Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification

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Sonic hedgehog (Shh) is a secreted signaling molecule for tissue patterning and stem cell specification in vertebrate embryos. Shh mediates both long-range and short-range signaling responses in embryonic tissues through the activation and repression of target genes by its Gli transcription factor effectors. Despite the well-established functions of Shh signaling in development and human disease, developmental target genes of Gli regulation are virtually unknown. In this study, we investigate the role of Shh signaling in the control of *Myf5*, a skeletal muscle regulatory gene for specification of muscle stem cells in vertebrate embryos. In previous genetic studies, we showed that *Shh* is required for *Myf5* expression in the specification of dorsal somite, epaxial muscle progenitors. However, these studies did not distinguish whether *Myf5* is a direct target of Gli regulation through long-range Shh signaling, or alternatively, whether *Myf5* regulation is a secondary response to Shh signaling. To address this question, we have used transgenic analysis with *lacZ* reporter genes to characterize an *Myf5* transcription enhancer that controls the activation of *Myf5* expression in the somite epaxial muscle progenitors in mouse embryos. This *Myf5* epaxial somite (ES) enhancer is Shh-dependent, as shown by its complete inactivity in somites of homozygous *Shh* mutant embryos, and by its reduced activity in heterozygous *Shh* mutant embryos. Furthermore, Shh and downstream Shh signal transducers specifically induce ES enhancer/luciferase reporters in Shh-responsive 3T3 cells. A Gli-binding site located within the ES enhancer is required for enhancer activation by Shh signaling in transfected 3T3 cells and in epaxial somite progenitors in transgenic embryos. These findings establish that *Myf5* is a direct target of long-range Shh signaling through positive regulation by Gli transcription factors, providing evidence that Shh signaling has a direct inductive function in cell lineage specification.

[Key Words: Sonic hedgehog; Gli; Myf5; somites; muscle specification]

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Sonic hedgehog (Shh) is a member of the Hedgehog (HH) family of signaling molecules. In vertebrates, Shh functions as a morphogen to mediate long-range and short-range signaling for dorsal-ventral patterning in the specification of neural, mesodermal, and endodermal stem cell lineages (Chiang et al. 1996; Ericson et al. 1996; Goodrich et al. 1997; Hebrok et al. 2000; Ramalho-Santos et al. 2000), for limb patterning (Riddle et al. 1993), for granular cell proliferation (Wechsler-Reya and Scott 1999), and for suppression of tissue apoptosis (Teillet et al. 1998). Mutations that disrupt Shh signaling cause human birth defects (Muenke and Cohen 2000) and cancer susceptibility in mice and humans (Goodrich and Scott 1998; Hahn et al. 1999). Hedgehog signaling in *Dro-*

sophila controls cuticular patterning in embryos and tissue patterning during later development (Dahmann and Basler 2000). HH signals are transduced to responding embryonic cells through HH binding to the Patched receptor (Ingham et al. 1991; Marigo et al. 1996a; Stone et al. 1996; Fuse et al. 1999), which is a negative regulator of HH signaling in the absence of ligand. HH binding to Patched blocks its repression of another transmembrane protein, Smoothened (Smo), which induces signal transduction by activating the nuclear translocation of Ci/Gli, zinc-finger transcription factors (van den Heuvel and Ingham 1996). In vertebrates, HH signal transduction is mediated through three Gli transcription factor effectors (Hui et al. 1994). *Drosophila* Ci has dual functions. As an unprocessed form, Ci mediates positive transcriptional responses to Hedgehog signaling activity, whereas its processed forms function as a transcription repressor (Porter et al. 1995; Aza-Blanc et al. 1997; Chen et al. 1999). In the vertebrate limb, Gli3 is differentially processed to form a transcription repressor under conditions

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of low Shh signaling, providing a mechanism controlling the differential activity of Gli3 in response to long-distance and short-distance Shh signaling (Wang et al. 2000). Long-distance HH signaling in embryos requires its autoprocessing and N-terminal cholesterol modification (Lewis et al. 2001; Zeng et al. 2001) as well as heparan-sulfate proteoglycans to promote its extracellular transmission (Bellaiche et al. 1998). Graded Shh signaling acts to mediate positive and negative regulation of neural tube patterning of neural cell fates (Roelink et al. 1995; Ericson et al. 1997; Briscoe and Ericson 1999), although the role of the Gli proteins in these processes remains unclear.

Although the importance of Shh signaling in development and human disease is well established, the transcriptional targets of Shh signaling through Gli transcription factors are virtually unknown. In response to signal transduction processes initiated by HH signaling, Gli/Ci transcription factors are thought to undergo nuclear translocation (Robbins et al. 1997; Sisson et al. 1997; Kogerman et al. 1999), leading to their binding to target sequence motifs in the regulatory elements of target genes (Kinzler and Vogelstein 1990; Von Ohlen et al. 1997). Gli1 and Gli2 function as positive transcription factor regulators, whereas processed Gli3 functions as a negative regulator (Sasaki et al. 1999; Wang et al. 2000). In *Drosophila* and vertebrates, components of the HH signal transduction pathway itself are subject to direct positive and negative feedback regulation. *Patched* expression is positively regulated by HH signaling through Gli/Ci regulation, providing a negative feedback mechanism for Shh signal transduction (Alexandre et al. 1996; Goodrich et al. 1996; Marigo et al. 1996b). In vertebrates, *Gli1* also is positively and directly regulated by HH signaling through essential Gli-binding sites (Lee et al. 1997; Dai et al. 1999), providing a mechanism for positive regulation of HH signaling. Only a few developmental regulatory genes have been identified as direct targets of Gli/Ci regulation. In *Drosophila*, HH signaling regulates *Wingless* in cuticle patterning (Heemskerk and DiNardo 1994) through Ci-binding sites (Von Ohlen et al. 1997). In vertebrate embryos, *HNF3-β* is an early developmental regulator of notochord and floor plate formation (Ang and Rossant 1994; Weinstein et al. 1994; Dufort et al. 1998), and its expression in the floor plate is controlled by Shh and Gli2 regulation (Ding et al. 1998; Matisse et al. 1998) through a floor plate enhancer that has an essential Gli-binding site (Sasaki et al. 1997).

