F., unpublished). We next tested whether *Gli1*, *Ccnd2* and *Hhip* are also direct targets of Shh signaling in the PSM. PSM explants were treated with Shh-N in the absence or presence of 1 μg/ml cycloheximide to inhibit protein synthesis. In the absence of protein synthesis Shh-N induces only the expression of *Gli1* and *Ccnd2*, but not of *Hhip* (Fig. 5E). Together with the Gli2 and ΔNG2 overexpression, these data support a linear pathway where Gli2 may be activated upon Shh signaling to induce *Gli1*, which in turn can induce the secondary target *Hhip*.

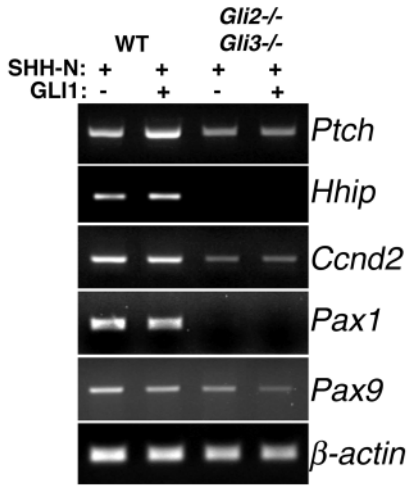

Fig. 6. Gli1 cannot restore Shh responsiveness in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ PSM. PSM from wild-type and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos was cultured with Shh-N in the presence (+) or absence (–) of Gli1 adenovirus for 24 hours. Induction of Shh target genes was assessed by RT-PCR. Infection with Gli1 adenovirus increased the expression of *Ptch* and *Hhip* but not other Shh target genes in wild-type PSM. In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ PSM infection with Gli1 adenovirus does not restore responsiveness of any Shh targets nor increase the expression of *Ptch* and *Hhip* (*n*=3).

