Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation

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Summary

The Gli family of zinc finger transcription factors are mediators of Shh signalling in vertebrates. In previous studies, we showed that Shh signalling, via an essential Gli -binding site in the *Myf5* epaxial somite (ES) enhancer, is required for the specification of epaxial muscle progenitor cells. Shh signalling is also required for the normal mediolateral patterning of myogenic cells within the somite. In this study, we investigate the role and the transcriptional activities of Gli proteins during somite myogenesis in the mouse embryo. We report that Gli genes are differentially expressed in the mouse somite. Gli2 and Gli3 are essential for *Gli1* expression in somites, establishing Gli2 and Gli3 as primary mediators and Gli1 as a secondary mediator of Shh signalling. Combining genetic studies with the use of a transgenic mouse line expressing a reporter gene under the control of the *Myf5* epaxial somite enhancer, we show that Gli2 or Gli3 is required for *Myf5* activation in the epaxial muscle progenitor cells. Furthermore, Gli3, but not Gli2 represses *Myf5* transcription in a dose-dependent manner in the absence of Shh. Finally, we provide evidence that hypaxial and myotomal gene expression is mispatterned in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ and *Gli3*$^{-/-}$*Shh*$^{-/-}$ somites. Together, our data demonstrate both positive and negative regulatory functions for Gli2 and Gli3 in the control of *Myf5* activation in the epaxial muscle progenitor cells and in dorsoventral and mediolateral patterning of the somite.

Key words: Myf5, Sonic hedgehog, Gli, Skeletal muscle, Somite, Mouse

Introduction

Progenitor cells for skeletal muscles of the head and the body arise from the head and prechordal mesoderm and the paraxial mesoderm, respectively (Christ and Ordahl, 1995; Noden, 1983). Specifically, the paraxial mesoderm is segmented into somites that form by budding off the anterior end of the presomitic mesoderm (Pourquie, 2001). Within the somite, precursor cells for the epaxial musculature (deep back muscles) originate from the medial edge of the dermamyotome, called the dorsal medial lip of the dermamyotome (DML), delaminate and involute inward to enter the epaxial myotome (Ben-Yair et al., 2003; Denetclaw et al., 2001; Denetclaw and Ordahl, 2000; Kalcheim et al., 1999). At the lateral edge of the dermamyotome, called the ventral lateral lip of the dermamyotome (VLL), cells involute inward to enter the lateral hypaxial myotome and form the ventral body wall and intercostal muscles (Cinnamon et al., 1999; Ordahl and Le Douarin, 1992). Ultimately, both epaxial and hypaxial myotomes merge and form a continuous layer of differentiated muscle cells. At the axial level of the limbs, lateral dermamyotomal cells delaminate and migrate to the limb buds to form appendage skeletal muscles. Finally, at the level of cervical/occipital somites, cells migrate anteriorly and form the hypoglossal chord, which will contribute to tongue and pharyngeal muscles (Mackenzie et al., 1998; Noden, 1983).

Genetic studies have established that the myogenic regulatory factor (MRF) Myf5 plays a key role in the specification of mesodermal cells to the different myogenic lineages, because *Myf5*-deficient mouse embryos display a significant delay in myogenesis until the onset of *Myod1* expression (Braun et al., 1994; Tajbakhsh et al., 1997) and in the absence of three of the four MRFs, *Myf5, Myod1* and *Mrf4* (*Myf6* – Mouse Genome Informatics) no skeletal muscle progenitor cell is formed (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). Furthermore, *Myf5*-deficient muscle progenitor cells at the DML fail to enter the epaxial myotome and migrate aberrantly in the dermatome and the sclerotome (Tajbakhsh et al., 1996). The temporal and spatial expression pattern of *Myf5* is consistent with its primary role in myogenic specification. *Myf5* is first found in the DML progenitor cells for epaxial muscles at E8.0 (embryonic day 8.0), followed by the VLL progenitor cells for hypaxial muscles at E9.75 (Ott et al., 1991; Tajbakhsh and Buckingham, 2000).

As expected for a protein acting upstream in the cascade of gene activation leading to skeletal muscle differentiation, *Myf5* transcriptional regulation is particularly complex and has only

been comprehensively described recently. Regulatory elements have been found over a region covering nearly 140 kb upstream and downstream of the *Myf5* transcriptional start (Buchberger et al., 2003; Carvajal et al., 2001; Hadchouel et al., 2003; Hadchouel et al., 2000; Summerbell et al., 2000). Each element appears to function specifically in the control of *Myf5* expression at discrete sites in the embryo, indicating that *Myf5* regulation is controlled independently in individual skeletal muscle progenitor domains. Consistent with this observation, distinct signals produced by tissues surrounding the somite have been found to activate *Myf5* expression in epaxial and hypaxial muscle progenitor cells (Borycki and Emerson, 2000). Notably, Wnt signals, secreted by surface ectoderm and neural tube cells, Sonic hedgehog (Shh), secreted by notochord and floor plate cells, and Bmp4, secreted by lateral plate cells, cooperate to induce and establish the somitic pattern of *Myf5* expression (Borycki et al., 1999; Dietrich et al., 1998; Ikeya and Takada, 1998; Pourquie et al., 1996; Tajbakhsh et al., 1998). In previous work, we have shown that Shh is required for *Myf5* activation in epaxial, but not in hypaxial muscle progenitor cells (Borycki et al., 1999; Kruger et al., 2001). Furthermore, Shh signalling appears to directly act on *Myf5* transcription in epaxial progenitor cells. Indeed, transgenic mice expressing a reporter gene under the control of the epaxial somite (ES) enhancer [also referred to as EEE, Early Epaxial Enhancer (Teboul et al., 2002)], located at –6.1 kb of the *Myf5* transcriptional start, recapitulates endogenous epaxial *Myf5* expression (Gustafsson et al., 2001; Teboul et al., 2002). In the context of a heterologous promoter, expression of this transgene is abolished in a $Shh^{-/-}$ background or following mutation of an internal Gli-binding site (Gustafsson et al., 2001), suggesting that Gli proteins, which mediate Shh signalling (Ingham, 1998; Ruiz i Altaba, 1997), directly control *Myf5* expression in epaxial muscle progenitor cells.

In the mouse, three Gli proteins, Gli1, Gli2, and Gli3 have been characterised (Hui et al., 1994). As is the case for Cubitus interruptus (Ci), the fly homologue of Gli, Gli2 and Gli3 can both activate and repress Shh target gene transcription in vitro (Dai et al., 1999; Sasaki et al., 1999). However, in vivo analyses of mouse mutants have shown that Gli2 acts primarily as a transcriptional activator (Ding et al., 1998; Matise et al., 1998), whereas Gli3 retains a bipotential activity, acting as transcriptional activator in motorneuron and ventral interneuron specification and as transcriptional repressor in dorsal interneuron specification (Bai et al., 2004; Litingtung and Chiang, 2000; Meyer and Roelink, 2003; Motoyama et al., 2003; Persson et al., 2002). Gli1 functions as a transcriptional activator of Shh target genes in vitro and in overexpression studies (Dai et al., 1999; Lee et al., 1997; Ruiz i Altaba, 1999; Sasaki et al., 1999). However, mouse mutants show that Gli1 is dispensable (Park et al., 2000), although it can mediate Shh signalling in the absence of Gli2 (Bai and Joyner, 2001; Park et al., 2000). Together, these observations indicate a complex interplay between Gli proteins in vivo.

Thus, a prediction from our previous studies of myogenesis in $Shh^{-/-}$ mice and ES trangenic mice would be that Gli proteins are required during somite myogenesis to mediate Shh signalling. To address this question and to investigate whether *Myf5* activation and somite patterning require the activator or the repressor function of Gli proteins, we have examined the expression pattern and the regulation of *Gli1*, *Gli2*, and *Gli3* during somite formation in the mouse embryo. We also carried out genetic studies using Gli and *Shh* mutant mice, as well as transgenic mice expressing *lacZ* under the control of the *Myf5* epaxial enhancer (ES), to test the redundant function and the transcriptional activity of Gli proteins in *Myf5* activation and somite patterning. We found that Gli2 or Gli3 is required for epaxial muscle progenitor cell specification and that Shh plays an essential role in this process to convert Gli3, but not Gli2 into a transcriptional activator. In addition, our study reveals an unexpected differential role for Gli2 and Gli3 along the antero-posterior axis in patterning the epaxial, hypaxial and myotomal compartments. Together, these data establish that Gli genes have essential specific and redundant functions during skeletal myogenesis.

