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Genomes & Developmental Control

Myf5 expression in satellite cells and spindles in adult muscle is controlled by separate genetic elements

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Abstract

The myogenic regulatory factor Myf5 is integral to the initiation and control of skeletal muscle formation. In adult muscle, *Myf5* is expressed in satellite cells, stem cells of mature muscle, but not in the myonuclei that sustain the myofibre. Using the $Myf5^{nlacZ/+}$ mouse, we now show that *Myf5* is also constitutively expressed in muscle spindles-stretch-sensitive mechanoreceptors, while muscle denervation induces extensive reactivation of the *Myf5* gene in myonuclei. To identify the elements involved in the regulation of *Myf5* in adult muscle, we analysed reporter gene expression in a transgenic bacterial artificial chromosome (BAC) deletion series of the *Mrf4/Myf5* locus. A BAC carrying 140 kb upstream of the *Myf5* transcription start site was sufficient to drive all aspects of *Myf5* expression in adult muscle. In contrast, BACs carrying 88 and 59 kb upstream were unable to drive consistent expression in satellite cells, although expression in muscle spindles and reactivation of the locus in myonuclei were retained. Therefore, as during development, multiple enhancers are required to generate the full expression pattern of *Myf5* in the adult. Together, these observations show that elements controlling adult *Myf5* expression are genetically separable and possibly distinct from those that control *Myf5* during development. These studies are a first step towards identifying cognate transcription factors involved in muscle stem cell regulation.
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Introduction

The myogenic regulatory factors (MRFs) Myf5, MyoD, myogenin and Mrf4, members of the basic helix-loop-helix superfamily of transcription factors, are integral to the initiation and control of skeletal muscle development. Myf5 and MyoD determine the myogenic lineage (Rudnicki et al., 1993; Tajbakhsh et al., 1996b), whereas myogenin is essential for muscle cell differentiation (Hasty et al., 1993; Nabeshima et al., 1993).

Myf5 is first expressed around 8.0 days post coitum (dpc) of mouse development (Ott et al., 1991) and is the first in a cascade of transcription factors that regulate the differentiation of skeletal muscle precursors. In the absence of Myf5, the initiation of myogenesis fails to occur until the myogenic program is rescued by the activation of MyoD at approximately 10.5 dpc (Braun et al., 1994). Furthermore, without functional Myf5 protein, cells that would normally undergo myogenic differentiation can adopt nonmuscle fates, before the onset of MyoD

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expression (Tajbakhsh et al., 1996b). Myf5 therefore can be considered the determination gene for skeletal myogenesis, and thus the identification of the signals that control its expression and maintenance is central to understanding skeletal myogenesis per se. Indeed, considerable effort has been made to determine the regulatory elements in the locus that are responsible for aspects of the developmental expression pattern of *Myf5* (Buchberger et al., 2003; Carvajal et al., 2001; Hadchouel et al., 2000, 2003; Patapoutian et al., 1993; Summerbell et al., 2000; Teboul et al., 2002; Zweigerdt et al., 1997) and *Mrf4* (Carvajal et al., 2001; Patapoutian et al., 1993; Pin and Konieczny, 2002; Pin et al., 1997). However, an equivalent systematic examination has not been made with respect to the transcriptional regulation of the locus in adult skeletal muscle, and thus the location and nature of the sequences responsible are unknown. Indeed, the combination of a lack of reliable Myf5 antibodies and the perinatal lethality of Myf5-null mice means that the expression and function of Myf5 in adult muscle remains unclear.

Adult skeletal muscle consists predominantly of myofibres, highly specialised syncitial structures that are sustained by hundreds of myonuclei. Although the myofibres are postmitotic, a pool of stem cells, termed satellite cells (Mauro, 1961), persists beneath the basal lamina that surrounds each myofibre (reviewed by Zammit and Beauchamp, 2001). During postnatal development, satellite cells divide to provide new myonuclei to the growing muscle fibres before becoming quiescent in mature muscle (Schultz et al., 1978). Satellite cells retain the ability to proliferate and differentiate in response to myonuclear turnover, further growth signals or trauma (Bischoff, 1986; Schmalbruch and Lewis, 2000; Snow, 1977, 1978). Significantly, many of the factors associated with myogenesis in the embryo, including the MRFs, are also involved in the control of satellite cell differentiation during regeneration of adult muscle (Fuchtbauer and Westphal, 1992; Grounds et al., 1992; Megeney et al., 1996).

The *Myf5*$^{nlacZ/+}$ mouse, in which one *Myf5* allele produces wild type protein while the other produces β-galactosidase, has proved an invaluable tool with which to explore the expression of *Myf5* (Beauchamp et al., 2000; Tajbakhsh et al., 1996b). During development in the *Myf5*$^{nlacZ/+}$ mouse, β-galactosidase is detectable in both myoblasts and myonuclei (Tajbakhsh et al., 1996a) but is subsequently down-regulated so that only quiescent satellite cells remain positive in adult muscle (Beauchamp et al., 2000; Heslop et al., 2001). However, Myf5 (in addition to MyoD and myogenin) is up-regulated during satellite cell activation induced by muscle damage (Cooper et al., 1999; Fuchtbauer and Westphal, 1992; Grounds et al., 1992), a process that can also be modeled in culture (Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2002). Interestingly, culture of *Myf5*$^{nlacZ/+}$-derived myofibres leads to the rapid reactivation of the *Myf5* locus in myonuclei (Zammit et al., 2002). While the relevance of this observation to the regulation of *Myf5* in vivo is unknown, it indicates that the full range of *Myf5* expression in adult may not yet have been realised.

Here, we further examine the expression pattern of *nlacZ* in adult *Myf5*$^{nlacZ/+}$ mice and show that in addition to quiescent satellite cells, *Myf5* is also robustly expressed in muscle spindles, stretch-sensitive mechanoreceptors responsible for sensing how far and fast a muscle is lengthened (reviewed by Maier, 1997; Zelena, 1994). We also show that muscle denervation leads to the rapid and conspicuous reactivation of *Myf5* in the majority of myonuclei. To identify the elements involved in the regulation of the various aspects of *Myf5* expression in adult muscle, we have analysed reporter gene expression in a transgenic bacterial artificial chromosome (BAC) deletion series of the *Mrf4/Myf5* locus, in which an *nlacZ* reporter gene has been targeted to the ATG of *Myf5* (Carvajal et al., 2001). A BAC carrying 140 kb upstream of the *Myf5* transcriptional start site was able to drive all aspects of *Myf5* expression in adult muscle. However, analysis of further deletions of the *Mrf4/Myf5* locus revealed that the elements controlling *Myf5* expression in satellite cells and satellite cell-derived myoblasts were genetically separable from those controlling expression in muscle spindles, with further elements responsible for myonuclear reactivation. The identification of the transcriptional activators of *Myf5* expression in the adult may reveal whether different regulatory pathways operate during embryogenesis and during adult homeostasis and regeneration.

