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The Wnt/β-catenin pathway regulates Gli-mediated *Myf5* expression during somitogenesis

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Canonical Wnt/β-catenin signaling regulates the activation of the myogenic determination gene *Myf5* at the onset of myogenesis, but the underlying molecular mechanism is unknown. Here, we report that the Wnt signal is transduced in muscle progenitor cells by at least two Frizzled (Fz) receptors (Fz1 and/or Fz6), through the canonical β-catenin pathway, in the epaxial domain of newly formed somites. We show that *Myf5* activation is dramatically reduced by blocking the Wnt/β-catenin pathway in somite progenitor cells, whereas expression of activated β-catenin is sufficient to activate *Myf5* in somites but not in the presomitic mesoderm. In addition, we identified Tcf/Lef sequences immediately 5′ to the *Myf5* early epaxial enhancer. These sites determine the correct spatiotemporal expression of *Myf5* in the epaxial domain of the somite, mediating the synergistic action of the Wnt/β-catenin and the Shh/Gli pathways. Taken together, these results demonstrate that *Myf5* is a direct target of Wnt/β-catenin, and that its full activation requires a cooperative interaction between the canonical Wnt and the Shh/Gli pathways in muscle progenitor cells.

KEY WORDS: Myogenic induction, *Myf5* activation, Wnt, β-catenin, Frizzled, Sonic Hedgehog, Gli, Ep enhancer

INTRODUCTION

Wnts are signaling molecules regulating different developmental processes, such as proliferation, asymmetric division, patterning and cell fate determination (Huelsken and Birchmeier, 2001; Moon et al., 1997) (see http://www.stanford.edu/~rnusse/wntwindow.html). Wnt/β-catenin signaling mediates β-catenin accumulation in the cytoplasm and translocation into the nucleus, where it forms a complex with the Tcf/Lef transcription factors to regulate the expression of target genes (Wodarz and Nusse, 1998). In addition, a 'non-canonical' Wnt/Ca^{2+} pathway leads to the activation of downstream genes involved in patterning (Kuhl et al., 2000; Miller et al., 1999). A Wnt/JNK pathway plays a role in cell polarity, asymmetrical cell division and apoptosis (Boutros et al., 1998; Lisovsky et al., 2002; Yamanaka et al., 2002). Complex crosstalk between these 'canonical' and 'non-canonical' pathways may regulate the cellular readout of Wnt signaling (Pandur et al., 2002). The role of the Wnt pathway in cell fate determination was demonstrated for neural crest cells (Brault et al., 2001; Dorsky et al., 2002) and for hematopoietic cells, for which a role in proliferation and stem cell self-renewal was also reported (Austin et al., 1997; Brandon et al., 2000; Reya et al., 2003). Wnt signaling is required for the specification of caudal neural cells (Nordstrom et al., 2002), for the proliferation of neural progenitor cells (Dickinson et al., 1994; Ikeya et al., 1997), for the differentiation of interneurons in the dorsal spinal cord (Muroyama et al., 2002) and for the specification of cells of the dorsal telencephalon (Gunhaga et al., 2003). Wnts have also been implicated in muscle progenitor cell determination (Borello et al., 1999b; Geetha-Loganathan et al., 2005; Munsterberg and Lassar, 1995; Schmidt et al., 2000; Tajbakhsh et al., 1998; Wagner et al., 2000), and muscle terminal differentiation and fiber patterning (Anakwe et al., 2003; Kardon et al., 2003).

Specification of somite cell fate depends upon paracrine factors secreted by adjacent tissues, such as the neural tube, notochord, surface ectoderm and lateral mesoderm. Sonic hedgehog (Shh) and Wnts have been investigated in detail as candidate molecules, although other signaling molecules, such as Noggin and Bmp (Reshef et al., 1998), and intra-cellular effectors, such as Creb (Chen et al., 2005), also play an important role in orchestrating the activation of myogenesis. Currently it is still unclear whether these molecules instruct naive cells, amplify a pool of committed progenitors and/or prevent their death (Borycki and Emerson, Jr, 2000; Cossu and Borello, 1999). We have previously shown in explant experiments that Wnt1, produced in the dorsal neural tube, induces myogenesis through the preferential activation of *Myf5*, whereas Wnt7a or Wnt6, produced in the dorsal ectoderm, preferentially activates *Myod* (Tajbakhsh et al., 1998). To dissect the molecular events that lead to *Myf5* activation during development, a detailed analysis of the regulatory regions influencing *Myf5* expression has been performed (Buchberger et al., 2003; Carvajal et al., 2001; Hadchouel et al., 2003; Hadchouel et al., 2000; Summerbell et al., 2000; Teboul et al., 2002). The *Myf5* locus demonstrates complex regulation: different enhancer modules were isolated that recapitulate the expression of *Myf5* in different domains of the developing embryo and at different stages of development. The epaxial enhancer (Ep) is necessary and sufficient for the expression of a *lacZ* reporter gene in the epaxial domain of the newly formed somites where *Myf5* is first expressed (Summerbell et al., 2000; Teboul et al., 2002). The activity of this enhancer has been reported to depend on a consensus binding sequence for Gli transcription factors, which transduces the Shh signal. In a *Shh* null background, this enhancer was shown to be inactive, suggesting that

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Shh is necessary for activation through the Ep enhancer (Gustafsson et al., 2002). However, other results (Teboul et al., 2003) indicated that the Gli site in the enhancer is involved in the maintenance, rather than the initiation, of Ep enhancer activity. The effect of Shh on epaxial activation of the *Myf5* gene has also been examined in the *Shh*$^{-/-}$ mutant (Borycki et al., 1999; Kruger et al., 2001), again with differing interpretations as to the effect on epaxial myogenesis. Analysis of *Gli* mutant embryos supports the view that Gli factors that respond to Shh signaling are involved in activation (McDermott et al., 2005).

Here, we report a series of experiments designed to examine the role of the Wnt canonical pathway in myogenic determination in the somite, and the interaction between Wnt and Shh signaling in this event. By refining the expression analysis of potential players in canonical Wnt signaling, and by using a combination of transfection assays, explant cultures and in vivo analysis, we demonstrate direct regulation of *Myf5* by canonical Wnt signaling. Furthermore, we show that direct activation is mediated by binding of the Tcf-Lef/β-catenin complex to the *Myf5* epaxial enhancer and to a newly identified element upstream of this enhancer. The binding of the Tcf-Lef/β-catenin complex and Gli1 to the extended Ep (EpExt) enhancer cooperatively activates the transcription of a reporter gene. Furthermore, deletion of the Tcf/Lef sites and the Gli site present in the enhancer abolishes activity in vivo. These results elucidate the molecular mechanism of the cooperative activation of *Myf5* by Wnt and Shh that is observed in explant culture (Munsterberg et al., 1995; Tajbakhsh et al., 1998).

MATERIALS AND METHODS

Mouse lines

The following mouse lines were used: *Myf5*$^{nlacZ/+}$ (Tajbakhsh et al., 1996), BAT-gal (Maretto et al., 2003) and β-catenin$^{floxed/+}$ (Brault et al., 2001). Heterozygous *Myf5*$^{nlacZ/+}$ or BAT-gal males were crossed with CD1 outbred female mice. To provide homozygous embryos, heterozygous mice were mated and the pregnant female was sacrificed at embryonic day E9.5. β-catenin$^{floxdel/floxed}$ mice were obtained by intercrossing β-catenin$^{floxdel/+}$ with β-catenin$^{floxed/floxed}$ mice. The null β-catenin$^{floxdel/+}$ allele resulted from crossing the β-catenin$^{floxed/+}$ with PGK-Cre mice (Lallemand et al., 1998).

