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Regulation of Skeletal Muscle Sarcomere Integrity and Postnatal Muscle Function by *Mef2c*▽

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Myocyte enhancer factor 2 (MEF2) transcription factors cooperate with the MyoD family of basic helix-loop-helix (bHLH) transcription factors to drive skeletal muscle development during embryogenesis, but little is known about the potential functions of MEF2 factors in postnatal skeletal muscle. Here we show that skeletal muscle-specific deletion of *Mef2c* in mice results in disorganized myofibers and perinatal lethality. In contrast, neither *Mef2a* nor *Mef2d* is required for normal skeletal muscle development in vivo. Skeletal muscle deficient in *Mef2c* differentiates and forms normal myofibers during embryogenesis, but myofibers rapidly deteriorate after birth due to disorganized sarcomeres and a loss of integrity of the M line. Microarray analysis of *Mef2c* null muscles identified several muscle structural genes that depend on MEF2C, including those encoding the M-line-specific proteins myomesin and M protein. We show that MEF2C directly regulates *myomesin* gene transcription and that loss of *Mef2c* in skeletal muscle results in improper sarcomere organization. These results reveal a key role for *Mef2c* in maintenance of sarcomere integrity and postnatal maturation of skeletal muscle.

The formation of skeletal muscle involves the specification of myogenic progenitor cells within the somites followed by the activation of a large array of muscle-specific genes through the synergistic activities of the MyoD and myocyte enhancer factor 2 (MEF2) families of transcription factors (9, 32). Members of the MyoD family of basic helix-loop-helix (bHLH) transcription factors, i.e., Myf5, MyoD, myogenin, and MRF4, are expressed specifically in skeletal muscle and are each capable of activating the muscle gene program when expressed in nonmuscle cells (reviewed in references 5, 41, 49, and 54). Loss-of-function studies have shown that that $MyoD^{-/-}$; $Myf5^{-/-}$ double-knockout mice fail to develop skeletal muscle (50), reflecting redundant roles of these genes in the establishment of the skeletal muscle lineage. *Mrf4* has also been implicated in specification of muscle cell fate (25), whereas *myogenin* is required for skeletal muscle terminal differentiation (23, 36).

The myogenic bHLH factors interact with MEF2 factors to cooperatively activate muscle-specific genes (32). MEF2 factors alone do not possess myogenic activity, but potentiate the activity of bHLH factors (32). The MEF2 proteins, MEF2A, -B, -C, and -D contain a conserved N-terminal MADS (MCM1, agamous, deficiens, SRF) domain and an adjacent MEF2-specific domain which, together, are necessary and sufficient for dimerization, cofactor interactions, and binding to the DNA consensus sequence $CTA(A/T)_4TAG$ (4, 33, 45, 46, 61).

Based on their expression patterns in vivo and activities in vitro, MEF2 factors are believed to function downstream of the bHLH transcription factors in the pathway for skeletal muscle development (18, 31, 34, 57). However, the promoters of the *myogenin* (12, 16, 19, 60) and *Mrf4* (8, 37) genes contain MEF2 binding sites that provide a mechanism for amplifying and maintaining their expression and stabilizing the muscle phenotype (34). The *Mef2c* gene also serves as a direct target of myogenic bHLH and MEF2 factors, which serve to further reinforce the decision of myoblasts to differentiate (57). Thus, the expression and activities of these two classes of myogenic transcription factors are intimately integrated through multiple regulatory mechanisms (34, 46, 57).

During mouse embryogenesis, MEF2 proteins display distinct but overlapping expression patterns in the skeletal muscle lineage, but unlike the myogenic bHLH transcription factors, MEF2 proteins are also expressed in other cell types, including neurons, cardiomyocytes, neural crest cells, chondrocytes, smooth muscle cells, and endothelial cells (6, 15, 20). *Mef2c* is the first member of the MEF2 family to be expressed in the myotome (at ca. embryonic day 9.0 [E9.0]), and its appearance lags approximately 18 h behind that of *Myf5*, the first bHLH myogenic regulator to be expressed (20). *Mef2a* and *Mef2d* are expressed after *Mef2c* (20).

Because of their overlapping expression patterns and common functions, it has been difficult to discern the functions of individual *Mef2* genes during different stages of mammalian development. However, loss of function of the single *Drosophila Mef2* gene has been shown to result in a block to differentiation of all muscle cell types (10, 27, 48), demonstrating the central role of MEF2 as a regulator of multiple muscle differentiation programs. Mice that lack *Mef2a* display an array of cardiovascular defects which cause most mice to die suddenly (38). Mice with homozygous mutations in *Mef2d* are viable (6), whereas mice lacking *Mef2c* die at E9.5 from cardiovascular defects (28, 29). The early lethality caused by the *Mef2c* loss-of-function mutation has therefore precluded analysis of its role in skeletal muscle at later developmental stages.

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In addition to its role in muscle development, MEF2 has been implicated in establishing the slow myofiber phenotype by serving as a target for calcium-dependent signaling to drive oxidative and slow-fiber-specific genes (17, 59). Recently, we showed that skeletal muscle-specific deletion of *Mef2c* in a mixed mouse genetic background results in a substantial reduction of slow skeletal muscle fibers, while overexpression of a superactive form of MEF2C (MEF2C-VP16) promotes the slow-fiber phenotype and enhances endurance exercise (47).