Materials and methods

Animal models

The *Myf5*$^{nlacZ/+}$ mouse (also called *myf-5*$^{a2+/-}$) has *nlacZ*-SV40poly(A)*RNApolII*/*Neo* targeted to the first exon of the *Myf5* gene, producing a fusion protein that consists of the first 13 amino acids of Myf5 and nuclear-localising β-galactosidase. *Myf5*$^{nlacZ/+}$ mice are viable and β-galactosidase reports expression of the endogenous *Myf5* locus (Tajbakhsh et al., 1996b).

The generation of mice transgenic for both plasmid (p) and BAC constructs containing defined regions of the *Mrf4*/*Myf5* locus has been described elsewhere (Carvajal et al., 2001; Summerbell et al., 2000). Four transgenic constructs were examined, their nomenclature indicating the extent of sequence 5′ (upstream) of the *Myf5* transcriptional start site (Fig. 1). Sequences 3′ range from approximately 50 to 5.4 kb downstream of the *Myf5* transcriptional start site (Fig. 1). The “AP” suffix indicates that the *Mrf4* promoter controls a membrane localised human *placental alkaline phosphatase* reporter gene (not the subject of this investigation), while “Z” means the *Myf5* promoter controls an *nlacZ* reporter gene. While the reporter genes are targeted to the ATG of the relevant gene, all downstream regulatory elements at the

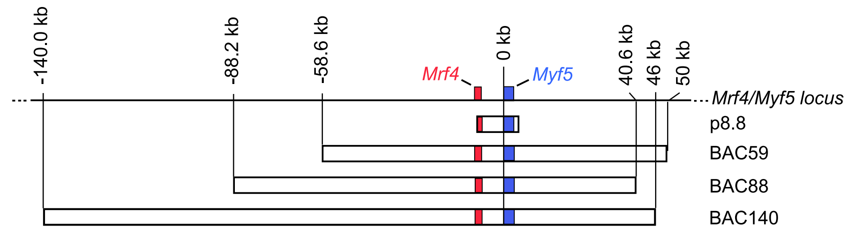

Fig. 1. Map of the *Mrf4/Myf5* locus and constructs used in the generation of transgenic mice. The *Mrf4* and *Myf5* genes are shown as red and blue boxes, respectively, with their transcription start sites separated by only 8.7 kb. The regions of the *Mrf4/Myf5* locus contained in the plasmid (p) and bacterial artificial chromosome (BAC) transgenic constructs are shown and the nomenclature indicates the extent of sequence upstream of the *Myf5* transcription start site (indicated as 0 kb).

locus are included. The adult mice used in this study were between 6 weeks and 4 months of age.

Crural muscle denervation

Mice were anaesthetised with hypnorm/hypnovel and a 1- to 2-cm incision made in the skin over the thigh. Blunt dissection was used to expose the sciatic nerve with minimal disruption to the blood supply, and a 2- to 5-mm segment of the nerve was excised. The skin incision was then closed with sutures and the animals kept warm and monitored until recovered from anaesthetic.

Tissue preparation and single fibre isolation

Mice were killed by cervical dislocation and the extensor digitorum longus (EDL), tibialis anterior (TA), soleus, diaphragm and intercostal muscles were all carefully removed. These were then either fixed for in toto revelation of β-galactosidase activity, snap frozen for cryosectioning or digested to isolate single myofibres.

The isolation of myofibres has been described in detail elsewhere (Rosenblatt et al., 1995). Briefly, muscles were incubated for 60 min at 37 °C in 0.2% (w/v) type 1 collagenase in supplemented Dulbecco's Modified Eagles' Medium [sDMEM; DMEM (Gibco) with 2 mM L-glutamine (Sigma) and 1% penicillin/streptomycin solution (Sigma)]. Myofibres were then isolated by trituration and either fixed for 2–5 min in 4% paraformaldehyde in PBS, or cultured, as described below.

Myofibre culture

Myofibres were placed in either 24 well Primaria plates (Marathon) or chamber slides (Labtec) coated with 1 mg/ml Matrigel (Collaborative Research Inc.). Plating medium [sDMEM with 10% (v/v) horse serum (PAA Laboratories) and 0.5% (v/v) chick embryo extract (CEE) (ICN Flow)] was added and the myofibres incubated at 37 °C in 5% CO_2. Under these conditions, myofibres adhere to the substrate and satellite cell-derived myogenic cells migrate away from the fibre and proliferate. Myofibres and cells were fixed in 2% paraformaldehyde/PBS for 2–5 min from 48 h to 7 days postplating.

Histochemistry

To visualise β-galactosidase activity, fixed muscles, myofibres and cells were incubated overnight at 37 °C in X-gal solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, 400 μg/ml X-gal and 0.02% IPEGAL CA-630 in PBS). Myofibres were then rinsed several times in PBS and mounted in DakoCytomation Faramount aqueous mounting medium.

Immunostaining

Fixed myofibres were permeabilised with 0.5% (v/v) Triton X-100/PBS and nonspecific antibody binding blocked by incubation in 20% (v/v) goat serum in PBS for at least 30 min. Primary antibodies used were monoclonal mouse anti-*Escherichia coli* β-galactosidase (clone 40-1A, Developmental Studies Hybridoma Bank; DSHB), monoclonal mouse anti-MyoD1 (clone 5.8a, DakoCytomation), monoclonal mouse anti-Pax7 (DSHB) monoclonal mouse anti-desmin (clone D33, DakoCytomation), rabbit polyclonal anti-*E. coli* β-galactosidase (Molecular Probes) and rabbit polyclonal anti-MyoD (Santa Cruz). Primary antibodies were applied overnight at 4 °C and then visualised by incubation with Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Myofibres or cells were then mounted in DakoCytomation Faramount fluorescent mounting medium containing 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain. Where secondary antibodies conjugated to HRP (DakoCytomation) were used, they were visualised using diaminobenzidine/hydrogen peroxide (Sigma).