Explants and infections

Embryos were dated taking day E0.5 as the morning of detection of the maternal vaginal plug. For most experiments, embryos at E9.5 (20 to 24 somites) were isolated in PBS.

The explant cultures were performed as described (Borello et al., 1999b; Tajbakhsh et al., 1998). In some experiments, somites were cultured in the presence of clones of C3H10T1/2 (ATCC number: CCL-226) previously selected for high-level expression of Frizzled truncated proteins (tested by western blot with an anti-HA antibody). Fz1ΔN (amino acids 1 to 237), Fz6ΔN (amino acids 1 to 163) and Fz7ΔN (amino acids 1 to 185) were cloned in the pCDNA3.1 (Invitrogen) expression vector in frame with an HA epitope sequence at the 3′ end. The Frzb1 construct was as previously described (Borello et al., 1999b). The tissue explants were seeded on a layer of C3H10T1/2 cells, to which they adhered within 10 minutes, and the dishes were then carefully transferred to the incubator. Control experiments with C3H10T1/2 cells transfected with the expression vector pCDNA3.1 alone had shown that C3H10T1/2 cells did not alter the extent of myogenic differentiation under these conditions.

Lentiviral vectors were prepared as described (Bonci et al., 2003; Dull et al., 1998; Follenzi et al., 2000). Dp and ΔC β-catenin, and CRE cDNAs were subcloned in the pRRLsin.PPT.CMV.NTRiresGFPpre plasmid using pENV instead of VSVG as an envelope protein for Dp and ΔC β-catenin lentiviral vectors. The Dp and ΔC β-catenin virus titers were 1×10^6 plaque forming units (PFU)/ml and the purified CRE titer was 5×10^9 PFU/ml. Control experiments indicated that, at the titer used, viral infection of somite and PSM explants did not induce cell death as measured by TUNEL assay (data not shown). After 6 hours of culture, the explants were infected with a multiplicity of infection (MOI) of 100 in RPMI/15% FCS overnight and 2 nM recombinant N-Shh was added (Incardona et al., 2000). The N-Shh recombinant protein was titrated on explants to determine a concentration that induced Myf5-positive cells. The following day the medium was changed and recombinant N-Shh was added at a final concentration of 2 nM. The explants were cultured for a total of 3 days. The significance of the data was verified using Student's *t*-test, considering $P<0.05$ as a significant value.

Cell transfection and luciferase assay

NIH3T3 cells (ATCC number: CRL-1658) were transfected with Lipofectamine Plus reagent (Invitrogen) with the expression vector for the full-length cDNA of mouse Lef1-HA, β-catenin (gifts of Dr Grosschedl, Max Planck Institute of Immunology, Germany), Tcf3 (gift of Dr Piccolo, University of Padua, Italy), Gli1 and human GLI3 (gifts of Dr Sasaki, Center for Developmental Biology, RIKEN, Japan), together with the enhancer fragments (see Fig. 6B), cloned in the pGL3 vector (Promega) with the TK minimal promoter upstream of the firefly luciferase gene. The vector phRL-TK (Promega) was co-transfected for normalization. The cells were transfected in OPTIMEM (Promega) following the manufacturer's instructions and then cultured in DMEM/10% FCS. Twenty-four hours after the transfection the cells were harvested in lysis buffer (Promega), and the luciferase and renilla luciferase activities were measured using the Dual Luciferase protocol (Promega). Reporter gene activities shown are the ratio of the average values of the reporter plus the indicated factors and the reporter alone, obtained from at least ten independent experiments.

Electrophoretic mobility shift assay (EMSA)

For this assay, Lef1-HA cDNA was cloned in the pCDNA3.1 vector and used as template for Lef1 protein obtained in vitro using the T7 TNT-coupled transcription/translation system (Promega). Translation efficiency was analyzed by western blotting with an anti-HA antibody. DNA probes used were complementary pairs of synthetic oligonucleotides derived from the EpExt enhancer: TBF1-2-3 (CCTCTCTTTCTTTGTTTCTTTCT-TTGTTTCTTTCTTTGTTTCTTTCT), TBF4 (CAAAAGAATTCTTTG-TTCATTTC) and TBF5 (TCATGTAAAAGTCAAAGGGAACCAAA).

DNA fragments were labeled by fill-in with Klenow enzyme and [α-^{32}P]dCTP (3000 Ci/mmol). In the competition assays, a 100-fold excess of unlabeled TBF1-2-3, TBF4 and TBF5, or TBF1-2-3mut (CCTCTC-TTTAAATCTTTCTTTAAATCTTTTAAATCTTTCTTTAAATCTTTCTT-CTTTCT), TBF4mut (CAAAAGAATTAAATCTTCTTCTTCATTTC) and TBF5mut (TCATGTAAAAGTTATTAAGAACCAA), oligonucleotides were added during the preincubation. For DNA binding, 5 μl of the TNT samples and 5 fmol of radiolabeled probe were combined in 10 mM Hepes/NaOH (pH 7.9), 50 mM NaCl, 5 mM EDTA, 1 mM DTT, 5% glycerol and 10 μg/ml of poly(dI:dC), and incubated on ice for 30 minutes. Samples were resolved on a 6% polyacrylamide gel in 0.5×Tris-Borate-EDTA and detected by autoradiography.

Immunocytochemistry

Immunocytochemistry on cultured cells was performed as described previously (Borello et al., 1999a; Brabletz et al., 2001; Tajbakhsh et al., 1998), using the following antibodies: MAb anti-β-galactosidase (1:100; Promega), MAb anti-CRE (1:200; Covance), MAb anti-Myf5 (undiluted; Santa Cruz), MAb anti-HA (1:200; Covance), MAb anti-β-catenin (1:50; Signal Transduction) and MAb anti-Myc (undiluted; a gift of F. Tato, University of Rome, Italy).

Whole-mount in situ hybridization (WHISH)

Embryos were prepared for WHISH as previously described (Borello et al., 1999b). Stained embryos were embedded in 25% gelatin and cut with a cryostat (Leica) at 15 μm. Photographs of whole-mount stained embryos were taken with a Leica stereomicroscope and section photographs were taken with a Leica DIC microscope.

Optical projection tomography (OPT)

Embryos selected for 3D imaging were embedded in 1% low-melting point agarose in water, dehydrated in methanol and cleared in Murray's clear (1:2 mixture of benzyl alcohol and benzyl benzoate). They were then scanned in

Table 1

Transgene	Transgenic embryos	β-gal⁺ cells in somites	
		E9.5 (23S-25S)	Earlier stages (7S-17S)
Ep	2	1^{+++}, 1^{+}	
EpE	6	2^{+++}, 2^{+}	2^{+++} (11S-12S)
ΔEp	4	4^{++}	
ΔEpGm	7	1^{+}, $2^{+/-}$, 1^{-}	$1^{+/-}$, 1^{+++} (17S), 1^{++} (7S)
ΔEpGT/Lm	4	4^{-}	

S, somites.
–, no expression in the somites.
+++, very strong expression in the somites.

an OPT apparatus and 3D reconstructions of the data produced as described by Sharpe et al. (Sharpe et al., 2002). The reconstructions were analysed using programs produced by the Edinburgh Mouse Atlas Project (http://genex.hgu.mrc.ac.uk).

