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Muscle Fiber Type Specific Activation of the Slow Myosin Heavy Chain 2 Promoter by a Non-canonical E-box

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Abstract

Different mechanisms control skeletal muscle fiber type gene expression at specific times in vertebrate development. Embryonic myogenesis leading to formation of primary muscle fibers in avian species is largely directed by myoblast cell commitment to the formation of diverse fiber types. In contrast, development of different secondary fiber types during fetal myogenesis is partly determined by neural influences. In both primary and secondary chicken muscle fibers, differential expression of the slow myosin heavy chain 2 (MyHC2) gene distinguishes fast from fast/slow muscle fibers. This study focused on the transcriptional regulation of the slow MyHC2 gene in primary myotubes formed from distinct fast/slow and fast myogenic cell lineages. Promoter deletion analyses identified a discrete 86bp promoter segment that conferred fiber type, lineage-specific gene expression in fast/slow versus fast myoblast derived primary myotubes. Sequence analysis and promoter activity assays determined that this segment contains two functional cis-regulatory elements. One element is a non-canonical E-box, and electromobility shift assays demonstrated that both cis-elements interacted with the E-protein, E47. The results indicate that primary muscle fiber type specific expression of the slow MyHC2 gene is controlled by a novel mechanism involving a transcriptional complex that includes E47 at a non-canonical E-box.

Keywords

Lineage; Myoblast; Embryonic; Myogenesis; Gene Regulation

Introduction

Skeletal muscles are comprised of muscle fibers with different patterns of gene expression that contribute to variation in contractile dynamics and metabolic characteristics. In general, muscle fiber types are categorized as fast or slow, depending on the expression of contractile protein genes, such as myosin heavy chain (MyHC) genes, that significantly determine muscle fiber function in the adult and during development [1,2]. Species differences exist with respect to muscle fiber type nomenclature, reflecting differences in MyHC gene

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expression. Whereas mammalian species typically have fast type IIa, IIb, and IIx and slow type I muscle fibers, avian skeletal muscle is comprised of fast and fast/slow fibers. The distinction between fast and fast/slow fibers in avian species is based principally upon regulated expression of the slow MyHC2 gene in fast/slow versus fast muscle fibers [3].

The development of these different muscle fiber types occurs across several stages of myogenesis [4]. The embryonic stage of myogenesis is characterized by the proliferation of embryonic myoblasts and their subsequent differentiation into primary muscle fibers of both epaxial and hypaxial musculature. In chicken development, embryonic myogenesis ceases at approximately embryonic day 8 (ED8). Fetal myogenesis then ensues. This latter stage of myogenesis is characterized by expansive proliferation of fetal myoblasts and their differentiation into secondary muscle fibers that constitute the majority of skeletal muscle mass. Finally, adult myogenesis occurs through satellite cell activation, proliferation, and differentiation in response to muscle degeneration or injury.

The diversity of fast and fast/slow muscle fiber types is evident at each stage of avian myogenesis. Primary muscle fibers, formed from embryonic myoblasts, are either fast or fast/slow from the time of their initial formation, both in vitro and in vivo [5,6]. The embryonic myoblasts that form fast and fast/slow primary muscle fibers are distinct in their lineal commitment to the formation of specific fiber types. Clonal populations of embryonic myoblasts consistently and stably formed either fast or fast/slow primary muscle fibers in vitro and in vivo [7,8]. This myoblast type-specific formation of different muscle fiber types is independent of functional innervation [9]. The intrinsic commitment of embryonic myoblasts to the formation of distinct muscle fiber types is a dominant feature of embryonic myogenesis.

In contrast to primary myogenesis, differentiation of distinct secondary muscle fiber types from fetal myoblasts is dependent on both cell lineage commitment and innervation [10]. Denervation and cross-reinnervation of developing and adult muscles typically leads to muscle fiber type transitions [11,12]. However, in both mammals and birds, not all muscle fiber types respond to altered innervation equally with fibers retaining diversity in MyHC gene expression. Some muscles in both mammals and birds are refractory to innervation-dependent muscle fiber type transitions [13]. For example, fetal avian myotubes in vitro do not express the slow MyHC2 gene. Innervation of these myotubes induced slow MyHC2 gene expression. However, the innervation-induced fiber type transition was also dependent on the origin of the myoblasts that formed the myotubes. Myoblasts from the fetal medial adductor muscle, a fast/slow muscle, formed myotubes that expressed the slow MyHC2 gene in response to innervation or electrical stimulation. Myoblasts from the exclusively fast fetal pectoralis muscle formed myotubes that did not express the slow MyHC2 gene when innervated or electrically stimulated at low, tonic stimulation frequencies [14,15].