To further explore the functions of *Mef2c* in developing skeletal muscle, we conditionally deleted a floxed *Mef2c* allele using two Cre recombinase transgenes that allow early versus late deletion of *Mef2c* in skeletal muscle. Here we show that early deletion of *Mef2c* results in neonatal lethality at postnatal day 1 (P1), while mice with a later deletion of *Mef2c* are viable. In mice with early deletion of *Mef2c*, skeletal muscle differentiates to form myofibers with abnormally assembled sarcomeres and weakened M lines. Microarray analysis revealed misregulation of genes encoding components of the sarcomere, including the M-line-specific proteins myomesin and M protein. Accordingly, we show that *Mef2c* directly regulates *myomesin* transcription in vivo. These results reveal an essential role for *Mef2c* in myofiber maturation and function and demonstrate an important role for MEF2 proteins in terminal differentiation through maintenance of muscle integrity.

MATERIALS AND METHODS

Plasmid constructs, tissue culture, and cell transfection. Wild-type *myomesin 1* (bp −1035 to +88) and *myomesin 2* (*M protein*) (bp −1058 to +6) (base pair numbering refers to the location of the DNA fragment relative to the transcriptional start site of the indicated gene) promoters were cloned into TOPO TA (Invitrogen). MEF2 and E-box sites were mutated by two-step PCR directed mutagenesis (*myomesin 1* MEF2, CTATATTTAT to CTGGGTTTAT; *myomesin 1* E box, CATGTG to TCTGTG; *myomesin 2* MEF2, CTAAATATAG to CTAGGGATAG). Primer sequences for promoter cloning and mutagenesis are as follows: *myomesin 1*, 5′-CTGGCCCTGACCGAATACCACCACCAAGG-3′ (forward) and 5′-CGAGGAGCAGGAGAGAATGAGGGCCCACC-3′ (reverse); *myomesin 2*, 5′-GCTGGCCTGCAGGTCAACCTCACGGAGGC-3′ (forward) and 5′-CTCCCTGCAGAGCTCTGTGCTTCCCCC-3′ (reverse); *myomesin 1* ΔMEF2, 5′-CCCCTCCCCATGTGCTGCTGGGTTTATCTGCCTTCCTGGCC-3′ and complement sequence; *myomesin 1* ΔE box, 5′-GGTTTGGGACTCCCCTCCCTCTGTGCTGCTATATTTATCTGC-3′ and complement sequence; *myomesin 2* ΔMEF2, 5′-GGAGAGGCAGTCCCTTGCCTGGGTATAGCACCTCCTCTGGCCATAA-3′ and complement sequence.

Wild-type and mutant promoters were then cloned into pGL3 and pGHlacZ vectors for luciferase reporter and in vivo expression, respectively.

COS cells and C2C12 myoblasts were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum and antibiotics as described previously (47). For transient-transfection assays, cells were plated and transfected 12 h later using Fugene (Roche) according to the manufacturer's instructions.

Generation of mutant and transgenic mice. Mice with a *Mef2c* allele flanked by loxP sites have been described (6). Skeletal muscle-specific transgenic mice expressing Cre recombinase under control of the *myogenin* promoter (26) or *MCK* promoter (11) have been described previously. Transgenic mice were generated as previously described (16). Staining of embryos for β-galactosidase was performed as previously described (16).

RNA in situ hybridization, histology, and electron microscopy. Embryos and tissue for histology were isolated in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde overnight, processed, and sectioned for hematoxylin and eosin (H&E) staining using standard procedures (52). In situ hybridization samples were processed in 0.1% diethylpyrocarbonate–PBS. ^{35}S-labeled RNA probes for Myf5, MyoD (30), and myogenin were generated using a Maxiscript kit (Amersham).

Electron microscopy was performed as previously described (26). Briefly, skeletal muscle from P1 pups was fixed overnight in 2% glutaraldehyde in PBS at 4°C and then postfixed in 1% OsO_4 and dehydrated in an ethanol series. Samples were then embedded in Spurr resin (Ted Pella, Inc., Redding, CA), stained with uranyl acetate and lead citrate, and sectioned at 80 nm.

RT-PCR, quantitative real-time PCR, and microarray analysis. Total RNA was extracted from wild-type and mutant skeletal muscle with Trizol reagent (Invitrogen). Four micrograms of RNA from each sample was used to generate cDNA using SuperScript II first-strand synthesis kit (Invitrogen). Reverse transcription-PCR (RT-PCR) for the deleted region of *Mef2c* was performed using the primer pair 5′-GATGAAGAAGGCTTATGAGCTGAGCGTGCTGTGCGACTGTGAG-3′ (forward) and 5′-CTGTTATGGCTGGACACTGGGATGGTAACTGGCATCTCAAAG-3′ (reverse).

Quantitative real-time PCR was performed using TaqMan one-step chemistry or SYBR green on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Predesigned intron-spanning primers were purchased from Applied Biosystems for TaqMan (myogenin, MyoD, Acta1, Myh7, myozenin 2, Myl4, Myl7, MEF2A, MEF2D, TnnI1, TnnI2, TnnT3, and GAPDH [glyceraldehyde-3-phosphate dehydrogenase]). Primer sequences for SYBR green are available upon request.