Generation and analysis of transgenic embryos

The EpExt and the Ep constructions were created by subcloning the EpExt and the Ep enhancers (Fig. 5), respectively, into pHGBnlacZ, which contains the *nlacZ* reporter gene driven by the human *β-globin* minimal promoter obtained from the plasmid BGZ40 (Yee and Rigby, 1993). The construction ΔEp was obtained by deleting the TBF4 and TBF5 sites from the Ep enhancer (Fig. 5). In the ΔEp construction, the Gli site was mutated [GACCACCAA to GACtgCagA; (Gustafsson et al., 2002)] to make the ΔEpGm plasmid. Finally, in the ΔEpGT/Lm plasmid, the non-consensus Tcf/Lef site (Fig. 5, dashed line) was deleted using as template the ΔEpGm plasmid.

Plasmid fragment purification was carried out as described (Kelly et al., 1995). Transgenic embryos were generated by the microinjection of purified plasmid DNA into fertilized (C57BL/6J×SJL) F2 eggs at a concentration of about 1ng/μl using standard techniques (Hogan et al., 1994). Injected eggs were re-implanted the same day or the day after the injection into outbred pseudo-pregnant foster mothers. Embryos were recovered at E9.5 days of gestation in most cases. Transient transgenic embryos were dated by taking the day of re-implantation as E0.5. Their age was estimated more precisely by counting the number of somites. Embryos were dissected in PBS, fixed in 4% paraformaldehyde for 10 minutes, rinsed three times in PBS and stained in X-gal solution (Tajbakhsh et al., 1996) at 37°C overnight.

DNA was prepared from X-gal-negative embryos and analyzed by PCR, using *nlacZ* primers. X-gal-stained embryos were examined as whole mounts or after cryostat sectioning into 10 μm sections, as described previously (Kelly et al., 1995). Table 1 summarizes the number of transgenic embryos and the transgene expression obtained with the different constructs.

RESULTS

Soluble forms of frizzled receptors inhibit the expression of *Myf5* in somite explants

The analysis of frizzled (*Fz*) gene expression patterns in newly formed somites showed that both *Fz1* and *Fz6* are expressed in the epaxial domain of the epithelial somite where *Myf5* is first activated, whereas *Fz7* is expressed in the hypaxial domain (Borello et al., 1999a). This direct correlation between *Fz1*/*Fz6* and *Myf5* expression suggests that these receptors might be involved in mediating Wnt activation of *Myf5* in the epaxial domain of the somite. To test this hypothesis, we overexpressed truncated forms of the Wnt receptor genes *Fz1*, *Fz6* and *Fz7* in somite explants and observed the effect on the subsequent activation of *Myf5* expression. These mutated receptors contain the extra-cellular Wnt binding domain, but are missing the transmembrane and cytoplasmic domains that anchor the receptor to the membrane and transmit a Wnt signal within the responding cell (Fig. 1B). We used these soluble mutated receptor forms to interfere with the endogenous Wnt-Fz1/Fz6 interaction that we postulated to be involved in activating the expression of *Myf5*. To examine possible effects on the

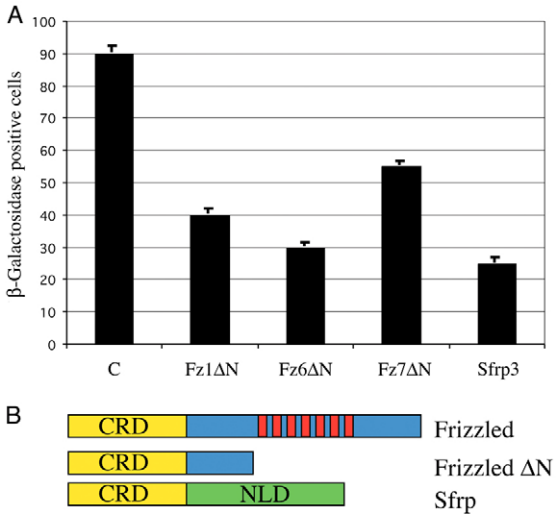

Fig. 1. Dominant-negative frizzled receptor mutants inhibit *Myf5* expression. (**A**) Quantitative analysis of *Myf5* activation in explants. PSM and the first somites (I-IV) of $Myf5^{nlacZ/+}$ targeted mice (Tajbakhsh et al., 1996) were co-cultured for 3 days with neural tube and C3H10T1/2 cells expressing Frizzled1ΔN (Fz1ΔN), Frizzled6ΔN (Fz6ΔN), Frizzled7ΔN (Fz7ΔN) or Sfrp3, or with control cells (C). Independent experiments (*n*=6) were performed in triplicate and averaged. (**B**) Schematic representation of the frizzled receptor structure compared with the frizzled ΔN mutants used in this assay and Sfrp molecules. CRD, cystein-rich domain; NLD, netrin-like domain; red bands, transmembrane domains.

Myf5 locus, presomitic mesoderm (PSM) and newly formed somite (I-V) explants were isolated from targeted $Myf5^{nlacZ/+}$ mouse embryos, allowing the visualization of *Myf5* expression by staining for β-galactosidase activity (Tajbakhsh et al., 1996). Co-culture of PSM or newly formed somites with neural tube explants led to β-galactosidase expression in responding cells (Fig. 1A, control), a quantifiable measure of *Myf5* activation. Explants were exposed to the soluble frizzled receptors and a natural, soluble, secreted Wnt binding protein, Sfrp3 (Frzb1), by co-culture with stably transfected C3H10T1/2 cells that secreted the respective proteins. Sfrp3 served as a control for the inhibition of *Myf5* expression in this assay, as we had previously demonstrated its ability to interfere with Wnt-ligand/frizzled-receptor interaction in somites, thereby downregulating the expression of *Myf5* (Borello et al., 1999b). In this assay all of the secreted Wnt binding proteins tested were found to interfere with the activation of *Myf5* by the neural tube, although those derived from *Fz1* and *Fz6*, the two frizzled genes expressed in the epaxial lip of the somite, had a significantly stronger effect (Fig. 1A). These data provide evidence supporting the idea that a frizzled-mediated Wnt signal from the neural tube contributes to *Myf5* activation.

The expression of Wnt effectors correlates with *Myf5* expression

We examined expression of the transcription factors that mediate canonical Wnt signaling in the nucleus. It has been previously reported that *Lef1* and *Tcf1* are expressed in the PSM but not in the somites (Galceran et al., 1999). We used optical projection tomography (OPT) (Sharpe et al., 2002) to scan whole-mount embryos hybridized with probes for *Lef1* and *Tcf1* to analyze the expression patterns in more detail. This technique allows computer reconstruction of the data for optical sectioning, allowing the

detection of low levels of expression. For data for *Lef1* see Fig. 2A-C. Panel A shows a lateral view of a lightly stained whole-mount hybridized embryo used for scanning; B and C, respectively, show sagittal and transverse optical sections through this specimen. The figures show high-level expression in the PSM as previously reported, but also localized expression in the dorsal domain of newly formed somites (Fig. 2B,C). Fig. 2F shows an optical section of *Tcf1* expression in newly formed somites in a plane similar to that shown in Fig. 2C. Expression is still mainly dorsal, but it is broader than in the case of *Lef1* and extends along the ventral edge. A low level of *Tcf3* expression is observed in newly formed somites (Fig. 2D,E), similar to that previously described by Galceran et al. (Galceran et al., 1999).

