Development 127, 2075-2087 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4332

Shh and Wnt signaling pathways converge to control *Gli* gene activation in avian somites

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Accepted 29 February; published on WWW 18 April 2000

SUMMARY

The regulation of the *Gli* genes during somite formation has been investigated in quail embryos. The *Gli* genes are a family encoding three related zinc finger transcription factors, Gli1, Gli2 and Gli3, which are effectors of Shh signaling in responding cells. A quail *Gli3* cDNA has been cloned and its expression compared with *Gli1* and *Gli2*. These studies show that *Gli1*, *Gli2* and *Gli3* are co-activated at the time of somite formation, thus providing a mechanism for regulating the initiation of Shh signaling in somites. Embryo surgery and paraxial mesoderm explant experiments show that each of the *Gli* genes is regulated by distinct signaling mechanisms. *Gli1* is activated in response to Shh produced by the notochord, which also controls the dorsalization of *Gli2* and *Gli3* following their activation by Wnt signaling from the surface ectoderm and neural tube. This surface ectoderm/neural tube Wnt signaling has both negative and positive functions in *Gli2* and *Gli3* regulation: these signals repress *Gli3* in segmental plate mesoderm prior to somite formation and then promote somite formation and the somite-specific activation of *Gli2* and *Gli3*. These studies, therefore, establish a role for Wnt signaling in the control of Shh signal transduction through the regulation of *Gli2* and *Gli3*, and provide a mechanistic basis for the known synergistic actions of surface ectoderm/neural tube and notochord signaling in somite cell specification.

Key words: *Gli* gene activation, Somite, Sonic hedgehog, Wnt, Quail

INTRODUCTION

In vertebrate embryos, paraxial mesoderm gives rise to multiple tissues, including skeletal muscles, cartilage, bones and dermis (Christ and Ordahl, 1995). The specification of these cell types requires multiple regulatory processes that are precisely coordinated with segmentation of the paraxial mesoderm to form somites (Borycki et al., 1997; Borycki and Emerson, 2000). Regulatory genes that control segmentation are expressed periodically in the segmental plate mesoderm and at the boundary between segmental plate mesoderm and newly formed somites (Palmeirim et al., 1997; McGrew and Pourquie, 1998). When somite formation initiates, the most anterior segmental plate mesoderm undergoes a mesenchymal-epithelial transition that leads to the formation of epithelial somites. In contrast to the process of segmentation, which is cell-autonomous, somite formation is controlled by unidentified extrinsic signals from the overlying surface ectoderm (Borycki et al., 1997; Sosic et al., 1997; Palmeirim et al., 1998). Following somite formation, cells in the newly formed somite acquire competence to respond to signals from surrounding tissues (Borycki et al., 1997). These signals positively and negatively control the dorso-ventral and medio-lateral patterning of gene expression in the somite (Munsterberg and Lassar, 1995; Pourquie et al., 1995; Pownall et al., 1996), leading to the determination of the different somite cell lineages (Lassar and Munsterberg, 1996; Borycki and Emerson, 2000; Dietrich, 1999).

Sonic hedgehog (Shh) produced by the ventral notochord and floor plate is essential for the induction of the muscle determination factors, *MyoD* and *Myf5*, and the sclerotome determination factor, *Pax1*, in somites of avian embryos (Johnson et al., 1994; Ebensperger et al., 1995; Munsterberg et al., 1995; Borycki et al., 1998). Additional signaling molecules, however, interact cooperatively with Shh to achieve proper activation and spatial patterning of gene expression and cell determination in somites. Wnt signals from the dorsal neural tube are required to maintain the epithelial architecture of the dermomyotome in the medial somite (Ikeya and Takada, 1998), and neural tube Wnts participate with Shh to control *MyoD* expression (Munsterberg et al., 1995) and to induce *Noggin*, which represses BMP4 signaling from the lateral plate to establish the medio-lateral somite axis (Hirsinger et al., 1997; Marcelle et al., 1997; Borycki and Emerson, 2000). Signals from the surface ectoderm are required for the survival and for the maintenance of epithelial organization of

dermomyotomal cells through the activation of *Pax3* (Fan and Tessier-Lavigne, 1994; Dietrich et al., 1997; Borycki et al., 1999b), and through the activation of *Paraxis* (Sosic et al., 1997), respectively. Finally, together with lateral plate BMP signals, they induce hypaxial lineage determination (Pourquie et al., 1996; Dietrich et al., 1998).

In order to identify the mechanisms that control the initiation of Shh signaling during somite formation, we have investigated the expression of *Gli* genes in somites of quail embryos. *Gli* genes are a family of three zinc finger transcription factor effectors that transduce Shh signals in responding cells. In mouse, human and *Xenopus*, three *Gli* genes have been identified: *Gli1*, *Gli2* and *Gli3* (Kinzler et al., 1988; Ruppert et al., 1990; Hui et al., 1994; Marine et al., 1997). These vertebrate *Gli* genes are homologues of the *Drosophila* transcription factor, Cubitus interruptus (Ci) (Alexandre et al., 1996). In avians, *Gli1* and *Gli2* have been previously identified (Marigo et al., 1996b). *Gli1* is preferentially activated in the ventral somite where *Pax1* is activated and sclerotome forms, whereas *Gli2* is first activated throughout the somite and then becomes restricted in its expression to the myotome in the dorsal somite (Borycki et al., 1998).

Here we report on the identification of an avian *Gli3* cDNA and present an experimental analysis of *Gli1*, *Gli2* and *Gli3* regulation during somite formation. These studies show that *Gli3* is co-activated with *Gli2* and *Gli1* at the onset of somite formation in quail embryos, providing a basis for the initiation of Shh signaling in newly formed somites (Borycki et al., 1998). Surprisingly, however, in vivo and in vitro experiments provide evidence that each of these *Gli* genes is controlled differently. *Gli2* and *Gli3* are regulated by Wnt signaling from the surface ectoderm and neural tube, and these signals are probably transduced through β-catenin. Surface ectoderm/neural tube Wnt signaling has both negative and positive functions that switch at the time of somite formation. In segmental plate mesoderm, Wnt signaling represses *Gli3*, but in segmental plate undergoing somite formation, Wnt signaling promotes somite formation and the activation of *Gli2* and *Gli3*. In contrast, *Gli1* activation is regulated by Shh signaling, which initiates at the time of somite formation and which also functions to dorsalize the expression of *Gli2* and *Gli3* to myotome progenitors during somite maturation. These findings, therefore, establish a role for Wnt signaling in the control of the Shh signaling pathway through the regulation of *Gli2* and *Gli3* activation, and provide a mechanistic basis for the known synergistic action of surface ectoderm/neural tube and notochord signaling in somite cell specification (Buffinger and Stockdale, 1994; Fan and Tessier-Lavigne, 1994; Munsterberg et al., 1995; Stern et al., 1995; Borycki et al., 1997, 1998, 1999a; Fan et al., 1997; Tajbakhsh et al., 1998).

MATERIALS AND METHODS

Cloning and sequencing of quail *Gli3*

A Hamburger-Hamilton (HH) stage-12 quail cDNA library cloned into pBK-CMV vector (Stratagene) was used as template in a Polymerase Chain Reaction (PCR) with a T3 primer and a degenerate primer designed from the published human, mouse and *Xenopus Gli3* sequences (Gli3-3′ZF: GCCTTCCCARTGGCAGTTTGTCTC; in these sequences, N=A,G,T,C; Y=C,T; R=A,G) to amplify a product of approximately 1300 bp. This PCR fragment was further purified by PCR amplification using the primer Gli3-3′ZF and a nested degenerate primer Gli3-5′ (Gli3-5′: CCNTACATYARYCCNTAYA-TGGAC), which produced a 900 bp cDNA fragment with 88% identity to human GLI3. This 900 bp quail Gli3 cDNA was then used as a probe to screen a stage-12 quail cDNA library. A full-length quail *Gli3* cDNA was isolated and sequenced on both strands (GenBank accession numbers: AF231111 and AF231112).

Cell culture

RatB1a fibroblasts (Finney and Bishop, 1993) were infected with the replication-defective retrovirus vector MV7 or with equivalent retroviruses carrying Wnt-1 or Wnt-4 cDNAs, as described (Jue et al., 1992). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL) containing 10% Fetal Calf Serum (FCS) (Gibco-BRL) and 1× Penicillin/Streptomycin/Fungizone (Gibco-BRL). 3 days before coculture with segmental plate explants, cells were trypsinized and transferred into 60 mm Petri dishes (Falcon) where they grew in suspension forming cell aggregates, which were cultured adjacent to segmental plate explants in collagen gels, as described below.