For microarray analysis, total RNA was extracted from wild-type or *Mef2c* SKM KO (skeletal muscle-specific deletion of *Mef2c*) E18.5 hindlimbs using Trizol reagent (Invitrogen). Microarray analysis was performed using the Mouse Genome 430 2.0 array (Affymetrix), and results were analyzed using PANTHER as previously described (35).

Reporter assays. The *myomesin 1*- and *2*-luciferase constructs contain a DNA fragment extending from bp −1035 bp to +88 and −706 to +6 from the *myomesin* and *M-protein* genes, respectively. The Myc-tagged MEF2C expression vector was described previously (44). COS cells in 24-well plates were transfected with 100 ng of reporter plasmids in the presence or absence of MEF2C (5 to 100 ng). The reporter assays were performed as previously described (14).

Gel mobility shift assays. Oligonucleotides corresponding to the conserved MEF2 binding site in the *myomesin 1* and *2* promoters, a mutated site, and a bona fide MEF2 site from the *MCK* enhancer were synthesized (Integrated DNA Technologies), annealed, labeled with [^{32}P]CTP using Klenow fragment, and purified with G25 columns (Roche). The following sequences were used: wild-type *myomesin 1*, 5′-GGGATGTGCTGCTATATTTATCTGCCTT-3′; *myomesin 1* ΔMEF2, 5′-GGGATGTGCTGCTACCGGTATCTGCCTT-3′; *muscle creatine kinase* (*MCK*), ′-GGGGATCGCTCTAAAAATAACCCTGTCG-3′; wild-type *myomesin 2*, 5′-GGGCCCTTGCCTAAATATAGCACCTCCT-3′; *myomesin 2* ΔMEF2, 5′-GGGCCCTTGCCTACCGGTAGCACCTCCT-3′.

Cell extracts were isolated from COS cells transfected with a myc-tagged MEF2C expression plasmid or empty vector. Reaction conditions were as previously described (13, 44). DNA-protein complexes were resolved on 5% polyacrylamide native gels.

RESULTS

Early embryonic deletion of *Mef2c* causes perinatal lethality. Mice with a homozygous null mutation of *Mef2c* exhibit early lethality at E9.5 due to cardiovascular defects (28, 29). To determine the function of *Mef2c* in skeletal muscle at later developmental stages, we deleted a floxed *Mef2c* allele (6) specifically in skeletal muscle by using the myogenin-Cre (Myo-Cre) transgene, which consists of a Cre recombinase expression cassette controlled by the *myogenin* promoter and the skeletal muscle-specific enhancer of the *Mef2c* gene. This transgene is expressed specifically in skeletal muscle beginning at E8.5 (26).

Skeletal muscle-specific deletion of *Mef2c* (*Mef2c* SKM KO) resulted in lethality at P1. This postnatal lethality was affected by genetic background. In a C57BL/6 mixed genetic background, 100% lethality was observed. However, other backgrounds (e.g., 129/SvEv) produced some viable *Mef2c* SKM KO mice, which display a fiber-type switching phenotype described previously (47). *Mef2c* SKM KO pups in the C57BL/6 mixed genetic background were slightly smaller than wild-type littermates (Fig. 1A) and died several hours after birth, always before P2. At P1, *Mef2c* SKM KO pups were mobile but lethargic compared to wild-type littermates and did not feed

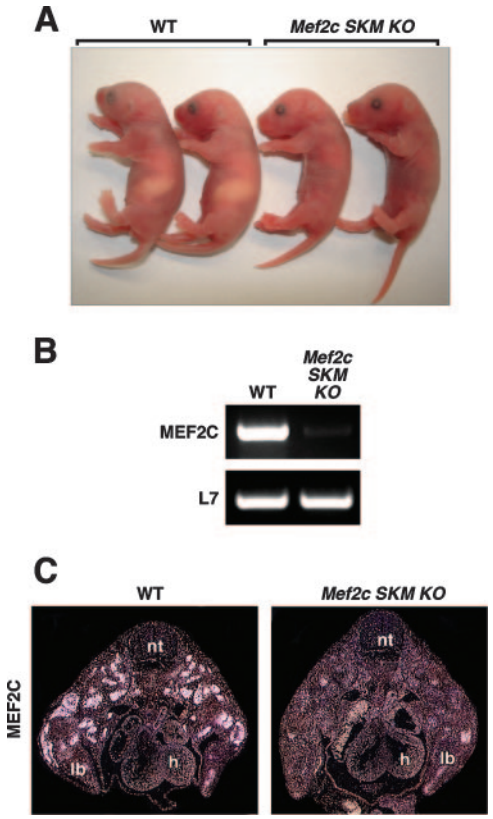


FIG. 1. Skeletal muscle deletion of *Mef2c*. (A) *Mef2c* SKM KO mice (*Mef2c*$^{fl/-}$; *Myo-Cre*) are smaller than wild-type (WT) littermates and do not feed (note the absence of milk in the stomach). (B and C) Analysis of efficiency of gene deletion in *Mef2c* SKM KO postnatal (B) and embryonic (C) muscles. (B) Total RNA was isolated from hind limb muscles of wild-type and *Mef2c* SKM KO mice at P1 and analyzed by semiquantitative RT-PCR for MEF2C mRNA. L7 served as a loading control. (C) In situ hybridization for MEF2C in transverse sections of E12.5 WT and *Mef2c* SKM KO embryos. nt, neural tube; h, heart; lb, limb.