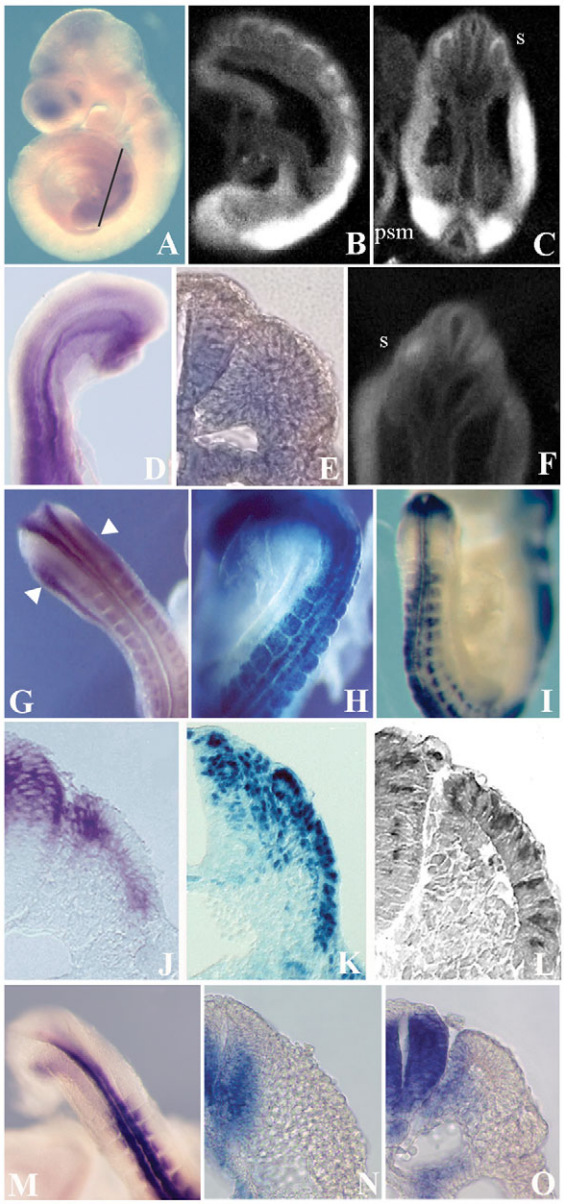

Fig. 2. Expression patterns of Tcf/Lef family members, β-catenin and *Gli1* in the PSM and newly formed somites at E9.5. (**A**) Lateral view of a lightly stained Lef1 whole-mount in situ hybridized embryo after OPT scanning and 3D reconstructions. (**B**,**C**) Optical sections through the resulting reconstruction: B is through the tail region, in the same plane of view as A, C is a transverse section, as indicated by the line in A. (**D**) An embryo hybridized to reveal Tcf3 expression in the newly formed somites. (**E**) Transverse section through the newly formed somites shown in D. (**F**) A similar section to C, through a Tcf1 hybridized embryo. (**G**) β-catenin WHISH; arrowheads indicate expression in the lateral mesoderm. (**H**,**I**) BAT-gal transgenic reporter mice: (H) β-galactosidase staining, (I) *lacZ* WHISH. (**J**,**K**) Transverse sections through newly formed somites in I and H, respectively. (**L**) A similar section to in J and K, immunostained for β-catenin. (**M**) *Gli1* WHISH. (**N**,**O**) Transverse sections through PSM (N) and newly formed somites (O) in M.

Expression of the β-catenin gene at the appropriate stage (E9.5; 20-24 somites) of development was also investigated by in situ hybridization (Fig. 2G). In partial agreement with the previously reported chick expression pattern (Schmidt et al., 2000), labeling was detected in newly formed somites and at a lower level in the PSM. Strong expression was observed in the lateral mesoderm. These expression patterns suggest that the Wnt/β-catenin nuclear effectors are strongly expressed only in the muscle precursor cells located in newly formed somites, where *Myf5* expression is first observed.

This analysis, however, shows only the localization of mRNA for Wnt effectors and does not indicate activity of the relative protein products. To address this, we analyzed a Wnt/β-catenin reporter mouse, BAT-gal, in which Tcf/β-catenin binding sites regulate expression of the *lacZ* gene (Maretto et al., 2003). Staining for β-galactosidase activity in such mice provides a means of detecting read-out of active canonical Wnt signaling. Staining of E9.5 embryos showed extensive reporter gene activity (Fig. 2H). Given the stability of the β-galactosidase protein, we also analyzed *lacZ* mRNA expression in these embryos by in situ hybridisation, to reveal more precisely the dynamics of reporter activation. This analysis showed that the reporter gene is strongly expressed in newly formed somites but only faintly in the PSM (Fig. 2I). Sections of E9.5 embryos showed that β-galactosidase expression (Fig. 2J) and activity (Fig. 2K) was localized predominantly in the epaxial lip of the somite. A modified immunohistochemical method to detect β-catenin protein (Brabletz et al., 2001) confirmed the accumulation of nuclear β-catenin in cells of the epaxial lip and the dermomyotome (Fig. 2L). Interestingly, the nuclear effector of the Shh pathway *Gli1* was expressed in the epaxial domain of the newly formed somites but not in the PSM (Fig. 2M-O), suggesting simultaneous and co-localized expression of Wnt/β-catenin and Shh/Gli pathway components in the newly formed somites.

Taken together with our previous analysis of the frizzled receptor genes, these data show localization of pathway effectors compatible with a role for canonical Wnt signaling in myogenic determination. Furthermore, they show that expression of *Lef1* is high in the PSM where β-catenin is not highly expressed and where the BAT-gal reporter gene is essentially silent. This opens the possibility that Lef1 alone might exert an inhibitory effect on target genes of the canonical Wnt pathway in this region when not complexed with β-catenin.

Overexpression of an activated form of β-catenin activates *Myf5* transcription

β-catenin accumulation in the nucleus is a necessary step for the activation of canonical Wnt target gene transcription. A constitutively active form of β-catenin (Dp) was produced by mutating the Gsk3 phosphorylation sites; this mutated form of the protein cannot be marked for degradation by Gsk3 phosphorylation even in the absence of a canonical Wnt signal. It therefore accumulates in the nucleus (Hsu et al., 1998). We infected PSM and somite explants of E9.5 embryos ($Myf5^{nlacZ/+}$) with a lentiviral

vector expressing this mutated form of β-catenin. We used the lentiviral system because it was found to be the most efficient system with which to deliver nuclear factors in embryonic cells (data not shown). Cells infected with the lentiviral vector could be identified by the expression of green fluorescent protein (GFP) (Fig. 3B,E), and activation of the *Myf5* locus was monitored by immunolabeling with an anti-β-galactosidase antibody (Fig. 3C,F). We found that somite explants showed a significant increase in the number of β-galactosidase-positive cells after infection of the vector expressing activated β-catenin (Fig. 3D-F,H), whereas PSM did not (Fig. 3A-C,G). When the infected explants were cultured in the presence of recombinant Shh, *Myf5*-positive cells appeared in the PSM and their total number was increased in the somites (Fig. 3G,H). A β-catenin form, with the same mutations in the Gsk3 phosphorylation sites as DP, which lacks in addition the transcriptional activation domain (ΔC), was used to abolish the Wnt/β-catenin-mediated transcriptional activation. *Myf5* expression was seen in the presence of Shh alone and was similar to the level seen with stabilized β-catenin in somites (Fig. 3H, Dp). This further implicates β-catenin transcriptional activity, together with Shh signaling, in the regulation of *Myf5* expression, and shows that somite explants respond to stabilized β-catenin alone. By contrast, PSM explants only respond when Shh is present. β-catenin has virtually no effect alone, possibly because of the repressor activity of Gli3 at this stage in the absence of Shh (McDermott et al., 2005). In somite explants, Gli proteins have responded to Shh and *Myf5* is already activated, so the situation is rather different.