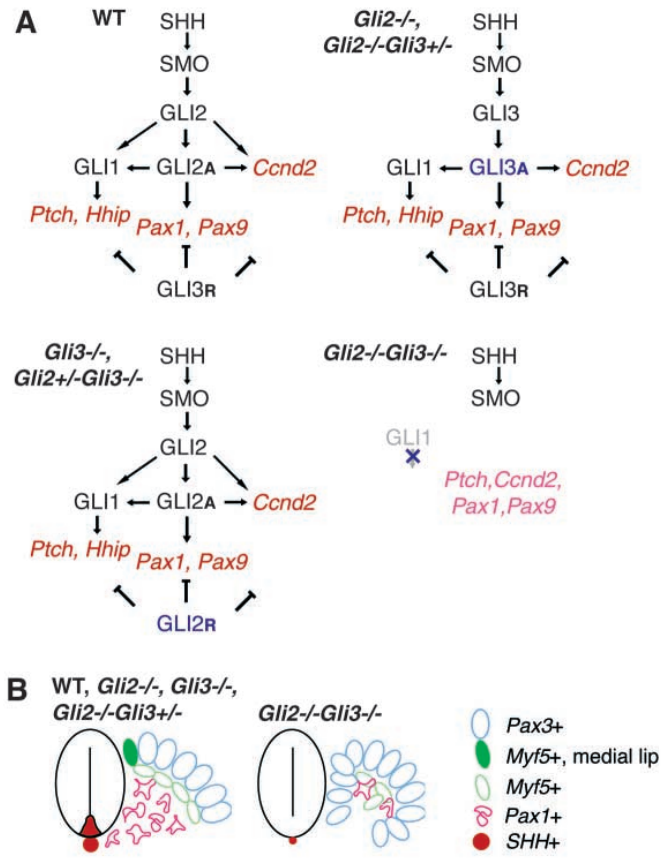

Fig. 7. A model for Gli-mediated Shh target gene induction in the somite. (A) In wild type, Shh signaling modifies Gli2 into a strong activator, Gli2A, to activate target genes, including *Gli1*. Gli1 acts secondarily to activate *Ptch* and *Hhip*. In the absence of Shh, or when overexpressed, Gli3 acts as a repressor, Gli3R. In $Gli2^{-/-}$ and $Gli2^{-/-}Gli3^{+/-}$, an activator function of Gli3 is revealed (in blue), to compensate for loss of *Gli2*. In $Gli3^{-/-}$ and $Gli2^{+/-}Gli3^{-/-}$, a repressor function of *Gli2* is revealed (in blue), to compensate for loss of Gli3. In wild-type embryos Gli2R and Gli3A may be present but play minor roles. This possibility is not illustrated for simplicity. In $Gli2^{-/-}Gli3^{-/-}$ embryos, both activator and repressor functions of Gli2 and Gli3 are lost resulting in a low level of Shh-independent expression of target genes. The low level of Gli1 in these embryos is non-functional (X). (B) The diagram illustrates somite morphology and gene expression in wild type and Gli mutants. Somite patterning and gene expression is largely normal in $Gli2^{-/-}$, $Gli3^{-/-}$ and $Gli2^{-/-}Gli3^{+/-}$ embryos (left). In $Gli2^{-/-}Gli3^{-/-}$ embryos, there is ventromedial expansion of *Pax3*-positive cells ($Pax3^{+}$), mixing of $Pax1^{+}$ and $Myf5^{+}$ cells, and expression of *Myf5* in the medial lip is lost (right).

Gli1 cannot restore Shh-responsiveness in $Gli2^{-/-}$ $Gli3^{-/-}$ PSM

Although PSM from $Gli2^{-/-}Gli3^{-/-}$ embryos does not activate Shh target genes in response to exogenous Shh-N, there is a low level of *Pax1* and *Pax9* expressed in $Gli2^{-/-}Gli3^{-/-}$ somites (Fig. 2E,E′). It is possible that *Gli1* expressed in the somites of $Gli2^{-/-}Gli3^{-/-}$ embryos can mediate some Shh target gene expression. We measured the level of *Gli1* expression in $Gli2^{-/-}Gli3^{-/-}$ embryos by quantitative real-time PCR and found it was reduced to 9% of wild-type levels (data not shown). To address whether this low level of *Gli1* is responsible for the *Pax1* and *Pax9* expression observed we tested whether expression of Gli1 in the $Gli2^{-/-}Gli3^{-/-}$ PSM could restore Shh-responsiveness. PSM explants from wild-type or $Gli2^{-/-}Gli3^{-/-}$ embryos were cultured in the presence of 500 ng/ml Shh-N and infected with either EGFP or Gli1 adenovirus for 24 hours. As shown in Fig. 6, infection with Gli1 adenovirus did not restore Shh-responsiveness in $Gli2^{-/-}Gli3^{-/-}$ PSM. We then extended these results by testing the Shh-responsiveness of somites from $Gli2^{-/-}Gli3^{-/-}$ embryos expressing endogenous *Gli1* (data not shown). $Gli2^{-/-}Gli3^{-/-}$ somites cultured with Shh-N also did not exhibit any Shh-responsiveness (data not shown). These findings demonstrate that in the absence of *Gli2* and *Gli3*, Gli1 cannot restore Shh-responsiveness in the somitic mesoderm. Surprisingly, we found that overexpression of Gli1 failed to increase *Ptch* and *Hhip* expression in $Gli2^{-/-}Gli3^{-/-}$ PSM, as it did in wild-type tissues (3.2- and 2.0-fold respectively), suggesting that Gli1 somehow requires *Gli2* or *Gli3* for transcriptional activity.

Discussion

Several reports have suggested Gli-independent mechanisms of Shh signaling in vertebrates (Krishnan et al., 1997a; Krishnan et al., 1997b; Lewis et al., 1999). However, our analysis of *Gli* function has unequivocally demonstrated a requirement for Gli2 and Gli3 in mediating Shh-induced sclerotomal gene expression. Through a combination of mutant studies and explant analysis, we reveal distinct functional activities for each *Gli* and identify cooperative roles for Gli genes in regulation of Shh target genes during mouse sclerotome development.

Skeletal defects in $Gli2^{-/-}$, $Gli3^{-/-}$ and $Gli2^{-/-}Gli3^{+/-}$ mice are likely due to loss of *Sox9* expression

We demonstrate that $Gli2^{-/-}$, $Gli3^{-/-}$ and $Gli2^{-/-}Gli3^{+/-}$ embryos have reduced expression of *Sox9* in the sclerotome of anterior somites at E9.5 and E10.5. A small reduction in *Sox9* expression can result in severe chondrogenic defects, as $Sox9^{+/-}$ cells cannot contribute to cartilage or bone in chimeric animals (Bi et al., 1999). We suggest that the loss of *Sox9* is thus a likely cause of the skeletal phenotypes observed in these mutants (Mo et al., 1997), as the domains of *Sox9* loss largely correspond to the specific skeletal abnormalities observed in each mutant. The normal *Sox9* expression in the early somites of these mutants suggests that the defect is due to an inability to maintain proper levels of *Sox9* expression in the mature somites. Recent work has shown that although Shh initiates *Sox9* expression, BMP signals maintain a positive regulatory loop leading to sustained *Sox9* expression (Zeng et al., 2002). Our data is consistent with either the establishment or maintenance of this positive regulatory loop being affected in these mutants.