Materials and methods

Mice

Gli2 and *Gli3XtJ* mice were maintained as heterozygous stocks and crossed to generate different mutant combinations. *Shh* mice (kindly provided by M. Maconochie), also maintained as heterozygous, were crossed to *Gli3XtJ* heterozygous mice to generate *Shh/Gli3* double mutant embryos, and to *Gli2* heterozygous mice to generate *Shh/Gli2* double mutant embryos. *Myf5* ES enhancer transgenic mice, which carry the reporter gene *lacZ* under the control of the tk viral promoter and the ES enhancer (Gustafsson et al., 2001), were crossed to $Gli2^{+/-}Gli3^{+/-}$ mice to produce $Gli2^{+/-}Gli3^{+/-}$*ESLacZ* mice. These mice were then bred to homozygocity. Embryonic day 0.5 was the day vaginal plugs were found. Embryos were harvested between E8.0 and E10.5 by Caesarian section, and genotyping was performed on yolk sac DNA by Polymerase Chain Reaction (PCR) with primers described previously (Chiang et al., 1996; Maynard et al., 2002; Mo et al., 1997).

Whole-mount in situ hybridization

Embryos were collected at various stages and fixed overnight at 4°C in 4% paraformaldehyde, washed in PTW (0.1% Tween 20 in PBS) and processed for in situ hybridisation according to the protocol described elsewhere (Henrique et al., 1995). To allow for quantitative comparisons, embryos were treated together in a single tube and staining allowed to develop for the same length of time. Digoxigenin (DIG)-labelled antisense riboprobes were generated from linearised plasmids containing inserts for *Myf5* (Ott et al., 1991), *Myod1* (Sassoon et al., 1989), *Pax1* (Deutsch et al., 1988), *Pax3* (Goulding et al., 1991), *Pax7* (Jostes et al., 1991), *Gli1* (Hui et al., 1994), *Gli2* (Hui et al., 1994), *Gli3* (Hui et al., 1994), myogenin (Sassoon et al., 1989), scleraxis (Cserjesi et al., 1995), paraxis (Burgess et al., 1995), *Lbx1* (Jagla et al., 1995), *Sim1* (Fan et al., 1996) and *Noggin* (McMahon et al., 1998). Stained embryos were photographed under a LEICA MZ12 stereomicroscope using a Spot digital camera (Diagnostic Instruments). Embryos were then embedded in 2% agarose in PBS and 80 μm transverse sections were performed using a vibratome. Sections were mounted on slides using Glycergel (Dako), and photographed under Nomarski optics on a LEICA DM-R microscope using a LEICA digital camera.

Results

Gli genes are expressed in distinct domains of somites

Using whole-mount in situ hybridisation, we examined the expression pattern of Gli genes during somitogenesis and compared it to that of the myotome-, syndetome- and sclerotome-specific genes *Myf5*, scleraxis, and *Pax1* (Brent et

al., 2003; Deutsch et al., 1988; Ott et al., 1991). Expression of Gli genes in the paraxial mesoderm begins at E.8.0 with the formation of the first somite pair (data not shown). *Gli1* is not detected in the pre-somitic mesoderm and its expression begins in somite I (Fig. 1A). In contrast, *Gli2* and *Gli3* are expressed in the anterior pre-somitic mesoderm (Fig. 1B,C). In newly-formed somites, *Gli1* is strongly expressed in the ventral medial cells surrounding the notochord and more diffusely in the dorsal sclerotome (Fig. 1D). As somites mature, *Gli1* expression spreads dorsally and laterally (Fig. 1G), overlapping with *Pax1* expression (Fig. 1J), which is specifically detected within the sclerotome and is excluded from the dermamyotome (Deutsch et al., 1988). Interestingly, *Gli1* expression is downregulated in cells surrounding the notochord in anterior somites of E10.5 embryos and in hindlimb somites of E11.5 embryos (data not shown), at the time condensation of mesenchymal cells occurs around the notochord (Hall and Miyake, 2000). At E9.5, *Gli2* is expressed throughout the newly formed somite in a domain encompassing both the sclerotome and the DML (Fig. 1B,E). In anterior somites, *Gli2* expression decreases in the sclerotome (Fig. 1H), although it is upregulated at later stages (E10.5 and E11.5) at the time *Gli1* expression is downregulated (data not shown). In anterior somites, *Gli2* (Fig. 1H) is expressed in a domain overlapping that of scleraxis and *Myf5*, which label the syndetome (Brent and Tabin, 2004) and the myotome (Ott et al., 1991), respectively (Fig. 1K,L). At E10.5, *Gli2* remains expressed in the DML of posterior, but not anterior somites (data not shown). At E9.5, *Gli3* is expressed abundantly in the lateral domain of newly formed somites and is weakly expressed in the sclerotome (Fig. 1C,F). *Gli3* is also expressed in the DML (Fig. 1F,I), although it is detected slightly later than *Gli2*. As somites mature, *Gli3* becomes restricted to the ventral dermamyotome and the myotome (Fig. 1I), in a domain that overlaps that of *Myf5* expression (Fig. 1L). In E10.5 and E11.5 embryos, *Gli3* expression remains high in the DML and in the VLL, but is downregulated in the central myotome (data not shown). These observations are consistent with Gli2 and Gli3 playing a role in *Myf5* activation in the DML and controlling epaxial and hypaxial myotome formation.

Gli2$^{-/-}$*Gli3*$^{-/-}$ mutant embryos fail to activate *Gli1* expression

We previously showed that in the avian somite *Gli1* expression is dependent on Shh signalling, and others showed that *Gli1* expression is downregulated in the neural tube of *Gli2*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mice (Bai et al., 2004; Borycki et al., 1998; Ding