We further investigated the requirement for β-catenin in the expression of *Myf5* by analyzing explants in a β-catenin null genetic background. We infected somite explants from β-catenin$^{floxdel/floxed}$ mice (Brault et al., 2001), co-cultured with neural

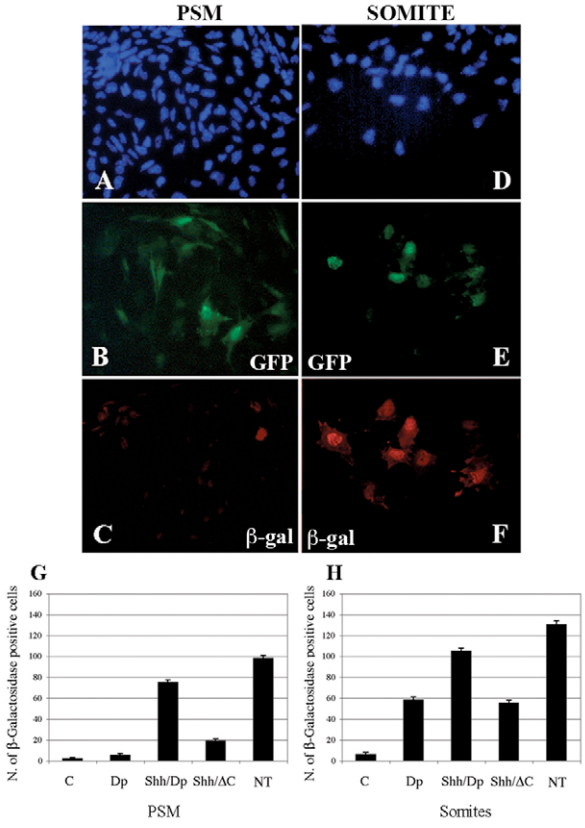

Fig. 3. β-catenin activation of *Myf5* expression in PSM and somite explants. (**A-F**) PSM explants (A-C) and somite explants (D-F) infected with a lentiviral vector carrying the cDNA for a stabilized form of β-catenin (Dp). (A,D) Hoechst staining of the nuclei. Infected cells were visualized by GFP fluorescence (B,E), and *Myf5*$^{nlacZ/+}$-expressing cells were detected by immunofluorescence with an anti-β-galactosidase monoclonal antibody (C,F). (**G**,**H**) Quantitative analysis of *Myf5*^{nlacZ} activation in infected PSM (G) and somite (H) explants. Explants were infected with Dp or ΔC β-catenin lentiviruses and co-cultured with or without 2 nM N-Shh recombinant protein (Shh). Explants co-cultured with neural tube (NT) were used as a positive control; explants infected with lentiviruses carrying *GFP* cDNA only were used as a negative control (C). Independent experiments (*n*=8) were performed in triplicate and averaged.

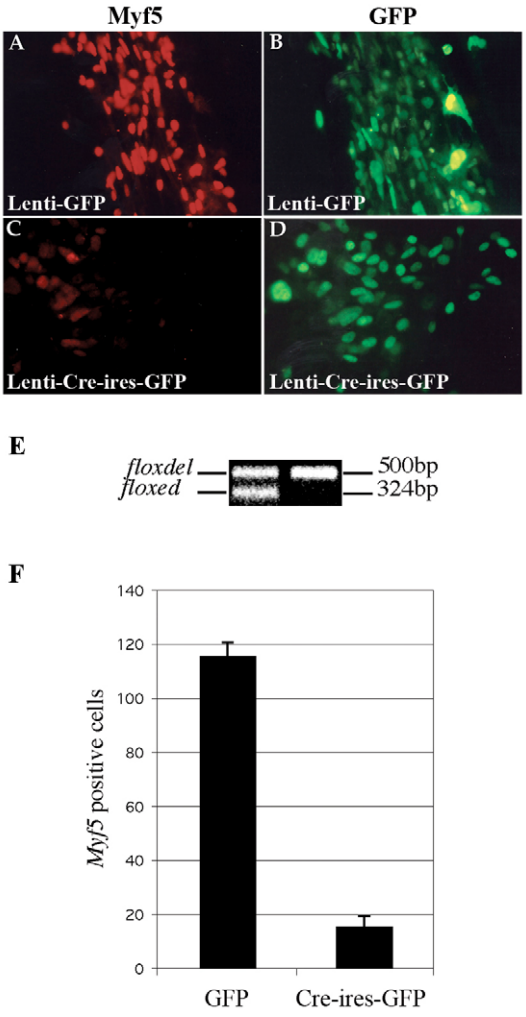

Fig. 4. β-catenin is required for *Myf5* activation in somite explants. (**A-D**) β-catenin$^{floxdel/floxed}$ somite explants were infected with a lentiviral vector carrying the cDNA for GFP (A,B) or CRE-IRES-GFP (C,D), and co-cultured with neural tube. Infected cells were visualized by GFP fluorescence (B,D) and *Myf5*-expressing cells were visualized with an anti-Myf5 monoclonal antibody (A,C). The GFP^{+} cells were also Cre^{+} when tested with an anti-Cre antibody (data not shown). (**E**) The absence of β-catenin in Cre-infected explants was demonstrated by PCR with specific oligonucleotides discriminating between the floxed and floxdel allele of β-catenin (Brault et al., 2001). (**F**) Quantification of *Myf5* activation. Independent experiments (*n*=8) were performed in triplicate and averaged.

tube, with a lentiviral vector expressing CRE recombinase (Fig. 4C,D). Cre-mediated deletion of the β-catenin gene, verified by PCR (Fig. 4E), induced a strong decrease in *Myf5* expression in the somites (Fig. 4C,D,F) when compared with a sister culture infected with a control lentiviral vector (Fig. 4A,B,F). When the experiment was repeated on PSM explants, we noticed cell death following Cre expression, probably due to the lack of cytoskeletal β-catenin with subsequent cell detachment from the substratum, not seen in somite explants (data not shown). However, cell death was never observed as a consequence of lentiviral infection (see Materials and methods).

Analysis of the epaxial enhancer sequence

To examine the molecular basis of the role of Wnt/β-catenin in *Myf5* activation, we analyzed transcriptional control sequences of the *Myf5* locus for Tcf/Lef binding sites. Previous work on the *Myf5* locus demonstrated that it is under a complex regulation (Buchberger et al., 2003; Hadchouel et al., 2003; Hadchouel et al., 2000; Summerbell et al., 2000; Teboul et al., 2002). Different enhancer modules recapitulate the expression of *Myf5* in different domains and at different stages of the developing embryo. One of these enhancers (named Ep) drives the expression of a *lacZ* reporter gene in the epaxial domain of newly formed somites where *Myf5* is first expressed (Summerbell et al., 2000; Teboul et al., 2002). The Ep sequence (Fig. 5A) shows a motif (TBF4) that matches the Tcf/Lef consensus (A/T)(A/T)CAAAG. At the 3′ end, another element (TBF5) has a single mismatch with a C instead of the first A/T of the Tcf/Lef binding consensus core. In addition, further analysis of the surrounding genomic sequence revealed three additional Tcf/Lef consensus sites (TBF1-TBF3) with an intersite spacing of 10 base pairs located upstream of the Ep enhancer (Fig. 5A). We have named the extended Ep enhancer, including the upstream potential binding sites TBF1-TBF3, EpExt.

The significance of these Tcf/Lef consensus sequences as potential targets of the Wnt/β-catenin pathway was first investigated by in vitro binding of Lef1. In an electrophoretic mobility shift assay, recombinant Lef1, tagged with the HA epitope (specifically recognized by an anti-HA antibody), bound efficiently to labeled oligonucleotides representing each of the putative target sites. The three closely linked binding sites (TBF1-TBF3) were assayed with a single oligonucleotide. The specificity of binding was confirmed by competition for Lef1 binding by excess unlabeled oligonucleotide, and by the failure of an oligonucleotide containing a mutated binding site to compete (Fig. 5B).