Surgeries and explant cultures

HH stage 12-13 quail embryos were obtained by incubating *Coturnix coturnix japonica* eggs for 45-48 hours at 38°C in humidified atmosphere. Embryos were transferred onto paper rings (Whatmann). Surgeries, including notochord separation, axial tissue separation and surface ectoderm removal were performed as previously described (Borycki et al., 1998). Embryos were then cultured for 7 or 16 hours using a modified New culture system, in presence of a mix of 90% thin albumin and 10% PBS/streptomycin. Segmental plate explants were dissected from quail embryos at HH stage 12-13. When indicated, surface ectoderm was removed after incubation for 45 seconds in Dispase I (Boehringer Mannheim). Explants were then cultured for 16 hours at 37°C in a CO_2 incubator on two different substrates: (1) on a 12-well plate coated with 1% agarose in DMEM-10% FCS (1:1); LiCl (Sigma) was added to the culture medium and the agarose at the final concentration of 10 mM when indicated. A range of LiCl concentrations from 2 to 20 mM was initially tested, and 10 mM LiCl gave optimal effects on gene expression with no observed cytotoxicity; or (2) embedded in a collagen gel (Boehringer Mannheim) according to the manufacturer's protocol, and cocultured adjacent to *Wnt*- or Mv7-expressing cell aggregates in the presence of DMEM-10% FCS. Embryos and explants were then transferred into an Eppendorf tube, rinsed with PBS, fixed at 4°C in 4% formaldehyde, 2 mM EGTA in PBS and analyzed by in situ hybridization, as described below.

Whole-mount in situ hybridization

Embryos and explants were fixed in 4% formaldehyde, 2 mM EGTA in PBS at 4°C for 16 hours and analyzed by in situ hybridization as previously described (Henrique et al., 1995) using DIG-labeled (Boehringer Mannheim) RNA probes. RNA probes were synthesized from chick *Gli1* and *Gli2* cDNA (Marigo et al., 1996b) and quail *Gli3* cDNA. Embryos were photographed using a video camera (Leica) and embedded in 4% low-melting point agarose (Sigma), and 100 μm transverse sections were cut using a Vibrotome 1000. Sections were photographed on a DIC microscope (Leica).

RESULTS

cDNA cloning and sequence analysis of a quail *Gli3*

Two chicken Gli proteins were previously identified (Marigo et al., 1996b) and classified as Gli1 and Gli2 (Borycki et al., 1998). As the mammalian *Gli* gene family comprises three members, we conducted a PCR-based cloning and library

Fig. 1. Amino acid sequence analysis and comparison of quail Gli3 with *Xenopus*, mouse and human Gli3. Quail, *Xenopus*, mouse and human Gli3 proteins are highly conserved and share 66-70% identity throughout the protein, with higher similarity at the N-terminal half of the protein. Dark gray shaded areas indicate residues that are conserved among Gli3 proteins. Light gray shaded areas indicate residues that are similar among Gli3 proteins. Homology domains, including the zinc finger domain (ZF), which have previously been described (Ruppert et al., 1990; Marine et al., 1997) are indicated by a black bar above the amino acid sequence. Phosphorylation sites for cAMP-dependent protein kinase (PKA) are indicated by boxes. Red arrows indicate the boundaries of the repressor domain of Gli3, as described by Sasaki et al. (1999). Green arrows indicate the boundaries of the binding domain for the transcriptional co-activator CBP, which also corresponds to the activation domain of Gli3, and PKA is thought to mediate cleavage in the region of aa 650-750 to produce a repressing form of Gli3, described by Dai et al. (1999). The cDNA we recovered from a stage-12 quail cDNA library contains an additional 65bp sequence, which introduces stop codons in the N-terminal domain of the protein (sequence in bracket; position 122). An in-frame methionine at position 201 (black arrowhead), surrounded by a conserved Kozak sequence for translational start (Kozak, 1989), could theoretically produce a truncated C-terminal protein without a functional N-terminal repressor domain. RT-PCR analysis shows that the major transcript found in the embryo does not have the 65 bp insertion and encodes a 1576 amino acid protein.

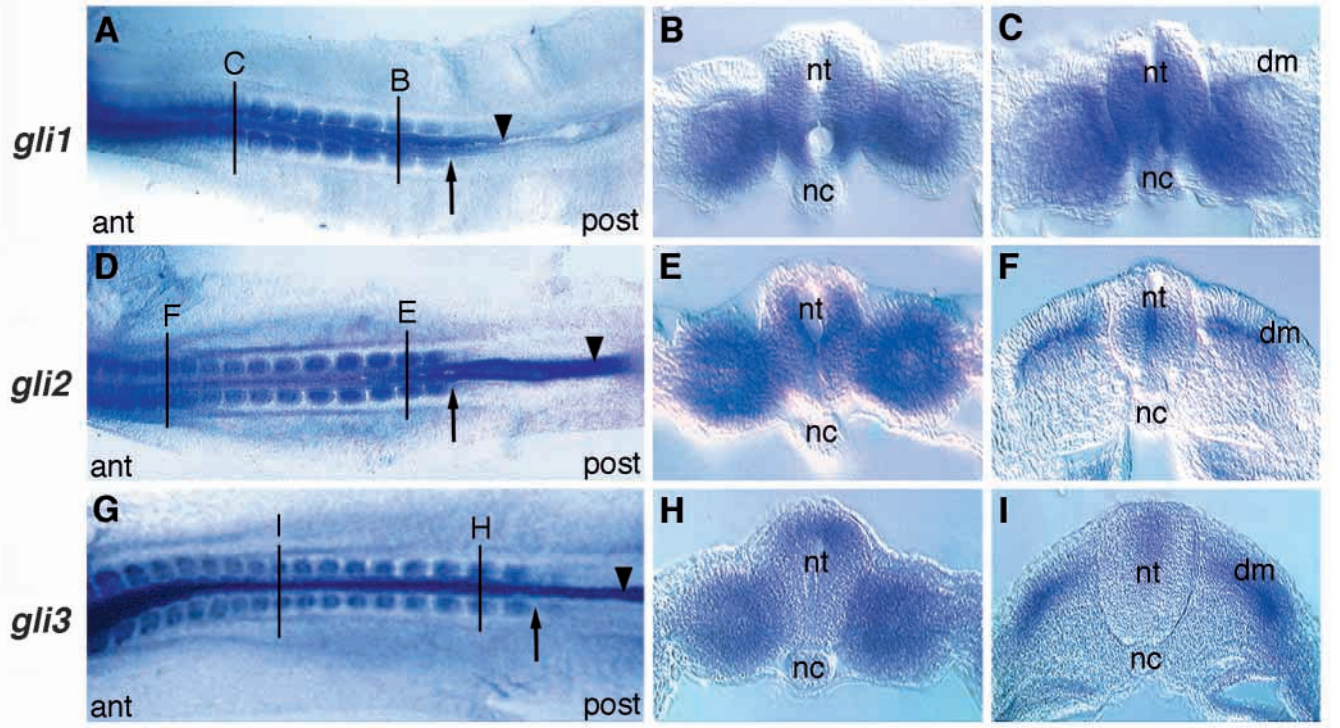

Fig. 2. *Gli1*, *Gli2* and *Gli3* regulation during somite formation. *Gli3* (G-I) expression at stage 12 was analyzed by whole-mount in situ hybridization of two quail embryos using DIG-labeled RNA probes, and compared to previously described *Gli1* (A-C) and *Gli2* (D-F) expression (Borycki et al., 1998). *Gli1, Gli2 and Gli3* genes are activated in coordination with somite formation (black arrow), whereas the expression of these *Gli* genes in the neural tube extends more posteriorly (black arrowhead). Transverse sections were cut at the indicated positions in A,D,G using a Vibrotome, following in situ hybridization. Expression of *Gli1* in somites remains localized to the medio-ventral domain during somite maturation (B,C), whereas *Gli2* and *Gli3* expression is first activated throughout newly formed somites (E,H), but then becomes rapidly restricted to the dorsal medial *MyoD* expressing domain in the prospective myotomal compartment (F,I). *Gli2* transcripts then become localized to the medial myotome (F), and *Gli3* transcripts become localized in the lateral myotome during later somite maturation (I). nt, neural tube; nc, notochord; dm, dermomyotome; ant, anterior; post, posterior. Magnification, 35× (A,D,G); 40× (B,C,E,F,H,I).