Reflecting the cellular differences in the regulation of muscle fiber type between embryonic and fetal stages of myogenesis and between fiber types themselves, the mechanisms that control slow MyHC2 gene transcription differ at embryonic and fetal stages of myogenesis and within different fiber types. The proximal 1358bp of DNA upstream from exon 1 of the slow MyHC2 gene control gene expression in fetal fast and fast/slow muscle fibers [16].

Within this transcriptional promoter region, functional E-boxes, a Myocyte Enhancer Factor 2 (MEF2) binding site, and a Nuclear Factor of Activated T Cells (NFAT) binding site regulate slow MyHC2 gene transcription in fast/slow medial adductor muscle fibers. NFAT-mediated transcriptional activation was found to be dependent on innervation. In contrast, slow MyHC2 gene expression in embryonic fast/slow muscle fibers was independent of innervation and NFAT activity [17]. The proximal 1358bp of the slow MyHC2 promoter was not sufficient to direct fiber type specific transcription in embryonic fast/slow versus fast muscle fibers.

The basic helix-loop-helix (bHLH) family of myogenic regulatory factors (MRFs) is required throughout myogenesis. In general, MyoD and Myf5 direct myogenic specification, and myogenin and MRF4 control myogenic differentiation [18–20]. Each of the MRFs bind to E boxes (CANNTG) in transcriptional control regions as heterodimers with Class I bHLH E proteins, including the E2A gene products, E12 and E47, HEB, daughterless, and E2-2 [21–23]. Studies in vitro and in vivo have indicated that the myogenic cell specific MRFs avidly heterodimerize with the more ubiquitously expressed E proteins and that MRF homodimerization was weak in the presence of the E box consensus binding site [22,24]. Binding site specificities of MRF:E protein heterodimers are also partly determined by nucleotide sequence within the E box consensus sequence. For example, MyoD and myogenin preferentially recognize the E box sequence CAGCTG [25]. Sequences flanking the consensus CANNTG binding site also contribute to MRF:E protein binding specificities [24]. Furthermore, interaction of specific MRFs with non-canonical E box sequences in the myogenin promoter has indicated greater diversity in MRF:E protein binding potential in the regulation of myogenesis [26].

In the present study, we investigated the transcriptional regulation of the slow MyHC2 gene in embryonic fast/slow versus fast myotubes formed from clonally derived embryonic myoblasts. Analysis of the slow MyHC2 promoter revealed that a specific region of 86bp in length controlled promoter activity in fast/slow versus fast myotubes. Furthermore, results indicate that a non-canonical E box sequence within this promoter segment regulates promoter activity and interacts with the E protein E47.

Materials and Methods

Cell Culture, DNA Transfection, and Promoter Activity Assays

Embryonic myoblasts were derived from ED4 limbs and cultured as previously described [8,17]. After 24 hrs, myoblasts were transfected with promoter-reporter DNA constructs using Lipofectamine 2000 (Invitrogen). The 6150SM2Luc DNA construct was previously described [17]. Transfections consisted of 3 μ g slow MyHC2 promoter-luciferase or promoterless-luciferase (pGL3Basic; Promega) DNA constructs plus 2 μ g pRLSV40 DNA (Promega). Cells were transfected for 5 hrs in medium without antibiotics at 37°C. Luciferase activities were measured 5 days after transfection. Firefly luciferase activities from slow MyHC2 promoter activities were normalized relative to constitutive expression of the Renilla luciferase gene from pRLSV40. Student's t-test was applied to determine statistical differences among mean luciferase activities. P values ≤ 0.05 were considered statistically significant.