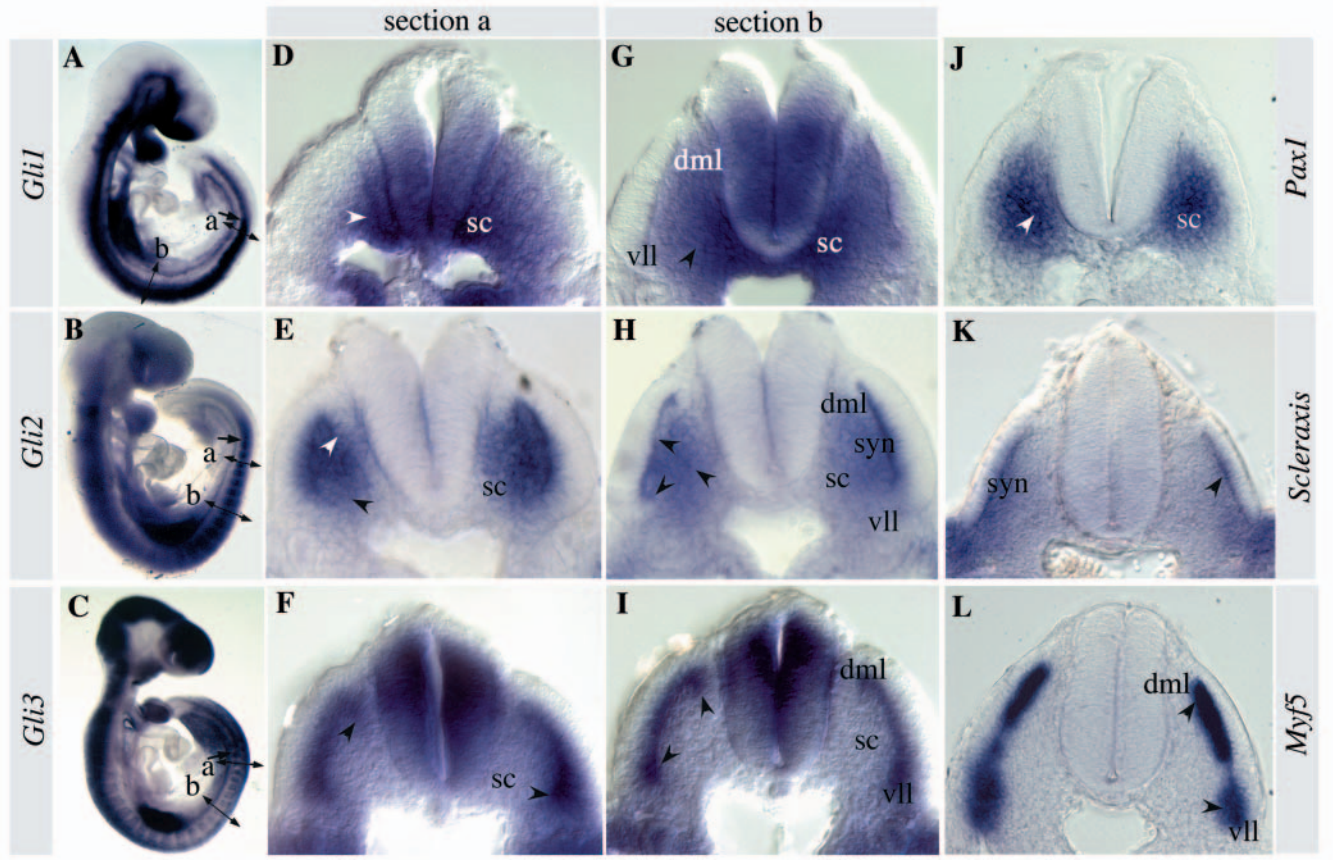

Fig. 1. Gli gene expression in mouse somites. The pattern of expression of *Gli1* (A,D,G), *Gli2* (B,E,H) and *Gli3* (C,F,I) was compared to that of the sclerotomal marker *Pax1* (J), the syndetomal marker scleraxis (K), and the myotomal marker *Myf5* (L) using whole-mount in situ hybridisation in E9.5 mouse embryos. *Gli1* expression is more abundant in the sclerotome (sc) and excluded from the dermamyotome, whereas *Gli2* and *Gli3*, although initially expressed throughout the somite, rapidly become restricted to the myotome and ventral dermomyotome. Black and white arrows point to Gli expression in the sclerotome (sc), which expresses *Pax1*, the dorsal medial lip (dml) and ventral lateral lip (vll) of the dermamyotome, which express *Myf5*, and the syndetome (syn), which expresses scleraxis.

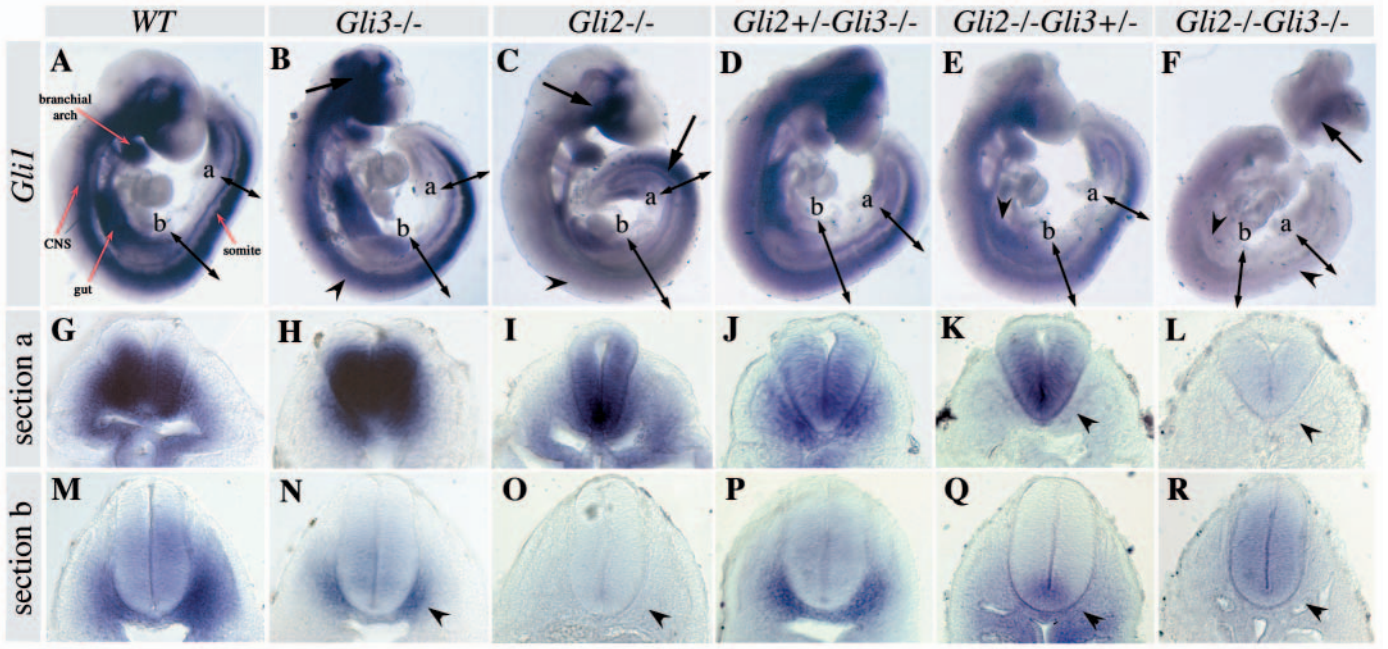

Fig. 2. *Gli1* expression in somites requires Gli2 or Gli3. *Gli1* expression was investigated in E9.5 wild-type (A,G,M), $Gli3^{-/-}$ (B,H,N), $Gli2^{-/-}$ (C,I,O), $Gli2^{+/-}Gli3^{-/-}$ (D,J,P), $Gli2^{-/-}Gli3^{+/-}$ (E,K,Q) and $Gli2^{-/-}Gli3^{-/-}$ (F,L,R) embryos using whole-mount in situ hybridisation. Transverse sections were performed at the level indicated by double black arrows. Black arrows indicate domains of *Gli1* expression that are unchanged or upregulated. Black arrowheads indicate loss of *Gli1* expression.

et al., 1998; Matise et al., 1998). To investigate whether somitic *Gli1* activation is controlled by Gli2 and Gli3, we analysed *Gli1* expression by in situ hybridisation of various Gli mutant embryos. We found that *Gli1* expression is nearly unchanged in $Gli3^{-/-}$ or $Gli2^{+/-}Gli3^{-/-}$ embryos (Fig. 2B,H,N,D,J,P). In contrast, *Gli1* is downregulated in anterior somites of $Gli2^{-/-}$ embryos (Fig. 2C,O), and loss of one *Gli3* allele in the *Gli2* mutant background nearly abolishes *Gli1* expression in somites, although CNS expression persists (Fig. 2E,K,Q). Noticeably, no *Gli1* transcript is detected in somites of $Gli2^{-/-}Gli3^{-/-}$ embryos (Fig. 2F,L,R), indicating that activation of *Gli1* requires Gli2 or Gli3. These data also reveal that $Gli2^{-/-}Gli3^{-/-}$ embryos are molecularly similar to $Gli1^{-/-}Gli2^{-/-}Gli3^{-/-}$ embryos. Therefore, no Gli-mediated target gene activation can occur in $Gli2^{-/-}Gli3^{-/-}$ somites.

Gli2 or Gli3 is required for *Myf5* activation in epaxial muscle progenitor cells

To address the function of Gli proteins in *Myf5* activation, we examined the expression of *Myf5* in wild-type, *Gli2*, *Gli3* and *Gli2/Gli3* mutant embryos by whole-mount in situ hybridisation. *Myf5* activation in the epaxial myogenic progenitor cells of the DML appears normal in E9.5 $Gli2^{-/-}$ and $Gli3^{-/-}$ embryos (data not shown), suggesting that Gli2 and Gli3 may be functionally redundant. We therefore examined compound *Gli2/Gli3* mutant embryos at E8.5, a stage at which *Myf5* expression is solely activated in the epaxial muscle progenitor cells of the DML, under the control of the epaxial somite enhancer (ES) (Gustafsson et al., 2001; Teboul et al., 2002). In wild-type embryos, *Myf5* transcripts are detected in seven out of 10-11 somites with strong expression in the six occipital somites (Fig. 3A,E,I). In contrast, *Myf5* activation is delayed in $Gli2^{-/-}Gli3^{-/-}$ embryos and only weak expression is observed in the occipital somites (Fig. 3D,H,L). Transverse sections show that $Gli2^{-/-}Gli3^{-/-}$ somites have an abnormal morphology and remain epithelial (Fig. 3H,L), and *Myf5* transcripts are observed along a ventral epithelial extension (Fig. 3L). Noticeably, somites retain their normal morphology in $Gli2^{-/-}Gli3^{+/-}$ and $Gli2^{+/-}Gli3^{-/-}$ embryos (Fig. 3G,K), and *Myf5* expression, although weaker, is activated on schedule in $Gli2^{-/-}Gli3^{+/-}$ and $Gli2^{+/-}Gli3^{-/-}$ embryos (Fig. 3B,C,F,G). Together, these data demonstrate that Gli2 or Gli3 is required for *Myf5* activation in the epaxial muscle progenitor cells, and that one copy of either Gli2 or Gli3 is sufficient to activate *Myf5*.