Gli2 can act as a repressor and *Gli3* can act as an activator for Shh target genes in the somites

Although Gli3 has been shown to activate *Gli1* and *Ptch* promoters in cultured cells (Dai et al., 1999; Shin et al., 1999), the physiological role of this activator function is not clear. We provide two lines of evidence supporting a Shh-induced activator function for *Gli3* in the somites. First, $Gli2^{-/-}Gli3^{+/-}$

embryos express levels of *Pax1* and *Pax9* comparable with wild-type levels. Second, *Gli2*$^{-/-}$*Gli3*$^{+/-}$ PSM can activate expression of Shh target genes while *Gli2*$^{-/-}$*Gli3*$^{-/-}$ PSM cannot. This indicates that one copy of *Gli3* is sufficient to mediate Shh-dependent gene induction.

Although Gli2 is generally considered to be an activator of Shh signaling, in vitro studies suggest that it may also possess repressor function (Sasaki et al., 1999). Recent studies in zebrafish embryos have uncovered a possible repressor function for Gli2 specifically in telencephalon and muscle development (Karlstrom et al., 2003; Wolff et al., 2003). We provide evidence of a repressor function for *Gli2* on direct targets of Shh in the sclerotome. Adenoviral overexpression of Gli2 in PSM can repress Shh induction of *Pax1* (Fig. 5C), and *Gli2*$^{+/-}$*Gli3*$^{-/-}$ PSM does not exhibit the same level of Shh target de-repression as *Gli2*$^{-/-}$*Gli3*$^{-/-}$ PSM (Fig. 4B; see Fig. S1D at http://dev.biologists.org/supplemental/), indicating that one copy of *Gli2* is sufficient to mediate some repressive functions similar to those carried out by *Gli3*. The finding that the activator *Gli1* can replace *Gli2* in vivo supports the notion that *Gli2* functions solely as an activator (Bai and Joyner, 2001). However, our data illustrate that Gli2 repressor function is probably masked by the strong Gli3 repressor and is revealed only in the absence of Gli3, or when overexpressed in vitro. Thus, in addition to their overlapping activator functions, we suggest Gli2 and Gli3 also share repressor functions during somite development in mice.

Gli genes and somite patterning

The temporal and spatial expression of Gli genes in the developing paraxial mesoderm is consistent with Gli2 and Gli3 mediating initial Shh-induced sclerotomal gene expression. Our overexpression and mutant analysis together support a model for *Gli* function in the somite as illustrated in Fig. 7A. In wild-type somites, Gli2 is modified to a strong activator (similar to ΔNG2) upon Shh signaling, and functions as the primary mediator of Shh signaling to promote both proliferation and patterning programs. Gli1 then acts downstream of Gli2, primarily activating Shh-dependent negative feedback mechanisms. However, as loss of *Gli1* does not affect somite development (Park et al., 2000), this function of Gli1 may be compensated by the activated form of Gli2. In the absence of Shh or when overexpressed, Gli3 represses Shh-induced programs. If Gli2 is lost, Gli3 activator function is revealed that compensates for the loss of Gli2. Conversely, in the absence of Gli3, Gli2 repressor function is revealed which compensates for the loss of Gli3. In the absence of both Gli2 and Gli3, the low level of Gli1 is non-functional and Shh responsiveness, as well as target repression, are lost, resulting in a low level of Shh-independent target gene expression.

Previous studies of Gli genes in the somites have centered on specification of myogenic precursors. In zebrafish *Gli1* and *Gli2* are required to specify most myogenic cell types in a semi-redundant fashion (Karlstrom et al., 2003; Wolff et al., 2003), whereas in the chick, *Gli3* is specifically upregulated in the myotome, suggesting it contributes to myogenesis (Borycki et al., 1998). Although *Myf5* has been shown to be a target of *Gli* regulation (Gustafsson et al., 2002), *Myf5* expression is detectable in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (Fig. 3F), suggesting a *Gli*-independent pathway for *Myf5* expression in the mouse. As roles for Gli genes in chick and zebrafish sclerotome are not known, our data provides the first description of Gli functions in sclerotome induction.

