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Foxj3 transcriptionally activates Mef2c and regulates adult skeletal muscle fiber type identity

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Abstract

The mechanisms that regulate skeletal muscle differentiation, fiber type diversity and muscle regeneration are incompletely defined. *Forkhead* transcription factors are critical regulators of cellular fate determination, proliferation, and differentiation. We identified a *forkhead*/winged helix transcription factor, Foxj3, which was expressed in embryonic and adult skeletal muscle. To define the functional role of Foxj3, we examined Foxj3 mutant mice. Foxj3 mutant mice are viable but have significantly fewer Type I slow-twitch myofibers and have impaired skeletal muscle contractile function compared to their wild type controls. In response to a severe injury, Foxj3 mutant mice have impaired muscle regeneration. Foxj3 mutant myogenic progenitor cells have perturbed cell cycle kinetics and decreased expression of Mef2c. Examination of the skeletal muscle 5′ upstream enhancer of the Mef2c gene revealed an evolutionary conserved *forkhead* binding site (FBS). Transcriptional assays in C2C12 myoblasts revealed that Foxj3 transcriptionally activates the Mef2c gene in a dose dependent fashion and binds to the conserved FBS. Together, these studies support the hypothesis that Foxj3 is an important regulator of myofiber identity and muscle regeneration through the transcriptional activation of the Mef2c gene.

Keywords

forkhead; Foxj3; Myocyte enhancer factor; Mef2c; Muscle fiber type; Muscle regeneration; Myogenesis; Gene disruption technologies

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Conflicts of interest

The authors declare no conflicts of interests.

Introduction

The molecular networks that govern muscle differentiation, fiber diversity and muscle regeneration remain incompletely defined. Previous studies have uncovered an essential role for the basic helix loop helix (bHLH) MyoD family members (i.e., MyoD, myf5, MRF4 and myogenin) and myocyte enhancer factor 2 (Mef2) factors, but the repertoire of interacting factors and upstream regulators that coordinately regulate skeletal muscle differentiation and fiber type specification remain an area of intense interest. Studies have demonstrated that the bHLH MyoD factors interact with Mef2 factors to synergistically coactivate the myogenic program. Global elimination of Mef2c results in early embryonic lethality due to cardiovascular defects (Lin et al., 1997). Conditional elimination of Mef2c in skeletal muscle revealed a role in fiber type specification as there was a significant decrease in Type I oxidative slow myofibers (Potthoff et al., 2007). While efforts have focused on downstream targets of Mef2c, few direct upstream regulators of Mef2c have been identified in the skeletal muscle lineage.

Forkhead/winged helix transcription factors perform an array of functions including fate specification, pattern formation, cellular proliferation, cellular differentiation and organogenesis (Lehmann et al., 2003). Members of the *forkhead*/winged helix transcription factors function through a DNA-binding dependent mechanism or alternatively through protein–protein interactions to regulate gene expression (Wijchers et al., 2006). For example, the *forkhead*/winged helix factor, Foxk1, is expressed in myogenic progenitor cells and functions as a cell cycle regulator by regulating the cyclindependent kinase inhibitor, $p21^{KIP}$(Garry et al., 2000). Mice lacking Foxk1 have perturbed skeletal muscle regeneration due to impaired activation/proliferation of the myogenic progenitor cell population (Hawke et al., 2003a). In addition to Foxk1, other *forkhead* factors including FoxO factors modulate cell signaling pathways, growth and atrophy of adult skeletal muscle (Sandri et al., 2004). These studies suggest that *forkhead* factors have critical roles in the regulation of skeletal muscle development and regeneration.

To further identify *forkhead* factors expressed in the myogenic lineages, we undertook a candidate based screen. Using this strategy, we identified Foxj3 as being significantly upregulated in differentiating myoblasts. To further explore the functional role of Foxj3 *in vivo*, we obtained a Foxj3 gene-targeted ES cell line and generated mutant mice that have a β-*geo* cassette flanked by two splice acceptors inserted into the Foxj3 locus. The resulting mutant Foxj3 allele produces a transcript that encodes exons 1–5 (1–176 amino acids) of the Foxj3 protein that lacks a transcriptional activation domain. Mice homozygous for both the mutant alleles (referred to as Foxj3m/m) are viable, but have impaired skeletal muscle contractility and decreased Type I oxidative myofibers compared to their wild type controls. In addition, Foxj3m/m mice have impaired skeletal muscle regeneration following injury, impaired cell cycle kinetics of the myogenic progenitor cell population and decreased expression of Mef2c. Examination of the 5′ upstream skeletal muscle enhancer of Mef2c revealed a highly conserved *forkhead* binding site (FBS). Transcriptional assays in C2C12 myoblasts demonstrated that Foxj3 activates a Mef2c-*luciferase* reporter and mutagenesis of the FBS in the Mef2c skeletal muscle enhancer ablates this transcriptional activation.

Together these studies reveal that Foxj3 is a transcriptional activator of Mef2c, and is an important regulator for adult muscle fiber type identity and skeletal muscle regeneration.

Results

We have utilized an array of techniques to define the expression of *forkhead* family members in the skeletal muscle lineage during development and regeneration. To complement these studies, we utilized a C2C12 myoblast differentiation assay to analyze gene expression during discrete phases of myogenesis (Shi and Garry, 2006). We identified a novel member of the *forkhead*/winged helix family, Foxj3, which was dynamically expressed during C2C12 myogenic differentiation using semi-quantitative RT-PCR (Fig. 1 and Supplemental Figure S1). This differentiation assay revealed that Foxj3 and Mef2c preceded myoglobin expression during myogenesis. These studies support the hypothesis that Foxj3 is expressed and regulated during myogenesis.

Generation of Foxj3m/m mice

To define the functional role of Foxj3, we generated Foxj3 mutant mice (referred to as Foxj3m/m) from a BayGenomics© ES cell "trapped" line (Stryke et al., 2003). The Foxj3 trapped ES cell line contained an insertion of a β-galactosidase/neomyocin (β-*geo*) cassette with two separate splice acceptors (Fig. 2A). The resulting mRNA predicted transcript encodes exons 1 through 5 and contains all but the last six residues of the *forkhead* DNA-binding domain. No transcripts were detected following exon 6 of the native Foxj3 gene (Figs. 2A–C). Previous studies using Gal4 transcriptional assays demonstrated that the carboxy-terminus harbors the activation domain of Foxj proteins and is essential for Foxj family of proteins functional activity (Pérez-Sánchez et al., 2000). Thus, we hypothesized that the fusion protein encoded from Foxj3m/m mice would result in a nonfunctional, transcriptionally inactive fusion protein. Additionally, we predicted that the Foxj3-β-gal fusion protein would recapitulate Foxj3 endogenous expression patterns.