To address whether the delay in epaxial *Myf5* activation observed at E8.5 persists at later stages, we examined E9.5 wild-type and mutant embryos. No epaxial *Myf5* transcript is detected in the three most newly formed somites in $Gli2^{-/-}Gli3^{-/-}$ mice (Fig. 3P), whereas *Myf5* is detected in DML cells of all wild-type somites (Fig. 3M). In $Gli2^{-/-}Gli3^{+/-}$ and $Gli2^{+/-}Gli3^{-/-}$ posterior somites, epaxial *Myf5* expression is reduced (Fig. 3N,O). Consistent with our observation on *Myf5* expression, activation of myogenin expression is delayed in $Gli2^{+/-}Gli3^{-/-}$ and $Gli2^{-/-}Gli3^{-/-}$ embryos (Fig. 5A″,A‴). It was not possible to assess later stages as double mutant embryos die between E9.5 and E10.5. To further demonstrate the requirement for Gli2 or Gli3 in *Myf5* activation and the determination of epaxial muscle progenitor cells, we crossed the ES/LacZ transgenic mice, which express the β-galactosidase reporter gene under the control of the *Myf5* epaxial somite enhancer (ES) (Gustafsson et al., 2001), into *Gli2/Gli3* mutant mice. Wild-type and $Gli2^{-/-}Gli3^{-/-}$ mutant embryos carrying the transgene were analysed at E9.5 for their

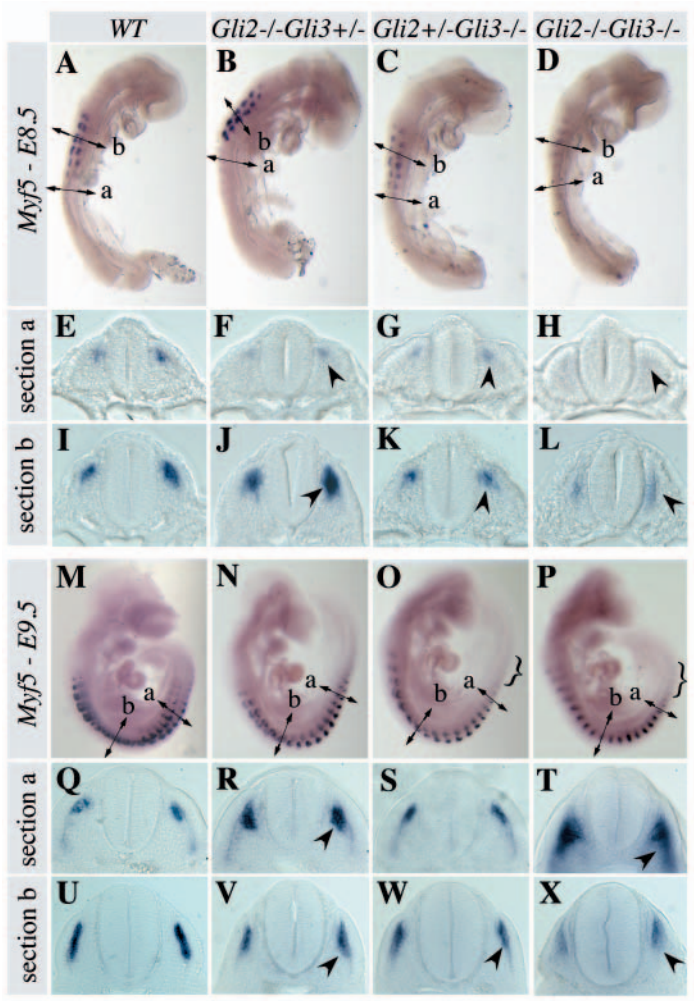

Fig. 3. *Myf5* activation is impaired in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos. *Myf5* expression was analysed by whole-mount in situ hybridisation at E8.5 (A-L) and E9.5 (M-X) in wild-type, *Gli2*$^{-/-}$*Gli3*$^{+/-}$, *Gli2*$^{+/-}$*Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos. Transverse sections were performed at the level indicated by black arrows. Black arrowheads point to the disrupted *Myf5* expression. Brackets indicate loss of *Myf5* expression in posterior somites. *Myf5* expression is not activated in posterior somites of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos and is reduced or delayed in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ and *Gli2*$^{+/-}$*Gli3*$^{-/-}$ embryos at E8.5 and E9.5. In addition, upregulation and ventral expansion is observed in E8.5 anterior somites and in E9.5 interlimb somites of *Gli2*$^{-/-}$*Gli3*$^{+/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos.

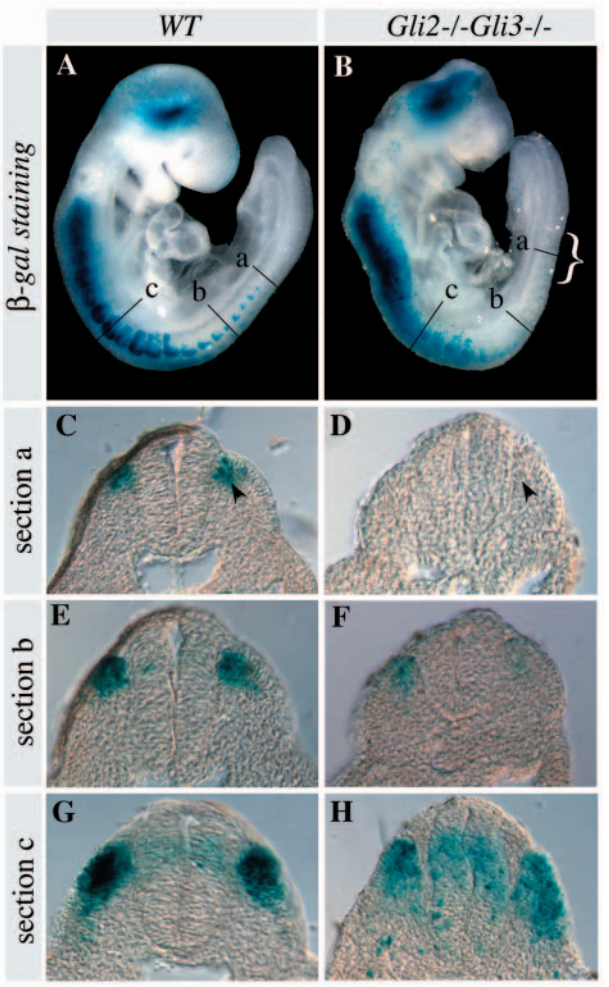

Fig. 4. *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos fail to activate the *Myf5* EE enhancer/lacZ transgene. X-gal staining of E9.5 wild-type (A,C,E,G) and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ (B,D,F,H) embryos was performed to assess the activity of the *Myf5* early epaxial enhancer. (A,B) Whole-mount X-gal staining shows that expression is lost in posterior somites (bracket). (C-H) Transverse sections were performed at the level indicated by the black line in posterior somites (C,D), interlimb somites (E,F) and anterior somites (G,H). In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, β-gal^{+} cells are absent from posterior somites (black arrowheads) and greatly reduced in anterior somites.

reporter gene expression (Fig. 4). Similar to endogenous *Myf5* expression, no β-gal staining is observed in the posterior somites of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (Fig. 4B,D), whereas wild-type embryos exhibit β-gal staining in the DML of all somites (Fig. 4A,C,E,G). Consistent with our in situ hybridisation results, this observation demonstrates that Gli2 and Gli3 play essential role in the specification of epaxial muscle progenitor cells via the direct control of the *Myf5* ES enhancer activity in newly formed somites. However, we observe weak β-gal staining in anterior somites, which is mainly ectopically located in the dorsal dermamyotome and in the myotome (Fig. 4F,H), indicating that in anterior somites additional factors may participate in *Myf5* activation and cooperate with Gli proteins to pattern correctly this expression along the dorsoventral and mediolateral axes.

Gli2$^{-/-}$*Gli3*$^{-/-}$ embryos exhibit abnormalities in the patterning of the myotome and hypaxial muscle progenitor cells

To further characterise the defects observed in *Gli2/Gli3* mutant somites, we performed in situ hybridisation using dermamyotomal, myotomal and hypaxial markers.