To analyze the ability of the Tcf/Lef binding sites within the extended Ep enhancer to drive reporter gene activity in response to Lef1 and β-catenin, we transfected NIH3T3 fibroblasts with β-catenin and Lef1 expression constructs, together with reporter constructs driven by various regions of the EpExt enhancer (Fig. 6A,B). Co-expression of β-catenin and Lef1 enhanced the expression of the luciferase gene driven by all tested Ep and EpExt enhancers possessing Tcf/Lef binding sites, when compared with the activity of the TK minimal promoter alone (Fig. 6A). The fragments containing only Tcf/Lef binding sites (Δ1-3) were activated by the Lef1/β-catenin complex, but to a lesser extent than the more complete enhancers. Fragment Δ4, containing the TBF5 Tcf/Lef binding site and the Gli site, had an intermediate activity. Surprisingly, the fragment containing the isolated Gli site alone (Δ5) mediated some transcriptional activity. This low activity might be due to inefficient binding of the Lef1/β-catenin complex to a non-consensus site identified in the fragment, under the conditions of this assay (Fig. 5A, dashed line; data not shown).

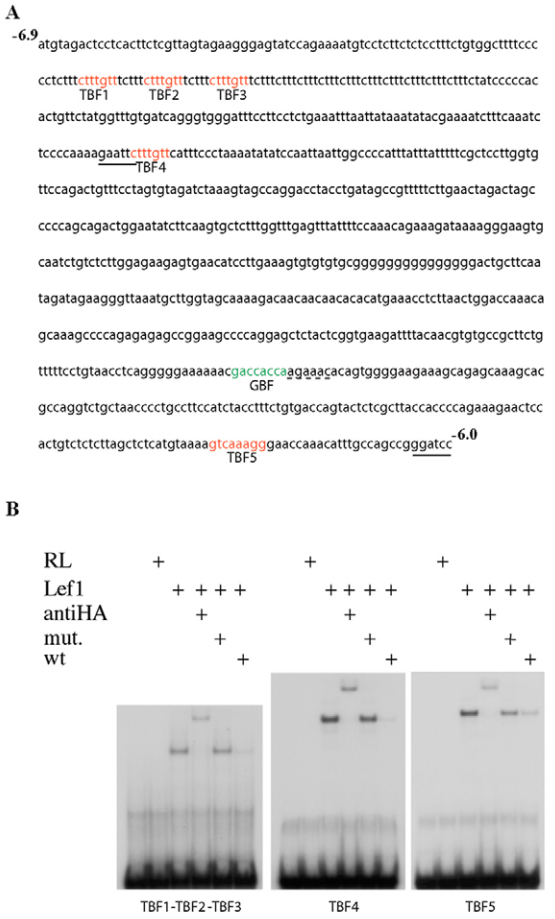

Fig. 5. Analysis of the Tcf/Lef binding sites in the EpExt enhancer regions. (**A**) The sequence of the EpExt enhancer. The Tcf/Lef binding sites are highlighted in red and the Gli binding site in green. The dashed line underlines a non-consensus Tcf/Lef site. Underlined are the *Eco*RI (–6.6 kb) and *Bam*HI (–6.0 kb) sites that indicate the boundaries of the originally defined Ep Enhancer. Nucleotide positions (in kb) relative to the *Myf5* coding sequence are indicated. (**B**) EMSAs performed with oligonucleotides of the Tcf/Lef binding sites (TBF1-2-3, TBF4 and TBF5), as indicated on the sequence in A, incubated with the reticulocyte lysate alone (RL) or with Lef1 recombinant protein (Lef1), in the presence of a specific antibody (antiHA) and a 100 molar excess of the mutated (mut.) or specific cold (wt) oligonucleotide.

Lef1/β-catenin and Gli1 cooperatively activate the extended Ep enhancer

A cooperative effect was demonstrated for Wnt1 and Shh in the activation of *Myf5* expression in somite explants (Munsterberg et al., 1995; Tajbakhsh et al., 1998), and we demonstrate here a strong activation of *Myf5* in somite explants when stabilized β-catenin is overexpressed in the presence of Shh (Fig. 3).

To investigate cooperation of the Wnt/β-catenin and Shh/Gli pathways at the transcriptional level, we compared the transcriptional activity of the Ep enhancer containing the Gli binding site (GBF) (Gustafsson et al., 2002; Teboul et al., 2003) to the EpExt enhancer described here that presents additional binding sites for canonical Wnt mediators (Figs 5, 6). In this assay system, we co-expressed different combinations of: (1) either stabilized (Dp) or dominant-negative β-catenin (ΔC); (2) either Lef1 or a dominant-negative form of Lef1 [Lef1 lacking the β-catenin binding domain (Tutter et al., 2001)]; and (3) either Gli1 or Gli3, mediators of the

Shh pathway. Gli1 was reported to activate Shh target genes, whereas Gli3 was shown to repress the transcription of some target genes (Ruiz i Altaba, 1999) and notably represses *Myf5* in the absence of Shh (McDermott et al., 2005).

Fig. 6C,D shows that in transfected NIH3T3 cells the combined effect of Wnt and Shh effectors is additive through the Ep enhancer but is clearly synergistic through EpExt. To further investigate the dynamics of co-operation between Wnt and Shh pathways in regulating *Myf5*, we co-transfected combinations of positive or negative mediators of each pathway (Fig. 6E,F). Again, the EpExt was more responsive to changes in the balance of contributory factors. Although the effect of inhibiting one or the other pathway on the Ep construct was small, a large reduction in transactivation was seen through EpExt. Another interesting difference between the two constructs was the effect of mutating β-catenin and Lef1. With a Lef1 dominant-negative mutant (N-terminal deletion) and Gli1, the enhancer activity is reduced almost to basal level. This Lef1 dominant-negative mutant was demonstrated to act as a strong repressor of the Wnt/β-catenin pathway (Billin et al., 2000). By contrast, the effect of a dominant-negative mutation in β-catenin (ΔC) was significantly less, indicating that either the Lef1/β-catenin complex or Gli are not sufficient to transactivate the enhancers when acting alone. Again this effect was more exaggerated with the EpExt construct (Fig. 6F). These data suggest that, in vitro, Wnt/β-catenin regulates the Shh-mediated transcriptional activation of the enhancer through two alternative Lef1 conformations: Lef1 bound to DNA and complexed with β-catenin acting as a cooperative factor for Gli-mediated transcriptional activation, or Lef1 bound to DNA without β-catenin acting as a repressor of Gli1 activation. This hypothesis is also supported by the finding that Lef1ΔN repression is partly reverted by TSA and valproic acid (data not shown), inhibitors of the HDAC proteins that were demonstrated to form a complex with Lef1 alone to block transcription at the DNA level (Billin et al., 2000), a process implicated in muscle development (Iezzi et al., 2002; Nervi et al., 2001). These results suggest that Lef1, complexed with co-repressors, can strongly inhibit the transcriptional activity of the EpExt enhancer; however, when complexed with an active form of β-catenin, Lef1 strongly activates transcription through cooperation with Gli1.