Shh signaling influences a diversity of developmental processes, including proliferation (Wechsler-Reya and Scott 1999) and apoptosis (Teillet et al. 1998; Borycki et al. 1999), which has led to controversy as to whether Shh has inductive or trophic signaling functions. Specifically, it has been unknown whether Shh signaling, through its Gli transcription factor effectors, regulates target genes that control cell lineage specification or, alternatively, regulates target genes that mediate the survival and proliferation of specific embryonic cell lineages. To understand the functions of Shh in muscle cell lineage specification, we have investigated the role of Shh signaling in

the control *Myf5*, a bHLH transcription factor gene that controls the specification of myogenic stem cell lineages in the vertebrate embryo (Rudnicki et al. 1993; Tajbakhsh et al. 1996b). *Myf5* is activated at different anatomical sites in the embryo under the control of distinct *cis*-acting regulatory elements (Hadchouel et al. 2000; Summerbell et al. 2000; Carvajal et al. 2001), responding to different developmental signals (Munsterberg et al. 1995; Borycki et al. 1998; Tajbakhsh et al. 1998). Genetic and explant induction experiments have established that Shh produced by the notochord is an essential signal for the activation of *Myf5* in epaxial muscle progenitors of the dorsal somite, but not in muscle progenitors at other sites of *Myf5* activation and myogenic specification in the embryo (Borycki et al. 1999). In *Shh* mutant embryos, *Myf5* fails to be activated specifically in the epaxial region of the somite, in the absence of localized defects in cell proliferation and apoptosis. However, these studies did not resolve whether *Myf5* is a direct target of Shh signaling through Gli regulation, or alternatively, whether *Myf5* regulation is a secondary response to Shh signaling.

To investigate the function of Gli transcription factors in *Myf5* regulation, we identified an *Myf5* epaxial somite (ES) enhancer and characterized its response to Shh signaling using a combination of transgenic and cell transfection approaches. This ES enhancer also was identified among a collection of regulatory elements in large-scale transgenic expression surveys of the 650-kb domain of the mouse *MRF4/Myf5* locus (Zweigerdt et al. 1997; Hadchouel et al. 2000; Summerbell et al. 2000; Carvajal et al. 2001). These enhancers control the activation and maintenance of *Myf5* and *MRF4* expression in limb, branchial arch, and epaxial and hypaxial muscle progenitors. The ES enhancer is unique in the *MRF4/Myf5* locus for its transcriptional activity in the epaxial muscle progenitors of the dorsal somite. In this study, we have investigated in detail the developmental activities of the ES enhancer in somites of developing wild-type embryos and homozygous and heterozygous *Shh* mutant embryos (Chiang et al. 1996), as well as in Shh-responsive 3T3 cells (Taipale et al. 2000), a culture model of somitic mesoderm progenitors (Taylor and Jones 1979). These studies show that Shh signaling controls the activation of the *Myf5* ES enhancer, both in epaxial muscle progenitor cells during somite formation and in 3T3 cells responding to Shh induction. Furthermore, Shh regulation of the ES enhancer in somites and in 3T3 cells is mediated through an essential Gli transcription factor binding site. These findings establish that *Myf5* is a direct target of long-range Shh signaling to control the specification of somite progenitor cells of the epaxial myogenic lineage. Furthermore, in contrast to the limb and neural tube, where long-range Shh signaling mediates negative regulation (Briscoe and Ericson 1999; Wang et al. 2000), we show that *Myf5* in the dorsal somite responds to Shh signaling through positive regulation by Gli transcription factors, establishing that long-range Shh signaling has a direct transcriptional function in *Myf5* regulation for specification of epaxial muscle progenitors.

Results

An Myf5 transcription enhancer specific for somite epaxial muscle progenitors

A BAC clone of the *MRF4/Myf5* locus was recovered from a mouse genomic DNA library, and subclones were characterized by sequence analysis and functional enhancer assays using transient transgenesis with a *lacZ* gene reporter expressed under the control of a heterologous viral tk promoter (Goldhamer et al. 1992). These studies identified a 651-bp enhancer located 6.6 kb 5' of the *Myf5* transcription start site (Tajbakhsh et al. 1996a) in the *MRF4/Myf5* intragenic region (Fig. 1). This 651-bp *Myf5* enhancer activates reporter expression in epaxial somite muscle progenitors as well as brain cells in 9.5-days-postcoitum (dpc) embryos (Fig. 2) and has extensive (86%) identity with the equivalent region 3' of rat *MRF4* (Pin et al. 1997), including a conserved sequence related to Gli binding-site sequences (Kinzler and Vogelstein 1990).

Histochemical analysis of β -galactosidase (β -gal) expression established that the *Myf5* ES enhancer/*lacZ* transgene is activated in somites of embryos as early as 8.25 dpc, when somites are first formed and chromosomal *Myf5* is first activated in epaxial muscle progenitors. *Myf5* activation is coordinated with somite formation, and expression persists in newly formed somites until 11.5 dpc, when somite formation is complete (Fig. 2A). Expression can also be detected in the brain at these stages of development (Daubas et al. 2000), as well as in the anterior neural tube in the two germ-line transgenic lines, E1 and E2, investigated. Neural tube expression

was not observed in transient transgenic embryos (see Fig. 6A below). Expression in the brain is localized to the region of the medial longitudinal fasciculus and mammillotegmental tracts, where *Myf5* is expressed (Daubas et al. 2000), although β -gal stained cells occupy a broader domain than reported by in situ hybridization assays of *Myf5* transcripts. In 8.25- to 9.5-dpc embryos, β -gal staining is abundant in somites along the entire anterior to posterior axis, including the newest formed somites in the posterior embryo (Fig. 2A, panels a–c). This reporter gene activity recapitulates chromosomal *Myf5* expression and precedes the expression of all of the other muscle regulatory factors, including *MRF4* (Ott et al. 1991; Tajbakhsh et al. 1997). At later developmental stages, β -gal activity is preferentially localized to the eight newest-formed somites, where *Myf5* is first activated (Tajbakhsh et al. 1996a), and activity is greatly diminished in the more mature anterior somites, in which only a few stained cells are detected in the myotome and dermatome (Fig. 2A, panels d,e). These somite expression patterns establish that the ES enhancer controls the transcriptional activation of *Myf5* in newly formed somites, but not the maintenance of its expression during somite maturation.