In interlimb and anterior somites of E9.5 *Gli2*$^{-/-}$*Gli3*$^{-/-}$ (Fig. 3T,X) and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ (Fig. 3R,V), but not *Gli2*$^{+/-}$*Gli3*$^{-/-}$ (Fig. 3S,W) embryos, *Myf5* expression is upregulated and shifted ventrally. Because *Myf5* becomes activated in the myotome and in the hypaxial muscle progenitor cells at E9.5, it is most likely that this observation indicates a function for

Gli2 and Gli3 in the formation and/or patterning of the myotome and the hypaxial somitic domain. Consistent with this possibility, *Myf5* transcripts take a central position in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites compared to wild-type somites (compare Fig. 3P to 3M), indicating that anterior and posterior somitic compartments are more severely affected. Furthermore, myogenin, whose transcripts mark myotomal cells as they delaminate from the DML and enter the epaxial myotome (Fig. 5A) (Sassoon et al., 1989), is mispatterned and found in a more ventral domain in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites (Fig. 5A′,A‴). Notably, myogenin transcripts are found in an overlapping domain with *Myf5,* along the ventral epithelial extension that characterizes the double mutant somite (compare Fig. 5A‴ and Fig. 3L). Finally, *Myod1* expression is

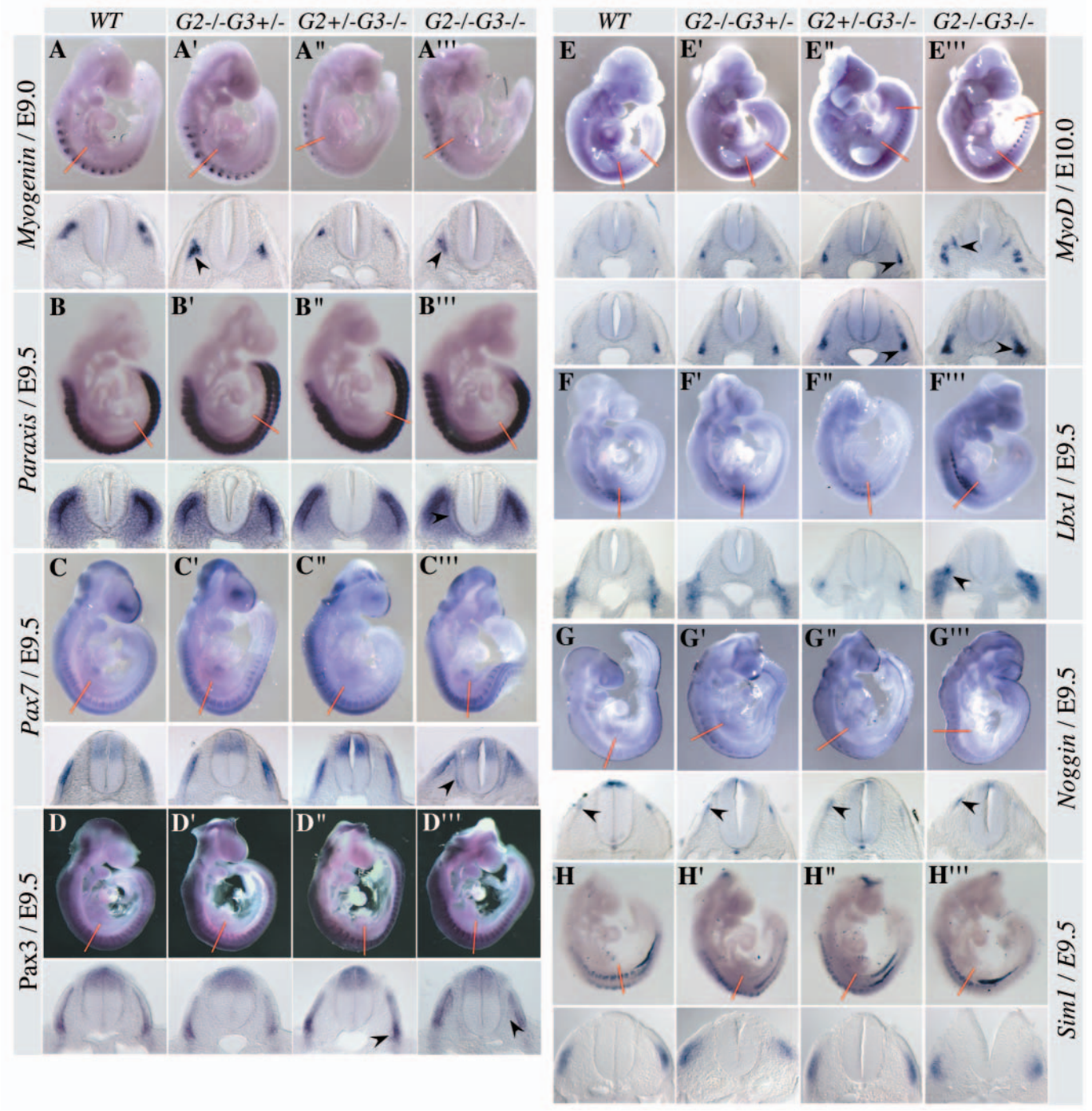

Fig. 5. *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos exhibit abnormalities in the formation of the myotome and hypaxial muscle progenitor cells. Expression of myogenin (A-A‴), paraxis (B-B‴), *Pax7* (C-C‴), *Pax3* (D-D‴), *Myod1* (E-E‴), *Lbx1* (F-F‴), *Noggin* (G-G‴), and *Sim1* (H-H‴) was assessed by whole-mount in situ hybridization in wild-type, *Gli2*$^{-/-}$*Gli3*$^{+/-}$, *Gli2*$^{+/-}$*Gli3*$^{-/-}$, and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos ranging from E9.0 to E10.0. Red lines indicate the position of transverse sections shown below the whole-mount image. Black arrowheads point to upregulation and/or misexpression of the gene studied. Note that *Noggin* expression is lost in the notochord of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos.

also precociously activated and misexpressed in a ventral domain of the epaxial myotome in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (Fig. 5E‴). These data are consistent with the idea that Gli2 and Gli3 are essential for the activation of *Myf5* in the epaxial muscle progenitor cells and the subsequent formation of the epaxial myotome, and are required for the normal dorsoventral patterning of the myotome.

To clarify the identity of the epithelial cells extending from the dorsal medial lip along the neural tube, we used the dermamyotomal markers paraxis, *Pax3* and *Pax7* (Burgess et al., 1995; Jostes et al., 1991). Paraxis, *Pax3* and *Pax7* are all expressed in the dermamyotome of wild-type embryos (Fig. 5B-D) and their expression is unchanged in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ and *Gli2*$^{+/-}$*Gli3*$^{-/-}$ embryos (Fig. 5B′,B″,C′,C″,D′,D″). In contrast, cells in the ventral epithelial extension of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites express paraxis *Pax3* and *Pax7*, although at lower levels than dorsal dermamyotomal cells (Fig. 5B‴,C‴,D‴). To investigate whether these cells express DML markers, we performed an in situ hybridisation using *Noggin*, which specifically labels DML cells from E9.5 onward (Fig. 5G) (McMahon et al., 1998). *Noggin* transcripts remain localised to the dorso-medial domain of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites and are not found in the epithelial cells that extend ventrally (Fig. 5G‴), indicating that these cells do not have the molecular identity of DML cells, although they do express dermamyotomal markers. *Noggin* expression has been proposed to provide a permissive environment for *Myf5* expression in DML cells (Hirsinger et al., 1997; Marcelle et al., 1997). However, we find here that *Myf5* expression in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mice occurs in a non-*Noggin*-expressing domain, indicating that antagonising Bmp4 signals is possibly necessary but not sufficient for *Myf5* activation in the dorsomedial somite.