(Fig. 1A). In contrast to the early perinatal lethality resulting from *Mef2c* deletion with Myo-Cre, mice with a later deletion of *Mef2c* beginning at ~E18.5 using a Cre transgene controlled by the *muscle creatine kinase* promoter (*MCK*-Cre) (11) were viable.

To verify the efficiency of *Mef2c* gene deletion by Myo-Cre, we performed RT-PCR and in situ hybridization for the deleted region of *Mef2c* in wild-type and *Mef2c* SKM KO skeletal muscles. As shown in Fig. 1B and C, *Mef2c* transcripts were efficiently deleted in muscles from neonates and embryos, respectively.

Notably, *Mef2a* or *Mef2d* homozygous mutant mice did not display skeletal muscle developmental defects (reference 47 and data not shown). We conclude that embryonic expression of *Mef2c* is specifically required for skeletal muscle development.

Loss of *Mef2c* results in myofiber disarray. Histological analysis showed that the hind limb muscles from *Mef2c* SKM KO pups at P1 were severely disorganized and fragmented (Fig. 2A). The diaphragms of *Mef2c* SKM KO mice were especially thin and lacking in well-developed myofibers, which is likely to be the cause of death (Fig. 2B). Notably, mutant myocytes were able to differentiate and fuse into myofibers (Fig. 2A), unlike muscles from *myogenin* knockout mice, which fail to fully differentiate and form very few myofibers in vivo (23). Skeletal muscle deletion of *Mef2c* with *MCK*-Cre did not disrupt myofiber organization (data not shown).

To define the time of onset of muscle defects in *Mef2c* SKM KO animals, we analyzed muscle at sequential developmental stages. Muscles appeared to be normal at E12.5, E14.5, and E16.5 (data not shown), whereas disorganization was apparent by E18.5 and became more severe by P1 (Fig. 2C and D).

Ultrastructural analysis showed that sarcomeres of skeletal muscle from *Mef2c* SKM KO mice at P1 were disorganized and fragmented compared to those from wild-type littermates (Fig. 3A). Fragmented myofibers along the M-line regions were especially apparent in the mutant (Fig. 3B), suggesting a weakening of the M-line structure, which is essential for maintenance of sarcomere integrity.

Abnormalities of muscle gene expression in *Mef2c* mutants. To determine whether the absence of *Mef2c* resulted in downregulation of myogenic bHLH transcription factors, which might cause the skeletal muscle abnormalities in SKM KO mice, we performed in situ hybridization for myogenin, Myf-5, and MyoD. Myogenic bHLH transcription factor expression patterns appeared to be unaltered in *Mef2c* SKM KO muscles at E9.5 and E12.5 (Fig. 4A) and E16.5 (data not shown). To provide a more quantitative analysis of bHLH expression in *Mef2c* mutant muscles, we analyzed the expression of myogenin, Myf-5, and MyoD in E12.5 wild-type and *Mef2c* SKM KO embryos by quantitative real-time PCR (Fig. 4B). Expression of myogenin and MyoD was slightly downregulated in *Mef2c*-deficient muscles.

To identify MEF2 target genes responsible for the *Mef2c* SKM KO phenotype, we performed expression profiling of skeletal muscle from wild-type and *Mef2c* SKM KO E18.5 hindlimbs. Using gene ontology analysis (55) of dysregulated transcripts, we analyzed whether certain pathways or biological processes were more sensitive to loss of *Mef2c* in skeletal muscle. This analysis revealed that the most significantly enriched biological processes, of downregulated genes, participate in muscle contraction (Fig. 4C). Down-regulated genes in this category were further analyzed by their annotated molecular function, demonstrating that the majority of these genes encode cytoskeletal proteins (Fig. 4D). Among the most dramatically downregulated genes were *myomesin 1* and *myomesin 2* (also referred to as *M protein*), which encode muscle-specific structural proteins that stabilize the sarcomere along the M line by forming an elastic (7), lattice structure that interacts with titin and myosin (2, 39, 40). This "elastic web" stabilizes muscles by diminishing thick filament displacement and by returning the sarcomere to its original state after contraction (1, 2). Myomesin proteins are expressed in all types of vertebrate striated muscle, and their importance is supported by their observed fixed expression ratio with myosin (2, 3). Moreover, myomesin proteins and the M line are crucial for sarcomere stability, since loss of M-line protein interaction with titin results in progressive sarcomere damage and lethality (43).

In addition to the *myomesin* genes, several additional structural, sarcomere, and sarcomere-associated gene products