Results

The expression of *Myf5* has been previously explored using the $Myf5^{nlacZ/+}$ mouse, in which one *Myf5* allele produces wild type Myf5 while the other produces β-galactosidase (Beauchamp et al., 2000; Tajbakhsh et al., 1996b). During the perinatal period, β-galactosidase is detectable in myoblasts and myonuclei (Tajbakhsh et al., 1996a) but is subsequently down-regulated in mature

myofibres so that only satellite cells contain β-galactosidase in adult muscle (Beauchamp et al., 2000; Heslop et al., 2001). This pattern of expression is maintained even in mice examined when over 6 months of age (Beauchamp et al., 2000).

Muscle spindles express Myf5

In this study, we initially examined a range of muscles from *Myf5*$^{nlacZ/+}$ mice and found strong *nlacZ* expression in muscle spindles (Fig. 2). Muscle spindles are stretch-sensitive mechanoreceptors composed of distinct small diameter muscle fibre types that lie in parallel with the myofibers (Fig. 2d) and are innervated by both sensory and motor axons. These muscle fibres and axons are enclosed in a fusiform capsule, which bulges at the midsection (equatorial region) of the spindle. The arrangement of the nuclei in this region allows these intrafusal muscle fibres to be classified into nuclear bag_1, nuclear bag_2 or nuclear chain intrafusal fibres (reviewed by Maier, 1997; Zelena, 1994). The nuclei of both nuclear bag and nuclear chain fibres had strong β-galactosidase activity, with marked *Myf5* expression in the equatorial and juxtaequatorial region, but less pronounced expression in nuclei of the polar regions (Figs. 2d and e).

Denervation leads to the reactivation of the Myf5 locus in myonuclei

We recently made the unexpected observation that the *Myf5* locus can be routinely reactivated in the myonuclei of

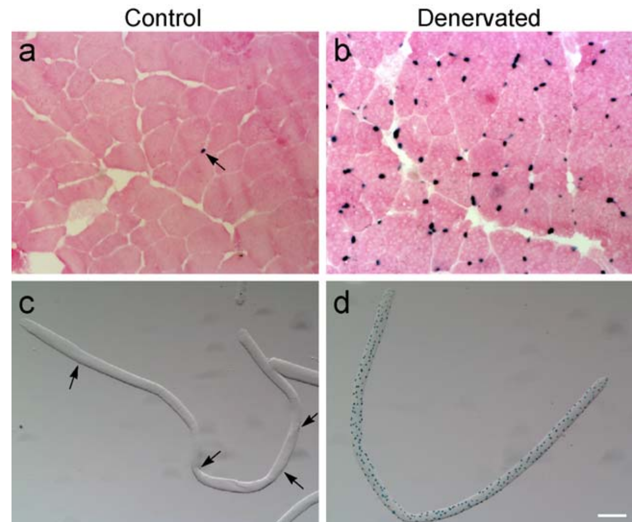

Fig. 3. In vivo muscle denervation leads to the reactivation of the *Myf5* locus in myonuclei. Adult *Myf5*$^{nlacZ/+}$ mice had the crural muscles of the right leg denervated by excising a portion of the sciatic nerve, while the left leg served as a control. A week after nerve section, the EDL, TA and soleus muscles from both legs were removed for analysis. Following X-gal colouration and eosin counterstain, cryosections of the control TA (a) or myofibres isolated from the control EDL muscle (c) only have β-galactosidase activity in quiescent satellite cells (arrow in a and c) and muscle spindles. However, cryosections of the denervated right TA (b) or myofibres isolated from the denervated EDL muscle (d) of the same animal, contained many myonuclei, which had reactivated the *Myf5* locus. Scale bar equals 30 μm for a and b and 60 μm for c and d.

EDL myofibres during culture (Zammit et al., 2002). Although previous studies have reported increased levels of *Myf5* transcript in denervated muscle (Buonanno et al.,

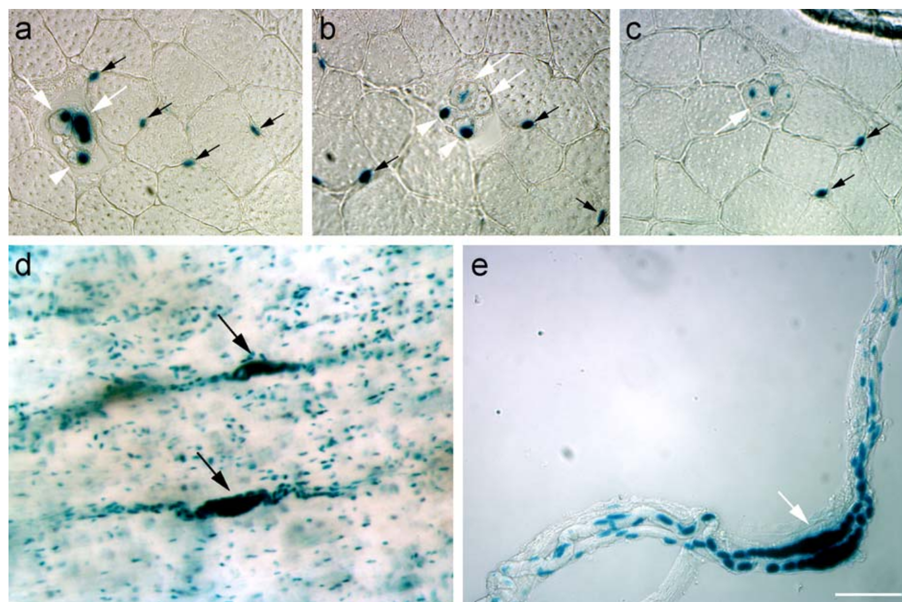

Fig. 2. Muscle spindles express *Myf5*. X-gal incubation of cryosections of adult *Myf5*$^{nlacZ/+}$ mouse soleus muscle (a, b and c) reveals β-galactosidase activity in muscle spindles (white arrows/heads) in both nuclear bag (a and b, white arrow) and nuclear chain fibres (a and b, white arrowhead), in addition to quiescent satellite cells (small black arrows). Muscle spindles can be clearly seen after X-gal incubation of the entire soleus muscle (arrow, d). The structure of the muscle spindles is better shown following their isolation from an EDL muscle by collagenase digestion (e) where nuclei in both the equatorial (arrow) and polar regions contain β-galactosidase. Scale bar equals 52 μm for a–c and 60 μm for e.