We then examined the specification, differentiation, and patterning of hypaxial muscle progenitor cells in Gli mutant embryos using *Pax3*, *Sim1*, *Myod1* and *Lbx1* expression. Hypaxial muscle progenitor cells originate from the *Pax3*-positive, *Sim1*-positive lateral dermamyotomal cells (Fig. 5D,H). As they delaminate from the dermamyotome to enter the hypaxial myotome or migrate to the limbs, they activate *Myod1* or *Lbx1*, respectively (Fig. 5E,F). Thus, *Myod1* is detected at E10.0 in the hypaxial muscle progenitor cells of interlimb somites (Fig. 5E) and this expression is immediately preceded by that of *Myf5* (Tajbakhsh and Buckingham, 2000). By E10.5, *Myod1* is detected in all hypaxial progenitor cells, including those of the body wall, limb and tongue muscles (Sassoon et al., 1989). In contrast, *Lbx1* is activated at E9.5 at the level of occipital and limb somites in a subset of hypaxial muscle progenitor cells (Fig. 5F) (Jagla et al., 1995; Uchiyama et al., 2000), which are committed to migrate to the limb bud or to the head (Brohmann et al., 2000; Dietrich et al., 1998; Gross et al., 2000). As expected from our previous analysis of *Shh* mutant mice (Borycki et al., 1999), none of the mutant combinations studied shows a defect in the onset of hypaxial muscle progenitor cell specification (Fig. 5D-D‴,E-E‴,F-F‴,H-H‴). Likewise, no change in *Myod1* and *Lbx1* expression pattern is observed in *Gli2*$^{-/-}$, *Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos at E9.5 and E10.0 (Fig. 5E′,F′ and data not shown). However, *Myod1* is upregulated in the hypaxial domain of *Gli2*$^{+/-}$*Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites, and both *Myod1* and *Lbx1* are upregulated and expand into the ventromedial somitic domain of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (Fig. 5E″,E‴,F″,F‴). Noticeably, no such upregulation occurs for *Pax3* and *Sim1* dermamyotomal expression in *Gli2*$^{+/-}$*Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites (Fig. 5D″,D‴,H″,H‴). This indicates that in the absence of Gli2 and Gli3, although hypaxial muscle progenitor cells form and are specified normally in the dermamyotome, they become unproperly patterned as they enter the hypaxial myotome or migrate into the limbs, as is the case for *Shh* mutant mouse embryos.

Gli3, but not Gli2 acts as a transcriptional repressor in the absence of Shh signalling

Gli2 and Gli3 proteins contain recognition sites for protein kinase A-dependent phosphorylation, a process that leads to the proteolytic cleavage of the full-length protein into a shorter N-terminal polypeptide containing a repressor domain in *Drosophila* (Chen et al., 1998; Epstein et al., 1996; Jia et al., 2002; Price and Kalderon, 2002). In vitro, both Gli2 and Gli3, but not Gli1 have been shown to act as transcriptional repressors (Dai et al., 1999; Sasaki et al., 1999). Shh signalling prevents this cleavage and converts Gli2 and Gli3 into transcriptional activators (Aza-Blanc et al., 2000). In vivo, only Gli3 was found to have repressor function and to require Shh signalling to be converted into a transcriptional activator (te Welscher et al., 2002; Wang et al., 2000). This implies that defects observed in dorsoventral patterning of the neural tube of *Shh* mutant mice can be partially rescued in *Shh/Gli3* compound mutant mice, as the result of the loss of a Gli3 repressor function (Litingtung and Chiang, 2000).

To test whether some of the somitic defects observed in *Shh* mutant mice are due to a repressor activity of Gli2 or Gli3, we analysed by in situ hybridisation the expression of *Myf5* and myogenin in *Shh*$^{-/-}$, *Gli3*$^{+/-}$*Shh*$^{-/-}$ and *Gli3*$^{-/-}$*Shh*$^{-/-}$ embryos, as well as in *Gli2*$^{+/-}$*Shh*$^{-/-}$ and *Gli2*$^{-/-}$*Shh*$^{-/-}$ embryos. As shown before (Borycki et al., 1999), *Myf5* expression is not activated in the epaxial muscle progenitor cells of the DML in *Shh*$^{-/-}$ embryos (Fig. 6F,J), although its activation comes on schedule in the hypaxial muscle progenitor cells (Fig. 6F,J,N,V,V′). In addition, hypaxial *Myf5* expression expands into the ventromedial somite (compare Fig. 6M and 6N,V′), and *Myf5* and *myogenin* expression is greatly reduced in occipital somites of *Shh*$^{-/-}$ embryos (Fig. 6B,F,R). Removal of one *Gli3* allele in the *Shh*$^{-/-}$ background restores *Myf5* expression in the DML epaxial muscle progenitor cells of interlimb somites, but not posterior somites (Fig. 6G,K). However, removal of both *Gli3* alleles in the *Shh*$^{-/-}$ background fully restores *Myf5* expression in DML muscle progenitor cells along the entire anteroposterior axis (Fig. 6H,L,P,T). Unlike for *Gli3*, loss of *Gli2* alleles in the *Shh*$^{-/-}$ background does not restore epaxial *Myf5* expression (Fig. 6U-X′). This indicates that Shh signalling is required to convert Gli3, but not Gli2 into a transcriptional activator for *Myf5* expression in DML cells. These data also suggest that DML cells of posterior and occipital somites are more sensitive to a ratio of Gli3 activator versus Gli3 repressor $\leqslant 1$ than are DML cells of interlimb somites.

Interestingly, *Gli3*$^{-/-}$*Shh*$^{-/-}$ interlimb somites show persisting ventromedial expansion of myotomal and hypaxial *Myf5* expression (Fig. 6P), whereas *Gli2*$^{-/-}$*Shh*$^{-/-}$ interlimb somites have a less pronounced expansion especially in cells surrounding the notochord (compare Fig. 6X′ to 6V′). These

data suggest that Shh signalling functions in a Gli3-independent manner in the mediolateral patterning of interlimb somites, presumably acting via a repressor function of Gli2.

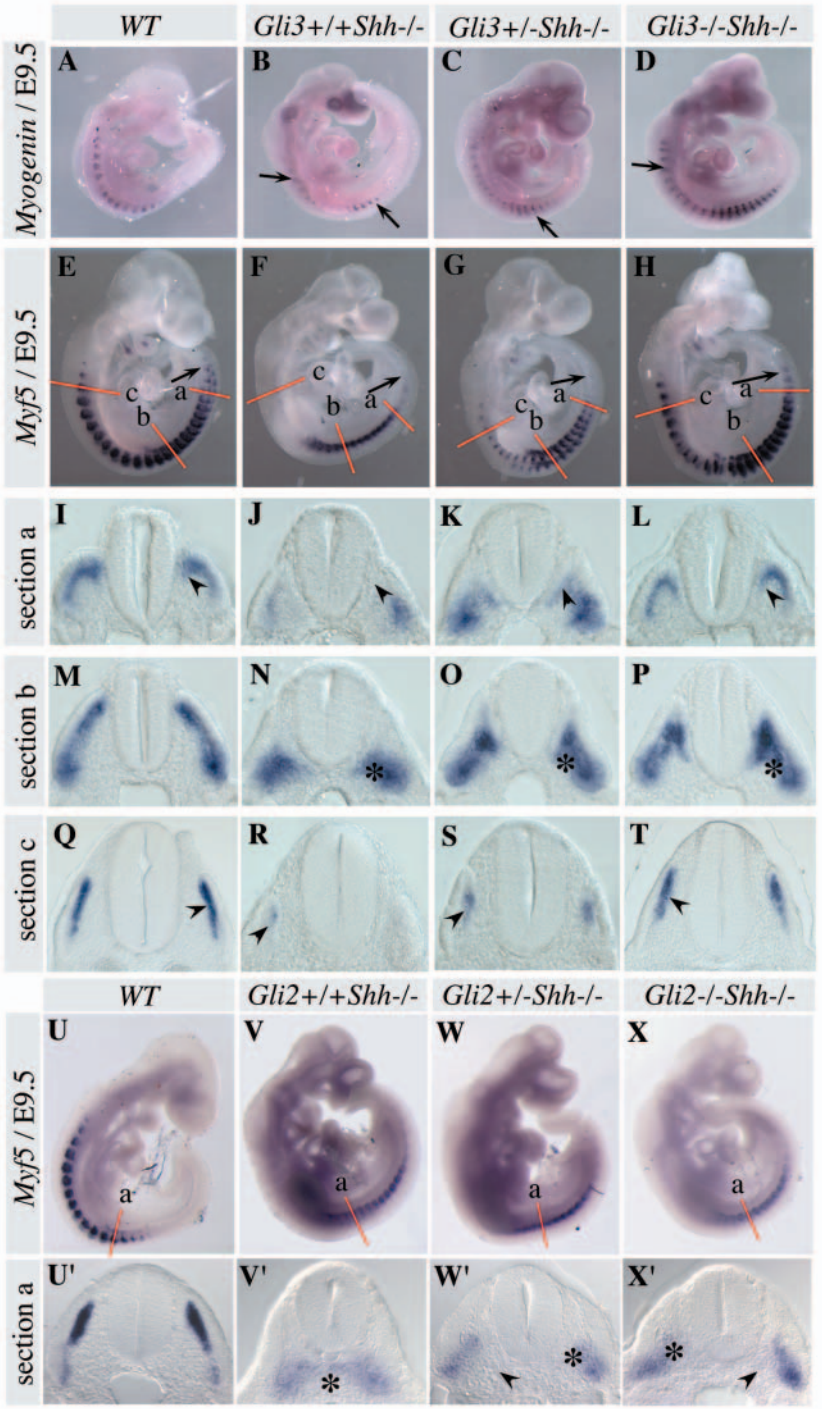

Fig. 6. Shh signalling converts Gli3, but not Gli2 into a transcriptional activator in somites. E9.5 wild-type (A,E,I,M,Q), $Shh^{-/-}$ (B,F,J,N,R,V,V′), $Gli3^{+/-}Shh^{-/-}$ (C,G,K,O,S), $Gli3^{-/-}Shh^{-/-}$ (D,H,L,P,T), $Gli2^{+/-}Shh^{-/-}$ (W,W′) and $Gli2^{-/-}Shh^{-/-}$ (X,X′) embryos were analysed by whole-mount in situ hybridisation for myogenin (A-D) and *Myf5* (E-X′) expression. Transverse sections were performed as indicated by red lines. Black arrows indicate loss of expression. Black arrowheads indicate domains of altered gene expression. Asterisks indicate the ventromedial expansion of *Myf5* expression.