To investigate the dorsal–ventral pattern of ES enhancer activity in somites, β -gal-stained embryos were sectioned along their anterior to posterior axes at 9.5 dpc, when somites along the entire axis are stained (Fig. 2A, panels c1–c3). Enhancer activity is detected in the first, newest-formed somite in the posterior embryo, and this expression is localized to the cluster of dorsal medial somite cells that are known to activate chromosomal *Myf5* for epaxial muscle progenitor cell specification

Figure 1. Organization of the *Myf5* ES enhancer in the *MRF4/Myf5* locus and the *lacZ* transgene. A 651-bp *Myf5* ES enhancer element is located 6.6 kb upstream of the *Myf5* transcription site, adjacent to the 3' end of the *MRF4* gene. The transcriptional activity of the ES enhancer was assayed using a *lacZ* transgene with a viral tk promoter, as shown. A candidate Gli-binding site (Gli^{wt} enh) is located at 470 bp in the enhancer. The core region of the Gli-binding site in the *Myf5* ES enhancer (red box) is variant at position 477 from the consensus Gli-binding site defined by

site selection (Kinzler and Vogelstein 1990) and at positions 472 and 477 from the Gli site in the *HNF-3 β* floor plate enhancer (Sasaki et al. 1997). Nucleotide substitution mutations introduced into this Gli sequence (Gli^{mut} enh) disrupt its Gli-binding and functional activity.

Figure 2. The *Myf5* ES enhancer/*lacZ* transgene is activated in the brain and in epaxial somites in coordination with somite formation. (A) ES enhancer/*lacZ* transgene expression was assayed by histochemical β-gal staining of whole-mount preparations of 8.25–11.5-dpc embryos recovered from E1 germ-line females, heterozygous for the ES enhancer/*lacZ* transgene. Specific staining was detected in the brain (red arrow) at all stages and in all somites along the anterior to posterior embryo axis at early stages (panels a–c), and predominantly in eight newest-born posterior somites in later stages (panels d,e). Ectopic dorsal neural tube staining is abundant in the E1 germ-line transgenic embryos (white arrow), but not in transient transgenic embryos (see Fig. 6A). At 8.25 dpc (a), staining is observed in the occipital somites, which are the first somites formed in the embryo, and at 8.75 dpc (b) and 9.5 dpc (c), staining is observed in somites along the entire axis, including the newest-formed posterior somites (red brackets). By 10.5 dpc, staining is predominant in the eight newest-formed posterior somites.

A 9.5-dpc embryo (c) was sectioned transversely along the anterior to posterior embryo axis, in the most posterior, newly formed epithelial somite (panel c1), an immediately anterior somite that has formed a dermatome and dorsal medial lip (c2), and an anterior somite that has initiated myotome formation (c3). (s) Sclerotome; (dm) dermatome; (my) myotome; (dml) dorsal medial lip; (nt) neural tube; (nc) notochord. (B) ES enhancer/*lacZ* transgene expression was assayed in embryos at 9.0 dpc by whole-mount in situ hybridization (ISH) using *Myf5* antisense DIG probes (panel a) and *lacZ* antisense DIG probes (panel b), and by whole-mount β-gal histochemical staining (panel c). Arrows and brackets demarcate the boundaries of *lacZ* transgene and *Myf5* transcript expression in the epaxial somite, as assayed by these different methods. *lacZ* transcripts expressed by the ES enhancer/*lacZ* transgene are localized only to the dorsal-most aspect of the epaxial somite (b), whereas β-gal enzyme activity in more anterior somites has a broader dorsal to ventral distribution, extending from the dorsal epaxial domain to a more ventral position where the myotome is forming.

(Tajbakhsh et al. 1997). Enhancer activity was not detected in the presomitic mesoderm at any developmental stage in transient or germ-line transgenic embryos. In more posterior somites, transgene expression is localized only to the dorsal medial lip (DML) of the dermatome, which is the proliferative source of progenitor cells that form the first myotomal epaxial muscles of the embryo. β-Galactosidase staining persists in these DML progenitors in more mature anterior somites that have initiated myotome formation, but abundant numbers of stained cells also populate the dorsal aspect of the myotome and the dorsal aspect of the overlying dermatome, which are formed by the dorsal and ventral migration of DML cells to these sites (Ordahl et al. 2001). Previously, β-gal staining of the dermatome ES enhancer/*lacZ* transgenic lines was interpreted as ectopic activity of the enhancer, which was thought to lack a negative regulatory

element (Summerbell et al. 2000). Alternatively, dermatome and myotome β-gal staining could reflect persistence of β-gal enzyme in the absence of sustained transcriptional activity of the ES/*lacZ* transgene. To distinguish these possibilities, we compared expression of the ES enhancer/*lacZ* transgene using β-gal enzyme staining with whole-mount in situ hybridization assays to detect β-gal transcripts (Fig. 2B, panels a–c). These experiments show that *lacZ* RNA transcripts are restricted to the DML in all somites, including anterior somites. Transcripts were not detectable in the dermatome or myotome, where β-gal enzyme activity accumulates and persists during somite maturation. Similar localization of β-gal transcripts was observed in studies of the expression of *lacZ* transgenes controlled by the larger 23-kb sequence upstream of the *Myf5* promoter, which included the ES enhancer (Hadchouel et al. 2000). These

findings establish that the *Myf5* ES enhancer is transcriptionally active only in the epaxial muscle progenitor cells of the DML, and its transcriptional activity is not maintained as DML cells migrate dorsally and ventrally to form both the myotome and dermatome, thus refuting the possibility that a negative regulatory element is necessary for localized ES enhancer expression in epaxial somite muscle progenitors.

The Myf5 ES enhancer has a functional Gli transcription factor binding site

The *Myf5* ES enhancer has a sequence element related to Gli-binding sequences (Fig. 1; Kinzler and Vogelstein 1990; Sasaki et al. 1997), suggesting that this enhancer is a direct target of Shh induction through Gli transcription factor regulation. As Gli binding sites in transcription enhancers have not previously been well documented, we performed electrophoretic mobility shift binding assays to test whether this *Myf5* sequence element binds Gli proteins. Myc-tagged Gli2 protein expressed in a reticulocyte expression system was reacted with radiolabeled oligonucleotides of the *Myf5* ES enhancer Gli sequence, and protein–DNA complexes were analyzed by electrophoretic mobility shift assays. We found that Gli2 protein produced in reticulocyte extracts forms stable DNA–protein complexes with the target *Myf5* enhancer sequence, and these complexes can be supershifted with anti-myc antibodies, showing that these complexes are formed by binding of the myc-tagged Gli2 protein to the *Myf5* enhancer sequence (Fig. 3A). Gli2 protein binding to the ES enhancer site is sequence-specific, based on competition with excess wild-type Gli oligonucleotide (Gli^{wt}), but not with excess Gli^{mut} oligonucleotides with base-substitution mutations that destroy the core Gli DNA-binding sequence (see Fig. 1). Nuclear Myc-tagged Gli1 and Gli2 proteins expressed in 293 cells and C2C12 myoblasts also bind specifically with Gli^{wt} oligonucleotides in these gel shift assays (data not shown), providing supporting evidence that Gli transcription factors can bind specifically to the Gli site in the *Myf5* enhancer.