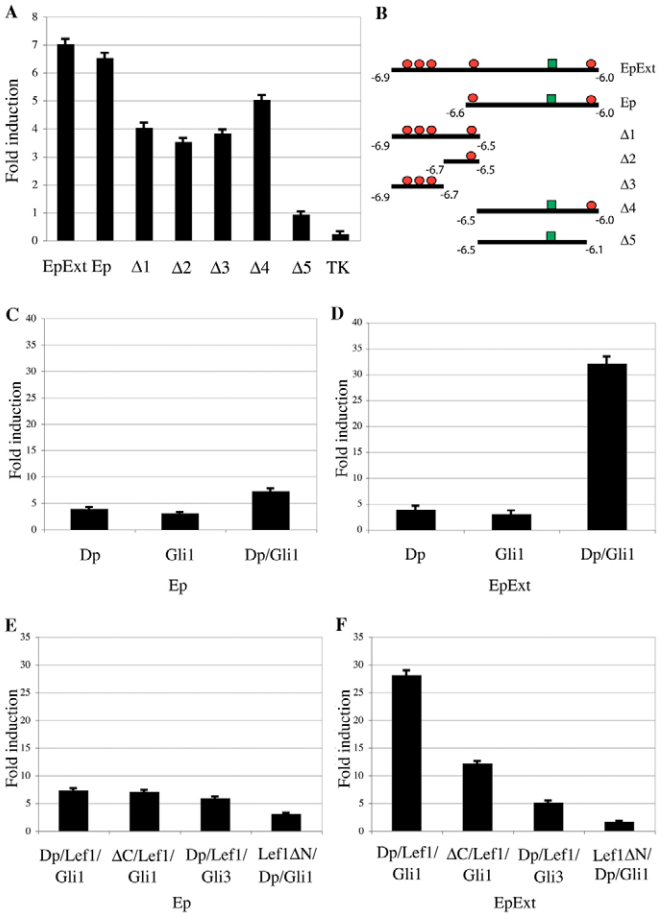

Fig. 6. Quantitative analysis of the transcriptional activity of the Lef/Tcf binding sites in the Ep and EpExt enhancers. (**A**) NIH3T3 cells were transfected with the reporter constructs indicated and described in Fig. 6B. TK, TK minimal promoter. (**B**) The deletions of the EpExt enhancer analyzed by luciferase assay. Tcf/Lef binding sites (red circles) and a Gli site (green rectangle) are indicated; numbers indicate nucleotide position in the genomic sequence, as in Fig. 5A. (**C-F**) NIH3T3 cells were transfected with either the Ep (C,E) or the EpExt (D,F) enhancer driving firefly luciferase and the expression vectors indicated below each bar on the graph. Dp, stabilized β-catenin; ΔC, dominant-negative β-catenin; Lef1ΔN, dominant-negative Lef1. The values of fold induction represent the ratio of the firefly luciferase activity of cells transfected with and without the β-catenin and Lef1 expression vectors, normalized to the activity of a control renilla luciferase-expressing vector. Independent experiments (n=10) were performed in triplicate and averaged.

The role of Tcf/Lef and Gli sites in the EpExt enhancer in vivo

The culture assay reported indicated that the extended enhancer EpExt mediates the expression of the *Myf5* gene by balancing activation and repression through Tcf/Lef binding sites, depending on the molecular context in the cell: i.e. repression in the absence of stabilized β-catenin and co-operative activation in the presence of β-catenin and Gli1. To examine the activation profile of this enhancer in vivo, transgenic embryos were produced by pronuclear injection of an *Ep-nlacZ* (Fig. 7A; Ep), or an *EpExt-nlacZ* construct (Fig. 7B; EpE), where the enhancer was cloned upstream of the *nlacZ* reporter gene under the control of the β-globin minimal promoter. Analysis of transgenic embryos at E9.5 showed robust expression in the epaxial domain of the newly formed somites. On sections, this domain of expression appeared more tightly restricted to the epaxial-most domain in *EpExt-nlacZ* transgenics (Fig. 7G,I) than in *Ep-nlacZ* transgenics (Fig. 7F,H). Expression of the *Ep-nlacZ* transgene extends more hypaxially, into a region where the endogeneous gene is not expressed (see also Teboul et al., 2002; Teboul et al., 2003). The role of Tcf/Lef binding sites was analyzed in a truncated version of the enhancer, ΔEp (Δ5), which has the 5′ and 3′ ends deleted, removing Tcf/Lef consensus sites. Transgene expression was reduced but still present (Fig. 7C) in these embryos. When the Gli site was mutated in ΔEpGm, activity was mainly undetectable in somites at E9.5, apart from a few labeled cells (Fig. 7D), although at earlier stages (7 or 17 somite embryos) more labeled cells were present in some embryos (see Table 1). This suggests that in this truncated Ep enhancer the Gli site may be less important initially. This sequence contains a non-consensus Tcf/Lef site (Fig. 5A, dashed line). When both this site and the Gli site were mutated (ΔEpGT/Lm), no β-galactosidase-positive cells were detected in the somites (Fig. 7E). This demonstrates that canonical Wnt signaling plays a crucial role in the expression of the Ep enhancer in the embryo.

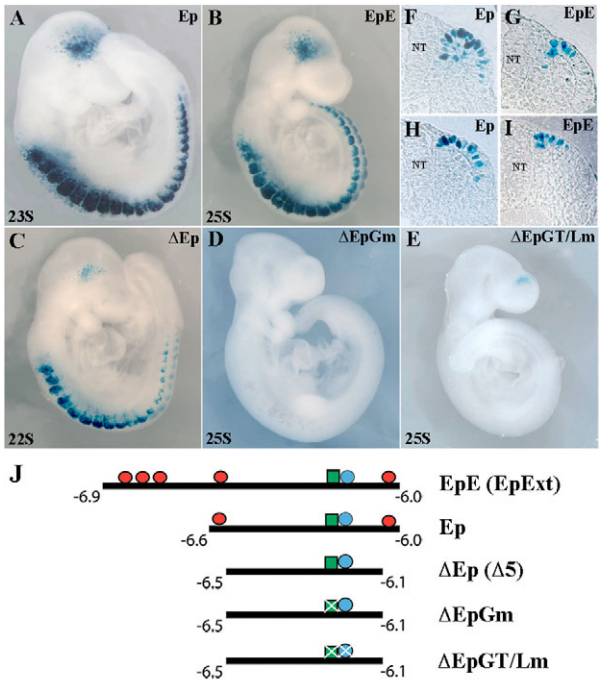

Fig. 7. Transgenic analysis of the role of the Tcf/Lef and Gli binding sites present in the epaxial enhancer of the *Myf5* gene. (**A-E**) X-gal staining of whole-mount embryos at E9.5, with the following enhancer sequences upstream of the β-globin promoter and the *nlacZ* reporter transgene: (A) the early epaxial enhancer (Ep), (B) the extended version of this enhancer (EpE), (C) the Ep enhancer with a 5′ and 3′ end deletion that removes the TBF4 and TBF5 Tcf/Lef binding sites (ΔEp), (D) the deleted ΔEp enhancer with the Gli site mutated (ΔEpGm), and (E) the deleted ΔEp enhancer with the Gli and remaining non-consensus Tcf/Lef binding site mutated (ΔEpGT/Lm). (**F**,**H**) Transverse sections of the embryo shown in A at two different levels in the interlimb region, showing extensive labelling of cells in the epithelial dermomyotome of somites. (**G**,**I**) Transverse sections of the embryo shown in B at two different levels in the interlimb region; labelled cells are restricted to the epaxial region of the dermomyotome, closest to the neural tube (NT). The number of somites (S) is indicated. (**J**) Schematic representation of the fragments of the epaxial enhancer used in the different transgenic constructs shown in A to I. EpE corresponds to the EpExt sequence and ΔEp to the Δ5 sequence presented in Fig. 6B. Red circles indicate the positions of the Tcf/Lef binding sites, the green rectangle indicates the position of the Gli binding site and the blue circle indicates the non-consensus Tcf/Lef binding site. White crosses indicate that the corresponding sites have been mutated.