Mutagenesis

Site-directed mutations were introduced into slow MyHC2 promoter-reporter DNA constructs by PCR amplification of template DNA using complementary mutagenic primers. Each reaction consisted of 200ng template DNA, 5ul 10X Pfu polymerase buffer, 100ng of each PCR primer, 2.5mM of each dNTP, 1ul PfuUltra High Fidelity DNA polymerase (Stratagene) in a total volume of 50ul. After amplification, 1ul DpnI was added and incubated with the reaction for 1hr at 37°C before bacterial transformation. DNA sequencing verified the generation of each mutation.

Electromobility Shift Assays

Nuclear cell extracts from differentiated cultures of fast and fast/slow myotubes were prepared as previously described [27]. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. The electromobility shift assay (EMSA) protocol was performed as previously described [28]. For EMSA supershift assays, 4ug E47 antibody (Santa Cruz) was added to the reaction prior to addition of the labeled probe and incubated on ice for 30 minutes. A 5% non-denaturing polyacrylamide gel, run at 200V for 90 minutes, resolved the protein-DNA complexes. The gel was dried and exposed to X-ray film at -80°C overnight before development.

Results

Localization of positive transcriptional control region

Previous studies determined that the 1358bp of slow MyHC2 DNA sequence positioned upstream from exon 1 was sufficient to direct slow MyHC2 promoter activity in innervated and stimulated secondary myotubes derived from fetal myoblasts [15,16]. However, it was insufficient to direct transcription in a fiber type specific manner in primary myotubes formed from embryonic myoblasts [17]. To determine the location of slow MyHC2 promoter regions that regulate gene expression in fast/slow versus fast primary myotubes, clonal populations of myoblasts, stably committed to the formation of fast/slow and fast myotubes [8,17], were transfected with a series of DNA constructs containing increasing lengths of slow MyHC2 promoter sequence coupled to the luciferase reporter gene. Transfected myoblasts were allowed to differentiate for 5 days, and slow MyHC2 promoter activities were measured and compared to the activity of the full length 6150SM2Luc construct (Figure 1). No significant difference in slow MyHC2 promoter activities was detected in fast/slow and fast myotubes transfected with 2279SM2Luc. Similarly, no differences of activities were detected with 2710SM2Luc. Promoter activities from both 2953SM2Luc and 2875SM2Luc approximated that of 6150SM2Luc. The 2796SM2Luc promoter-reporter construct demonstrated the most significant changes in promoter activity. Relative to 6150SM2Luc, its promoter activity was increased by 71%, suggesting that a negative regulatory element resides between 2875bp and 2796bp in the respective constructs. Importantly, a 91.7% reduction in slow MyHC2 promoter activity was detected by omission of the 86bp between 2796SM2Luc and 2710SM2Luc in fast/slow myotubes. All slow MyHC2 promoter-reporter DNA constructs displayed minimal promoter activities in fast primary myotubes. These results indicated that a positive transcriptional element that

directs fiber type specific expression of the slow MyHC2 gene is located between 2796bp and 2710bp within the respective DNA constructs.

Protein-DNA complex formation at the regulatory region

Experiments were performed to determine the location(s) within the 86bp segment of the slow MyHC2 promoter between 2796bp and 2710bp that interacts with components in protein nuclear extracts. Four doublestranded oligonucleotides (probes 1–4), each 28bp in length with 10bp overlaps, were synthesized. These oligonucleotides spanned 80 of the 86bp between positions 2796 and 2710 (Supplement Figure 1). Each probe was incubated with nuclear extracts from fast/slow and fast primary myotubes, and protein-DNA complexes were resolved in non-denaturing polyacrylamide gels (Figure 2). Probes 1 and 4 formed protein-DNA complexes with components of nuclear extract from fast/slow myotubes. These complexes migrated in the gel with different relative mobilities, suggesting that the protein component(s) within the complexes formed with probes 1 and 4 were different. Probes 2 and 3 did not form detectable complexes with the same extract. No protein-DNA complexes were detected with any of the four probes incubated with nuclear extract from fast primary myotubes.

To more narrowly define the interactions of Probes 1 and 4 with the nuclear extract proteins, the electromobility shift assays were repeated using oligonucleotides with mutated sequences as potential DNA competitors of protein-DNA complex formation with the respective wild type oligonucleotide probe (Supplement Figure 2). Formation of the protein-DNA complexes with Probe 1 was effectively competed by the wild type oligonucleotide and mutant oligonucleotides 3, 4, and 5 (Figure 3A). Mutant oligonucleotides 1 and 2 demonstrated minimal competition with Probe 1. Similarly, two mutant oligonucleotides did not compete with Probe 4 for complex formation. Mutant oligonucleotides 3 and 4 did not reduce complex formation, whereas mutant oligonucleotides 1, 2, and 5 effectively reduced complex formation. These results established discrete binding sites within the slow MyHC2 promoter sequence for nuclear factors in fast/slow myotubes.