screen to isolate a 5025 bp full-length quail *Gli3* cDNA clone. Sequence analysis reveals that this quail clone is homologous to vertebrate *Gli3* cDNAs (Fig. 1). Quail Gli3 encodes a 1576 amino acid protein that has 66% identity with *Xenopus* Gli3, 69% identity with mouse Gli3 and 70% identity with human Gli3. Quail Gli3 also has 98-99% sequence homology with human, *Xenopus* and mouse *Gli3* in the zinc finger domain and displays highly conserved sequences in the N- and C-terminal domains (Fig. 1), some of which have been described previously (Ruppert et al., 1990; Marine et al., 1997). The quail *Gli3* cDNA we isolated has an additional 65 bp insertion in its 5′ region, probably resulting from alternative splicing (Fig. 1). RT-PCR analysis, using primers on either side of the insertion site, was performed on RNA recovered from whole embryos and from dissected somites at HH stage 12. A minor amplified PCR product identified includes the 65 bp insertion found in the *Gli3* cDNA sequence, whereas the major amplified PCR product lacks this insertion (data not shown), supporting the view that this insertion is an alternatively spliced exon. The minor *Gli3* transcripts with this insertion sequence are enriched in somite transcripts. The translation product encoded by this cDNA has three in-frame stop codons that would lead to the production of an N-terminal Gli3 fragment (Fig. 1). The 3′ region of this cDNA also has an in-frame methionine and Kozak sequence that could be utilized to produce the Gli3 C-terminal protein (Kozak, 1989) that would lack most of the N-terminal repressor domain (Dai et al., 1999). It remains to be investigated whether these variant Gli3 proteins are produced in somites.

Gli1, *Gli2* and *Gli3* are activated in coordination with somite formation, but have distinct somite domains of expression

Whole-mount in situ hybridization methods were used to compare the expression of *Gli3* with *Gli1* and *Gli2* during somite formation in stage-12 quail embryos (Fig. 2). We observed that *Gli3*, like *Gli1* and *Gli2* (Borycki et al., 1998), is undetectable in the segmental plate mesoderm, but is activated to high levels in paraxial mesoderm initiating somite formation (Fig. 2A,D,G). *Gli3*, like *Gli2*, is expressed throughout the newly formed somite, with higher expression in the domain surrounding the somitocoele and lower expression in the lateral somite (Fig. 2E,H). In contrast, *Gli1* expression is restricted to the ventromedial somite domain encompassing the domain of *Pax1* expression, but including the more dorso-medial domain of *MyoD* activation (Fig. 2B,C). The spatial patterning of *Gli1* expression remains unchanged during somite maturation, but *Gli2* and *Gli3* expression becomes dorsalized to the dorso-medial domain of *MyoD* expression and myotome formation (Fig. 2F,I). Significantly, *Gli2* and *Gli3* expression are patterned identically in posterior somites; however, as somites mature along the antero-posterior axis,

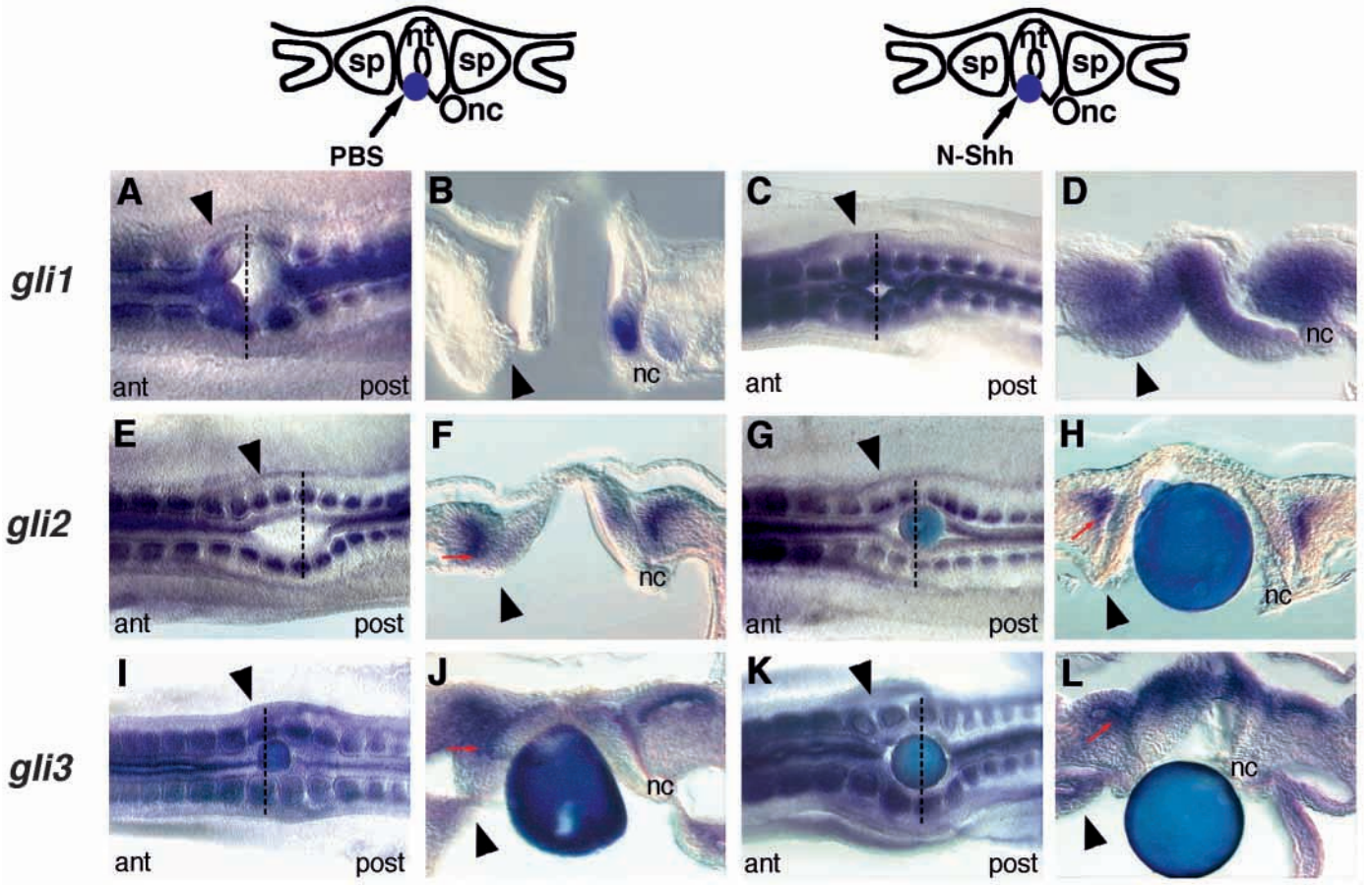

Fig. 3. Sonic hedgehog function in *Gli* regulation and patterning during somite formation. Notochord and floor plate were separated from the segmental plate on one side of the embryo by a surgical incision along the ventral neural tube in the region of segmental plate mesoderm. A bead soaked in PBS (A,B,E,F,I,J) or in a solution of 100 μg/ml purified N-Shh (C,D,G,H,K,L) was inserted in the neural tube, and embryos were cultured for 16 hours. Expression of *Gli1* (A-D), *Gli2* (E-H) and *Gli3* (I-L) was assessed by whole-mount in situ hybridization followed by transverse sectioning. (A,B) As previously described (Borycki et al., 1998), *Gli1* is not expressed in somites or neural tube on the operated side without associated notochord (black arrowhead), indicating that *Gli1* expression requires notochord signals. (C,D) Implantation of a bead soaked in N-Shh restores and upregulates *Gli1* activation in somites and neural tube on the operated (black arrowhead) and control side of the embryo, indicating that Shh is sufficient for *Gli1* activation. (E,F) After notochord ablation surgery, *Gli2* expression fails to become dorsally restricted (red arrow) on the operated side (black arrowhead), establishing that the notochord is required for the dorsalization of *Gli2* transcripts in somites. (G,H) Implantation of a bead soaked in N-Shh restores *Gli2* dorsalization in somites (red arrow) on the operated side (black arrowhead), showing that Shh controls *Gli2* dorsalization. (I,J) *Gli3* expression fails to become dorsally restricted (red arrow) on the operated side (black arrowhead), indicating that notochord is required for *Gli3* dorsalization. (K,L) Implantation of a bead soaked in N-Shh restores *Gli3* dorsalization in somites (red arrow) on the operated side (black arrowhead), establishing that Shh is sufficient for *Gli3* dorsalization. Dashed lines indicate sites of transverse sections. ant, anterior; post, posterior; nc, notochord. Magnification, 35× (A,C,E,G,I,K); 40× (B,D,F,H,J,L).