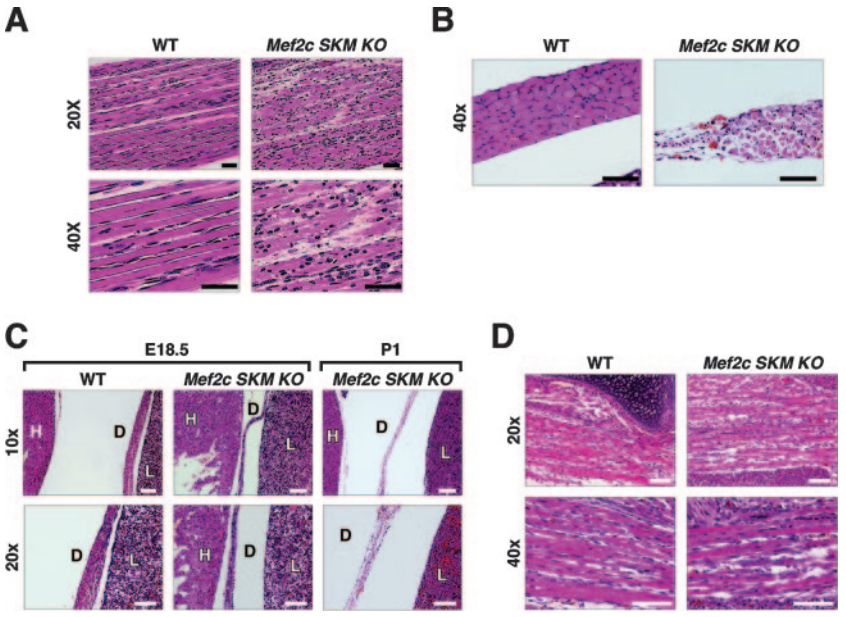


FIG. 2. Myofiber disarray in *Mef2c*-deficient muscles. Hind limb muscles and diaphragms of wild-type (WT) and *Mef2c* SKM KO (*Mef2c*$^{fl/-}$; *Myo-Cre*) mice are shown. (A) H&E staining of histological sections of representative hind limb muscle from P1 WT and *Mef2c* SKM KO mice (magnifications, ×20 and ×40; bars, 300 nm). (B) Thinned diaphragm and degenerated fibers in *Mef2c* SKM KO mice compared to WT littermates at P1 (magnification, ×40; bars, 100 nm). (C) Diaphragms from E18.5 *Mef2c* SKM KO mice are thinner than diaphragms from wild-type controls but less disorganized than *Mef2c* SKM KO P1 diaphragms (H, heart; D, diaphragm; L, liver; bars, 300 and 200 nm for magnifications of ×10 and ×20, respectively). (D) H&E staining of histological sections of representative hind limb muscle from E18.5 WT and *Mef2c* SKM KO mice at E18.5 (magnifications, ×10 and ×20; bars, 300 nm.). *Mef2c*-deficient muscles at E18.5 display minor defects in disorganization and fragmentation compared to those of WT littermates.

were misregulated in *Mef2c* SKM KO muscles including myozenin 1 and 2 (also termed calsarcin 2 and 1, respectively), actin, myosin, myotilin, and muscle creatine kinase (MCK) (Fig. 4E). MEF2 proteins were previously shown to be important for expression of thick filament proteins in vivo (24) but were only slightly downregulated in *Mef2c* SKM KO muscles. Myozenin 1 and 2 are Z-line-interacting proteins that are important stress sensors that link calcineurin with the sarcomere (21). Additionally, *myotilin*, which encodes an actin cross-linking protein necessary for sarcomere assembly (51), was downregulated in *Mef2c* SKM KO muscles.

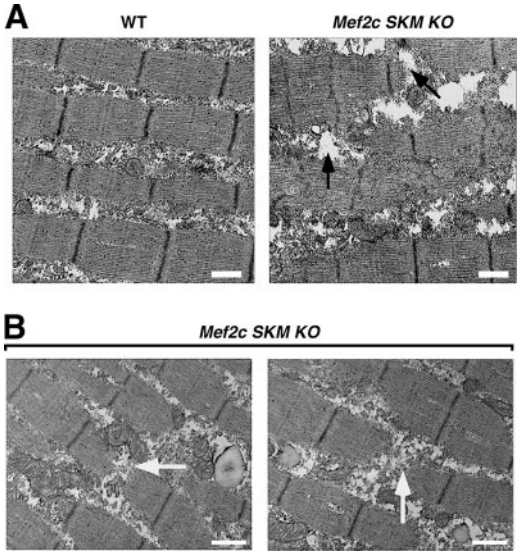


FIG. 3. Disorganized sarcomeres in *Mef2c* SKM KO muscles. (A) Hind limb muscle from P1 wild-type (WT) and *Mef2c* SKM KO (*Mef2c*$^{fl/-}$; *Myo-Cre*) mice were analyzed by electron microscopy. The sarcomeres in *Mef2c* mutant mice are disorganized and fragmented compared to those in WT littermates. Magnifications are shown on the left (magnification, ×30,000; bar, 500 nm). (B) Higher magnification of alternative regions reveal severed M lines as indicated by the arrows (magnification, ~×40,000; bar, 500 nm).

Transcripts encoding the bHLH transcription factors MyoD and myogenin were slightly downregulated at E18.5, as detected by quantitative real-time PCR, while *Myf5* transcript levels were unchanged (Fig. 4E). Known MEF2 target genes (e.g., *Bop*, *Srpk3* [*MSSK*], and *desmin*) were also slightly downregulated in *Mef2c*-deficient muscles (Fig. 4B and data not shown).

The *myomesin 1* and *2* genes are direct targets of MEF2C. Myomesin 1 and 2 play a crucial role in maintaining sarcomere organization (1), suggesting that their downregulation could be causal in the *Mef2c* SKM KO phenotype. We therefore searched the *myomesin* promoters for conserved MEF2 sites that might control their expression in skeletal muscle. As shown in Fig. 5A, ClustalW analysis revealed conserved consensus MEF2 sites located immediately upstream of both genes. In addition, the *myomesin 1* promoter contains a conserved MEF2 site directly adjacent to an E box, and this region confers transcriptional regulation to the *myomesin 1* gene (53).