Foxj3 mutant mice are viable

Mice homozygous for the Foxj3-β-*geo* targeted allele were generated from matings of heterozygote mice. Initially, our studies utilized the C57/B6J:129OlaP2Hsd mixed strain; however, we have backcrossed the original chimeric mice with inbred C57/B6J and 129OlaP2Hsd strains separately and over six generations and we observed no strain variances. Mice homozygous with the Foxj3 mutant alleles, generated from heterozygote matings, were viable and born at normal Mendelian ratios (Supplemental Figure S2). Semiquantitative RT-PCR of gastrocnemius skeletal muscle from wildtype and homozygous Foxj3 mutant mice revealed that expression of the Foxj3 mRNA transcript was as predicted limited to exons 1–5, and that the carboxy-terminal activation domain in exons 8–12 were not transcribed (Fig. 2C).

Foxj3m/m mice have impaired skeletal muscle regeneration following injury

As previously described (Landgren and Carlsson, 2004), Foxj3 was expressed in the myogenic lineage during embyogenesis (Supplemental Figure S3) and in the in vitro myogenic differentiation assay (Fig. 1 and Supplemental Figure S1). We then examined the

ability of Foxj3m/m mice to regenerate their skeletal muscle following a severe myonecrotic injury which destroys approximately 90% of the adult skeletal muscle. In response to this cardiotoxin-induced injury, wild type skeletal muscle regenerates within a 2-week period and has restoration of its architecture. In contrast, Foxj3 mutant mice had a severe regenerative impairment that was evident at 2 and 3 weeks following delivery of cardiotoxin. We observed persistent myonecrosis and widespread replacement of the myofibers with adipocytes (Fig. 2D). Using electron microscopy, we observed the presence of myogenic progenitor cells (i.e., satellite cells) in the unperturbed Foxj3m/m skeletal muscle and there were no significant differences in their number compared to the gender and age matched control mice (Supplemental Figure S4 and data not shown).

Foxj3 mutant myogenic progenitor cells have an increased proliferative capacity

To determine the proliferative capacity of the mutant and wild type myogenic progenitor cell population, we performed cell cycle kinetics assay and an in situ proliferation assay. We isolated myogenic progenitor cells from wild type and mutant skeletal muscle and analyzed the cell cycle kinetics in asynchronized proliferating cells. The mutant myogenic progenitor cells had a significant decrease in the G0/G1 phase and a significant increase in the G2/M phase as assayed by flow cytometry ($p<0.05$; $n=3$ littermate pairs) (Figs. 3A and B). These cell cycle results were further supported using the proliferation marker Ki67 (which labels all proliferating cells) in regenerating Foxj3 mutant vs. control skeletal muscle performed 5 days following cardiotoxin induced injury. These results demonstrate an increased proliferative capacity, as measured by Ki67 expression (Fig. 3C) that represented a 65% increase compared to the wild type control ($p<0.05$; $n=3$) (Fig. 3D). Additionally, most of the Ki67-positive cells were also positive for MyoD expression, which supports the notion that the impaired regenerative defect may also be due in part to increased proliferation of the mutant myogenic progenitor cells (Fig. 3E). Importantly, we observed no significant differences in Ki67 expression in unperturbed Foxj3 mutant and wild type control skeletal muscle (data not shown). These results, in combination with the studies revealing no significant differences in the number of myogenic progenitor cells using electron microscopy or TUNEL-positive cells in Foxj3m/m vs. control unperturbed skeletal muscle further support the conclusion that Foxj3 is important in cell cycle regulation as opposed to having a role in the maintenance of the myogenic progenitor cell population (Supplemental Figure S4).

shRNA knockdown of endogenous Foxj3 results in increased myoblast proliferation

To validate the in vivo increased myoblast proliferation phenotype observed in the injured Foxj3m/m mice, we undertook shRNA knockdown of endogenous Foxj3 in C2C12 myoblasts. Using four separate shRNA and an empty vector control, we observed knockdown of Foxj3 transcript in two out of the four shRNA constructs examined (Fig. 4A). Knockdown of endogenous Foxj3 in C2C12 myoblasts resulted in increased proliferation of Foxj3 myoblasts and an induction of cells in the proliferative phase of the cell cycle (Figs. 4B and C). No evidence of cellular death was observed in any of the samples ($n=3$ replicates, performed in three separate experiments).

Foxj3m/m skeletal muscles have decreased Type I fibers and impaired contractility

We next examined the fiber type diversity of the oxidative, slow twitch soleus muscle and the glycolytic fast twitch EDL muscle in adult Foxj3m/m male mice using metachromatic ATPase fiber type analysis. We observed that both the soleus and EDL muscles of the Foxj3m/m mice had decreased numbers of oxidative Type I fibers and significantly increased numbers of Type IIa/b fibers compared to their wild type controls (Figs. 5A–C; n=3; *, p<0.05).

We next assessed the ability of the muscles to produce stress (force normalized to muscle cross sectional area; Grange et al., 2002) to determine if any functional deficits existed in the Foxj3m/m muscles resulting from the shift in fiber types. Compared to WT controls, Foxj3m/m EDL muscles (Fig. 5D) had decreased stress (with increased stimulation frequency compared to the wild type control; n=6; p<0.05), but stress was minimally effected in the soleus (Fig. 5E). These results establish that Foxj3 is important for fiber type diversity and physiological function of skeletal muscle.

Foxj3 functions as a transcriptional activator of gene expression

We next undertook a series of transcriptional assays to evaluate the functional role of Foxj3 as a modulator of gene transcription. We utilized an *in vitro* Gal4-UAS-luciferase reporter-based assay in C2C12 myoblasts (Chang et al., 2005). The Foxj3 full length construct (containing the entire open reading frame) and the $Foxj3_{1–176\ aa}$ mutant transcript were fused to the Gal4-DNA binding domain and transfected with a UAS-*luciferase* reporter into C2C12 myoblasts. Overexpression of the full-length Gal4-Foxj3 and the truncated transcripts were expressed in the nuclear compartment (Supplemental Figure S5A and S5B). Overexpression of the full length Gal4-Foxj3 construct was capable of activating the reporter 12-fold in a dose-dependant fashion (Supplemental Figure S5C; n=3;*, p<0.05) whereas the $Foxj3_{1–176\ aa}$ mutant transcript had no transcriptional activity)(Supplemental Figure S5D). These results support the hypothesis that Foxj3 functions as a transcriptional activator of gene expression.