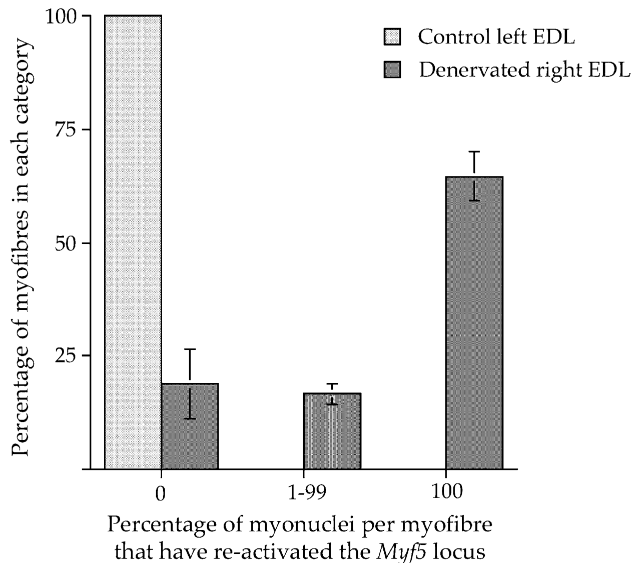

Fig. 4. Denervation leads to myonuclear reactivation of the *Myf5* locus. Myofibres were isolated from the EDL muscles of *Myf5*$^{nlacZ/+}$ mice that had been denervated for a week. The percentage of myonuclei per myofibre with β-galactosidase activity was then estimated following incubation in X-gal. Myofibres (81.3% ± 7.7%) had myonuclear reactivation of *Myf5*, with 64.6% ± 5.3% showing β-galactosidase activity in all myonuclei, whereas the myonuclei of control EDL myofibres never contained β-galactosidase. Values are the mean ± SEM of data pooled from approximately 50 myofibres from each of 3 adult *Myf5*$^{nlacZ/+}$ mice.

1992), this may have been due to up-regulation during satellite cell activation. We therefore wanted to determine whether *Myf5* was also reactivated in myonuclei in response to denervation in vivo.

The crural muscles of the right leg of 9 adult *Myf5*$^{nlacZ/+}$ mice were denervated by the excision of a 2- to 5-mm length of sciatic nerve in the thigh. At various times after nerve section, the right denervated TA, EDL and soleus were removed together with the contralateral control muscles and either cryosectioned or digested with collagenase to isolate single myofibres (Fig. 3). A week after nerve section, the denervated muscles showed β-galactosidase in many myonuclei (Fig. 3b), while activity was restricted to satellite cells and muscle spindles in the contralateral controls (Fig. 3a). Isolation of myofibres revealed the extent of the myonuclear reactivation in individual denervated myofibres (Fig. 3d). Analysis of approximately 50 myofibres isolated from each of 3 denervated EDL muscles showed some myonuclear reactivation in 81.3 ± 7.7% of myofibres, of which 64.6% ± 5.3% exhibited reactivation in all myonuclei (Fig. 4). In contrast, control myofibres showed no myonuclear β-galactosidase activity (Fig. 4). Similar results were obtained with denervated soleus muscles, with 77.8% (n = 2 animals) of myofibres showing myonuclear *Myf5* reactivation. Importantly, the reactivation of *Myf5* was widespread and not limited to myonuclei near the endplate (Fig. 3d). Although muscles were routinely examined after a week, extensive myonuclear reactivation of *Myf5* was also observed 48 h after denervation (data not shown).

The coordination of separate genetic elements control Myf5 expression in adult muscle

Extensive transgenic studies have identified distinct and separable regions of the *Mrf4/Myf5* locus that are responsible for the spatial and temporal activation and maintenance of *Myf5* expression during embryogenesis (Buchberger et al., 2003; Carvajal et al., 2001; Hadchouel et al., 2000, 2003; Patapoutian et al., 1993; Summerbell et al., 2000; Teboul et al., 2002; Zweigerdt et al., 1997). Since multiple sequences are responsible for the full developmental expression pattern of *Myf5*, it was of interest to determine whether the coordination of separate genetic elements was also required for *Myf5* expression in adult. We therefore analysed the musculature from a panel of mice transgenic for defined portions of the *Mrf4/Myf5* locus (Fig. 1). The results are summarised in Table 1. It should be noted that the embryonic and foetal expression patterns of these transgenic lines have already been

Table 1
Summary of the *Myf5* expression pattern in adult muscle of mice transgenic for defined regions of the *Mrf4/Myf5* locus

Transgenic line (line number)	Muscle spindles	Satellite cells in vivo	Satellite cell-derived myoblasts	Myonuclear reactivation
BAC140APZ (3)	+	+	+	+
BAC140APZ (4)	+	+	+	+
BAC140APZ (5)[a]	+	−	−/+	−
BAC88APZ (1)	+	−/+	−/+	−/+
BAC88APZ (2)[b]	+	−/+	−/+	−
BAC88APZ (3)[b]	+	−	n/d	n/d
BAC59APZ (2)	+	−/+[c]	−/+	−
BAC59Z (2)	+	+	−/+	+
BAC59Z (3)	+	−/+	−/+	+
BAC59Z (4)	+	−/+	−/+	+
P8.8Z (16)	−	−	−	−
P8.8Z (35)	−	−	−	−

Where + means widespread expression, −/+ means occasional positive cells, − means no expression and n/d is not determined.
[a] Expression down-regulates during development, atypical of other BAC140 lines.
[b] Diaphragm and intercostals not examined.
[c] Concentrated at the presumptive neuromuscular junction.

described by Carvajal et al. (2001) or Summerbell et al. (2000), except for line BAC140APZ(5), which was not available at the time.