To test whether Gli binding to the ES enhancer Gli-binding site forms an active transcription complex, Shh-responsive 3T3 cells (Taipale et al. 2000) were cotransfected with luciferase reporter genes of 8× multimerized wild-type (8× Gli^{wt}) and mutant (8× Gli^{mut}) Gli-binding sites, with Gli1 or Gli2 expression vectors, and with a Renilla reference plasmid (Fig. 3B). The 8× Gli^{wt} reporter is transactivated 500-fold by Gli1 and 30-fold by Gli2 expression vectors, whereas the 8× Gli^{mut} is not transactivated by either Gli1 or Gli2. These biochemical and functional studies, therefore, establish that the *Myf5* ES enhancer has a functional Gli-binding site for Shh regulation.

Shh signaling controls Myf5 ES enhancer activation and endogenous Myf5 epaxial expression

To investigate whether Shh signaling regulates *Myf5* ES enhancer activity, we crossed the *Myf5* ES enhancer/*lacZ* transgene into *Shh* mutant mice (Chiang et al.

Figure 3. Identification of a functional Gli-binding site in the *Myf5* ES enhancer. (A) Electrophoresis mobility shift assays show that Myc-tagged Gli2 protein expressed in reticulocyte lysates (TNT lysate) binds and forms a gel shift complex with a ³²P-radiolabeled, 32-bp oligonucleotide of the ES enhancer wild-type Gli site (mGli2-myc). A 100-fold excess of wild-type Gli site (Gli^{wt}) oligonucleotide competes for Gli2-myc binding to radiolabeled wild-type oligonucleotide, whereas mutant Gli site (Gli^{mut}) oligonucleotide does not compete for binding. Anti-Myc antibodies (myc Ab) supershift Gli2-myc gel shift complexes whereas control antibodies (mouse IgG) have no effect, showing that Gli2 is a major component of these gel shift complexes. (B) Luciferase reporters of 8× multimerized wild-type Gli binding site (8× Gli^{wt}) and 8× mutant Gli binding site (8× Gli^{mut}) from the *Myf5* ES enhancer were cotransfected into 3T3 cells with mouse mGli1 or mGli2 expression vectors. Results are expressed as fold induction, which is the ratio of luciferase activity induced in Gli-transfected cells relative to basal luciferase activity in control transfected 3T3 cells. Both Gli1 and Gli2 are strong transcription activators of the 8× Gli^{wt} reporter, but not the 8× Gli^{mut} reporter, showing that the ES enhancer Gli-binding site can form active transcription complexes with Gli transcription factors synthesized in vivo.

1996), which were then mated to recover litters of 9.0–9.5-dpc heterozygous (+/-) and homozygous (-/-) *Shh* mutant embryos carrying the reporter construct for whole-mount analysis of transgene expression (Fig. 4A). All *Shh*^{-/-} embryos analyzed (*n* = 3) show a complete absence of β-gal staining in all somites, including newly formed posterior somites, where the enhancer is first activated in wild-type embryos (Fig. 4A, panel c). Brain transgene expression also was eliminated in all *Shh*^{-/-} embryos, but ectopic neural tube transgene expression persisted at high levels, providing a convenient internal control for transgene activity in mutant embryos. Significantly, we found that *Myf5* ES enhancer activity is greatly diminished in somites of all *Shh*^{+/-} mutant embryos analyzed (*n* = 4). Expression was most severely lost in the posterior, newest-formed somites, which had little or no transgene expression (Fig. 4B, cf. panels c and d). Neural tube and brain expression remains high in *Shh*^{+/-} embryos relative to reduced somite expression (Fig. 4B, cf. panels a and b). In heterozygous mutant embryos, β-gal staining, although reduced, continues to be restricted to the DML, consistent with the normal dorsal pattern of ES enhancer transgene activity. Dorsal dermomyotome and myotome morphology are normal in heterozy-

gous *Shh* mutant embryos (Borycki et al. 1999), providing additional evidence that the ES enhancer activation is controlled by Shh signaling and that defective *Myf5* activation in *Shh* mutant embryos is not a result of primary defects in somite maturation.

To investigate whether Shh signaling is required for epaxial expression of the endogenous *Myf5* gene at the time of first somite formation, whole-mount in situ hybridization assays were performed in 8.75-dpc *Shh*^{+/+} and *Shh*^{-/-} embryos. In wild-type embryos at this developmental stage, *Myf5* expression is restricted to the epaxial domain of newly formed somites (Fig. 4C, panel a). In *Shh*^{-/-} embryos, *Myf5* expression distributed diffusely, at low levels, most prominently in the four most anterior occipital somites (Fig. 4C, panel b). This low-level expression is not localized to the epaxial domain in the dorsal somite, but is distributed throughout the medial and lateral aspects of the somite, consistent with our previously reported observations of *Myf5* expression in newly formed somites of *Shh*^{-/-} embryos at 9.75 dpc (Borycki et al. 1999). Reduced levels of *Myf5* expression were also reported in the most anterior occipital somites of *Smoothed* mutant embryos, which also are disrupted in Shh signaling (Zhang et al. 2001).