Gli2 expression becomes predominant in the medial myotome, whereas *Gli3* expression becomes predominant in the lateral myotome (Fig. 2F,I). In addition, *Gli3*, but not *Gli2*, is strongly expressed in the posterior mesoderm and tail bud (Fig. 5B,E) and thus could have a specific function in the early processes of mesoderm development. The tail bud expression of *Gli3* in stage-13 embryos overlaps with the domain of expression of *Bmp4* and *Hoxd10*, two target genes of Shh signaling that are implicated in gut formation, but is not expressed in the caudal intestinal portal domain where Shh is expressed (Roberts et al., 1995). In the neural tube, *Gli3* expression extends more posteriorly than does *Gli2* or *Gli1* (Figs 2A,D,G, 5B,E), but expression of all three *Gli* genes in the neural tube extends more posteriorly in stage-12 embryos than their expression in paraxial mesoderm, which is delimited by the boundary of somite formation. The posterior expression of these *Gli* genes as well as *Ptc1* in the neural tube provides evidence that the notochord is producing functional levels of Shh in the posterior embryo where somites have not yet formed (Borycki et al., 1998). *Gli1* expression is predominant in the ventral neural tube, *Gli2* expression is predominant in the central neural tube, and *Gli3* expression is restricted to the dorsal-most neural tube (Fig. 2B,E,H), as reported in the mouse embryo (Hui et al., 1994; Lee et al., 1997; Sasaki et al., 1997).

The notochord and Shh are not required for the *Gli2* and *Gli3* activation in somites, but are required for their dorso-ventral patterning

Surgical and Shh bead implantation experiments were undertaken to investigate the role of the notochord and Shh in

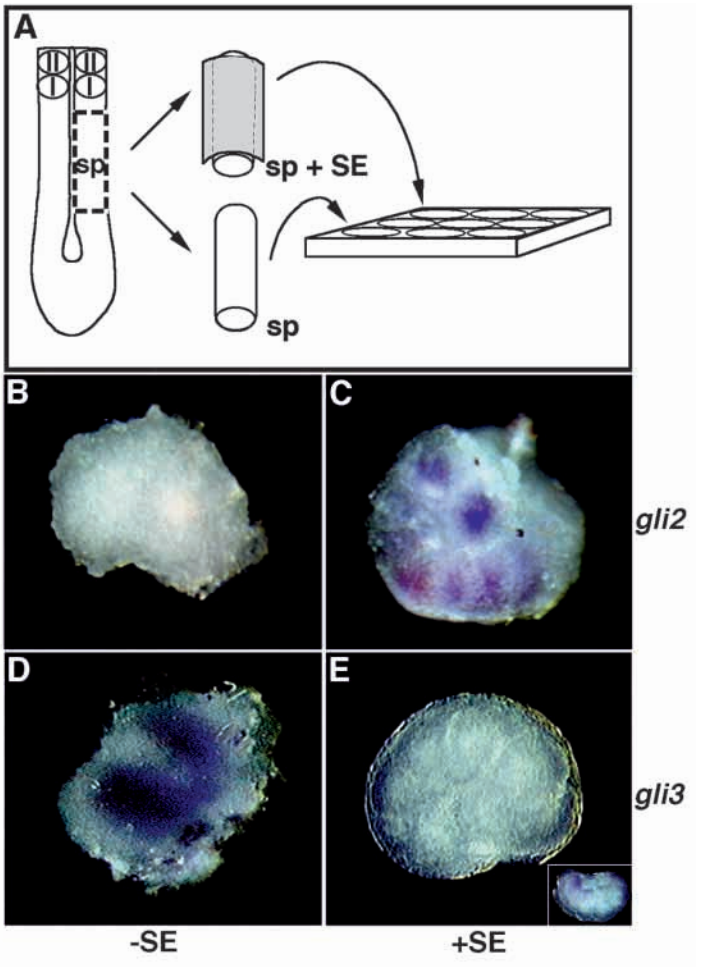

Fig. 4. Surface ectoderm differentially controls *Gli2* and *Gli3* expression and somite formation. (A) Segmental plate (sp) from stage-12 quail embryos was explanted onto agarose-coated plates and cultured for 16 hours in the presence (C,E) or absence (B,D) of surface ectoderm (SE). Explants were then analyzed by whole-mount in situ hybridization using *Gli2* (B,C) or *Gli3* (D,E) DIG RNA probes. (B,C) As previously described (Borycki et al., 1998), surface ectoderm positively controls mesodermal *Gli2* activation in coordination with somite formation. Expression of *Gli2* is detected only in explants cultured in the presence of surface ectoderm (C), but not in explants cultured in the absence of surface ectoderm (B). (D,E) Surface ectoderm negatively controls mesodermal *Gli3* expression. Explants cultured in the absence of surface ectoderm activate higher levels of *Gli3* (D) than do explants cultured associated with surface ectoderm (E). Staining reactions of explants in D and E were developed for the same period of time (4 hours). Insert in E shows *Gli3* expression in explants with surface ectoderm, as revealed after longer development in the staining solution. Magnification, 60×.

the control of *Gli* genes during somite formation. Previously, we reported that Shh produced by the notochord is required for the activation of *Gli1* expression during somite formation (Borycki et al., 1998). To examine whether notochord Shh signals control *Gli2* and *Gli3* activation, we surgically separated the notochord and floor plate from the paraxial unsegmented mesoderm on one side of stage-12 quail embryos, leaving the contralateral side as a control. Embryos were then cultured for 16 hours, during which time somites formed around the site of surgery, and *Gli* gene expression was assayed by in situ hybridization (Fig. 3). In contrast to *Gli1* activation, which is blocked by notochord separation (Fig. 3A,B), we found that *Gli2* and *Gli3* activation does not require notochord signals (Fig. 3E,F,I,J). However, we observed that the notochord is required for the dorsalization of *Gli2* and *Gli3* expression, which normally occurs after somite formation (Fig. 3F,J). To determine whether Shh signaling is responsible for the dorsalization of *Gli2* and *Gli3*, we surgically separated the notochord from the segmental plate mesoderm on one side of the embryo and implanted a bead soaked in a solution of purified N-terminal fragment of Sonic hedgehog (N-Shh), which has been shown to convey Shh signaling activity (Riddle et al., 1993; Marti et al., 1995; Roelink et al., 1995). In situ hybridization analysis of embryos 16 hours after culture revealed that N-Shh restores *Gli1* activation, as we have previously reported (Borycki et al., 1998) (Fig. 3C,D), and also restricts *Gli2* and *Gli3* expression to the dorsal, myotome-forming region of the somite (Fig. 3G,H,K,L). We conclude that Shh signaling is not required for *Gli2* and *Gli3* activation during somite formation, but is required for the dorsalization of *Gli2* and *Gli3* expression during somite maturation.

Surface ectoderm/neural tube Wnts, transduced through β-catenin, control *Gli2* and *Gli3* and somite formation

Additional surgical and explant studies were undertaken to investigate the roles of surface ectoderm and neural tube in *Gli2* and *Gli3* regulation during somite formation. Explants of segmental plate mesoderm from stage-12 quail embryos were cultured for 16 hours in presence or in the absence of overlying ectoderm (Fig. 4A), and *Gli2* and *Gli3* expression was assayed by in situ hybridization. As previously reported (Borycki et al., 1998), surface ectoderm induces both somite formation and *Gli2* expression in segmental plate explants (Fig. 4B,C). In contrast, we found that *Gli3* is activated to high levels in paraxial mesoderm explants after removal of surface ectoderm (Fig. 4D,E). To determine whether surface ectoderm signals actively repress *Gli3* expression in the segmental plate mesoderm, we preformed in vivo surgical experiments. Surface ectoderm overlying the posterior segmental plate mesoderm and axial tissues were separated on one side of stage-12 embryos (Fig. 5A), as we found that the neural tube provides redundant signals for *Gli2* and *Gli3* regulation (Borycki et al., 1998). Embryos were cultured for 7 hours in order to allow the surgery site to remain at the level of the segmental plate mesoderm. When *Gli2* and *Gli3* expression was assayed by in situ hybridization, we observed that *Gli3* is prematurely activated to high levels in the segmental plate mesoderm on the operated side, whereas *Gli3* is not detected in the segmental plate mesoderm on the unoperated control side (Fig. 5B,C). Transverse sections reveal that, on the operated side, *Gli3* is expressed throughout the segmental plate mesoderm (Fig. 5D). In contrast, *Gli2* is not expressed in segmental plate mesoderm in absence of surface ectoderm and axial tissues (Fig. 5E-G), demonstrating that distinct mechanisms control *Gli2* and *Gli3* expression: surface ectoderm and neural tube signals positively control the activation of *Gli2* expression and the initiation of somite formation, whereas *Gli3* expression is a cell-autonomous property of the segmental plate mesoderm in