Multiple transgenic mouse lines were examined for each construct. To first obtain a global view of the expression pattern in the transgenic mice, diaphragm, intercostal, soleus and TA muscles were dissected, immediately fixed and incubated in X-gal. This procedure was used to examine expression in muscle spindles and satellite cells. The results obtained from the examination of axial musculature agreed with those made in limb muscle. Myofibres were then isolated from the EDL and soleus muscles, which together

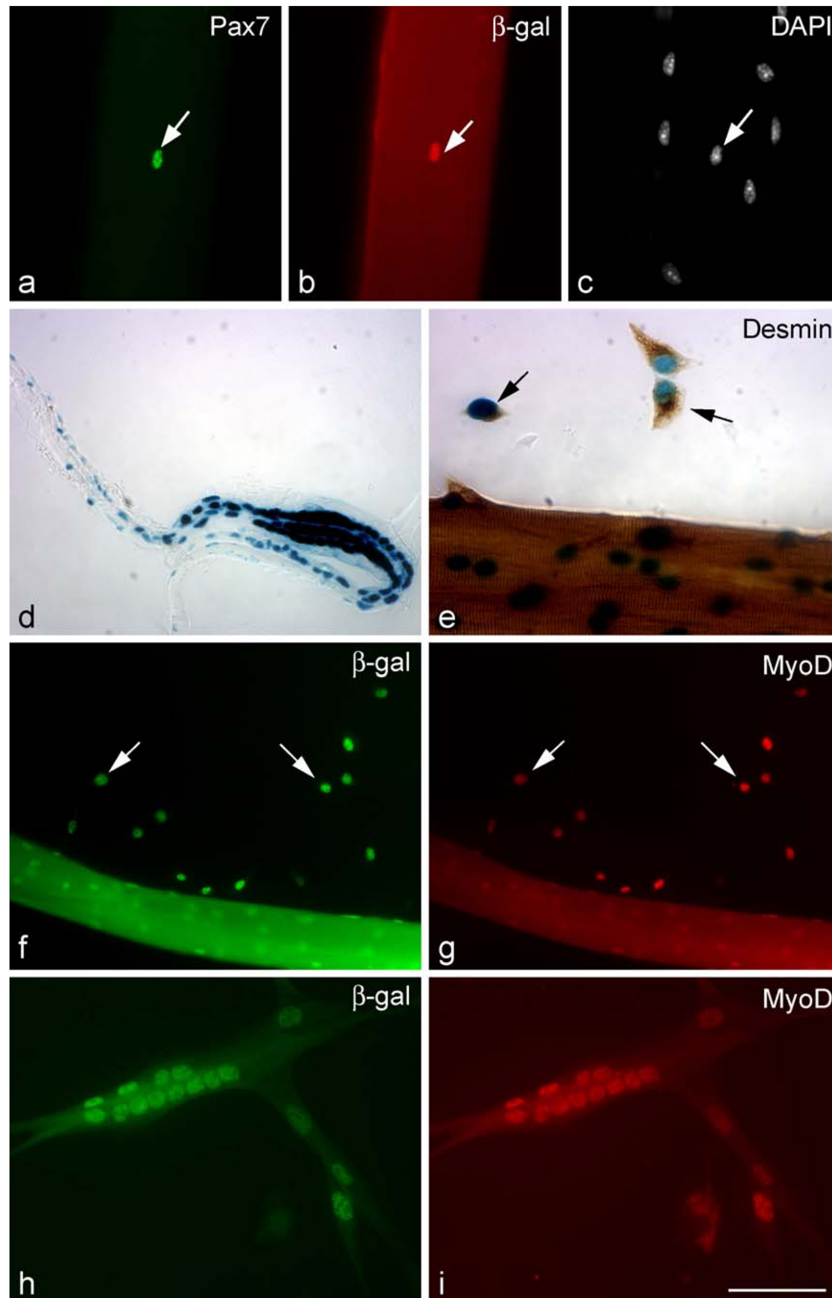

Fig. 5. The 140 kb upstream of *Myf5* sufficient to control all aspects of *Myf5* expression or regulation in adult muscle. Immunostaining of EDL myofibres isolated from a BAC140APZ(4) mouse demonstrated that quiescent satellite cells (arrowed in a, b and c), identified by Pax7 expression (a), can contain β-galactosidase (b), whereas there is no reporter gene product in myonuclei, revealed by a DAPI counterstain (c). Isolated muscle spindles of BAC140APZ(4) mice also had strong β-galactosidase activity in nuclei following incubation in X-gal (d). When isolated myofibres are cultured, satellite cells activate and migrate onto the matrigel surface (Rosenblatt et al., 1995). Following X-gal incubation of EDL cultures fixed after 72 h, widespread β-galactosidase activity was observed in these cells, which were confirmed as satellite cell-derived myoblasts by desmin immunostaining (arrow, e). Immunostaining of soleus (f and g) myofibre cultures after 72 h and EDL (h and i) myofibres cultured for 7 days showed β-galactosidase protein in satellite cell-derived myoblasts (arrow, f) and satellite cell-derived myotubes (h), both of which had high levels of MyoD protein (g and i). Myonuclear reactivation of the *Myf5* locus was also observed in cultured myofibres isolated from EDL muscle (e). Scale bar equals 50 μm except for d, f and g where it equals 100 μm.

contain the four main skeletal muscle fibre types (I, IIa, IIx and IIb), and cultured to assay expression in satellite cell-derived myoblasts and myonuclear reactivation of *Myf5*.

BAC140

BAC140APZ containing 140 kb of sequence 5′ to the *Myf5* transcription start site was found to be sufficient to drive *nlacZ* expression in muscle spindles in both axial and limb muscles of all lines examined (Fig. 5d). In 2/3 lines, this BAC was also able to drive all other aspects of *Myf5* regulation in adult muscle (Fig. 5). Widespread β-galactosidase activity was observed in satellite cells in diaphragm (Fig. 6), intercostal, soleus and TA muscles. Immunostaining of isolated myofibres with Pax7 showed the presence of β-galactosidase protein in satellite cells (Figs. 5a–c). When isolated myofibres are cultured, satellite cell-derived myoblasts emigrate from the myofibre and proliferate on the matrigel substrate. EDL and soleus myofibre cultures fixed after 72 h were surrounded by many *nlacZ* expressing cells, confirmed as myoblasts by the presence of desmin and MyoD proteins (Figs. 5e–g). EDL and soleus cultures examined 1 week after plating contained myotubes with multiple β-galactosidase-positive nuclei (Figs. 5h and i). Extensive reactivation of the *Myf5* locus was observed in EDL myofibres after culture (Fig. 5e). Thus, sequences within BAC140APZ are sufficient to drive the normal expression pattern of *Myf5* in adult muscle.