Figure 4. Activation of the *Myf5* ES enhancer/luciferase transgene in somites and the brain is disrupted in homozygous and heterozygous *Shh* mutant embryos. (A) (panel a) Wild-type embryos (*Shh*^{+/+}) and embryos that were (panel b) heterozygous *Shh* mutant (*Shh*^{+/-}) and (panel c) homozygous *Shh* mutant (*Shh*^{-/-}) were stained for β-gal to assay *lacZ* transgene activity in the brain (red arrow), somites (red bracket), and neural tube (white arrow). All embryos are heterozygous for the ES enhancer/*lacZ* transgene. ES enhancer activity is completely absent in somites and the brain of homozygous mutant embryos and greatly reduced in heterozygous mutant embryos, even under extended staining conditions, whereas ectopic neural tube expression persists. (B) (panels a,c) Heterozygous *Shh* mutant (+/-) and (panels b,d) wild-type (+/+) embryos were assayed for β-gal staining and then sectioned to localize staining in somites and neural tube in anterior (a,b) and posterior (c,d) regions of the embryo. In *Shh*^{+/-} embryos, *lacZ* transgene activity is reduced in the anterior somites (a) and is almost entirely absent in the most posterior somites (c). (dml) Dermomyotome; (s) somite; (nt) neural tube. (C) (panel a) Wild-type *Shh*^{+/+} and (panel b) homozygous mutant *Shh*^{-/-} embryos at 8.75 dpc were assayed for *Myf5* expression by in situ hybridization. In wild-type embryos (a), *Myf5* is activated exclusively within the epaxial domain of newly formed somites, whereas in *Shh*^{-/-} embryos (b), *Myf5* expression is disrupted in the epaxial domain of newly formed somites. *Myf5* expression distributed diffusely, at low levels, particularly in the four most anterior occipital somites (red bracket). This low-level expression is not localized to the epaxial domain in the dorsal somite, but is distributed throughout the medial and lateral aspects of the somite. Staining in the head of *Shh*^{-/-} embryos is not reproducible.

To further test whether the ES enhancer is a target of Shh signaling, we investigated the Shh signaling response of the ES enhancer in Shh-responsive 3T3 cells (Taipale et al. 2000). For these experiments, the *Myf5* ES enhancer, cloned into a luciferase reporter gene with a δ -crystalline promoter, was cotransfected into 3T3 cells along with a Renilla reference reporter and expression vectors encoding Shh signaling components. Shh inducers included Shh, an oncogenic mutant form of Smoothed (SmoM2) that constitutively activates Shh signal transduction (Taipale et al. 2000), and Gli1 and Gli2. Each of these Shh signaling components was found to activate the *Myf5* ES enhancer (Fig. 5a), establishing that Shh has an inductive function in *Myf5* ES enhancer regulation. The role of Shh signaling in *Myf5* ES enhancer activation is further supported by results of Shh inhibitor studies. The Shh induction of the *Myf5* ES enhancer was completely inhibited by forskolin, an antagonist of Shh signaling (Fig. 5b; Fan et al. 1995), and by cyclopamine, a compound that disrupts the capacity of Smo to mediate Shh signal transduction (Fig. 5c; Taipale et al. 2000), further supporting the hypothesis that Shh signaling specifically induces ES enhancer activation.

Shh induction of the Myf5 ES enhancer is mediated through its Gli-binding site

To determine whether Shh regulates *Myf5* ES enhancer activation through Gli transcription factors, we first

used 3T3 transfection assays to compare the Shh induction of the *Myf5* ES enhancer luciferase reporters with a wild-type (Gli^{wt} enh) or a mutant (Gli^{mut} enh) Gli-binding site. These studies show that mutations of the Gli-binding site that block DNA binding block transactivation of the *Myf5* ES enhancer by Shh, SmoM2, Gli1, and Gli2 (Fig. 5a–c). Furthermore, forskolin and cyclopamine do not further reduce the activity of the Gli mutant enhancer below basal activity in the absence of Shh signaling, providing evidence that these inhibitors directly block Shh induction and do not inhibit other activities required for reporter gene response (Fig. 5b,c).

We next used transient transgenic assays to determine whether the Gli site in the *Myf5* ES enhancer was required for reporter expression in the epaxial somite. *Myf5* ES enhancer/*lacZ* constructs containing either wild-type (Gli^{wt} enh) or mutant (Gli^{mut} enh) Gli-binding sites were introduced into embryos by pronuclear injection, and β -gal expression in somites and the brain was assayed in 8.5- and 9.5-dpc embryos (Fig. 6) as well as at 10.5 dpc (data not shown). These studies showed that mutation of the Gli-binding site in the ES enhancer completely blocks reporter activation in the epaxial somite in all transgenic embryos examined. Therefore, the Gli-binding site in the ES enhancer transgene has an essential positive regulatory function for enhancer activation in newly formed somites at multiple developmental stages. Significantly, mutation of the Gli site in the ES enhancer does not cause misexpression of the enhancer at other tissue sites of Shh signaling in the embryo such

Figure 5. Shh signaling activates the *Myf5* epaxial enhancer through its Gli-binding site in 3T3 cells. *Myf5* ES enhancer/luciferase reporter plasmids with wild-type (Gli^{wt}) and mutant (Gli^{mut}) Gli sites were cotransfected into cultures of 3T3 cells with Gli1, Gli2, Shh, and activated Smoothed (SmoM2) expression vector plasmids. The luciferase activities of the ES enhancer/luciferase transgene in response to induction by different Shh signaling components are expressed as fold induction, which represents the luciferase activity of the wild-type or mutant ES enhancer/luciferase reporter in response to inducers relative to its activity in transfected control cells without inducers. (a) In untreated, control cultures, the wild-type ES enhancer was induced by Shh, activated SmoM2, Gli1, and Gli2; and this Shh induction was blocked by mutation of the Gli-binding site in the ES enhancer. (b) In cultures treated with 10 μ M forskolin, an antagonist of Shh signaling (Fan et al. 1995), the wild-type ES enhancer was completely blocked in its response to Shh and activated SmoM2, significantly reduced in its response to Gli1 and Gli2, and the Gli mutant ES enhancer was completely unresponsive to induction by the signaling components. (c) In cultures treated with 1 μ M cyclopamine, wild-type ES enhancer is completely blocked in its response to Shh, and reduced but not blocked in its activation by SmoM2, Gli1, and Gli2, consistent with activity of cyclopamine as an inhibitor of Smo function (Taipale et al. 2000).