The first ~1,000 bp and 700 bp of the *myomesin 1* and *2* promoters, respectively, were cloned into a luciferase reporter and found to be responsive to MEF2 when cotransfected into COS cells (Fig. 5B). Mutation of the MEF2 sites in both promoters abolished responsiveness to MEF2 (Fig. 5B). In addition, when C2C12 cells were transfected with these lucif-

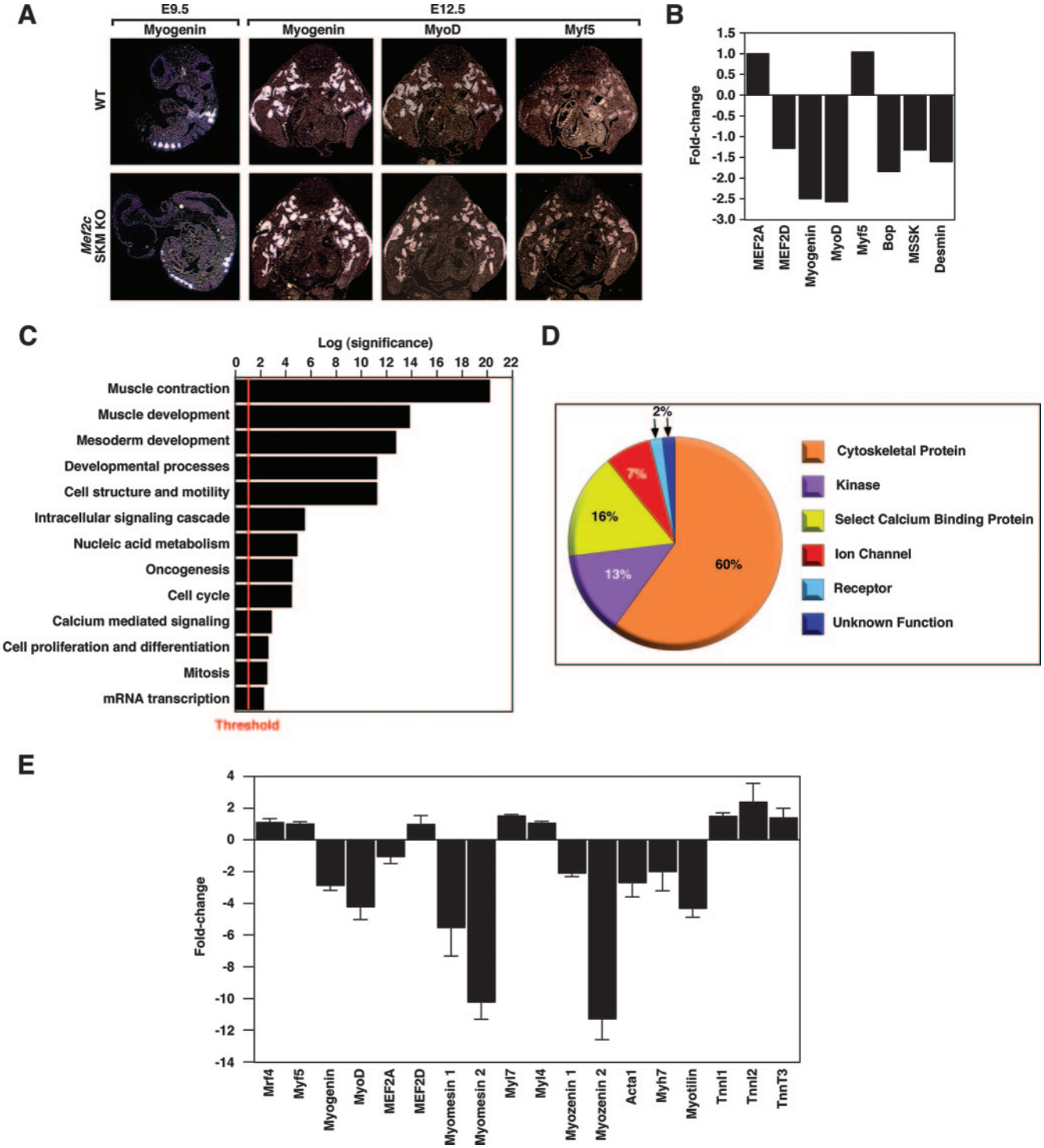
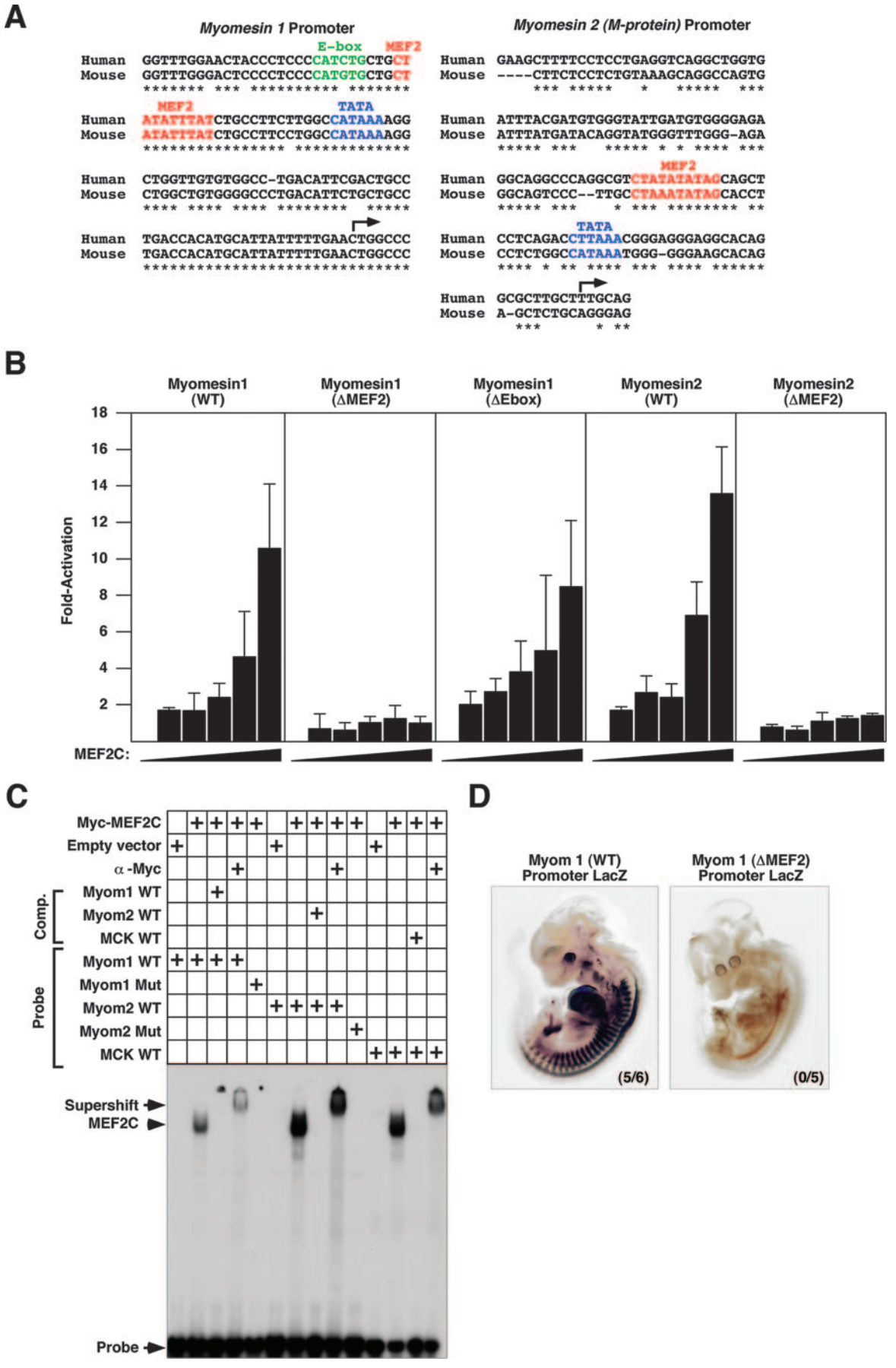