Sequence analysis identified candidate transcription factor binding sites within the 86bp segment of the slow MyHC2 promoter. Particular focus was placed on DNA sequence within Probes 1 and 4 involved in the formation of protein-DNA complexes with the fast/slow primary myotube nuclear extract. A non-canonical E-protein binding site (GCCAGCTCC) was identified in Probe 1 DNA sequence. This sequence overlapped the mutated sequences within mutated oligonucleotides 1 and 2 (Mut1 and Mut2) of Probe 1 that did not effectively compete for formation of the protein-DNA complexes shown in Figure 3A. Probe 4 DNA sequence contained candidate binding sites for Pax6 (GTGAC) and STAT4 (ATTTCC). These candidate binding sites did not overlap with the mutated sequences within mutated oligonucleotides Mut 3 and Mut4 of Probe 4 that did not effectively compete with wild type Probe 4 in Figure 4.

To identify the nuclear factor that interacted with Probes 1 and 4 within the 86bp slow MyHC2 promoter segment, electromobility shift experiments were repeated with an antibody directed against the E-protein, E47. As shown in Figure 3B, addition of the E47 antibody resulted in a further shift of the protein-DNA complexes formed between the

nuclear extract and Probe 1. Interestingly, the E47 antibody also caused a further shift of the protein DNA complexes formed with Probe 4. This interaction was not predicted since Probe 4 sequence did not contain an identified candidate E-protein binding site.

Functional Analysis of E47 Binding Site

Slow MyHC2 promoter-reporter DNA constructs containing Probe 1 Mut1 sequence and Probe 4 Mut3 sequence were individually transfected into fast/slow myogenic cell cultures to assess the function of the E47 binding site in directing slow MyHC2 gene transcription (Figure 4). Activity of the slow MyHC2 promoter with Probe 1 Mut1 sequence was reduced to $45.7 \pm 8.3\%$ ($p < 0.01$) compared to wild type promoter activity. Mutation of the sequence within Probe 4 caused a reduction of promoter activity to $58.8 \pm 10.7\%$ ($p < 0.01$) compared to the wild type promoter. These results indicate that both identified sites within the 86bp segment of the slow MyHC2 promoter are functional and act as positive transcriptional regulatory sites in fast/slow primary muscle fibers.

Discussion

Understanding of the cellular and molecular mechanisms that control skeletal muscle fiber type has progressed significantly. Much of this advancement in our knowledge has focused on transcriptional regulators of genes differentially expressed in specific fiber types. The transcriptional regulators Six1 and Eya1 activate the aldolase A promoter in fast twitch glycolytic muscle fibers and partly induce a slow-to-fast fiber type transition in slow muscle fibers that overexpress Six1/Eya1 [29]. Conversely the fast muscle fiber phenotype is controlled by suppression of the slow muscle fiber phenotype through the transcriptional repressor Sox6 [30]. Together with the transcription factor Prdm1 in zebrafish, Sox6 expression causes a slow-to-fast fiber type transition as well [31]. Perhaps in balance with these transcription factors that augment the fast muscle fiber phenotype, the calcineurin/NFAT pathway can promote the slow muscle fiber phenotype. The calcium responsive phosphatase calcineurin controls nuclear import of NFAT which in turn can function as a transcriptional activator of the slow myogenic program [32–34]. Importantly, these studies have principally focused on fetal, postnatal, and adult muscle fibers.

Much less is known regarding the mechanisms that establish initial diversity in primary muscle fibers. Previous studies using clonally derived fast and fast/slow myogenic cells indicated that the slow MyHC2 promoter was regulated quite differently in embryonic versus fetal slow muscle fibers. In fetal muscle fibers, the slow MyHC2 promoter was activated via an NFAT binding site in response to innervation and electrical stimulation [15,16]. In contrast, NFATc1 repressed slow MyHC2 promoter activity in embryonic fast/slow myotubes [17]. This NFAT binding site is located within the proximal slow MyHC2 promoter sequence that is sufficient to drive fiber type specific gene expression in fetal, secondary muscle fibers, but is not sufficient to do so in embryonic primary muscle fibers. Therefore, the mechanisms of transcriptional regulation of the slow MyHC2 gene and muscle fiber type are different between primary and secondary muscle fibers.