Figure 6. The *Myf5* ES enhancer activation in somites and the brain is mediated through its Gli-binding sequence. The *Myf5* ES enhancer/*lacZ* constructs containing either a wild-type (*Gli*^{wt} enh) or mutant (*Gli*^{mut} enh) Gli-binding site were introduced into embryos for transient transgenesis by paranuclear injection. Embryos were recovered at 8.5 and 9.5 dpc, genotyped to identify transgenic embryos, and assayed for reporter expression by β -gal staining. (A, left panel) A 9.5-dpc embryo expressing a wild-type *Gli*^{wt} enh transgene with reporter activity in somites and brain (red arrow). (Right panel) A 9.5-dpc *Gli*^{mut} enh transgenic embryo with ectopic transgene expression in the ventral somite (white arrow), but not in the dorsal epaxial somite. This embryo was the only one of 24 *Gli*^{mut} enh transgenic embryos to show ectopic staining in somites. (B) Tabular summary of the *Gli*^{wt} enh and *Gli*^{mut} enh transgene expression in somites of 8.5- and 9.5-dpc transgenic embryos. Epaxial somite expression of the Gli mutant enhancer is completely blocked. Only one 9.5-dpc *Gli*^{mut} enh embryo (*), shown in A, had ectopic expression in the somite.

as the neural tube, establishing that this Gli site does not function to repress ES enhancer activity in other cell lineages. Together, these transgenic and 3T3 transfection studies establish that *Myf5* ES enhancer activation in the epaxial somite is mediated through long-range Shh signaling, under the positive control of the Gli transcription factors.

Discussion

This study reports the identification of a Shh-regulated enhancer that controls activation of *Myf5*, a master regulatory gene for specification of epaxial skeletal muscle progenitor cells (Rudnicki et al. 1993; Tajbakhsh et al.

1996b). The *Myf5* ES enhancer activates reporter expression in the epaxial muscle progenitors of newly forming somites from 8.25 dpc, when somites are first formed in the developing embryo, until 11.5 dpc, when somite formation is complete. The activity of ES enhancer for epaxial somite regulation is unique in a 650-kb region around the *MRF4/Myf5* locus, which has been shown to include other enhancers that direct *Myf5* transgene expression to all of the other domains of muscle progenitor specification in the embryo, including branchial arches, limb bud, and the hypaxial somite (Patapoutian et al. 1993; Zweigerdt et al. 1997; Hadchouel et al. 2000; Summerbell et al. 2000). Because *Myf5* is an essential regulator of epaxial muscle specification, our finding that the *Myf5* ES enhancer is a direct target of Shh signaling through an essential Gli-binding site provides the first evidence that Shh has a direct inductive function in the process of cell lineage specification. In addition to epaxial somite expression, the ES enhancer also controls *Myf5* expression in the longitudinal tracks of the developing brain (Daubas et al. 2000). The brain activity of the ES enhancer also is mediated through its Gli-binding site, and although the domain of ES enhancer/*lacZ* reporter activity is broader than the expression of *Myf5* transcripts, these brain cells are near a source of Shh, indicating that the transcriptional activity of the ES enhancer in the brain also is controlled by Shh signaling.

Our findings show that *Myf5* ES enhancer is under positive regulation by Shh signaling and Gli regulation through a Gli site that we identified in DNA/protein binding and functional transcription assays. This Gli-binding site is required for enhancer activity in somites and the brain at both 8.5 and 9.5 dpc, and for Shh induction in 3T3 cells. Significantly, mutation of the Gli site did not lead to ectopic enhancer activity in the somite or other embryonic tissues, indicating that the enhancer is subject to positive regulation by Gli transcription factors. Because this Gli site is essential to mediate reporter expression in the somite, other regulatory elements must also be present within the enhancer to restrict its activity to the epaxial somite, either by repressing enhancer activity in other Shh-responsive tissues such as the neural tube, or by functioning cooperatively with Glis to activate the enhancer specifically in the DML. It remains to be determined which Gli transcription factor regulates ES enhancer activation. *Gli1*, *Gli2*, and *Gli3* are all expressed in somites (Hui et al. 1994). In avian embryos, the expression of the three Gli transcription factor genes initiates in newly forming somites, immediately preceding *Myf5* activation, and *Gli2* and *Gli3* transcripts become localized to the epaxial muscle progenitor cells of the dorsal somite, at the site of *Myf5* activation (Borycki et al. 2000). Genetic studies show that *Gli1*, *Gli2*, and *Gli3* have redundant functions in both neural and somite development in the mouse (Mo et al. 1997; Motoyama et al. 1998; Park et al. 2000), indicating that these *Gli* genes could also be redundant for *Myf5* regulation. Identification of the specific Glis that function together with somite transcription factors to activate the ES enhancer in epaxial somite progenitors

will provide a basis for an understanding of the molecular mechanisms that control the tissue specificity of Shh signaling responses.

The *Myf5* ES enhancer is dependent on Shh signaling for its activation in epaxial myogenic progenitor cells of somites throughout the embryo axis, as shown by the loss of the ES enhancer/*lacZ* transgene expression in somites of *Shh*^{-/-} embryos. The somites of *Shh* mutant embryos form normally and undergo dermatome epithelialization and DML formation, but fail to express *Myf5* specifically in the epaxial somite domain. Significantly, *Shh*^{-/-} embryos exhibit normal cell survival and proliferation in the dorsal somite (Borycki et al. 1999). Therefore, the loss of transgene expression in the *Shh* mutant embryos cannot be attributed to defects in the survival or proliferation of *Myf5*-expressing cells in the newly formed somite. During later stages of somite maturation in *Shh*^{-/-} mutant embryos, the dorsal aspects of the dermatome and myotome are absent. Similarly, in *Myf5*^{-/-} mutant embryos, dorsal somite cell migration and dermatome and myotome formation are also blocked (Tajbakhsh et al. 1996b). Our findings and those of recent lineage marking studies (Ordahl et al. 2001) now show that the DML cells contribute dorsal growth of both the myotome and the dermatome. Thus, we can now explain the absence of the medial dermatome and myotome in *Shh*^{-/-} embryos as a specific defect in the Shh-controlled specification of the DML cells to form these lineages, rather than a degenerative defect in somite maturation. It also is noteworthy that the activity of the ES enhancer is greatly reduced in *Shh*^{+/-} embryos, in which somite formation and maturation are entirely normal, further supporting the specificity of Shh signaling for *Myf5* epaxial somite regulation. Previously, we noted that *Myf5* transcript expression, as assayed by in situ hybridization, was unaffected in somites of *Shh*^{+/-} embryos (Borycki et al. 1999), which contrasts with our current finding that ES enhancer activity, assayed by the β -gal staining assay, is significantly reduced in *Shh*^{+/-} embryos. These differing results may reflect improved quantitative sensitivity of the β -gal-staining assay for transgene activity. Alternatively, transcriptional or posttranscriptional compensatory mechanisms may modulate *Myf5* expression in response to reduced levels of Shh, and these mechanisms may not be operative for the ES enhancer transgene and its β -gal transcript.