FIG. 4. Analysis of muscle gene expression in *Mef2c* mutants. (A and B) Expression of myogenin, MyoD, and Myf5 in wild-type (WT) and *Mef2c* SKM KO (*Mef2c*$^{fl/-}$; *Myo-Cre*) embryos by in situ hybridization at E9.5 (sagital sections) and E12.5 (transverse sections) (A) and quantitative real-time PCR of E12.5 WT and *Mef2c* SKM KO embryos (B). (C) Gene ontology analysis (PANTHER) of misexpressed genes in *Mef2c* SKM KO E18.5 hind limbs. Significantly ($P < 0.05$) enriched biological processes are shown. The $-\log$ (P value) is plotted with the threshold set to 1.3 [$-\log(0.05)$] (red line). (D) Molecular functions were assigned to the genes in the most significantly enriched biological process "muscle contraction" in panel C. (E) Validation of microarray targets and analysis of gene expression of structural candidate genes by quantitative real-time PCR. Error bars indicate standard deviations.


A
Myomesin 1 Promoter
E-box
MEF2
Human GGTTTGGAACTACCCTCCCCATCTGCTGCT
Mouse GGTTTGGGACTCCCCTCCCCATGTGCTGCT
MEF2
TATA
Human ATATTTATCTGCCTTCTTGGCCATAAAAGG
Mouse ATATTTATCTGCCTTCCTGGCCATAAAAGG
Human CTGGTTGTGTGGCC-TGACATTCGACTGCC
Mouse CTGGCTGTGGGGCCCTGACATTCTGCTGCC
Human TGACCACATGCATTATTTTTGAACTGGCCC
Mouse TGACCACATGCATTATTTTTGAACTGGCCC
Myomesin 2 (M-protein) Promoter
Human GAAGCTTTTCCTCCTGAGGTCAGGCTGGTG
Mouse ----CTTCTCCTCTGTAAAGCAGGCCAGTG
Human ATTTACGATGTGGGTATTGATGTGGGGAGA
Mouse ATTTATGATACAGGTATGGGTTTGGG-AGA
MEF2
Human GGCAGGCCCAGGCGTCTATATATAGCAGCT
Mouse GGCAGTCCC--TTGCCTAAATATAGCACCT
TATA
Human CCTCAGACCTTAAACGGGAGGGAGGCACAG
Mouse CCTCTGGCCATAAATGGG-GGGAAGCACAG
Human GCGCTTGCTTTGCAG
Mouse A-GCTCTGCAGGGAG
B
Myomesin1 (WT)
Myomesin1 (ΔMEF2)
Myomesin1 (ΔEbox)
Myomesin2 (WT)
Myomesin2 (ΔMEF2)
Fold-Activation
18
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MEF2C:
C
Myc-MEF2C
Empty vector
α -Myc
Comp.
Myom1 WT
Myom2 WT
MCK WT
Probe
Myom1 WT
Myom1 Mut
Myom2 WT
Myom2 Mut
MCK WT
Supershift
MEF2C
Probe
D
Myom 1 (WT) Promoter LacZ
Myom 1 (ΔMEF2) Promoter LacZ
(5/6)
(0/5)