Mef2c is a direct downstream target gene of Foxj3 in myoblasts

To identify potential downstream target genes of Foxj3, we utilized oligonucleotide microarray technology to examine dysregulated genes in Foxj3m/m myogenic progenitor cells. We identified over 900 genes that were significantly dysregulated (p<0.005) greater than two-fold and validated several of the most dysregulated transcripts by semiquantitative RT-PCR (Fig. 6A). Analysis of the Foxj3 dysregulated transcripts suggested that this *forkhead* protein regulated a Mef2c-dependent pathway as a number of the downregulated transcripts contained Mef2c-binding elements within their promoters. Recent studies have validated a number of these downregulated transcripts (troponins I and T, myomesin2) as direct Mef2c downstream target genes (Blais et al., 2005). Myocyte enhancer factor 2C (Mef2c) was downregulated more than 10-fold in the Foxj3 mutant myoblasts compared to the wild type controls. In addition, we observed that Mef2c target genes were also downregulated including Tnni1 and Tnnt2 (Fig. 6A). Upon examination of the 5′ upstream promoter of Mef2c, we identified a highly conserved *forkhead* binding site within the skeletal muscle enhancer region (−1.1 kb /+77 bp, Wang et al., 2001) (Fig. 6B). Using

transgenic technology, Wang and colleagues demonstrated that this 1.2 kb skeletal muscle enhancer region directs lacZ reporter expression exclusively in embryonic and adult skeletal muscle lineages. To confirm our hypothesis that Foxj3 was able to bind to the evolutionary conserved FBS and was a direct regulator of Mef2c *in vivo*, we performed a chromatin immunoprecipitation (ChIP) assay and PCR with primers specific for the FBS located in the Mef2c 5′ upstream skeletal enhancer region. When we overexpressed the Foxj3-GFP fusion construct in C2C12 myoblasts, and purified the bound chromatin, the segment containing the FBS in the 1.2 kb Mef2c skeletal muscle enhancer was amplified, but not in the negative IgG control (Fig. 6C). These studies support the conclusion that Foxj3 binds to the Mef2c promoter in vivo.

Based on the results of the ChIP assay and the evolutionary conserved region of the Mef2c promoter, we fused the −1.1 kb/+77bp (referred to as 1.2 kb Mef2c) to a *luciferase* reporter and transfected C2C12 myoblasts with increasing amounts of Foxj3. Using these transcriptional assays, we observed that Foxj3 trancriptionally activated the 1.2 kb Mef2c-*luc* up to 12-fold in a dose dependant fashion (Fig. 6D and E). The $Foxj3_{1–176\ aa}$ mutant transcript that was produced in the Foxj3m/m mice had essentially no transcriptional activity (Supplemental Figure S5E and S5H). Using site-directed mutagenesis, we mutated the *forkhead* binding site (FBS) in the 1.2 kb Mef2c-luc reporter, and noted that overexpression of Foxj3 in C2C12 myoblasts lacked any transcriptional activity (Figs. 6D and E). Collectively, these studies support the conclusion that Foxj3 is a direct upstream regulator of the Mef2c gene.

Discussion

Molecular networks coordinately regulate skeletal myogenesis during embryogenesis and regeneration of adult skeletal muscle. Many of these transcriptional networks (MyoD family members, Mef2 factors, Pax3, Pax7, Foxk1, etc.) have been defined using gene disruption strategies that confirmed or uncovered novel essential roles in developing and regenerating muscle (reviewed by Shi and Garry, 2006). While intense interest has focused on downstream pathways that impact muscle differentiation and fiber type specification, the upstream regulators are incompletely defined. In the present study, we have begun to further decipher the regulators of the myogenic program by making three principal findings. First, we utilized molecular technologies to generate Foxj3 mutant mice (not Foxj3 null mice) that were viable. Importantly, we recognize that this genetic model may produce a truncated functional Foxj3 protein. As Foxj3 is expressed in skeletal muscle, we examined the skeletal muscle regenerative capacity in Foxj3 mutant mice. We observed that mutant Foxj3 mice had impaired muscle regeneration.

Another *forkhead* transcription factor, Foxk1, has been implicated as essential for normal skeletal muscle regeneration (Garry et al., 2000). However, the Foxj3m/m regenerative phenotype exhibits considerable differences with the Foxk1 null phenotype. Foxk1 is restricted to the MPC population and mice lacking Foxk1 have perturbed skeletal muscle regeneration due to decreased MPCs and an inability to efficiently activate the MPC population (Garry et al., 2000). In contrast, these studies emphasize that while the absence

of either *forkhead* factor results in impaired muscle regeneration, the underlying mechanism is distinct for both Foxj3 and Foxk1.

A second major finding of the present study is that Foxj3 is an important cell cycle regulator. While no proliferative changes were observed in the unperturbed Foxj3m/m skeletal muscle, our in vivo studies (following cardiotoxin injury) and *in vitro* studies (isolation and activation of the MPC population) demonstrate that Foxj3 is an antiproliferative factor. While the mechanistic detail regarding Foxj3′s antiproliferative role is incompletely defined, one possible mechanism involves decreased Mef2c expression. Previous studies have demonstrated that Mef2c interacts with the RNA helicase, CHAMP (Mov10l1), which induces the cyclin dependent kinase inhibitor $p21^{KIP}$ (cdkn1a) in striated muscle to promote cellular quiescence (Liu and Olson, 2002). Many other *forkhead* factors have repressive and permissive (Foxk1) influences on cell cycle kinetics and therefore have important regulatory roles during embryogenesis, growth and regeneration. Our studies have further defined a novel *forkhead* transcription factor that regulates cell cycle progression in the muscle lineage.

A third major finding of this study is that Foxj3 is a transcriptional activator of Mef2c. The Fox transcription factor family has a number of members such as the FoxO family that directly regulate gene expression and impact muscle growth and function. For example, Foxo3 has been shown to transcriptionally activate the ubiquitin ligase, atrogin-1 gene that results in atrophy of the myofibers (Sandri et al., 2004). In the present study, we utilized transcriptome analysis, transcriptional assays, mutagenesis and binding studies to support the conclusion that Foxj3 is a direct upstream activator of the Mef2c gene.

Mice lacking Mef2c are embryonic lethal at E9.5 due to cardiovascular defects (Lin et al., 1997). While the role of Mef2c in the heart has been elucidated in the past few years, the exact role of Mef2c in skeletal myogenesis is unclear (reviewed by Berkes and Tapscott, 2005). A recent study utilizing a temperature sensitive Drosophila Mef2 orthologue (*dMEF2*), surprisingly revealed that dMEF2 was not essential for adult myogenesis (Baker et al., 2005). While flies lacking dMEF2 had severe neurological deficits and an inability to fly, they had normal patterning of skeletal muscle. However, studies in vertebrates support a role for Mef2c in skeletal myogenesis.