In addition to the Shh-dependent activation of *Myf5* in the epaxial somite progenitors, through the ES enhancer, Shh-independent signals activate *Myf5* in other muscle progenitor lineages in the embryo. This Shh-independent regulation is mediated by the other *Myf5* transcription enhancers that have been identified in a 650-kb region around the *MRF4/Myf5* locus (Zweigerdt et al. 1997; Hadchouel et al. 2000; Summerbell et al. 2000; Carvajal et al. 2001). These enhancers direct *Myf5* activation in the myogenic progenitor cell domains that form and express *Myf5* in wild-type and *Shh* mutant embryos, including the progenitor cells of the hypaxial somite, the limb, and the branchial arches (Borycki et al. 1999). These transcription enhancers are controlled by devel-

opmental signals such as Wnts (Tajbakhsh et al. 1998). Our earlier findings established that *Myf5* is activated in all of these muscle progenitor cell domains, except the epaxial somite domain, in 9.75-dpc and 10.50-dpc *Shh* mutant embryos (Borycki et al. 1999). In this study, we examined *Myf5* expression in earlier 8.75-dpc *Shh* mutant embryos, which are forming the first somites. These findings confirm our earlier work showing that *Myf5* expression is specifically disrupted in the dorsal epaxial domain somites of 8.75-dpc *Shh* mutant embryos, which, as previously reported, express low levels of *Myf5* in the medial and lateral somite domains. Based on analysis of medial and lateral somite marker genes, medial-lateral somite patterning is disrupted in *Shh* mutant embryos such that lateral somite genes, including hypaxial *Myf5*, are expressed medially, suggesting that low-level *Myf5* expression in somites of *Shh* mutant embryos reflects activity of the hypaxial *Myf5* enhancer on these lateral signals. In 8.75-dpc embryos, reduced levels of *Myf5* expression also are observed in the most anterior four occipital somites of *Shh* mutant embryos as well as *Smoothed* (*Smo*) mutant embryos, which are defective in all HH signaling (Zhang et al. 2001). A distal *Myf5* enhancer located at -58 to -48 kb is active in occipital somites and could serve this function (Hadchouel et al. 2000). This same enhancer regulates the *Myf5* expression in the myotome of the medial somite and may contribute to the low level of *Myf5* expression observed in the medial somites of *Shh*^{-/-} embryos (Kruger et al. 2001).

Epaxial muscle progenitors in the dorsal somite are located at a significant distance from the notochord source of Shh, indicating that epaxial *Myf5* expression is controlled by long-range Shh signaling through the ES enhancer. The ES enhancer apparently is regulated positively and directly by Shh signaling, as this signaling activity is mediated through a Gli-binding site that is essential for ES enhancer activity. Disruption of this Gli-binding site does not lead to ectopic expression of the ES enhancer, indicating that this Gli-binding site is not used to repress *Myf5* expression in other Shh-responsive tissues such as the neural tube. Long-range transport of Shh in somites has not been investigated, but in the limb, long-range Shh signaling requires N-terminal processing and cholesterol modification (Lewis et al. 2001; Zeng et al. 2001). The levels of Shh in the responding dorsal somite would need to be sufficiently high to elicit a positive regulatory response, which would presumably include limiting the processing of Gli3 into its repressor form, which is thought to occur under conditions of low-level Shh signaling (Wang et al. 2000).

Our ES enhancer transgene expression results show that *lacZ* transcripts are localized to the DML in both newly formed and mature somites of wild-type embryos, indicating that the Shh signaling is restricted to the DML and that enhancer activity is not maintained as cells, marked by persistent β -gal enzyme, migrate from the DML to form the dorsal aspects of the dermatome and the myotome. DML cells that form the myotome maintain *Myf5* transcription, likely through the activity of a myotome-specific maintenance enhancer located

Figure 7. A signal enhancement model for localized Shh signaling for epaxial muscle specification and dermatome and myotome differentiation. This model proposes that medial lateral patterning of the dermomyotome in the dorsal somite is controlled by opposing Shh and BMP signals in the dorsal somite. Opposing Shh and BMP signals also control dorsal ventral patterning of the neural tube (NT) specification (Roelink et al. 1995), and the specification of sclerotome progenitors in the ventral somite (McMahon et al. 1998). In the dorsal somite, epaxial myogenic specification is controlled by long-distance Shh induction of *Myf5* through positive Gli regulation of the *Myf5* ES enhancer. Positive Gli regulation of *Myf5* in the DML is controlled by the localized expression of Noggin, which inhibits the repressive activity of BMP signals prevalent in the dermomyotome, thus allowing high signal transduction activity in response to low levels of Shh from the distant notochord (NC). Subsequently, *Myf5*-expressing DML cells migrate dorsally into a repressive high BMP environment that represses ES enhancer activity and *Myf5* expression, allowing these cells to assume a dermatome fate. DML cells that migrate ventral laterally also lose ES enhancer activity,

but these myotome progenitor cells activate a myotome-specific, *Myf5*-maintenance enhancer (Hadchouel et al. 2000) to maintain high-level *Myf5* transcription to promote their specification to the myogenic lineage and their differentiation to form the epaxial myotomal muscles.

–58 to –48 kb 5' of *Myf5* (Hadchouel 2000). Presumably the sustained expression of *Myf5*, under the control of this maintenance enhancer, would promote the specification of myotome progenitors to the myogenic lineage. In DML cells that form the dermatome, the ES enhancer is inactive and *Myf5* transcription ceases as these cells become committed to the dermatome lineage. Specific mechanisms, therefore, must exist to enhance and localize long-distance Shh signaling for Gli activation of the *Myf5* ES enhancer specifically in DML progenitors and to suppress its response to Shh signaling as DML cells migrate to the dorsal myotome and dermatome. One possibility is that the Shh signaling response of the ES enhancer in the DML is controlled by opposing Shh and BMP signals, which are known to function together in the specification of the epaxial and hypaxial progenitor cells in the medial and lateral somite (Fig. 7). When BMP signaling in the somite is blocked, *Myf5* expression is ectopically expressed throughout the dermomyotome; and when BMP signaling in the somite is enhanced, *Myf5* expression in the DML is repressed and hypaxial genes are activated ectopically in the epaxial domain (Pourquie et al. 1996). Furthermore, the DML is protected from the repressive activity of BMP on Shh signaling by the localized expression of Noggin, a potent BMP inhibitor that is activated in the DML in response to Wnt signaling from the dorsal neural tube (Hirsinger et al. 1997; Marcelle et al. 1997; Reshef et al. 1998). In the mouse, *Noggin* expression is essential for epaxial somite specification (McMahon et al. 1998). According to this mechanism, the localized expression of Noggin or another BMP inhibitor in the DML would enhance the response of these cells to low levels of Shh signals from the ventral notochord. Shh signaling and ES enhancer

activity would be inhibited as DML cells migrate outside of the BMP inhibitor domain to form the myotome and dermatome. The discovery that the *Myf5* ES enhancer is a direct target of Shh signaling and Gli regulation provides a molecular approach to test this and other models of Shh function in embryo patterning and cell lineage specification.