Shh functions are divided preferentially amongst different Gli genes in the somite

Although previous analyses of *Gli2* and *Gli3* have revealed cooperative functions in several contexts, including the neural tube, lung, trachea and teeth (Hardcastle et al., 1998; Motoyama et al., 1998; Motoyama et al., 2003; Persson et al., 2002), how multiple Gli proteins cooperate together in mediating Shh response is not well understood. Our analysis of Gli function extends the previous studies by addressing the tissue autonomous contributions of each Gli gene to regulation of several direct Shh targets in the somitic mesoderm. Using mutant explants, we find that Shh targets display different sensitivities to loss of each Gli gene. For example, *Pax9* is the most sensitive to loss of Gli repressor function, as it is abnormally expressed in the absence of Shh in *Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ PSM (Fig. 4B). By comparison, *Ptch* is the most sensitive to loss of Gli2 activator function, as it cannot be induced in *Gli2*$^{-/-}$ PSM by Shh. Thus, Gli genes may mediate Shh induction of target genes in at least two ways. One way is for Shh to inhibit formation of Gli repressors, which may be the primary method of Shh induction of *Pax9*. A second way is for Shh to stabilize formation of Gli activators, which compete with Gli repressors for target gene activation. This is consistent with the regulation we observe for *Ptch*, which cannot be activated in the absence of *Gli2* until one copy of *Gli3* is lost, reducing the concentration of Gli repressor. For *Pax1* and *Gli1* a small amount of activator, such as that possibly formed by Gli3 in the presence of Shh in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos, is sufficient to mediate gene expression. This demonstrates additional distinctions in the functional output of each Gli and suggests a previously unappreciated ability of Gli genes to distinguish between different promoter/enhancer contexts.

Mis-patterning of the *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somite results from a complete loss of responsiveness to Hedgehog signaling

Our data suggest that the somite phenotypes of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos result from a loss of Shh responsiveness and *Gli3* repressor function. However, the somitic phenotypes observed in these embryos are more severe than those of *Shh*$^{-/-}$*Gli3*$^{-/-}$ embryos. Comparisons of *Shh* and *Smo* mutants have revealed a compensatory role in the somite for another HH family member, Indian hedgehog (Ihh). As *Smo* mutants lose all Hh-responsiveness, they cannot be compensated by Ihh, and thus exhibit more severe phenotypes than *Shh* mutants (Zhang et al., 2001). We suggest that *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos also lose all Hh-responsiveness based upon the following evidence. First, *Pax1* expression in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites is weaker than that in *Shh*$^{-/-}$*Gli3*$^{-/-}$ somites, suggesting a lack of rescue by Ihh. Second, we observe ectopic *Pax3* in the ventromedial somite of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, consistent with recent studies of neural tube patterning in *Smo*$^{-/-}$ chimeras where dorsal markers are ectopically expressed in the most ventral regions (Wijgerde et al., 2002). Last, we find *Myf5* and *Pax1* both expressed in the mesenchymal cells of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites, suggesting mixing of *Pax1*-positive and *Myf5*-positive cells (illustrated in Fig. 7B). This is consistent with the mixing of ventral cell types

observed in the neural tube of *Smo*$^{-/-}$*Gli3*$^{-/-}$ embryos (Wijgerde et al., 2002). Thus, the patterning and gene expression observed in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites probably represents a default state, independent of all Hh signaling and Gli repressive activity.

How does transcriptional activation by Gli1 occur?

We present the unexpected result that in the absence of *Gli2* and *Gli3*. Gli1 cannot transcriptionally activate its downstream targets *Ptch* and *Hhip* in the PSM. This suggests that Gli1 transcriptional activation somehow requires either *Gli2* or *Gli3*. This is surprising in light of previous studies that strongly suggest direct transcriptional activation by Gli1. For example, Gli1 has been shown to bind a conserved *Gli* enhancer sequence in vitro (Sasaki et al., 1997). Furthermore, *Gli1* can largely replace *Gli2* function in vivo, suggesting a level of functional equivalency between the two Gli genes (Bai and Joyner, 2001). However, our result does not necessarily contradict these findings. One possibility is that Gli1 activation requires physical interaction with Gli2 or Gli3. Alternatively, Gli2 or Gli3 is required to provide a downstream factor necessary for Gli1 activator function. Further molecular and biochemical analyses of the Gli1 transcriptional activation mechanism will help to explain our finding.

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