Recent studies undertaken by Hughes and colleagues demonstrated that simultaneous morpholino knockdown of zebrafish Mef2c and Mef2d resulted in a loss of thick filament proteins and the disruption of sarcomeric structure (Hinits and Hughes, 2007). In addition, it has been conclusively demonstrated that Mef2c is an essential upstream transcriptional activator of troponins in skeletal muscle (Di Lisi et al., 1998; Blais et al., 2005) and myofiber identity (Wu et al., 2000). Conditional transgenic technologies have revealed a broader role for Mef2c in cellular maintenance in various lineages. The conditional deletion of Mef2c in skeletal muscle lineages using a MCK-*Cre* transgenic line resulted in a severe decrease of type 1 fibers (Potthoff et al., 2007). Potthoff and colleages clearly demonstrated that HDAC-mediated inhibition of Mef2c was the essential regulatory step in the conversion of oxidative fibers to slow-twitch, non-oxidative, fasttwitch fibers. The authors further demonstrated that the conditional loss of Mef2c in the skeletal muscle lineage, results in

decreased expression of structural Mef2c target genes, including troponins I and T and myomesin2.

In the Foxj3m/m skeletal muscle, we observed decreased expression of Mef2c as well as decreased expression of the Mef2c downstream target genes. The decreased expression of Mef2c and its downstream targets resulted in perturbed fiber type specification and impaired contractility. The decrease in overall maximum stress output in Foxj3 mutant mice could be the result of the decreased levels of troponins as a consequence of Mef2c downregulation. This could lead to decreased activation of the contractile apparatus. For reasons that are not clear, the decrease in stress was greater in the EDL than the soleus of the Foxj3 mutant mice. Together these results establish Foxj3 is a modulator of myofiber identity through a likely MEF2-dependent pathway.

Collectively, these studies have identified a novel *forkhead* transcriptional regulator, Foxj3 for Mef2c gene expression. Moreover, Foxj3 mutant mice demonstrate a phenotype consistent with decreased Mef2c expression and emphasize the role of Foxj3 in skeletal muscle biology including cell cycle kinetics, fiber type diversity and muscle regeneration.

Methods

Knockout mice

Foxj3 mutant mice were generated from the BayGenomics Foxj3-trapped ES-cell line (XL913) of the129P2OlaHsd strain (Stryke et al., 2003). After ES cell expansion and sequence confirmation, the Foxj3-trapped ES cells were injected into blastocysts, and chimeric mice were born to foster mothers. Progeny that were greater than 90% chimeric were then mated to both C56/B6J (Jackson Laboratory) and the 129P2OlaHsd (Harlan) mice to ensure germline transmission. Unless specified, wild type littermates from Foxj3 heterozygote crosses were used for all experiments (See Supplemental Materials and Methods for genotyping primers).

Mice were kept on a 4% fat irradiated chow (Harlan) diet and exposed to standard 12-h light/dark cycles. Mouse weights were recorded on a Mettler PE 200 scale. Mice were housed in a sterile, pathogen-free animal facility. All animal protocols were approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center at Dallas.

Immunohistochemistry/histochemistry of histological sections

All tissues were perfusion fixed using 4% paraformaldehyde at 4 C and washed in 1 PBS before sectioning. Sections were stained with hemotoxylin and eosin, nuclear fast red, and other antisera as previously described (Meeson et al., 2007).

Skeletal muscle tissues/sections for fiber type analysis were performed following cryoembedding of tissues in OCT (Tissue Tek) and Gum Tragacanth (Sigma) mix as previously described (Meeson et al., 2007). The protocol for metachromatic ATPase fibertyping has been previously described (Garry et al., 1996).

Primers

All primers used in this study were from Operon, and RTPCR primer sequences can be found in the Supplemental Materials and Methods section.

Plasmids

The murine Foxj3 gene (NM_172699; NCBI Gene Bank) consisting of a 1869 base pair open reading frame was cloned into the pCDNA3.1-NT-GFP (Invitrogen), pM1 (Gal4 vector) and the pCDNA3.1-N-myc plasmids using standard techniques. Foxj3 constructs that contained the amino terminus and the DNA-binding domain ($Foxj3_{1–176aa}$) were cloned in frame in the same expression plasmids. The Gal4-UAS-luciferase system has been previously described (McKinsey et al., 2006). More detailed information about the plasmids used in these studies is available upon request.

The Mef2c skeletal muscle-specific promoter (−1.1 kb/+77 bp) containing the *forkhead* binding site has been previously described (Wang et al., 2001). The promoter region was amplified from genomic DNA and cloned into the pGL3-basic promoter with a minimal TATA promoter (pGL3-TATA). Mutagenesis of the *forkhead* binding site in the Mef2c-luciferase promoter construct was performed using the QuikChange II Site-Directed Mutagenesis Kit (Strategene). The Mef2c FBS was mutated using the mutant primer 5′ TAATAGGAACAGGTGGGCGGCTACAAAGC 3′ and its reverse compliment.

Cell culture and transfection assays

Unless otherwise noted, all transfections were performed using C2C12 myoblasts grown in 20% fetal calf serum/DMEM (Gibco). Transfections of 10^5 C2C12 myoblasts were performed in six-well plates using a Lipofectamine (Invitrogen) kit, and total protein was harvested 48 h post-transfection. Up to 2 μg of plasmid DNA was transfected in serum-free Optimem media (Gibco). Luciferase assays were performed using a Luciferase Assay System (Promega) following the manufacturer's instructions. All fold changes were normalized to a lacZ loading control, as previously described (Meeson et al., 2007). Myotube differentiation was promoted by exposing 80% confluent myoblast cultures to differentiation medium (DMEM supplemented with 2% heat inactivated horse serum, antibiotics, insulin and transferrin) as previously described (Hawke et al., 2003b).

Antisera

The following antisera were used in these studies: mouse monoclonal anti-Ki67 serum (Nova Castra; 1:1,000 dilution), rabbit anti-GFP serum (Molecular Probes; 1:1,000 dilution) and mouse monoclonal anti-MyoD serum (Nova Castra, 1:500 dilution). The secondary antisera utilized in these studies included a FITC conjugated goat anti-mouse serum (1:50 dilution; Jackson ImmunoResearch) and a rhodamine conjugated goat anti-rabbit serum (1:50 dilution; Jackson ImmunoResearch).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the ChIP Kit-M for mammalian cells (Progeneron, Inc). C2C12 myoblasts (2×10^6) were transfected with 4 μg

Foxj3-GFP, and harvested for nuclear protein 48 h post-transfection. Nuclear lysates were sonicated using a 550 Sonic Dismembrator (Fisher Scientific) sonicator and immunoprecipitated with 6 μg of anti-GFP serum (Molecular Probes) overnight at 4 C. Selected samples were incubated with IgG (Sigma) as an internal control. Lysates were then bound on UltralLink Immobilized Protein A/G beads (Pierce), and chromatin was then purified using Tripure (Roche) before elution in sterile water. 6 μL of five-fold diluted input and antibody-bound chromatin were used in the PCR reaction with primers specific for the region in the Mef2c skeletal muscle enhancer containing the *forkhead* binding site (see Supplemental Materials and Methods).