Materials and methods

Identification of an *Myf5* epaxial somite transcription enhancer

A mouse BAC 129/SvJ genomic DNA library (Genome Systems) was screened with *Myf5* PCR primer probes to identify clones including the *MRF4/Myf5* locus. Subclones that included the linked *MRF4* and *Myf5* genes were analyzed by DNA sequencing and transgenic analysis using *lacZ* reporter genes and transient transgenic injection. Transgene expression was assayed by whole-mount histochemical assays of β -gal as well as by whole-mount in situ hybridization with DIG *lacZ* probe, as described previously (Goldhamer et al. 1995; Borycki et al. 1999). The *lacZ* reporter gene has a viral tk promoter driving transcription of a *lacZ* gene encoding β -gal with a nuclear localization domain (Goldhamer et al. 1992). DNA sequence and transgenic analyses identified a 651-bp *Bam*HI–*Eco*R1 fragment in the *MRF4/Myf5* intragenic region, located –6.6 kb 5' of the *Myf5* promoter, immediately 3' of the *MRF4* gene and 5' of a previously identified branchial arch enhancer (Patapoutian et al. 1993). This region was highly conserved with rat intragenic sequence (Pin et al. 1997) and contained a candidate Gli-binding site, identified with the PatSearch 1.1 transcription site search program. Two germ-line transgenic lines carrying the –6.6-kb epaxial somite (ES) enhancer, E1 and E2, were produced in CD1 mice for developmental and genetic studies. Transgene expression was identical in both germ-line transgenic lines, including epaxial somite and brain expression, and ectopic expression in the anterior neural tube, which was not observed in transient

transgenic embryos. Embryos were recovered from pregnant females between 8.0 and 11.5 dpc for β -gal staining in whole-mount histoenzymatic assays (Goldhamer et al. 1995). Embryos were sectioned transversely along the anterior to posterior embryo axis using a vibratome. For genetic studies, heterozygous *Shh* mutant mice (Chiang et al. 1996) in a CD1 background were crossed with *Myf5* ES enhancer transgenic lines heterozygous for the transgene, to generate homozygous *Shh* mutant mice that carried the *Myf5* ES enhancer transgene. These lines were bred to produce homozygous and heterozygous *Shh* mutant embryos for β -gal staining to assay *Myf5* ES enhancer transgene activities.

Luciferase reporter genes and DNA transfection assays

Myf5 ES enhancer/luciferase reporter plasmids were generated by subcloning the 651-bp *Myf5* ES enhancer sequence, with a wild-type (Gli^{wt} enh) or a mutant (Gli^{mut} enh) Gli-binding site, into the p δ 51lucII luciferase reporter, which has a δ -crystalline promoter with low basal activity in DNA transfection assays. 8 $\times$ Gli/luciferase reporter plasmids were generated by subcloning into the p δ 51lucII luciferase reporter 8 $\times$ multimers of the wild-type (8 $\times$ Gli^{wt}, 5'-AACGACCACCAAGAAACA-3') and mutant (8 $\times$ Gli^{mut}, 5'-AACGACTgCagAGAAACA-3') Gli-binding site present in the 651-bp *Myf5* ES enhancer. These multimers were generated as direct repeats by oligonucleotide synthesis. Mouse Gli2 expression vector was prepared by subcloning Gli2 (Sasaki et al. 1999) into pGEM myc tag vectors, producing C-terminal-tagged pGEM Gli2-myc.

DNA transfection assays were performed by plating confluent 3T3 cultures into 6- or 12-well plates at a 1:6 dilution in DMEM supplemented with 10% calf serum. After 18 h, cells were co-transfected with Renilla luciferase plasmid (pRL-TK, Promega; 5% w/w DNA), and *Myf5* ES enhancer luciferase or 8 $\times$ Gli luciferase reporter plasmids (45%), and Gli1, Gli2, *Shh*, or SmoM2 expression plasmids (50%), using Eugene 6 (Roche) transfection reagent (1 μ g/well in 6-well plates or 500 ng/well in 12-well plates) in a 3:1 ratio (v/w) of reagent to DNA. Two h after addition of DNA, cells were rinsed and cultured in 0.5% serum medium either containing forskolin or cyclopamine or without (control). After 24 h cells were lysed and luciferase assays were performed as described previously (Dhoot et al. 2001). Luciferase activity in each culture sample was normalized to Renilla luciferase activity to correct for variations in transfection efficiency.

Electrophoretic mobility shift assays

Gli2 protein was synthesized by TNT coupled transcription/translation (Promega), using pGEM-Gli2-myc plasmid as a template. For electrophoretic mobility shift assays, 5 μ L of Gli2-myc translation extracts were incubated on ice for 45 min with 5×10^4 cpm of ³²P-labeled double-stranded oligonucleotide of the Gli wild-type (Gli^{wt}, 5'-GGGAAAAACGACCACCAAGAAACACAGT-3') and Gli mutant (Gli^{mut}, 5'-GGGAAAAACGACTgCagAGAAACACAGT-3') binding sites in 20 μ L containing 20 mM Tris (pH 7.9), 80 mM NaCl, 1 mM β -mercaptoethanol, 0.1 mg/mL of bovine serum albumin, and 10% (v/v) glycerol. In competition assays, 100-fold excess of unlabeled competitor Gli^{wt} or Gli^{mut} oligonucleotide was added during preincubation. In supershift assays, 4 μ g of Myc monoclonal antibody (9E10) was added to the binding mixture on ice for 1 h prior to the addition of radiolabeled probe. Bound complexes resolved by electrophoresis on 5% polyacrylamide gels in 0.25 $\times$ TBE buffer, and radioactivity was detected by autoradiography.

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