This study was initiated to identify a site within the slow MyHC2 promoter that controls promoter activity in fast/slow primary muscle fibers. Since the promoter sequence contained

within the 2279SM2Luc promoter-reporter construct was not sufficient to direct fiber type specific gene expression in these primary muscle fibers, the experimental approach focused on more upstream promoter sequence contained within 6150SM2Luc. Deletion analysis identified an 86bp segment of the slow MyHC2 promoter that conferred significant promoter activity in fast/slow versus fast primary myotubes in vitro. This segment of the promoter contained two sites that interacted with a component(s) within nuclear extracts from fast/slow myotubes, but not fast myotubes. Mutation of these sites significantly reduced promoter activity indicating that both sites were functional in fast/slow myotubes. Sequence analysis identified a candidate E-protein binding site within one of the two DNA elements that formed protein-DNA complexes with fast/slow myotube nuclear extract. A protein component within the protein-DNA complex at this binding site was shown to be E47. Interestingly, E47 also interacted with the second element within the 86bp promoter segment.

The protein binding site located within Probe 1 DNA sequence of the 86bp slow MyHC2 promoter segment contains a non-canonical E-box. This site interacts with E47. Non-canonical E-box sequences have been located in other myogenic genes. The myogenin gene contains a non-canonical E-box that participates in myogenic specification and therefore downstream myogenic differentiation [18]. This site appears to be a target for MyoD binding and is potentially a focal point within the myogenin locus for MyoD-mediated recruitment of SWI/SNF chromatin remodeling enzymes and subsequent gene activation. The capacity of MyoD to interact with the non-canonical E-box in the myogenin promoter is dependent upon a neighboring cis-regulatory site with which the homeodomain-containing factors Pbx1/Meis1 interact [18,26]. MyoD interacted with this transcriptional complex as a heterodimer with E12.