Primary MPC isolation, culture and immunohistochemical analyses

As previously described (Meeson et al., 2007), primary MPC cultures were established following the isolation of MPCs from hindlimb skeletal muscle of 2-day-old neonatal mice. Cells were preplated and cultured in F-10 growth medium (Gibco/Invitrogen) supplemented with 25 ng/ml fibroblast growth factor (Invitrogen). WT and Foxj3m/m MPCs were isolated from postnatal day 2 littermate pups generated from Foxj3 heterozygote breeders. Asynchronously dividing WT and Foxj3m/m MPCs were fixed for 10 min with 4% paraformaldehyde and immunostained as previously described (Meeson et al., 2007).

Electron microscopy and satellite cell quantitation

As previously described (Meeson et al., 2007), tibialis anterior muscles from 3 to 4 month old male adult WT and Foxj3m/m mice were harvested, perfusion fixed with 3% gluteraldehyde and postfixed with buffered 1% osmium tetroxide. Samples were then dehydreated with ethanol, embedded in Spurr resin, polymerized, sectioned and placed on copper grids. Sections were examined using a JEOL 1200 EXII TEM. MPCs were quantified using criteria as outlined previously (Garry et al., 1997).

Muscle physiology protocol

Both extensor digitorum longus (EDL) and soleus muscles were dissected from anesthetized WT control and Foxj3m/m mice aged 130–190 days (2 mg xylazine–20 mg ketamine per 100 g of body mass i.p.). Muscles were incubated at 30 °C in an oxygenated (95% O_2–5% CO_2) physiological salt solution (PSS; pH 7.6) containing (in mM):120.5 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 20.4 $NaHCO_3$, 1.6 $CaCl_2$, 1.2 NaH_2PO_4, 10.0 dextrose, and 1.0 pyruvate. Muscles were prepared for isometric contractions using a dual-mode servomotor system (300B, Aurora Scientific) as previously described (Wolff et al., 2006). Resting muscle force was set at 1.00 ± 0.05 g, (i.e., L_0, muscle length at which twitch force was maximal) that was automatically maintained by a stepper motor (Wolff et al., 2006). The stepper motor was temporarily inactive during isometric contractions. Dynamic Muscle Control software (DMC Version 4.1.6, Aurora Scientific) was used to record force output in response to electrical activation of the muscles via closely flanking platinum electrodes. Each EDL and soleus muscle was subjected to a force-frequency (F-F; 1, 30, 50, 80, 100, 150 Hz) protocol. Forces for the F-F protocol were converted to stress, or force normalized to muscle cross sectional area, as described (Grange et al., 2002). Physiological data were analyzed using a one-way ANOVA. (JMP 7.0).

Semiquantitative RT-PCR

Total RNA was extracted from cells and homogenized tissues using Tripure (Roche). 1 μg of total RNA was reverse transcribed using the SuperScript First Strand Synthesis kit (Invitrogen). cDNA was then diluted ten-fold in water, and PCR amplified using Go Taq Master Mix (Promega), and visualized on 1% agarose gels stained with ethidium bromide.

shRNA experiments

Four separate sets of short hairpin RNA (shRNA; HuSH-29 kit, Origene) directed against murine Foxj3 were cloned into the pRS plasmid under the U6 promoter and used for transfection assays in C2C12 myoblasts. The empty vector (pRS) and a nonsense (pRS-GFP) plasmid were used as negative controls. Effectiveness of the Foxj3 knockdown was tested in C2C12 myoblasts using both semiquantitative RT-PCR for endogenous Foxj3. 0.5 μg of shFoxj3 or vector was transfected into C2C12 myoblasts using lipofectamine (Invitrogen) in serum free Optimem (Gibco) media for 4 h. After 4 h, the transfection media was replaced with growth media containing 20% FBS in DMEM (Gibco). Cells were harvested 48 h post transfection for RNA, protein, and cellular content in a manner previously described (Meeson et al., 2007). Real-time PCR of Foxj3 was run following reverse transcription of 1 μg of total RNA (Superscript II kit/Invitrogen) using SYBRGreen Master Mix (Applied Biosystems). Samples were run on an ABI Prism 7900 light cycler and data were analyzed as previously described (Meeson et al., 2007).

cDNA microarray data

Total RNA was harvested from wild type and Foxj3m/m myoblasts, and labeled for Affymetrix arrays as previously described (Goetsch et al., 2003). cDNA microarray data can be accessed at the NCBI Gene Expression Omnibus (accession number GSE10628).

Cardiotoxin-induced muscle regeneration

Cardiotoxin (Calbiochem) was delivered intramuscularly into the gastrocnemius muscle of adult transgenic mice as previously described (Meeson et al., 2007).

Statistical analysis

All non-physiological *p*-values were calculated using Student's *t*-test (two-tailed) analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.ydbio.2009.11.015.

Nonstandard abbreviations

Fox	Forkhead box
FBS	forkhead binding site
Mef2c	Myocyte enhancer factor 2c
luc	luciferase
β-gal	β-galactosidase
bHLH	basic Helix-Loop-Helix
H&E	hemotoxylin and eosin
NFR	nuclear fast red
WT	wild type
GFP	green fluorescent protein
DMEM	Dulbecco's modification of eagle media
ES	embryonic stem
ChIP	chromatin immunoprecipitation
EDL	extensor digitorum longus
TA	tibialis anterior
MCK	muscle creatine kinase

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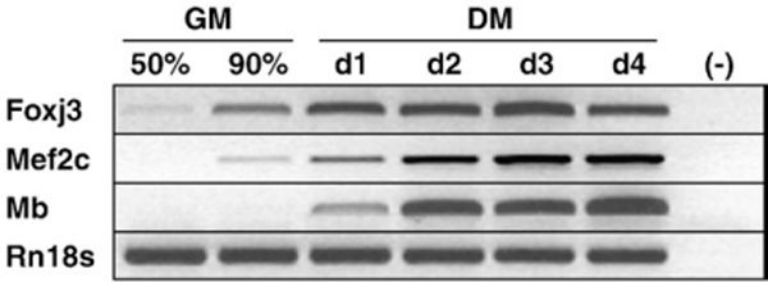


Fig. 1.

Foxj3 is expressed in C2C12 myoblasts. Semiquantitative RT-PCR analysis of transcript expression during C2C12 differentiation from myoblasts (50% or 90% confluency, cultured in growth medium or GM) to myotubes (day 1 or d1 to day 4 or d4, cultured in differentiation medium or DM) demonstrates that Foxj3 is expressed in myoblasts prior to myoglobin (Mb) expression. Rn18s was used as a loading control. (−) represents a negative control (lacking reverse transcriptase).