Discussion

Through a detailed analysis of single and compound Gli mutant mice, together with the analysis of transgenic mice carrying a reporter gene under the control of the *Myf5* epaxial enhancer (ES), we demonstrate that Gli2 or Gli3 is required to specify epaxial muscle progenitor cells and to maintain proper mediolateral and dorsoventral patterning of the dorsal somite (data are summarised in Table 1). Our data also reveal that the formation of distinct myogenic lineages within the somite requires a combination of activating and repressing Gli functions along the antero-posterior axis of the embryo.

Shh-dependent activation of *Gli1* expression is mediated by Gli2 and Gli3 in somites

Gli1, Gli2 and *Gli3* expression is activated in coordination with somite formation in the mouse embryo, with *Gli2* and *Gli3* expression beginning just before somite formation. Thus, as in the avian embryo (Borycki et al., 1998), Gli-dependent Shh response is linked to somitogenesis in the mouse embryo. This suggests that similar mechanisms control Gli gene expression in the mouse and in the bird, where we showed that Shh signals control Gli1 activation, and Wnt signals control Gli2 and Gli3 activation (Borycki et al., 1998).

Gli genes are differentially expressed in the somite, with *Gli1* restricted to the ventral somite, and *Gli2* and to a lesser extent *Gli3,* initially expressed throughout the somite and then predominantly found in the dorsal somite. This expression pattern is consistent with our findings that Gli2 and Gli3 are required for *Gli1* activation and that Gli3 can only compensate for the loss of Gli2 in newly formed somites. However, Gli3 acts as a weak transcriptional activator, because the presence of one *Gli3* allele in a *Gli2* mutant background is not sufficient to activate *Gli1* expression in newly formed somites. The overlapping expression of *Gli2* and *Gli3* in the dorsal somite also accounts for their functional redundancy during somite myogenesis, although their respective preponderance in the medial and lateral somite is consistent with independent functions in mediolateral patterning of the somite.

Gli2 or Gli3 is required for the specification of epaxial muscle progenitor cells

We provide several lines of evidence that Gli2 or Gli3 is required to mediate Shh signalling in the specification of epaxial muscle progenitor cells from the DML. First, as for $Shh^{-/-}$ embryos, *Myf5* expression is not activated in DML cells of $Gli2^{-/-}Gli3^{-/-}$ embryos, whereas activation

Table 1. Summary of the myogenic phenotypes observed in the different compound mouse mutants analysed in this study

Mouse mutants	Epaxial	Hypaxial
Gli2$^{-/-}$*Gli3*$^{+/-}$	Somite morphology normal *Myf5* activation in DML cells reduced Epaxial myotome expands ventrally	Normal
Gli2$^{+/-}$*Gli3*$^{-/-}$	Somite morphology normal *Myf5* activation in DML cells is delayed Epaxial myotome is delayed and reduced	Ventromedial expansion of hypaxial myotome (dermomyotomal markers *Pax3* and *Sim1* not affected)
Gli2$^{-/-}$*Gli3*$^{-/-}$	Somite morphology altered (ventral epithelial extension of dermomyotome) *Myf5* activation in DML cells is delayed Epaxial myotome is delayed, reduced and expands ventrally Premature activation of *Myod1*	Ventromedial expansion of hypaxial myotome (dermomyotomal markers *Pax3* and *Sim1* not affected) Upregulation of hypaxial migratory cells (Lbx1-positive)
Shh$^{-/-}$	Somite morphology is altered (loss of medial dermomyotome) No *Myf5* activation in DML cells No epaxial myotome	Ventromedial expansion of interlimb hypaxial myotome and dermomyotomal markers *Pax3* and *Sim1*
Shh$^{-/-}$*Gli3*$^{-/-}$	Somite morphology restored Normal *Myf5* activation in DML cells and in epaxial myotome Ventral expansion of interlimb epaxial myotome	Ventromedial expansion of interlimb hypaxial myotome Dermomyotomal markers not tested
Shh$^{-/-}$*Gli2*$^{-/-}$	Somite morphology is altered (loss of medial dermomyotome) No *Myf5* activation in DML cells No epaxial myotome	Reduced expansion of interlimb hypaxial myotome (shift from ventromedial to dorsomedial) Dermomyotomal markers not tested.

occurs, albeit at lower levels, in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ and *Gli2*$^{+/-}$*Gli3*$^{-/-}$ embryos. Second, no β-Gal+ cell is observed in newly formed somites of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos crossed into the *Myf5 ES/LacZ* transgenic mice, which express the reporter gene in the epaxial muscle progenitor cells (Gustafsson et al., 2001; Teboul et al., 2002). Third, Shh signalling is necessary to convert Gli3R into Gli3A for *Myf5* activation in DML cells. This establishes that Gli2 and Gli3 both act as transcriptional activators of *Myf5* expression in DML cells and are required for the transcriptional activity of the *Myf5* ES enhancer (Fig. 7). These data are consistent with our previous findings that Shh signalling is necessary to initiate epaxial *Myf5* expression, and that the Gli binding site located in the *Myf5* ES enhancer is essential for the enhancer activity in posterior somites (Gustafsson et al., 2001). In contrast, the present data do not support previous findings by Teboul et al., that the Gli binding site in the *Myf5* ES enhancer is required for the maintenance of epaxial *Myf5* expression but not for its initiation (Teboul et al., 2003). We believe this discrepancy reflects the different behaviour of the transgenic constructs used, which in the latter case, contains upstream of the *Myf5* promoter both the branchial arch enhancer and the *Myf5* ES enhancer (Teboul et al., 2003). It is possible that cross-talk between enhancers, yet to be characterised, occurs and alters the activity of the ES enhancer.

At E8.5, weak *Myf5* expression can still be detected in anterior somites of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, but in a more ventral position. This expression has also been observed in *Shh*$^{-/-}$ and *Smo*$^{-/-}$ embryos (Gustafsson et al., 2001; Kruger et al., 2001; Zhang et al., 2001). The origin of these *Myf5* transcripts remains to be identified. We believe that Gli2 and Gli3 are required for the activation of *Myf5* but in the absence of Shh signalling, *Myf5* expression in the epaxial progenitor cells can be activated with delay in a Shh-independent manner (Fig. 7). Indeed, we find that *Myf5* expression in E9.5 *Gli2*$^{-/-}$*Gli3*$^{-/-}$ anterior somites maps to the ES enhancer, indicating that other factors interacting either with the Gli binding site or with another site can drive *Myf5* expression at later stages. Further characterisation of the *Myf5* ES enhancer is required to elucidate the molecular mechanisms that control its activity. In particular, it will be of interest to investigate whether other Gli-related transcription factors can bind the consensus Gli site in the ES enhancer. Candidate proteins are members of the Zic family of transcription factors, which bind consensus Gli binding sites with a lower affinity than Gli proteins (Mizugishi et al., 2001), and can physically associate with Gli (Koyabu et al., 2001). Alternatively, the residual *Myf5* expression observed at E8.5 in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ somites could result from the deregulated activity of a distinct enhancer and for instance, derive from the upregulation of the myotome enhancer (Buchberger et al., 2003; Hadchouel et al., 2003). In agreement with this hypothesis, we find that *Myf5* and myogenin expression overlaps in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ occipital somites. The comparative analysis of E8.5 *Shh*$^{-/-}$ or *Smo*$^{-/-}$ and wild-type embryos carrying a transgene with the full *Myf5* regulatory region, in which the ES enhancer has been deleted, will be critical to definitely rule out this possibility.