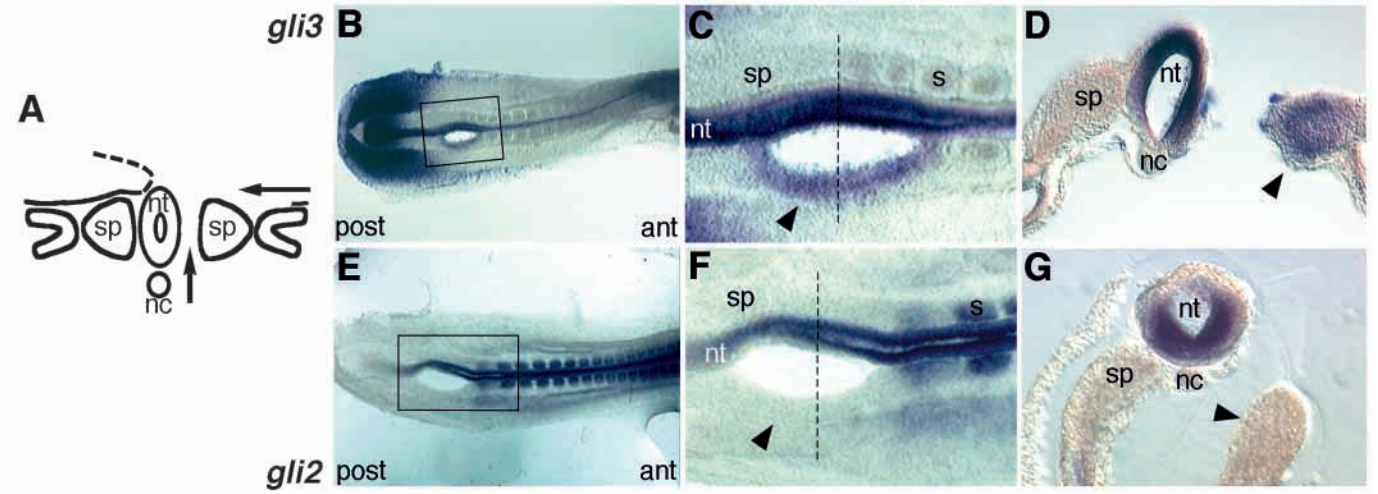

Fig. 5. In embryos, surface ectoderm represses *Gli3* expression in the segmental plate mesoderm. (A) Surface ectoderm and axial tissues were separated from segmental plate (sp) of stage-12 quail embryos (Borycki et al., 1998). Embryos were then cultured for 7 hours and analyzed by whole-mount in situ hybridization using *Gli3* (B-D) and *Gli2* (E-G) DIG RNA probes. (B,C) Expression of *Gli3* is prematurely activated in the segmental plate on the operated side (black arrowhead) whereas the control unoperated side does not express *Gli3*. Note that *Gli3* expression is readily detected on the operated side of the embryo even though development of the in situ hybridization color reaction was stopped before *Gli3* is normally detected in somites on the control side of the embryo. (D) Transverse section at the level of the surgery showing that *Gli3* is expressed in the operated segmental plate (black arrowhead) in a similar manner to newly formed somites (see Fig. 2H). (E,F) Expression of *Gli2* is not induced by surface ectoderm removal (black arrowhead). Note that, in this experiment, the development of the color reaction was prolonged until somitic expression of *Gli2* is detected. (G) Transverse section at the level of the surgery shows no expression of *Gli2* in the segmental plate of the operated embryos (black arrowhead). Boxes in B and E indicate the magnified region shown in C and F. Dashed lines indicate sites of transverse sections. nt: neural tube; nc: notochord. Magnification, 15× (B,E); 40× (C,D,F,G).

the absence of surface ectoderm. *Gli3* activation at the time of somite formation, therefore, involves a derepression mechanism that must be mediated by a change in surface ectoderm signaling or in the response of the newly forming somite to these signals.

Wnt signaling controls *Gli2* activation and *Gli3* repression

To identify the surface ectoderm signaling molecules responsible for the regulation of *Gli2* and *Gli3* expression during somite formation, we undertook a candidate molecule approach. Wnts are known surface ectoderm and neural tube signaling molecules (Parr et al., 1993; Parr and McMahon, 1995; Cadigan and Nusse, 1997; Willert and Nusse, 1998; Eastman and Grosschedl, 1999). To test whether Wnt proteins control *Gli* gene expression in the segmental plate mesoderm, we cocultured quail segmental plate mesoderm explants without surface ectoderm in collagen gels, either with RatB1a fibroblast cells stably infected with a MV7 control vector or with this vector expressing mouse Wnt1 or Wnt4. Explants without surface ectoderm cocultured with MV7-expressing RatB1a fibroblasts activate *Gli3* expression (Fig. 6D), and do not express *Gli2* (Fig. 6A). When explants are cocultured with Wnt1-expressing RatB1a fibroblasts, *Gli2* expression is induced to high levels throughout the unsegmented paraxial mesoderm in greater than 80% of explants (Fig. 6B). Wnt4-expressing RatB1a fibroblasts also induce *Gli2* expression in segmental plate explants in absence of surface ectoderm, but to a lower level than Wnt1-expressing cells (Fig. 6C). Wnt1-expressing RatB1a cells maintain the repression of *Gli3* in segmental plate explants (Fig. 6E), which would express high levels of *Gli3* in the absence of surface ectoderm, whereas Wnt4-expressing cells are less potent repressor of *Gli3* (Fig. 6F). These data, therefore, establish that Wnt1 and Wnt4 can provide both positive and negative signaling activities for *Gli2* activation and *Gli3* repression.

LiCl can mimic surface ectoderm signaling in *Gli2* and *Gli3* regulation and the initiation of somite formation

Wnt1 and Wnt4 transduce their signals through inhibition of Glycogen Synthase Kinase-3 beta (GSK-3β), which results in the stabilization of β-catenin in responding cells, leading to formation of nuclear β-catenin/LEF1/tcf transcription factor complexes (Willert and Nusse, 1998; Eastman and Grosschedl, 1999). β-catenin-dependent Wnt signaling can be mimicked by LiCl treatment, which also acts to increase β-catenin by inhibiting GSK-3β β (Klein and Melton, 1996; Hedgepeth et al., 1997). To test whether activation of *Gli2* and *Gli3* is controlled by a β-catenin-dependent signaling mechanism, as predicted by the activities of Wnt1 and Wnt4 on paraxial mesoderm explants, we cultured segmental plate mesoderm explants from stage-12 quail embryos, without surface ectoderm, in the presence or absence of 10 mM LiCl. Consistent with the explant assays described above, we found that, in absence of LiCl, explants activate *Gli3* at high levels (Fig. 7E) but do not form somites, retain a characteristic rough morphology, and do not express *Gli2* (Fig. 7A). However, treatment of explants with 10 mM LiCl induces somite structures, as attested by the epithelial shape and the condensation of explant cells, and by the segmented expression pattern of *Gli2* and *Gli3* in these explants (compare Fig. 7B′,F′ with 7A′,E′). The somite structures formed in LiCl-treated explants are not as well organized as observed in explants