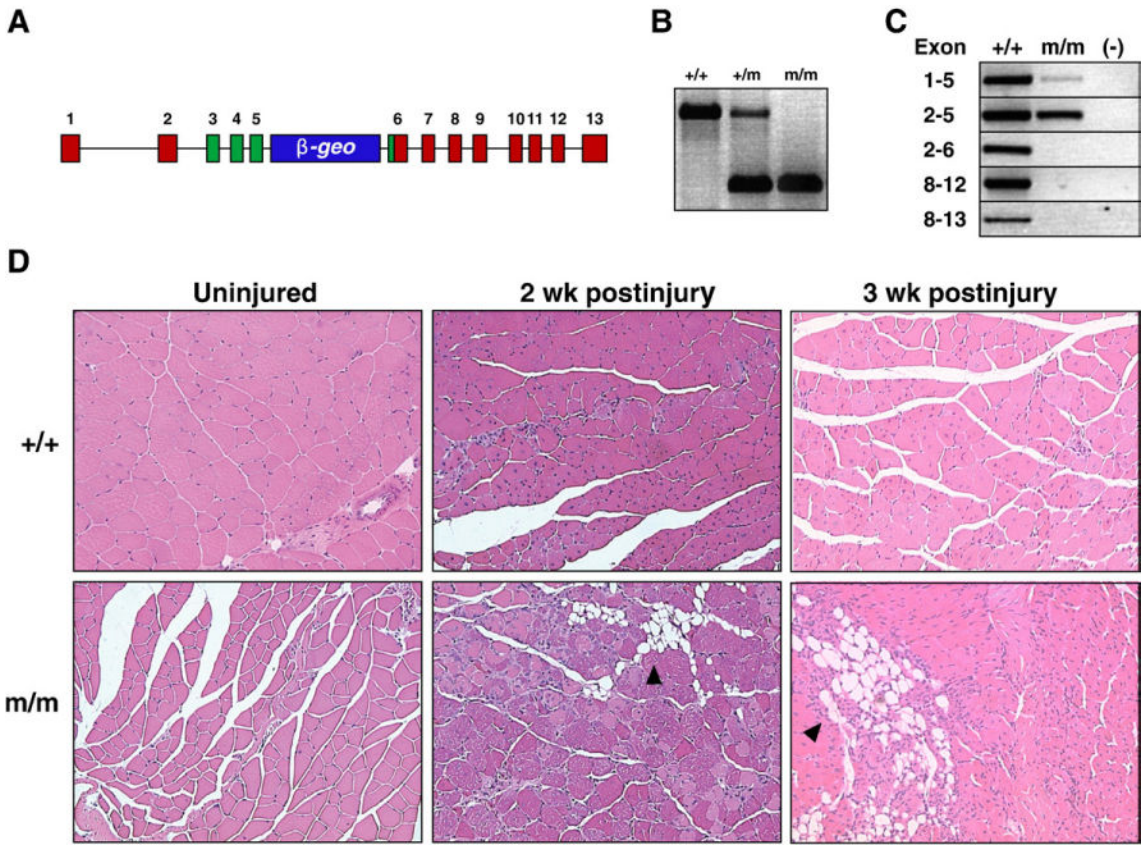


Fig. 2.

Foxj3m/m mice have impaired skeletal muscle regeneration. (A) Schematic outlying the Foxj3-β-geo “gene trapped” locus. (B) PCR genotyping of tails from Foxj3 mice. (C) The Foxj3 mutant transcript in Foxj3m/m gastrocnemius cDNA does not encode beyond exon 5 using RT-PCR techniques. Note that the transcript is amplified up to exon 5 in the Foxj3m/m muscle; however, only the wild type transcript can be amplified from exon 6 and beyond. (D) Histological sections of WT (+/+) and Foxj3m/m gastrocnemius skeletal muscle uninjured, 2 weeks, and 3 weeks post-cardiotoxin injury from 3-month-old male littermates. Note that the wild type skeletal muscle is fully regenerated with restoration of skeletal muscle architecture within two weeks (note central nuclei representing regenerated myofibers). Conversely, theFoxj3m/m cardiotoxin injured skeletal muscle fails to fully regenerate up to 3 weeks postinjury. Black arrowheads mark areas of necrosis and adipogenesis that form and replace myofibers following injury in the Foxj3m/m mice.