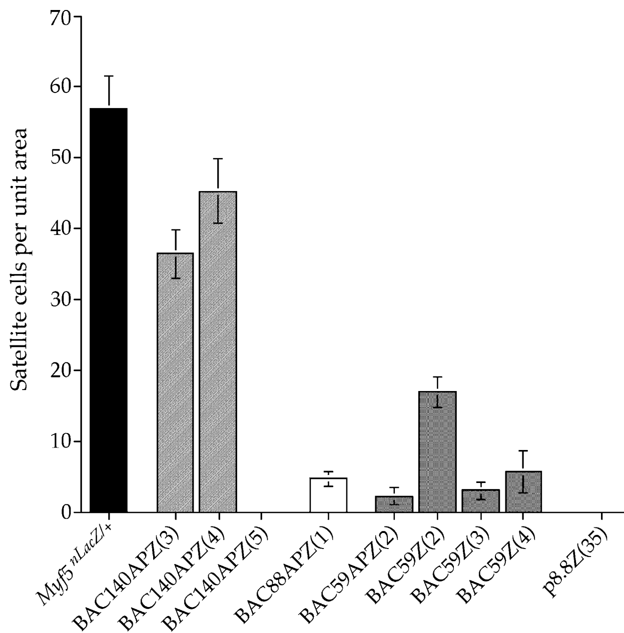

Fig. 6. Elements within the −140 to −88 kb interval drive widespread expression in satellite cells. Diaphragms were dissected, immediately fixed, incubated in X-gal and cleared in 90% glycerol/PBS. The number of β-galactosidase containing nuclei was counted per unit area (6.25×10^4 μm^2) from several fields per diaphragm. The data from multiple animals from each transgenic line were then pooled and expressed as mean ± SEM. BAC140 was able to drive widespread expression in satellite cells, but the deletion of the approximately 52 kb located between −140 and −88 kb resulted in the loss of expression in the majority of satellite cells.

BAC88

All three BAC88APZ lines investigated consistently showed muscle spindle expression in all muscles examined (Figs. 7a and b). However, both whole muscles and freshly isolated myofibres contained only rare β-galactosidase-positive cells (Figs. 6 and 7a). In culture, few satellite cell-derived myoblasts and myotubes contained β-galactosidase activity while the vast majority were negative (Fig. 7c). No consistent myonuclear reactivation of *Myf5* was observed (data not shown). These observations indicate that the sequences that drive widespread and robust *Myf5* expression in satellite cells in vivo and satellite cell-derived myoblasts reside in the 52 kb interval between −140 and −88 kb upstream of *Myf5*.

BAC59

The four BAC59 lines examined, carrying 59 kb upstream of *Myf5*, contained both limb and axial muscle spindles with robust β-galactosidase activity (Fig. 7d). Furthermore, BAC59APZ and BAC59Z (2/3) lines expressed *nlacZ* in only a few satellite cells in vivo and satellite cell-derived myoblasts, being essentially the same as the BAC88APZ lines (Fig. 6). The β-galactosidase-positive satellite cells in BAC59APZ muscle however were concentrated, possibly reflecting the position of endplates (Fig. 7e). One of the four BAC59 lines (BAC59Z(2)) showed more widespread *nlacZ* expression in satellite cells in vivo (Fig. 6) and positive myonuclei in some freshly isolated myofibres. However, even in this line, few desmin-positive satellite cell-derived myoblasts also contained β-galactosidase (Figs. 7f and g) similar to the other BAC59 lines. All three BAC59Z lines exhibited strong myonuclear reaction in EDL myofibres during culture (Fig. 7f).

p8.8Z

p8.8Z (corresponding to Construct 1 in Summerbell et al., 2000) was the smallest construct examined in this study, and includes most of the coding sequences of *Mrf4*, the intergenic region and *Myf5* down to the 3′UTR (Fig. 1). No β-galactosidase activity was detected in whole muscles (Figs. 6 and 7h), cultured myofibres or satellite cell-derived myoblasts (Fig. 7i) in the two lines examined. This was not unexpected since this construct is unable to drive expression in developing limbs or indeed, even maintain correct *Myf5* expression at most sites beyond approximately 12.5 dpc (Summerbell et al., 2000). Thus, the muscle spindle element is probably located between −59 and −8.8 kb upstream of *Myf5*.

Discussion

Myf5 is considered the determination gene for skeletal myogenesis. Accordingly, the expression pattern of *Myf5* during mouse development has been analysed in great detail but has received less attention in adult tissue. Previously, the