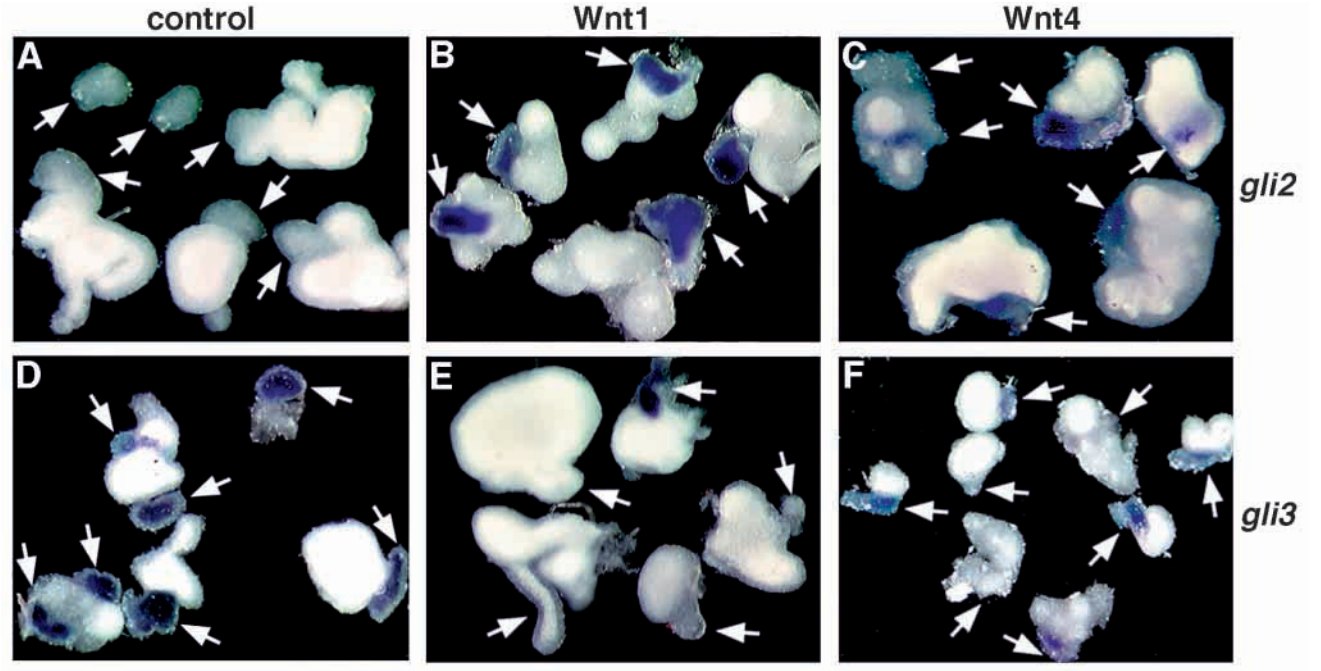

Fig. 6. Activation of *Gli2* and repression of *Gli3* by Wnt1 and Wnt4 in segmental plate mesoderm explants. Segmental plate explants without surface ectoderm from stage-12 quail embryos were embedded in collagen gel with aggregates of RatB1a fibroblasts expressing a control vector Mv7 (A,D) or Wnt1 (B,E) or Wnt4 (C,F). Explants were cultured for 16 hours and analyzed by in situ hybridization with *Gli2* (A-C) and *Gli3* (D-F) RNA probes. (A) *Gli2* is not expressed in explants cocultured with cells expressing the control vector, but is activated to high levels in presence of Wnt1-expressing cells (B), and at lower levels in response to Wnt4-expressing cells (C). (D) Expression of *Gli3* is prematurely induced in explants cultured in absence of surface ectoderm with control cells. Wnt1 (E) and to a lower extent, Wnt4 (F), repress *Gli3* expression in segmental plate explants. White arrows indicate explants adjacent to RatB1a cells. Magnification, 48×.

associated with surface ectoderm (compare Fig. 7B′,F′ with 7C′,G′), suggesting that additional surface ectoderm signals are required to complete the process of somite formation. LiCl also activates *Gli2* and *Gli3* expression in a segmental pattern (Fig. 7B,F), as well as maintains the repression of *Gli3* in unsegmented regions of explants (Fig. 7F). Given the activity of LiCl as an inhibitor of GSK-3β, these data indicate that *Gli2* and *Gli3* activation during somite formation is controlled by β catenin-dependent Wnt signaling. In contrast to Wnt1 and Wnt4 activities, LiCl treatment activates *Gli2* locally in somite segments, but not in the unsegmented regions, indicating that LiCl treatment can coordinate *Gli2* and *Gli3* activation with somite formation. Therefore, during the process of somite formation, Wnt/β-catenin signaling undergoes a negative to positive switch from repression of *Gli3* in segmental plate mesoderm to activation of somite formation and *Gli2* and *Gli3* in newly formed somites.

To examine whether *Gli2* and *Gli3* expression is a downstream response to the induction of somite formation by LiCl, we compared the activity of LiCl on *Gli2* and *Gli3* expression in unsegmented paraxial mesoderm explants that were maintained in association with their overlying surface ectoderm, which induces robust somite formation. Explants associated with surface ectoderm were found to express much higher levels of *Gli2* (Fig. 7D) and *Gli3* (Fig. 7H) in response to LiCl than did untreated explants (Fig. 7C,G), even though somite formation was equivalent in treated and untreated explants (Fig. 7D,H versus Fig. 7C,G). The higher levels of *Gli2* and *Gli3* expression in LiCl-treated explants provides evidence that both *Gli2* and *Gli3* can respond to LiCl, independent of the initiation of somite formation. The enhanced expression of *Gli3* in somites formed in LiCl-treated explants in contact with surface ectoderm indicates that *Gli3* expression is also subject to positive regulation through β-catenin, following its derepression at the time of somite formation. β-catenin, therefore, has a central role coordinating the activation of both *Gli2* and *Gli3* with the initiation of somite formation.

DISCUSSION

Gli genes are co-activated during somite formation

In this study, we have investigated the regulation of *Gli* genes during somite formation in quail embryos. We report that *Gli1*, *Gli2* and *Gli3* are co-activated in temporal coordination with the process of somite formation. The activation of these *Gli* genes coincides with the initiation of Shh responsiveness in newly formed somites, as indicated by the activation of a set of Shh-dependent genes that includes *Ptc1*, which is a known Shh-responsive gene (Chen and Struhl, 1996; Goodrich et al., 1996; Marigo et al., 1996a), as well as *Myf5*, *MyoD* and *Pax1*, which control somite cell specification (Borycki et al., 1998, 1999a). Considering that molecular and genetic evidence indicate that Gli transcription factors are effectors of Shh signal transduction in vertebrates (Hynes et al., 1997; Lee et al., 1997; Platt et al., 1997; Sasaki et al., 1997, 1999; Ding et al., 1998; Matise et al., 1998), these findings support a model for the initiation of Shh responsiveness in newly formed somites through the coordinated activation of this set of *Gli* genes.