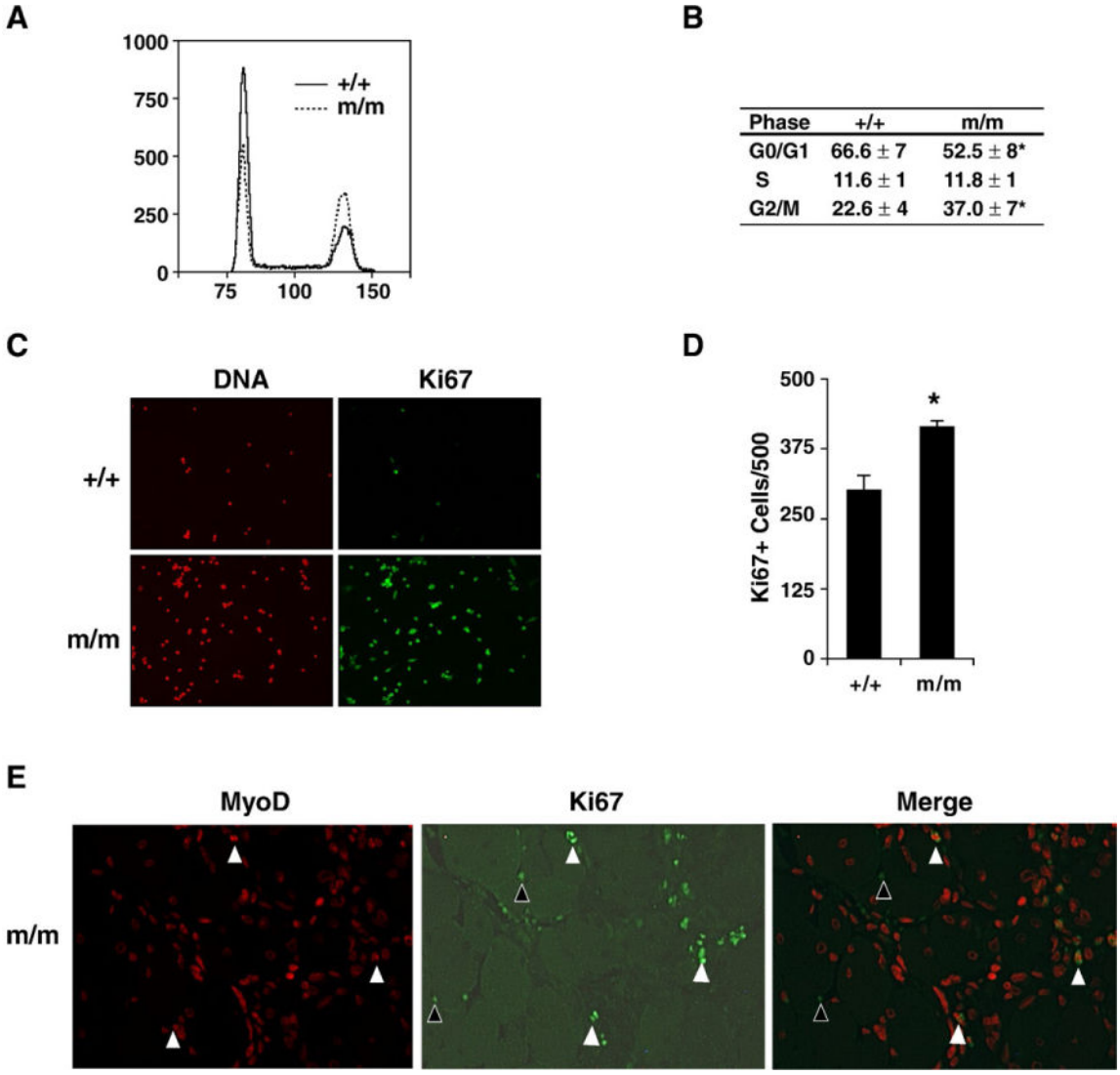
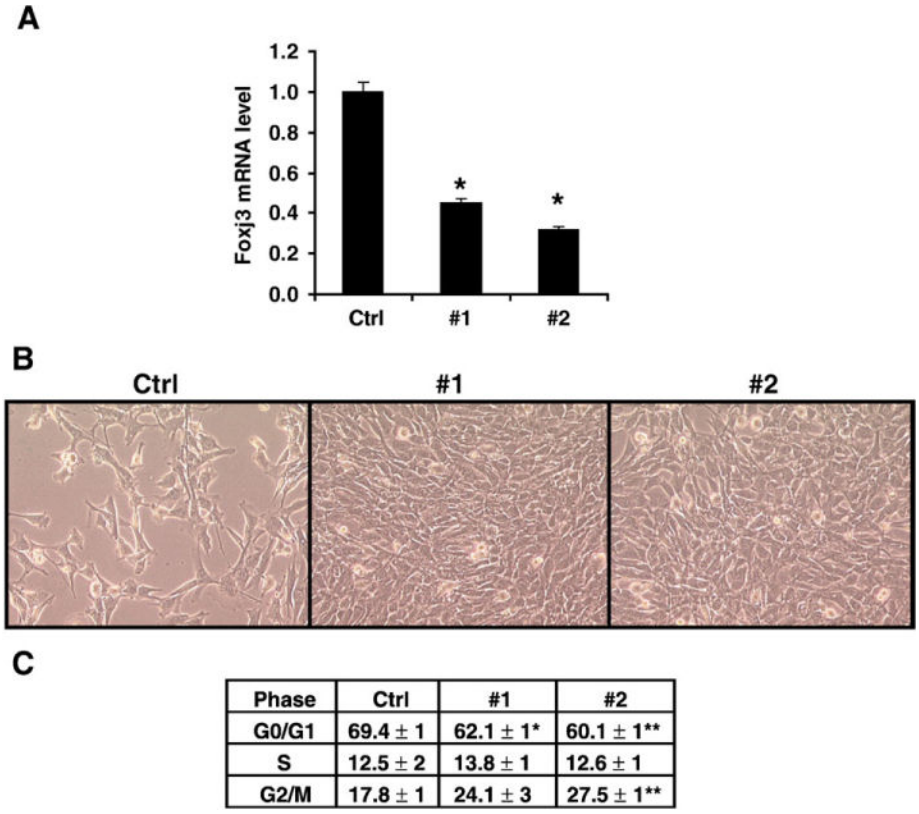


Fig. 3.

Foxj3m/m MPCs have perturbed cell cycle kinetics. (A) Flow cytometry profile of wild type and Foxj3m/m MPCs reveals that mutant MPCs have decreased number of cells in the G0/G1 stage and increased cells in the G2/M stage of the cell cycle. (B) Table outlines quantitative results of experiments undertaken in panel A representing the average cell cycle kinetics of WT and Foxj3m/m MPCs (n=3 separate experiments; *p<0.003). (C) Ki67 immunostaining of WT and mutant injured skeletal muscle reveals increased proliferation in the absence of Foxj3 following injury. (D) Quantitation of the Ki67 immunostaining reveals approximately a 65% increase in cellular proliferation in the Foxj3m/m injured skeletal muscle (*p<0.05). (E) Immunohistochemical staining of MyoD (red) and Ki67 (green) expression following injury. White arrowheads indicate that a population Ki67 positive cells are also positive for MyoD expression. A sub-population of cells are also positive for Ki67 (black arrowheads) but not myogenic (MyoD negative) in regenerating skeletal muscle.



Phase	Ctrl	#1	#2
G0/G1	69.4 ± 1	62.1 ± 1*	60.1 ± 1**
S	12.5 ± 2	13.8 ± 1	12.6 ± 1
G2/M	17.8 ± 1	24.1 ± 3	27.5 ± 1**

Fig. 4.

shRNA knockdown of endogenous Foxj3 results in increased myoblast proliferation. (A) Real-time PCR confirming knockdown of endogenous Foxj3 transcript in cells transfected with shRNA directed against Foxj3 (n=3 replicates; *p<0.05). (B) Phase microscopy of C2C12 myoblasts 48 h post-transfection of both control (pRS; empty vector) and shRNA directed against mouse Foxj3; shRNA #1 and #2. Note increased number of myoblasts with knockdown of Foxj3. (C) Table summarizing the results of the FACS histogram profiles of the C2C12 myoblasts transfected with control and shRNA Foxj3 vectors (n=3 replicates; *p<0.03; **p<0.003).