The ventral position of epaxial *Myf5* transcripts in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ reveals that dermamyotomal and myotomal cells are mislocated in the ventromedial somite. This observation correlates with the reduction or loss of *Pax1*-expressing cells in the ventral somite of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mice (Buttitta et al., 2003), indicating that normal dorsoventral patterning of the somite is disrupted in the absence of Gli proteins. This defect is reminiscent of the ventral shift of neuronal cells in the developing central nervous system of *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mice (Wijgerde et al., 2002). This suggests that, as was demonstrated in the neural tube (Jessell, 2000), somitic cells adopt a specific cell fate along the ventro-dorsal axis according to the level of Hedgehog signalling transduced (Fig. 7).

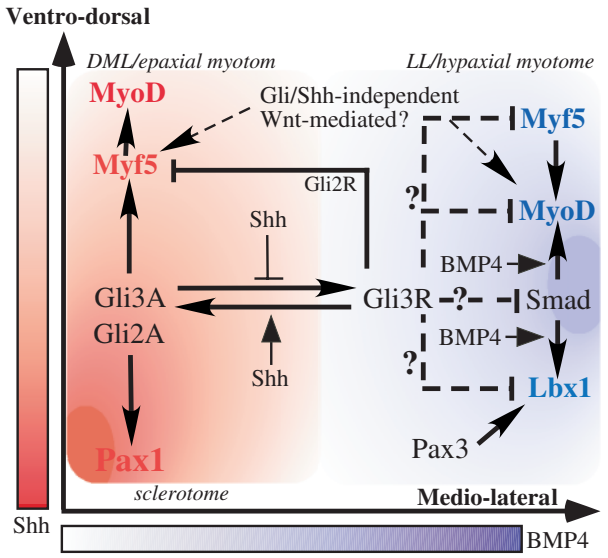

Fig. 7. Model for Gli2 and Gli3 function in somite myogenesis. Dorsoventral and mediolateral patterning of the somite is dictated in part by the opposing action of a ventral gradient of Hedgehog signalling (in red) and a lateral gradient of Bmp4 signalling (in blue). In the medial somite, the combined activity of Gli2A and Gli3A, converted from Gli3R into Gli3A by Shh, leads to the activation of *Myf5* in DML cells and the activation of *Pax1* in sclerotomal cells. *Myf5* is also a target of Gli2A and Gli3A in the epaxial myotome, although a repressor function of Gli3 is revealed in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos. In the lateral somite, characterised by low levels of Shh signalling and high levels of Bmp4 signalling, *Myod1* and *Lbx1* expression is controlled by Gli3R and Smad. We suggest that Gli3R could directly control *Lbx1*, *Myf5* and *Myod1* levels, and/or could interfere with Bmp4 signalling via an interaction with Smad in the control of *Lbx1*, *Myf5* and *Myod1* levels in the VLL and hypaxial myotome. Additional Gli/Shh-independent mechanisms of *Myf5* activation are active in both the medial and lateral somite, possibly mediated by Wnt and/or FGF signalling.

Dual function of Gli2 and Gli3 in somite myogenesis

Biochemical and in vitro studies have shown that Gli2 and Gli3 can act as transcriptional activators and repressors (Ruiz i Altaba, 1999; Sasaki et al., 1999), but until recently it was thought that in vivo, Gli2 had only activator function and Gli3 had only repressor function (Bai et al., 2002; Ding et al., 1998; Litingtung and Chiang, 2000; Matise et al., 1998; Wang et al., 2000). Our data show that Gli2 and Gli3 have both activator and repressor function in the dorsal somite depending on the genetic context. For instance, Gli2 represses hypaxial gene expression in the absence of Gli3, as illustrated by the gradual increase in hypaxial gene expression in *Gli2*$^{+/-}$*Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (Fig. 7). Conversely, Gli3 activates epaxial gene expression in the absence of Gli2, as *Myf5* expression, although reduced, is still observed in posterior somites of *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos and is lost in *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos (Fig. 7). These results are in line with recent studies showing that Gli3 has activator function in the specification of floor plate and V3 interneurons in the neural tube (Bai et al., 2004), and in the specification of sclerotomal cells in the somite (Buttitta et al., 2003).

Our data clearly indicate that the failure to activate *Myf5* in the epaxial somitic domain of *Shh*$^{-/-}$ embryos is due to the repressor activity of Gli3, as we observe a progressive restoration of *Myf5* expression in *Gli3*$^{+/-}$*Shh*$^{-/-}$ and *Gli3*$^{-/-}$*Shh*$^{-/-}$ embryos. Moreover, Gli3 repressor clearly acts in a concentration-dependent manner on *Myf5* activation (Fig. 7), and accounts for the more severe phenotype observed in *Shh*$^{-/-}$ mice compared to *Gli2*$^{-/-}$*Gli3*$^{-/-}$ mice. Similar effects have been reported in the dorso-ventral patterning of the neural tube and in the anteroposterior patterning of the limb (Litingtung and Chiang, 2000; Litingtung et al., 2002; te Welscher et al., 2002), revealing that repression of target genes by Gli3 in the absence of Shh signals constitutes a fundamental mechanism in tissue patterning. This remarkable result also indicates that *Myf5* activation can occur in the absence of both Shh and Gli signalling, as Gli1 and Gli2 proteins are thought to not be active in the absence of Shh. This observation is in line with previous studies showing that combinatorial Wnt and Shh signalling activate *Myf5* expression (Munsterberg et al., 1995; Tajbakhsh et al., 1998), and suggests that in the absence of Gli activity, other transcription factors activate the *Myf5* ES enhancer and *Myf5* transcription. Crossing *Gli3/Shh* mutant mice into the *ES/lacZ* transgenic background would confirm that this is the case. Nevertheless, the fact that the *Myf5* ES enhancer has a single Gli site suggests that the variable phenotype observed in our compound mutants can only be explained if activator and repressor forms of Gli, alone or in association with partners, bind the Gli binding site with different affinities.

Gli2 and Gli3 have a central role in the patterning of the myotome and hypaxial muscle progenitor cells

In a previous report, we showed that in the absence of Shh, hypaxial *Myf5* expression expanded into the ventromedial somite (Borycki et al., 1999). Here, we find that expression of *Myf5* and myogenin in epaxial myotomal cells is upregulated and expands ventrally in *Gli2*$^{-/-}$*Gli3*$^{+/-}$ embryos, and expression of *Myf5, Myod1* and *Lbx1* in hypaxial muscle cells is upregulated and expands medially in *Gli2*$^{+/-}$*Gli3*$^{-/-}$ and *Gli2*$^{-/-}$*Gli3*$^{-/-}$ embryos, indicating that in addition to their role in epaxial muscle cell determination, Gli2 and Gli3 function also in dorsoventral and mediolateral patterning of the somite. Moreover, this somite patterning defect persists in *Gli3*$^{-/-}$*Shh*$^{-/-}$ interlimb somites, in agreement with the idea that Gli3 and possibly Gli2, act synergistically in the lateral somite to repress the hypaxial programme. The exact mechanism of Shh/Gli function in mediolateral patterning remains to be determined, and in particular, whether Gli repressors act directly or indirectly on the hypaxial/myotomal genes. Evidence for a direct role would require that similar studies to that presented here are performed using the hypaxial *Myf5* enhancer. In the avian somite, evidence for an indirect role proposes that mediolateral patterning is established via counteracting activities between a lateral Bmp4 gradient originating from the lateral mesoderm and a medial Shh gradient originating from the axial mesoderm (Hirsinger et al., 1997; Marcelle et al., 1997). It is still not clear how Bmp4 and Shh counteract each other, but one possibility could be that Gli3R interacts with Smad proteins, which mediate Bmp4 signalling in the control of hypaxial gene expression (Fig. 7) (Liu et al., 1998). Finally, mispatterning could result from the deregulated cell division of specific muscle progenitor cell subpopulations (i.e. epaxial myotome and hypaxial cells). In this view, Shh signalling has

previously been shown to control cell cycle progression via the regulation of cyclins (Bai et al., 2004; Barnes et al., 2001; Kenney and Rowitch, 2000), and is involved in the balance of proliferation/differentiation of limb muscle cells (Amthor et al., 1999; Duprez et al., 1998). Future experiments are required to investigate the molecular factors controlling the activity of the enhancers of hypaxial and myotomal genes and the possible cooperation of Smad and Gli proteins, as well as the relationship between Shh signalling and cell cycle regulators in skeletal muscle progenitor cells.

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