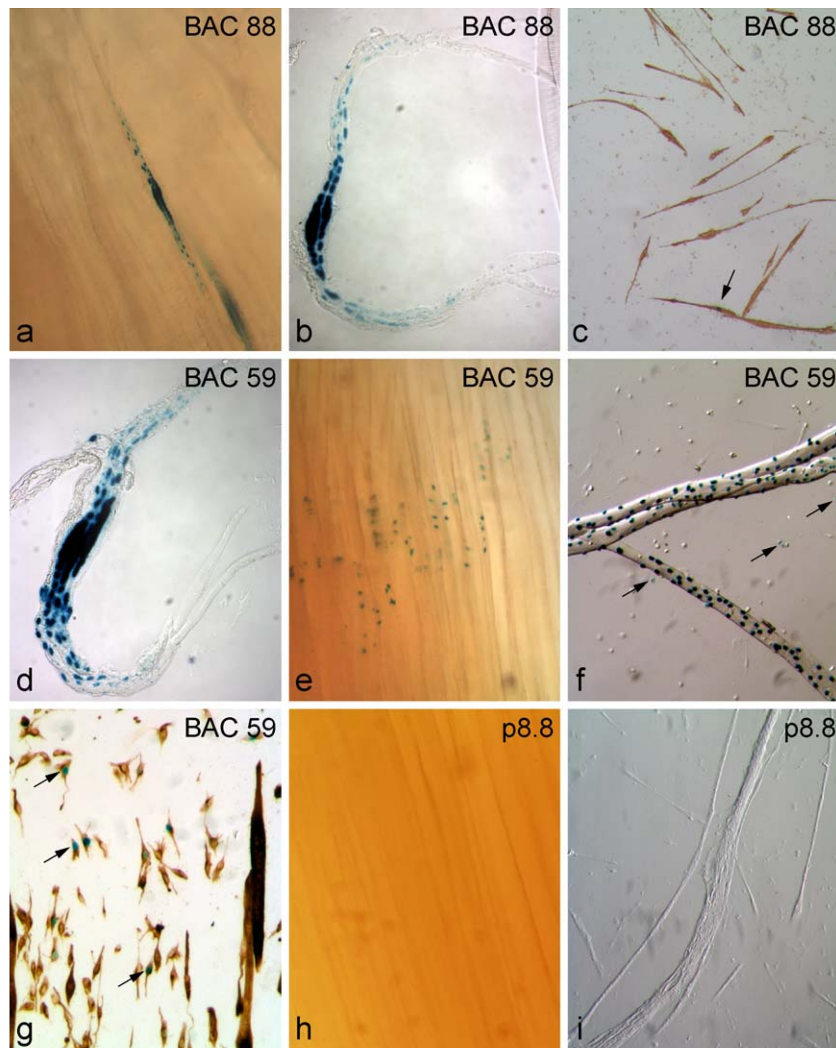

Fig. 7. The element driving constitutive muscle spindle expression of *Myf5* resides between −59 and −8.8 kb upstream of *Myf5*. Incubation in X-gal demonstrates that BAC88APZ(1) drives muscle spindle, but not satellite cell, expression (a and b). Immunostaining showed that only rare desmin-positive satellite cell-derived myoblasts and myotubes had β-galactosidase activity (arrow) after incubation in X-gal (c). BAC59APZ (2) had strong expression of *nlacZ* in muscle spindles (d), but there was little expression in satellite cells, apart from some located at the presumed neuromuscular junction of TA muscle (e). BAC59Z (2) also had β-galactosidase activity in muscle spindles and satellite cells and showed widespread myonuclear reactivation in culture (f), yet few satellite cell-derived myoblasts expressed the transgene (arrow, f). Following X-gal incubation of EDL cultures, the low number of β-galactosidase-positive cells was confirmed as satellite cell-derived myoblasts by desmin immunostaining (arrow, g). The smallest construct examined p8.8Z, containing 8.8 kb upstream of *Myf5*, failed to drive expression in either muscle spindles or satellite cells in vivo (h) and, when myofibres were cultured, in either satellite cell progeny or control myonuclear reactivation (i).

Myf5 locus was shown to be active in adult in quiescent satellite cells, satellite cell-derived myoblasts (Beauchamp et al., 2000; Heslop et al., 2001; Zammit et al., 2002) and certain structures of the CNS (Daubas et al., 2000). Here, we identify the muscle spindle as another major site of *Myf5* expression in adult muscle. Muscle spindles are stretch-sensitive mechanoreceptors that sense how far and fast a muscle is lengthened (reviewed by Zelena, 1994). They are composed of distinct intrafusal muscle fibre types that lie within a fusiform capsule and are innervated by both sensory and motor axons. The nuclei of both nuclear bag and nuclear chain intrafusal fibres express *Myf5*. Nuclear bag$_2$ fibres form first and are possibly derived from the same pool of myofibres that will form adult extrafusal fibres, with innervation dictating their fate. Mononucleated cells are then recruited to form nuclear bag$_1$ and nuclear chain intrafusal fibres (Kozeka and Ontell, 1981; reviewed by Maier, 1997; Zelena, 1994). Muscle spindles have been shown to express developmental isoforms of certain muscle genes, for example, maintaining neonatal myosin heavy chain (MyHC) expression, in addition to containing slow-tonic MyHC, an isoform unique to spindles in the limb and trunk musculature (Pedrosa and Thornell, 1990; Pedrosa-Domellof et al., 1991; Walro and Kucera, 1999). Myf5 therefore may be required to maintain this unique combination of developmental and adult muscle protein isoforms. Indeed, the higher *Myf5* expression at the equatorial or juxtaequatorial regions is reflected in the regionalised

distribution of certain MyHC isoforms (Pedrosa and Thornell, 1990; Pedrosa-Domellof et al., 1991; Walro and Kucera, 1999).

nlacZ is expressed in myonuclei of extrafusal myofibres of *Myf5*$^{nlacZ/+}$ mice during the perinatal period (Tajbakhsh et al., 1996a), but not in the adult. Here we show that following muscle denervation, the *Myf5* locus is reactivated in myonuclei. Previous studies of whole muscle extracts have shown that *Myf5* transcript is elevated in denervated muscle (Buonanno et al., 1992; Duclert et al., 1991). Our data reveal the localisation and extent of this response and show that the vast majority of myofibres reactivate *Myf5*. Importantly, in most fibres, all myonuclei respond and not just those located at or near the motor end plate. Therefore, Myf5, together with MyoD and myogenin (Weis, 1994) are all reinduced in myonuclei following denervation. Interestingly, denervation accompanied by electrical stimulation reduces the accumulation of MRF transcripts (Buonanno et al., 1992). The expression of these myogenic transcription factors is probably involved in the reactivation or redistribution of downstream muscle genes in response to denervation. For example, the acetylcholine receptor (Schuetze and Role, 1987) and N-CAM (Covault and Sanes, 1985) are no longer restricted to the vicinity of the endplate but spread over the surface of a denervated myofibre. Indeed, MRF directly regulates acetylcholine receptor subunit gene expression (Piette et al., 1990). Interestingly, although myonuclei are differentiated and postmitotic, they are still able to traverse the G0/G1 boundary and serum stimulation initiates a typical immediate early response before arrest in G1 (Tiainen et al., 1996). As *Myf5* is cell cycle regulated (Kitzmann et al., 1998), its reactivation in myonuclei could also be a consequence of this aborted cell cycle response.