Several aspects of the regulatory mechanisms that control the myogenin promoter and the slow MyHC2 promoter are similar. Both transcriptional regulatory regions contain functional, non-canonical E-boxes. In the myogenin promoter, the non-canonical E-boxes overlap in the CAACAGCTT sequence [18]. The slow MyHC2 promoter appears to have a single non-canonical site, GCCAGCTCC. In both promoters, the E-box is closely flanked by a non-E-box regulatory element. Nonetheless, this neighboring element forms a transcriptional complex that includes an E-protein, E12 at the myogenin locus and E47 at the slow MyHC2 promoter site. The protein complex at the non-canonical E-box and Pbx1/Meis1 binding site in the myogenin promoter appears to mark the locus for chromatin remodeling and subsequent transcriptional activation. Further studies are needed to determine whether the non-canonical E-box and the neighboring cis-element in the slow MyHC2 promoter also mark it for a similar mode of transcriptional regulation of fiber type specific genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Reiser PJ, Moss RL, Giulian GG, Greaser ML. Shortening velocity in single fibers from adult rabbit soleus muscles is correlated with myosin heavy chain composition. *J Biol Chem.* 1985; 260:9077–9080. [PubMed: 4019463]
2. Reiser PJ, Greaser ML, Moss RL. Myosin heavy chain composition of single cells from avian slow skeletal muscle is strongly correlated with velocity of shortening during development. *Dev Biol.* 1988; 129:400–407. [PubMed: 3417046]
3. Reid SK, Kennedy JM, Shimizu N, Stewart A, Vrbova G, Zak R. Regulation of expression of avian slow myosin heavy-chain isoforms. *Biochem J.* 1989; 260:449–454. [PubMed: 2764882]
4. Stockdale FE. Mechanisms of formation of muscle fiber types. *Cell Struct Funct.* 1997; 22:37–43. [PubMed: 9113388]
5. Crow MT, Stockdale FE. Myosin expression and specialization among the earliest muscle fibers of the developing avian limb. *Dev Biol.* 1986; 113:238–254. [PubMed: 3943663]
6. Miller JB, Stockdale FE. Developmental origins of skeletal muscle fibers: clonal analysis of myogenic cell lineages based on expression of fast and slow myosin heavy chains. *Proc Natl Acad Sci.* 1986; 83:3860–3864. [PubMed: 3520558]
7. Miller JB, Stockdale FE. Developmental regulation of multiple myogenic cell lineages of the avian embryo. *J Cell Biol.* 1986; 103:2197–2208. [PubMed: 3782296]
8. DiMario JX, Fernyak SE, Stockdale FE. Myoblasts transferred to the limbs of embryos are committed to specific fibre fates. *Nature.* 1993; 362:165–167. [PubMed: 8383807]
9. Stockdale FE. The myogenic lineage: evidence for multiple cellular precursors during avian limb development. *Proc Soc Exp Biol Med.* 1990; 194:71–75. [PubMed: 2190235]
10. Wigmore PM, Evans DJ. Molecular and cellular mechanisms involved in the generation of fiber diversity during myogenesis. *Int Rev Cytol.* 2002; 216:175–232. [PubMed: 12049208]
11. McLennan IS. Neural dependence and independence of myotube production in chicken hindlimb muscles. *Dev Biol.* 1983; 98:287–294. [PubMed: 6873458]
12. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. *Physiol Rev.* 2011; 91:1447–1531. [PubMed: 22013216]
13. Condon K, Silberstein L, Blau HM, Thompson WE. Differentiation of fiber types in aneural musculature of the prenatal rat hindlimb. *Dev Biol.* 1990; 138:275–295. [PubMed: 2318339]
14. DiMario JX, Stockdale FE. Both myoblast lineage and innervation determine fiber type and are required for expression of the slow myosin heavy chain 2 gene. *Dev Biol.* 1997; 188:167–180. [PubMed: 9245520]
15. Crew JR, Falzari K, DiMario JX. Muscle fiber type specific induction of slow myosin heavy chain 2 gene expression by electrical stimulation. *Exp Cell Res.* 2010; 316:1039–1049. [PubMed: 20070941]
16. Jiang H, Jordan T, Li J, Li H, DiMario JX. Innervation-dependent and fiber type-specific transcriptional regulation of the slow myosin heavy chain 2 promoter in avian skeletal muscle fibers. *Dev Dyn.* 2004; 231:292–302. [PubMed: 15366006]
17. Theobald J, DiMario JX. Lineage-based primary muscle fiber type diversification independent of MEF2 and NFAT in chick embryos. *J Muscle Res Cell Motil.* 2011; 31:369–381. [PubMed: 21290171]
18. Berkes CA, Tapscott SJ. MyoD and the transcriptional control of myogenesis. *Semin Cell Dev Biol.* 2005; 16:585–595. [PubMed: 16099183]
19. Buckingham M. Skeletal muscle formation in vertebrates. *Curr Opin Genet Dev.* 2001; 11:440–448. [PubMed: 11448631]
20. Sabourin LA, Rudnicki MA. The molecular regulation of myogenesis. *Clin Genet.* 2000; 57:16–25. [PubMed: 10733231]
21. Davis RL, Cheng PF, Lassar AB, Weintraub H. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell.* 1990; 60:733–746. [PubMed: 2155707]

22. Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell*. 1991; 66:305–315. [PubMed: 1649701]
23. Weintraub H, Dwarki VJ, Verma I, Davis R, Hollenberg S, Snider L, Lassar A, Tapscott SJ. Muscle-specific transcriptional activation by MyoD. *Genes Dev*. 1991; 5:1377–1386. [PubMed: 1651276]
24. Blackwell TK, Weintraub H. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by DNA site selection. *Science*. 1990; 250:1104–1110. [PubMed: 2174572]
25. Cao Y, Yao Z, Sarkar D, Lawrence M, Sanchez GJ, Parker MH, MacQuarrie KL, Davison J, Morgan MT, Ruzzo WL, Gentleman RC, Tapscott SJ. Genome-wide MyoD binding in skeletal muscle cells: a potential for broad cellular reprogramming. *Dev Cell*. 2010; 18:662–674. [PubMed: 20412780]
26. Heidt AB, Rojas A, Harris IS, Black BL. Determinants of myogenic specificity within MyoD are required for noncanonical E box binding. *Mol Cell Biol*. 2007; 27:5910–5920. [PubMed: 17562853]
27. Dignam JD, Martin PL, Shastry BS, Roeder RG. Eukaryotic gene transcription with purified components. *Meth Enzymol*. 1983; 101:582–598. [PubMed: 6888276]
28. Hellman LM, Fried MG. Electromobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat Protoc*. 2007; 2:1849–1861. [PubMed: 17703195]
29. Grifone R, Laclef C, Spitz F, Lopez S, Demignon J, Guidotti JE, Kawakami K, Xu PX, Kelly R, Petrof BJ, Daegelen D, Concordet JP, Maire P. Six1 and Eya1 expression can reprogram adult muscle from the slow-twitch phenotype into the fast-twitch phenotype. *Mol Cell Biol*. 2004; 24:6253–6267. [PubMed: 15226428]
30. Hagiwara N, Ma B, Ly A. Slow and fast fiber isoform gene expression is systematically altered in skeletal muscle of the Sox6 mutant, p100H. *Dev Dyn*. 2005; 234:301–311. [PubMed: 16124007]
31. Jackson HE, Ono Y, Wang X, Elworthy S, Cunliffe VT, Ingham PW. The role of Sox6 in zebrafish muscle fiber type specification. *Skelet Muscle*. 2015; 5:2. [PubMed: 25671076]
32. Calabria E, Ciciliot S, Moretti I, Garcia M, Picard M, Dyar KA, Pallafacchina G, Tothova J, Schiaffino S, Murgia M. NFAT isoforms control activity-dependent muscle fiber type specification. *Proc Natl Acad Sci*. 2009; 106:13335–13340. [PubMed: 19633193]
33. Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, Williams RS. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev*. 1998; 12:2499–2509. [PubMed: 9716403]
34. Naya FJ, Mercer B, Shelton J, Richardson JA, Williams RS, Olson EN. Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *J Biol Chem*. 2000; 275:4545–4548. [PubMed: 10671477]

Figure 1.

Detailed deletion analysis of the slow MyHC2 promoter. Slow MyHC2 promoter-reporter DNA constructs containing 2279bp, 2710bp, 2796bp, 2875bp, 2953bp, and 6150bp of slow MyHC2 DNA upstream from the firefly luciferase gene were transfected into fast/slow (black bars) and fast (gray bars) myoblasts. The promoterless pGL3Basic negative control vector and pSV40RL positive control vector were also transfected. After 5 days, luciferase activities were measured and normalized to activity from the full-length 6150SM2Luc construct in fast/slow myotubes. Bars represent the means of promoter activities $\pm$ standard error of the mean (error bars). Significant differences in means was determined by Student's two tailed t-test with $p = 0.05$, $n=5$.

Figure 2. Electromobility shift analysis. Nuclear extracts (N.E.) from fast/slow myotubes (left panel) and fast myotubes (right panel) were incubated with oligonucleotide probes 1–4 and resolved in non-denaturing polyacrylamide gels.

Figure 3. Competitive electromobility shift analysis. A. Nuclear extract (N.E.) from fast/slow primary myotubes was incubated with radiolabelled Probe 1 (left panel) or Probe 4 (right panel) and 200 molar excess of unlabelled wild type (WT) or mutated (Mut1, etc) oligonucleotides. Protein-DNA complexes were resolved in non-denaturing polyacrylamide gels. B. Nuclear extract (N.E.) from fast/slow primary myotubes was incubated with Probe 1 and Probe 4. Some of the reactions contained either non-specific antibody (N.S. Ab) or E47 antibody. Protein-DNA complexes were resolved in non-denaturing polyacrylamide gels.

Figure 4. Functional analysis of E47 binding sites. Slow MyHC2 promoter-reporter DNA constructs harboring mutated sequences of Probe 1 Mut1 and Probe 4 Mut3 were transfected into fast/slow embryonic myoblasts. After 5 days, luciferase activities were measured and normalized to activity from the full-length 6150SM2Luc construct in fast/slow myotubes. Bars represent the means of promoter activities $\pm$ standard error of the mean (error bars). Significant differences in means was determined by Student's two tailed t-test with $p < 0.05$, $n=5$.