DISCUSSION

How Wnt signaling leads to activation of *Myf5*

The mechanism of Wnt-mediated activation of *Myf5* is still unknown. In order to elucidate how Wnt signaling leads to the activation of *Myf5* in progenitor cells, we first investigated whether those frizzled receptors that are expressed in the epaxial lip of newly formed somites (Fz1 and Fz6) might transduce Wnt signaling. We showed that truncated, secreted forms of these receptors significantly inhibit *Myf5* activation. By contrast, truncated Fz7, which is not expressed in the epaxial lip, had only a modest effect, indicating a certain degree of specificity of this inhibition. Although the specificity of different frizzled receptors for activation of the canonical Wnt/β-catenin pathway is relatively poorly understood, it was reported that Fz1, but not Fz6, could stimulate the classic β-catenin pathway (Kuhl et al., 2000). We therefore investigated whether β-catenin and the canonical pathway are implicated in activation of *Myf5* in the epaxial region of the somite. Notably, we found that β-catenin is expressed in newly formed somites, but only weakly in the PSM, from which somites are formed. By contrast, the β-catenin cofactor *Lef1* was strongly expressed in the PSM and in the newly formed somites. The other members of the *Lef1* HMGB1 protein super-family, *Tcf1* and *Tcf3*, were also expressed in the PSM and the newly formed somites, although at a lower level than was *Lef1*. These patterns of expression correlate with the activity of *lacZ* in the transgenic Wnt reporter mouse BAT-gal, where the *lacZ* reporter gene is under the control of Tcf/Lef binding sites and responds to transduction of a canonical Wnt signal in the embryo (Maretto et al., 2003). The analysis of the *lacZ* expression pattern of the BAT-gal embryos clearly demonstrated that the Wnt/β-catenin pathway is active in the epaxial domain of the newly formed somites. The Wnt/β-catenin pathway was much less active in the PSM. β-galactosidase staining was clearly detectable, probably reflecting β-galactosidase stability, as there is strong expression in the tail-bud. These data suggest that the Wnt/β-catenin pathway may play a role in the regulation of *Myf5*. The overexpression of stabilized or dominant-negative forms of β-catenin in somite and PSM explant cultures clearly demonstrated that β-catenin is required for *Myf5* activation in the somites. These data were further confirmed by examining *Myf5* activation in a β-catenin null background, showing that, in the absence of β-catenin, *Myf5* activation was dramatically reduced in explants of somites (~90%). Expression of a stabilized form of β-catenin induced *Myf5* activation in explants of somites but not of PSM.

To explain this result, we analyzed the expression of the Shh effector Gli1 in the embryo. Gli1 has been shown to be a read out of Shh signaling, mediated by Gli2 and Gli3, which activate *Gli1*, leading to *Myf5* expression in the somite (McDermott et al., 2005). We show that *Gli1* is strongly expressed in the epaxial domain of the somites and not in the PSM. This suggests a cooperation between Wnt and Shh pathways in *Myf5* activation in the epaxial domain of the somites. Indeed, the addition of Shh to explants co-cultured with the neural tube, as a source of Wnts, readily led to *Myf5* activation. By contrast, expression of a dominant-negative version of β-catenin strongly reduces the Shh and Wnt cooperative effect in somite and to a lesser extent in PSM explants. Together, these data demonstrate a crucial role for the Wnt/β-catenin pathway in the regulation of *Myf5*, acting co-operatively with the Shh/Gli pathway.

Wnt and Shh converge on the same epaxial enhancer

To investigate the mechanism of Wnt/β-catenin in *Myf5* activation, we analyzed the early epaxial enhancer where a Gli site regulates transcription (Gustafsson et al., 2002; McDermott et al., 2005; Teboul et al., 2002). This region contains two canonical Tcf/Lef binding sites, as well as three additional sites at the 5′ end, described in this paper. In our assay, all these sites were able to bind Lef1 specifically and mediated Lef/β-catenin transcriptional activation in transiently transfected non-myogenic cells.

In the extended *Myf5* enhancer EpExt, a dominant-negative form of Lef1 strongly inhibits transcriptional activation, supporting the notion that integrity of the Lef/β-catenin complex is essential in this context. Although deletion analysis revealed a similar level of activation for the different Tcf/Lef binding sites, co-expression of a constitutively active β-catenin gene and *Gli* revealed a striking synergistic effect on the extended epaxial enhancer in comparison with the epaxial enhancer, where the effect was additive. This is in

keeping with previous observations on the co-operative effects of Wnt and Shh signaling in explant cultures (Munsterberg et al., 1995; Tajbakhsh et al., 1998). We suggest that the role of the additional Tcf/Lef binding sites of the EpExt enhancer is to finely tune the Shh signal during the activation of *Myf5*. The alternative binding of Lef1 complexed with either Groucho repressor factors or with the activator β-catenin may induce conformational changes in the region of the EpExt enhancer, allowing Gli factors to bind to DNA only when β-catenin is part of the complex. This mechanism of co-regulation was demonstrated for the *Cdx1* promoter, where the specificity of transcriptional activation is generated by TCF4E, inducing a promoter topology compatible with the binding of a transcriptionally active multiprotein complex (Hecht and Stemmler, 2003). In transgenic embryos, we observed that *lacZ* expression under the control of the EpExt enhancer was more restricted to the epaxial domain of the somite, whereas the Ep enhancer directed broader transgene expression in the dermomyotome (Teboul et al., 2002). This suggests that the EpExt element may provide the correct balance of elements to ensure *Myf5* activation in an appropriately restricted domain. Furthermore, deletion analysis demonstrated that both the Tcf/Lef sites and the Gli site play a role in the expression of the transgene, in keeping with the explant and cell culture experiments.

Our results show that, in the absence of both the Gli and the Tcf/Lef binding sites that we have identified, activation does not occur, indicating that both of these signaling pathways are necessary for the onset of myogenesis in the epaxial domain of the somite. Deletion of a non-consensus Tcf/Lef site immediately adjacent to the Gli site was necessary to completely abolish the basal level of transcription observed both in vitro (Fig. 6A, Δ5) and in vivo (Fig. 7D,E). Other sub-optimal Tcf/Lef consensus sites that we have not analysed may also contribute to activation of the enhancer by Wnts. However, our results indicate that a threshold level of Tcf/β-catenin input is necessary for activation, and that epaxial expression of the transgene is optimal in the presence of more than one site, together with the Gli binding sequence.

A unifying model for *Myf5* activation in the epaxial domain of newly formed somites

The data reported in this work, together with previous reports, allows us to propose a relatively simple model for the activation of *Myf5* by Gli and Lef/β-catenin-mediated signaling in newly formed somites. We do not suggest that this working model is complete; other unidentified factors may also contribute to this system. Indeed, in vivo, the Gli site in the epaxial enhancer may respond to other signals (Teboul et al., 2003). In the absence of both Shh and Gli3, epaxial *Myf5* expression is observed, suggesting that Gli activity, required for expression, may depend on other signal pathways (McDermott et al., 2005), which may involve Wnt signaling.

We suggest that in the PSM in the absence or presence of a low level of β-catenin, Lef1 and/or other Tcfs act as transcriptional repressors, or at least fail to support full transcriptional activity. At the onset of somitogenesis, the activation of *Gli1*-mediated signal together with the Wnt/β-catenin pathway leads to the correct activation of *Myf5* transcription in the epaxial domain.

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