It has been suggested that *nlacZ* expression in quiescent satellite cells could be a result of the specific *Myf5*$^{nlacZ/+}$ allele (Chargé and Rudnicki, 2004). We have now shown that BAC140 is also able to drive consistent *nlacZ* expression in this cell population. Crucially, this construct does not contain a constitutively active *neo* selection gene, which can occasionally cause aberrant expression of the targeted allele (e.g., Fiering et al., 1995) or of neighbouring genes (Olson et al., 1996). Furthermore, since the reporter gene was targeted to the translational start point of *Myf5*, no Myf5 protein sequences are fused to β-galactosidase. Expression in quiescent satellite cells of *Myf5*$^{nlacZ/+}$ mice is therefore directed by sequences also present in BAC140. These observations indicate that the *Myf5* gene is active in satellite cells, but that the protein is probably below the level of detection for localisation using currently available antibodies (discussed by Zammit et al., 2002). However, Myf5 protein has been detected in uninjured muscle (Sakuma et al., 1999). The function of Myf5 in satellite cells is probably related to the maintenance of their undifferentiated, but myogenic, status: Without Myf5, muscle progenitors can adopt nonmyogenic fates (Tajbakhsh et al., 1996b), although the presence of Myf5 does not initiate differentiation in the absence of MyoD, myogenin and Mrf4 (Valdez et al., 2000). Additionally, high Myf5 protein levels are associated with arrested primary myoblasts and G0 in the myogenic cell line C2 in vitro (Kitzmann et al., 1998). It is also possible that the *Myf5* locus is maintained in a transcriptionally active state but that no Myf5 protein is produced. In response to activating cues, the locus will then be able to respond rapidly to provide Myf5 protein.

The elements of the *Mrf4/Myf5* locus responsible for the activation and maintenance of *Myf5* expression during embryogenesis are being defined (Buchberger et al., 2003; Carvajal et al., 2001; Hadchouel et al., 2000, 2003; Patapoutian et al., 1993; Summerbell et al., 2000; Teboul et al., 2002; Zweigerdt et al., 1997). A BAC containing 140 kb upstream of the *Myf5* transcription start site contains all the elements necessary to control the expression of *Myf5* during development (Carvajal et al., 2001). Here we show that this BAC also contains the elements required to regulate *Myf5* expression in adult muscle: namely, it drives expression in muscle spindles, quiescent satellite cells, satellite cell-derived myoblasts and controls myonuclear reactivation.

Using a panel of transgenic mice carrying defined regions of the *Mrf4/Myf5* locus, we have mapped the sequences responsible for the control of *Myf5* expression in muscle spindles to the −59 to −8.8 kb interval. Nevertheless, we cannot discount the possible existence of enhancer elements driving this expression situated downstream of *Myf5* in the +5.4 to +40.6 kb interval. A number of regulatory sequences driving embryonic expression have been localised to the −59 to −8.8 kb interval although BAC59 fails to maintain axial expression of *Myf5* from 12.5 dpc (Carvajal et al., 2001). Since we have observed muscle spindles both in limb and axial musculature, this would suggest that the elements driving this aspect of adult expression are independent from those driving the embryonic pattern. It is thought that spindles persist in an "immature" state (e.g., maintenance of developmental MyHC isoforms) under the influence of factors derived from afferent innervation. Since innervation of skeletal muscle is a relatively late occurrence in development (15.5–16.5 dpc), and no elements within BAC59 are active in the axial musculature at this stage, it is likely that *Myf5* expression is controlled by a separate circuit that operates in muscle spindles at later developmental stages and maintains this expression into the adult, contributing to the "immature" status of these structures. Unraveling the signaling pathways that maintain *Myf5* expression in these very specialised structures will provide a further insight into their complex biology. In addition, these transgenic mouse lines provide an excellent model in which the visualisation of muscle spindles is straightforward, even in whole muscles, and allows their distribution or morphology to be easily studied.

Sequences within BAC59 and BAC88 are able to drive *Myf5* expression in very limited numbers of satellite cells. It

is known that at any time a small percentage of satellite cells in mature muscle are cycling (Schmalbruch and Lewis, 2000). It is thus possible that the low numbers of β-galactosidase-positive cells detected in BAC88 and BAC59 correspond to this active cell population. The addition of further upstream sequences results in widespread expression, suggesting that a second element is required to fully recapitulate the endogenous expression pattern of the *Myf5* gene in satellite cells. These therefore may not only mark those cells actively dividing, but also truly quiescent cells. This second element, located in the −140 to −88 kb interval, is also responsible for driving expression in satellite cell-derived myoblasts. Therefore, there may be an element required to maintain the locus in a transcriptionally active state and thus able to respond to external cues in order activate the myogenic program in newly formed myoblasts.

While this is the first study of the regions of the *Mrf4/Myf5* locus responsible for directing *Myf5* expression in adult muscle, previous studies have defined areas essential for correct developmental expression. It has been shown that *Myf5* expression is regulated in a modular fashion, with different enhancers driving expression in specific cell precursor populations. Some enhancers, such as the early epaxial enhancer, seem to operate individually (Teboul et al., 2002) while the expression pattern at particular anatomical locations, such as the limb, require the concerted action of more than one of these elements (Hadchouel et al., 2003). This modular regulation at the anatomical level is further complicated by the existence of additional modules controlling temporal expression. Indeed, deletion of the axial maintenance element (−88 to −63 kb) results in down−regulation of *Myf5* expression in axial structures (Carvajal et al., 2001), while deletion of the limb element abolishes early but not late *Myf5* appendicular expression (Hadchouel et al., 2003). This shows that initiation of *Myf5* expression can be driven by enhancers separate from those required for maintenance or later induction of expression in the same anatomical structures. Our data show that the regulation of *Myf5* in satellite cells and their derivatives also seems to be modular. The differences in numbers of *nlacZ* expressing cells observed between BAC140 and the smaller BACs could be the result of the difference between induction and maintenance, although whether the enhancers controlling this expression coincide with those involved in embryonic regulation remain to be determined.

Finally, what controls the reactivation of the *Myf5* locus in the myonuclei of myofibres during culture, and presumably after denervation? BAC140 (2/3) and BAC59 (3/4) drive consistent induction of *nlacZ* in the myonuclei, while BAC88 (2/2) did not. This suggests that there is a positive element within BAC59 and a negative element present between −88 and −59 kb, the activity of which is controlled by a further positive element in the −140 to −88 kb interval. It is also possible that this expression is regulated by a single far downstream enhancer as BAC88 extends to +40.6 kb while BAC140 and BAC59 extend to +46 and +50 kb, respectively, and thus this element could be located within this interval.

In summary, we have shown that multiple elements are required to regulate *Myf5* in adult skeletal muscle, and that separate elements are active in different cell populations, similar to the developmental control of *Myf5* expression. Additionally, different circuits may operate at adult and embryonic stages. Further experiments will be used to subdivide and further characterise these elements and should provide the first steps to unravel the signaling pathways involved in the specification, maintenance and activation of muscle satellite cells.

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