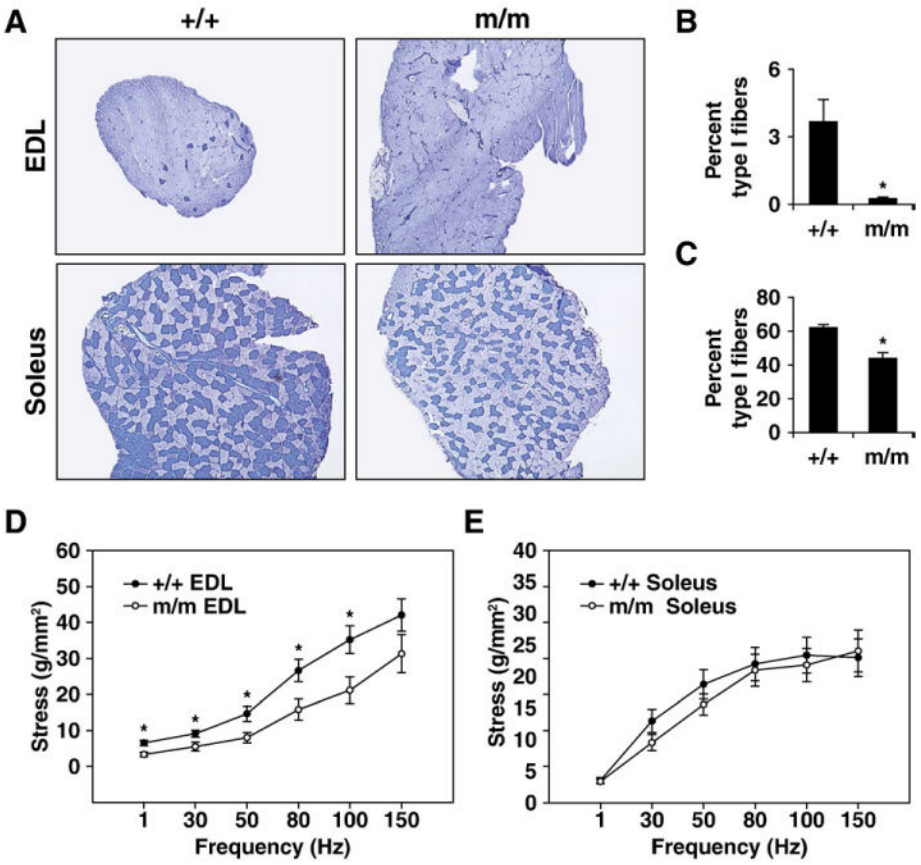


Fig. 5.

Foxj3 is essential for the determination of adult myofiber identity. (A) Metachromatic ATPase staining of EDL and soleus skeletal muscles of WT and Foxj3m/m mice. Foxj3m/m muscles have decreased numbers of Type 1 oxidative slow-twitch fibers and increased numbers of Type IIa/b fast twitch fibers. (B) Quantitation of Type 1 fibers in wild type and Foxj3m/m EDL muscles (n=4 separate muscles; 3 littermate pairs; *p<0.05) reveals decreased Type 1 fibers compared to WT control. (C) Quantitation of Type 1 fibers in wild type and Foxj3m/m soleus muscles (n=4 separate muscles; 3 littermate pairs; *p<0.05) reveals decreased Type 1 fibers compared to WT control. (D and E) Isolated wild type and Foxj3m/m EDL (D) and soleus (E) skeletal muscle stress-frequency responses. Both the EDL and soleus muscles of the Foxj3 mutant mice showed a right shift in the stress–frequency relationship compared to their wild type littermates (n=4 separate muscles; 3 littermate pairs; *p<0.05).

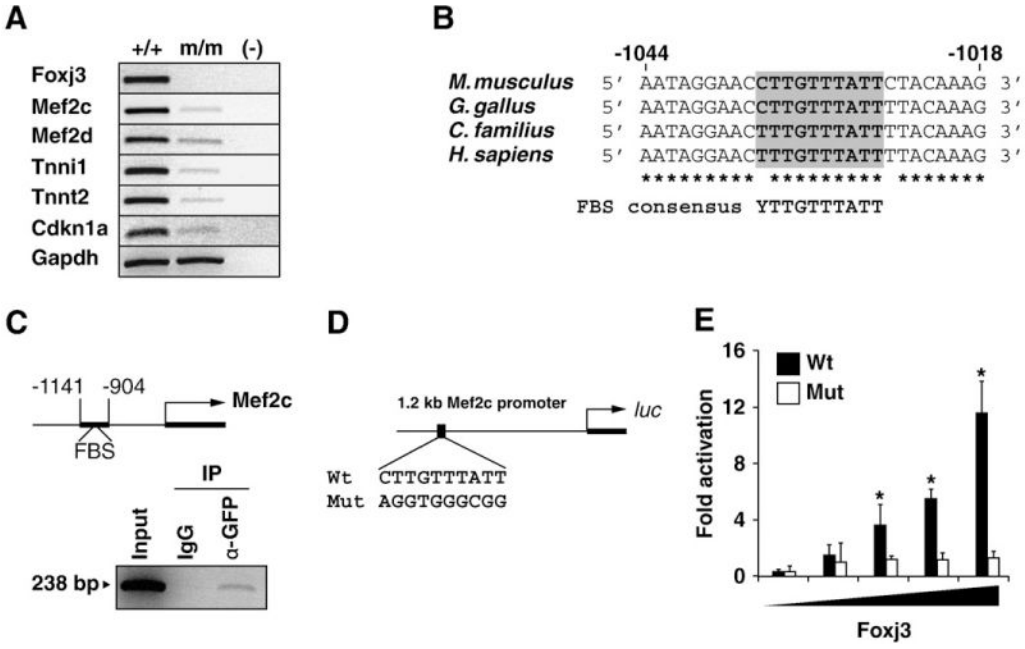


Fig. 6.

Mef2c is a direct downstream target of Foxj3 in skeletal muscle. (A) Semi-quantitative RT-PCR analysis of WT and Foxj3m/m MPCs reveals decreased expression of Mef2c and Mef2c-dependent target genes in the absence of Foxj3. Decreased expression of Cdkn1a or $p21^{KIP}$ corresponds to the increased proliferative capacity of the mutant MPCs as demonstrated in Fig. 3A. The housekeeping gene *Gapdh* is used as the loading control. (B) Homology alignment of the 5′ upstream Mef2c promoter reveals an evolutionarily conserved *forkhead* binding site or FBS. (C) Chromatin immunoprecipitation (ChIP) of Foxj3 using primers for the FBS in the Mef2c skeletal muscle enhancer indicate that Foxj3 directly binds to the FBS. IgG and 10% total chromatin (Input) are shown as controls. (D) Schematic outlining the Mef2c upstream fragment that harbors the FBS (and corresponding mutant construct) fused to the luciferase (luc) reporter constructs that were used in the transcriptional assays in panel E. (E) Foxj3 transcriptionally activates the Mef2c-*luc* skeletal muscle enhancer reporter in a dose dependent fashion in C2C12 myoblasts ($n=3$; $^*p < 0.005$). Mutagenesis of the FBS ablates the ability of Foxj3 to transcriptionally activate the Mef2c-luc reporter.