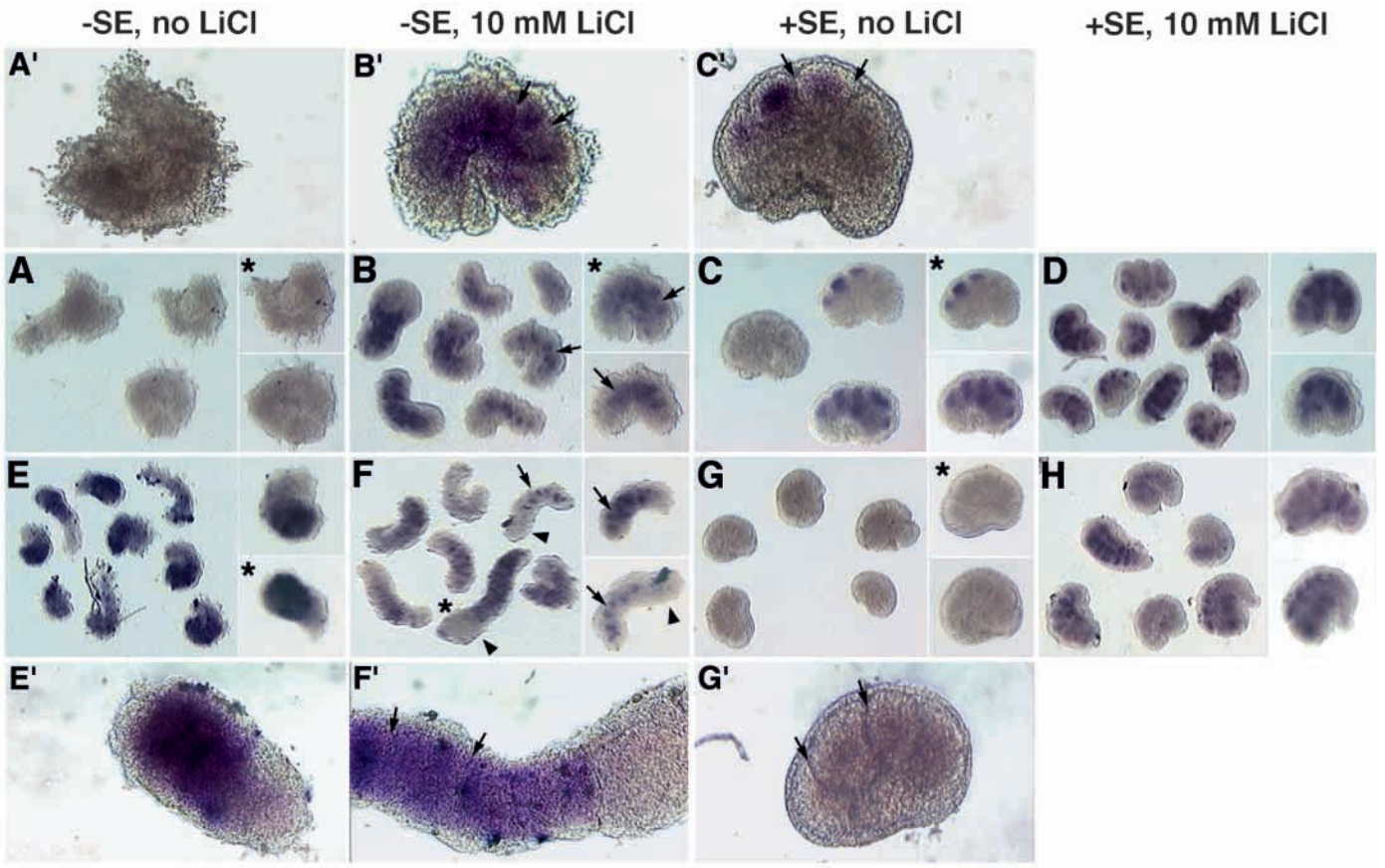

Fig. 7. LiCl induces somite formation and the somite-specific activation of *Gli2* and *Gli3* in segmental plate explants. (A-D) In situ hybridization analysis of *Gli2* expression in segmental plate explants from stage-12 quail embryos cultured in the absence (A,A′,B) or presence (C,C′,D) of surface ectoderm (SE), and in the presence (B,B′,D) or absence of 10 mM LiCl (A,C,C′). Addition of LiCl to explants cultured in absence of SE (B,B′) promotes *Gli2* activation as well as induces somite formation (black arrows). Addition of 10 mM LiCl to explants cultured in presence of SE (D) upregulates *Gli2* expression to levels higher than detected by surface ectoderm alone (compare C and D). (E-H) In situ hybridization analysis of *Gli3* expression in segmental plate explants from stage-12 quail embryos cultured in the absence (E,E′,F) or presence (G,G′,H) of surface ectoderm (SE), and with (F,F′,H) or without (E,E′,G) LiCl. In the absence of SE (E,E′), *Gli3* is expressed throughout explants; but in the presence of LiCl (F,F′), *Gli3* expression is restricted to LiCl-induced somites (arrows) and continues to be repressed in unsegmented regions of explants (arrowheads). A′, B′, C′, E′, F′ and G′ are phase micrographs (magnification, 140×) of explants marked with asterisks in A, B, C, E, F and G, respectively. Magnification, 60×.

Once these *Gli* genes are expressed, they can then respond to Shh, which is constitutively expressed by the underlying notochord along the axis of the embryo. Somites, therefore, provide the first example of an embryonic tissue in which Shh transduction is controlled at the level of *Gli* gene regulation. It remains to be determined whether the Shh-responsive genes such as *MyoD* and *Pax1*, which become activated following somite formation, are direct targets of Gli transcription factors or whether these genes are regulated by downstream effectors of Shh signaling. Investigations of the expression, processing and functional activities of Gli proteins and splicing variants, such as described in this study, will be required to define the gene targets of Gli transcription factors in newly formed somites.

Distinct signaling mechanisms regulate *Gli* genes during somite formation

Embryo surgery and segmental plate mesoderm explant studies reveal that distinct signaling mechanisms regulate each of the three *Gli* genes in somites. *Gli2* and *Gli3* are subject to positive and negative regulation, respectively, by Wnt signaling from the surface ectoderm and neural tube. LiCl studies provide supporting evidence that Wnt regulation of *Gli2* and *Gli3* is mediated by β-catenin, which coordinates the activation of these genes with the initiation of somite formation. Prior to this study, there has been no information on the mechanisms that regulate *Gli2* and *Gli3* transcription and on the role of *Gli* regulation in the control of Shh signaling. Therefore, future investigations of the functions of Wnts and β-catenin in the control of *Gli2* and *Gli3* in other Shh-responsive tissues, including the nervous system and limb (Hui et al., 1994; Marigo et al., 1996b; Lee et al., 1997; Sasaki et al., 1997), will be of interest to determine if these mechanisms for *Gli* regulation are widely utilized. In contrast to *Gli2* and *Gli3*, which are controlled by surface ectoderm/neural tube Wnt signaling, *Gli1* activation is controlled by Shh signaling from the notochord. The cooperative interactions of surface ectoderm/neural tube and notochord signaling in the activation of the three *Gli* genes provides a molecular explanation for earlier findings that these tissues and Shh and Wnt signaling

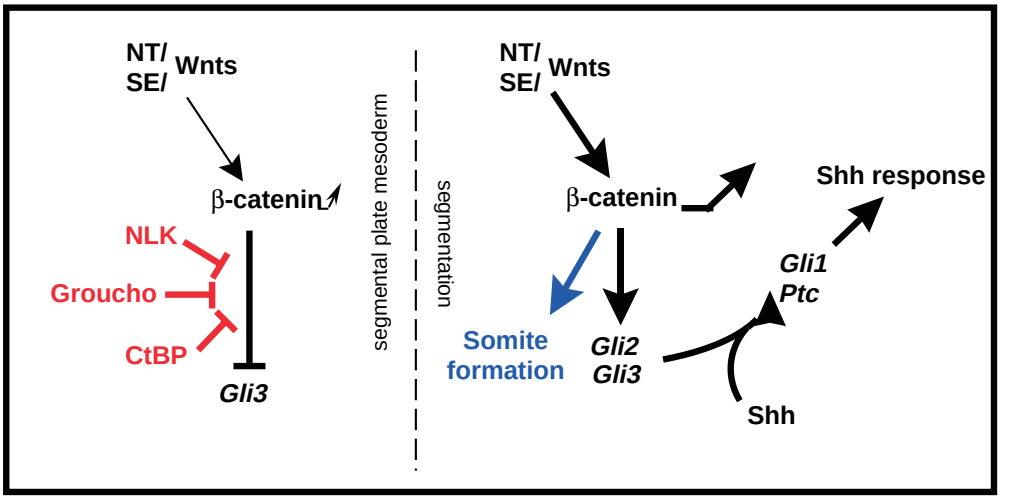

Fig. 8. Model for Wnt and Shh signaling in the control of *Gli* gene activation during somite formation. In segmental plate mesoderm, *Gli3* is maintained in a repressed state by Wnt signaling through β-catenin. When anteriormost segmental plate mesoderm initiates somite formation, Wnt/β-catenin signaling undergoes a negative to positive switch, leading to derepression of *Gli3*, to the initiation of somite formation, and to activation of the somite-specific expression of *Gli2* and *Gli3*. This switch in Wnt/β-catenin function could be mediated by transcription cofactors such as Groucho, NLK and CtBP (Fisher and Caudy, 1998; Brannon et al., 1999; Ishitani et al., 1999; Meneghini et al., 1999) that are known to control the transcription activities of β-catenin/LEF1/tcf complexes in segmental plate mesoderm. The process of somite formation and the regulated expression of β-catenin cofactors would be under the control of the segmentation genes (Palmeirim et al., 1998). Quantitative changes in Wnt signaling at the time of somite formation, resulting from the activation of Wnt expression in the neural tube (Hirsinger et al., 1997; Marcelle et al., 1997) and loss of Wnt inhibitors in newly forming somites (Hoang et al., 1998), would mediate increased levels β-catenin. This high level of β-catenin would participate in both the cytoplasmic cell adhesion processes to initiate somite formation as well as in new β-catenin/LEF1/tcf transcription complexes for *Gli2* and *Gli3* activation. The Gli2 and Gli3 proteins produced in newly formed somites would then become activated as nuclear transcription factors in response to Shh that is produced by the notochord, leading to their participation in the activation of Shh response genes, including *Gli1* and *Ptc1*.

function synergistically to regulate the expression of somite-specific genes (Buffinger and Stockdale, 1994; Fan and Tessier-Lavigne, 1994; Munsterberg et al., 1995; Munsterberg and Lassar, 1995; Stern et al., 1995; Borycki et al., 1997, 1998; Fan et al., 1997; Tajbakhsh et al., 1998).

Somite formation is accompanied by a switch in Wnt signaling

The results of our in vivo and in vitro studies reveal that *Gli3* is repressed by surface ectoderm/neural tube signals in segmental plate mesoderm, whereas somite formation and *Gli2* and *Gli3* activation are under the positive control of surface ectoderm/neural tube signals. Therefore, the surface ectoderm/neural tube signaling that regulates *Gli2* and *Gli3* activation undergoes a switch at the time of somite formation. Our Wnt coculture experiments provide evidence that Wnts are the surface ectoderm/neural tube signaling molecules that mediate *Gli3* repression in segmental plate mesoderm as well as the activation of *Gli2* and *Gli3* to initiate Shh signaling at the time of somite formation. In support of the essential role played by Wnt signaling in somites, injection of Frzb-1 expressing cells into the placenta of a pregnant female mouse blocks the Shh-dependent activation of *Myf5* activation in the somites of embryos (Borello et al., 1999). As both Wnt1 and Wnt4 transduce their signaling activity through β-catenin, by inhibition of GSK-3β (Willert and Nusse, 1998; Eastman and Grosschedl, 1999), these findings indicate that both the repressing and the activating functions of Wnt signaling in *Gli* gene regulation are mediated through β-catenin.

A molecular model for a switch in Wnt signaling at the time of somite formation is shown in Fig. 8. This model proposes that this switch is controlled by a regulated change in the association of β-catenin/LEF1/tcf transcription complexes with cofactors such as Groucho, NLK and CtBP (Fisher and Caudy, 1998; Brannon et al., 1999; Ishitani et al., 1999; Meneghini et al., 1999). These cofactors modulate whether β-catenin/LEF1/tcf transcription complexes have repressing or activating gene regulatory functions. Presumably, the segmentation genes, which control the initiation of somite formation (Palmeirim et al., 1998), could mediate this switch in β-catenin function.

Gli gene regulation by Wnt signaling from surface ectoderm and neural tube is coordinated with the initiation of somite formation. We observed that, although Wnt1 and Wnt4 can repress *Gli3* and activate *Gli2* in explant coculture assays, LiCl can induce the entire repertoire of signaling activities of surface ectoderm/neural tube on segmental mesoderm, including somite formation and the regulated activation of *Gli2* and *Gli3*. A previous report has shown that β-catenin levels increase in segmental plate cells prior to somite formation in chick embryos, and that LiCl treatment induces ectopic somite formation and β-catenin activation in segmental mesoderm (Linask et al., 1998). A number of explanations can account for the observed differences in the activities of LiCl and Wnts in our assays. One possibility is that the Wnts produced in vivo by the surface ectoderm and neural tube have more potent signaling activity or are produced at higher levels than the Wnt1 and Wnt4 produced in our coculture assays, leading to higher accumulation of β-catenin to levels required for somite formation and *Gli* regulation. Consistent with this possibility, LiCl is a chemical inhibitor of GSK-3β that bypasses all upstream processes required for Wnt signaling, and in this study, its activity has been optimized to mediate a maximal response for somite formation and *Gli* activation. Alternatively, surface ectoderm and neural tube may provide signals, in addition to Wnts, that are required for somite formation and *Gli* regulation, and LiCl may be able to activate the entire array of responses directed by these signals. Finally, we do not exclude the possibility that matrix factors present in collagen gels used to assay Wnt activity in our Wnt coculture assays

may selectively prevent somite epithelialization. Collagen is not used in LiCl treatment experiments, in which segmental mesoderm explants are maintained on agarose substrates. Consistent with this possibility, collagen has previously been shown to interfere with *Pax1* and *Sim1* expression in somite explant cultures (Fan and Tessier-Lavigne, 1994). In any case, our findings provide clear evidence that Wnts acting through β-catenin are critical signals that regulate both somite formation and *Gli2* and *Gli3* activation.

The accumulation of β-catenin in newly forming somite cells could be regulated in vivo by mechanisms that regulate the levels of Wnt synthesis or the activities of Wnts. At this time, we do not know which Wnts are produced by surface ectoderm and neural tube to regulate somite formation and *Gli* activation. Wnt1 and Wnt4, which have activities in our induction coculture assays, are reasonable candidates, based on their expression in surface ectoderm (Fan et al., 1997) and neural tube (Parr and McMahon, 1994). It is notable that several different *Wnt* genes, including *Wnt1* and *Wnt4*, are activated in the neural tube at the level along the embryo axis where somites are forming (Hirsinger et al., 1997; Marcelle et al., 1997), providing a source of new Wnts and increased levels of Wnt signaling to neighboring segmental plate cells that are initiating somite formation. In addition, Wnt signaling to newly forming somites could also be increased by a regulated reduction in the expression of inhibitors of Wnt signaling such as Dickkopf-1 (dkk-1), WIF-1, Cerberus and members of the Frizzled related protein (FRP) family (Finch et al., 1997; Leyns et al., 1997; Wang et al., 1997; Glinka et al., 1998; Hsieh et al., 1999; Piccolo et al., 1999). These inhibitors bind to Wnts and block their interactions with receptors. In support of this possibility, such Wnt inhibitors have been shown to be expressed at high levels in segmental plate mesoderm and their expression markedly decreases at the boundary of somite formation (Hoang et al., 1998). The enhanced Wnt signaling to newly forming somites, as a result of decreased expression of Wnt inhibitors and increased Wnt expression in the adjacent neural tube, could contribute to the observed accumulation of β-catenin in newly forming somites (Linask et al., 1998). These increased levels of β-catenin, in turn, would promote the cell adhesion processes of somite formation through its interaction with N-cadherins (Bellairs, 1985; Duband et al., 1987; Hatta et al., 1987), as well as promote the transcriptional activation of *Gli2* and *Gli3* through the formation of β-catenin/LEF1/tcf transcription complexes. These findings, therefore, provide evidence that Wnt signal transduction, like Shh signal transduction, is highly regulated during somite formation, and this regulation plays a central role in coordinating the process of somite formation with the regulated activation of *Gli2* and *Gli3* to initiate Shh signaling.

Shh control of *Gli1* and the dorsalization of *Gli2* and *Gli3*

In contrast to *Gli2* and *Gli3*, which we show are regulated by Wnt/β-catenin signaling, *Gli1* activation in somites is shown to be controlled by Shh signaling. Shh also controls *Gli1* expression in other tissues that are responsive to Shh signaling, including the limb and neural tube (Marigo et al., 1996b; Lee et al., 1997; Sasaki et al., 1997). As *Gli1* is activated in newly formed somites when *Gli2* and *Gli3* are activated, *Gli1* may be regulated by *Gli2* or *Gli3*, through Shh signaling. In support of this model, the mouse *Gli1* promoter has Gli binding sites and is transactivated by Gli3 (Dai et al., 1999) and *Gli2* mutant mice do not activate *Gli1* expression in the floor plate of the neural tube (Ding et al., 1998; Matise et al., 1998). An alternative possibility is that other Shh transcription effectors control *Gli1* activation in somites, for instance COUP-TFII (Krishnan et al., 1997). In either case, the regulatory elements that regulate *Gli1* activation are subject to Shh-dependent regulation in coordination with somite formation, which places *Gli1* expression in somites downstream in the Shh signaling pathway initiated at somite formation (Fig. 8). As Shh signaling also dorsalizes *Gli2* and *Gli3* expression following their activation in somites, the maintenance of *Gli1* expression in the ventral somite is independent of *Gli2* and *Gli3*, regardless of their potential roles in *Gli1* activation. Gli1 could mediate the dorsalization of *Gli2* and *Gli3* expression directly, or through downstream Shh effectors. Notably, recent data report evidence for Shh-mediated activation of *Sfrp2*, a Wnt1- and Wnt4-specific antagonist, in the ventral somite (Lee et al., 2000), providing a mechanism by which Shh could mediate *Gli2* and *Gli3* dorsalization during somite maturation. The roles of *Gli1* in sclerotome specification in the ventral somite, and *Gli2* and *Gli3* in myotome specification in the dorsal somite, remain to be discovered.

We thank Lori Mendham for her technical assistance, and the members of the Emerson laboratory for suggestions and support. We also thank Peter Klein for his suggestions and advice, Ivan Bergstein for providing Wnt4 cDNA, Andrew McMahon for a gift of purified recombinant N-Sonic hedgehog protein, and Clifford Tabin for sharing sequences before publication and for critical reading of this manuscript. This work was supported by grants from the NICHD and the Muscular Dystrophy Association to C. P. E., and from the NCI to A. M. C. B, who is a recipient of an Irma T. Hirschl career scientist award.

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