erase constructs and allowed to differentiate, luciferase activity was observed with the wild-type *myomesin* promoter but was significantly reduced with the promoters containing a mutated MEF2 site (data not shown). Mutagenesis of the E box in the *myomesin 1* promoter reduced but did not eliminate luciferase activity (Fig. 5B).

DNA binding assays using extracts from COS cells transfected with a Myc-MEF2C expression plasmid confirmed that the MEF2 sites in the *myomesin 1* and *2* promoters were bona fide MEF2 sites (Fig. 5C). The MEF2 consensus sequences from the *myomesin 1* and *2* promoters bound MEF2C comparably to the canonical MEF2 site from the *MCK* enhancer (22) (Fig. 5C). This DNA-protein complex was abolished in the presence of excess competitor (unlabeled cognate DNA sequence) and was supershifted by an anti-Myc antibody, whereas a sequence containing a mutated MEF2 site was unable to compete for MEF2C binding (Fig. 5C).

To analyze the importance of the MEF2 site in the *myomesin 1* promoter in vivo, we generated transgenic mice with a *lacZ* reporter gene linked to the *myomesin 1* promoter. As seen in Fig. 5D, the wild-type *myomesin 1* promoter directed expression in the somites, limb muscles, and heart (Fig. 5D) at E11.5. Expression in all of these cell types was abolished by a mutation in the MEF2 site (Fig. 5D). These findings support the conclusion that MEF2C directly activates myomesin gene transcription in vitro and in vivo.

DISCUSSION

The results of this study show that skeletal muscle-specific deletion of *Mef2c* results in hypoplastic myofibers, disorganized sarcomeres, and defects of the M line that cause perinatal death. *Mef2c*-deficient muscles showed reduced expression of genes encoding important structural proteins, including myomesin and M protein, which are localized to the M line and maintain sarcomere integrity. Moreover, our results demonstrate that MEF2C directly regulates *myomesin* gene transcription. We conclude that *Mef2c* plays an essential role in the perinatal regulation of genes necessary for proper sarcomere assembly and maintenance of myofiber integrity.

Distinct functions of MEF2 factors in skeletal muscle. Given the early and specific expression of *Mef2c* in the skeletal muscle lineage and the ability of MEF2C to synergistically activate myofiber genes with members of the MyoD family, it is surprising that *Mef2c*-deficient myocytes undergo early steps of differentiation, including myofiber formation. It seems likely that other MEF2 proteins compensate for the loss of *Mef2c* during early stages of embryonic and fetal myogenesis. However, we should point out that we have also found that skeletal muscle deletion of both *Mef2c* and *Mef2d* does not exacerbate the *Mef2c* SKM KO phenotype (data not shown), raising the possibility that residual levels of *Mef2a* or *Mef2b* are adequate to support initial steps in muscle development.

Mef2c-deficient muscles display minor defects late in embryonic development but rapidly degenerate immediately after birth. The relatively late onset of the *Mef2c* mutant muscle phenotype, in which *Mef2c* was deleted with the Myo-Cre, is surprising considering that this transgene causes gene deletion at E8.5 in muscle. However, this timing may reflect the relative lack of contractility or weight-bearing stress on skeletal muscle before birth. Consistent with this, expression profiling of *Mef2c* SKM KO muscles revealed misregulated genes associated with muscle contraction and stress responsiveness. Therefore, although MEF2C is removed early in skeletal muscle development, the genes which it regulates (e.g., *myomesin*) function later to maintain sarcomere integrity and muscle function, possibly explaining why severe defects are not observed until birth. In addition, the lack of muscle defects in mice in which *Mef2c* is deleted later (~E18.5) demonstrates the importance of activation of these MEF2 targets early in development and their integration and association with the sarcomere. Moreover, this importance of MEF2C early in skeletal muscle development may be similar to the case for cardiac muscle, in which MEF2C is important early (29) but is dispensable later in development (56).

It is intriguing that the absence of MEF2C results in such a specific diminution of *myomesin* expression, despite the continued expression of *Mef2a* and *Mef2d*. We suggest two potential explanations for this finding. (i) The *myomesin* genes might be exquisitely sensitive to the level of MEF2 expression, irrespective of the isoform, such that residual *Mef2a* and *-d* cannot drive expression of these genes in the absence of *Mef2c*. (ii) MEF2C may be specifically required for *myomesin* expression because of a function not shared with the other MEF2 isoforms, perhaps mediated by a unique structural domain.

Recently, knockdown of *mef2c* and *mef2d* in zebrafish was reported to disrupt muscle function and sarcomere assembly as a result of diminished expression of thick filament proteins (24). In contrast, our results demonstrate that loss of *Mef2c* alone is sufficient to disrupt sarcomere assembly. The differences between the zebrafish and mouse phenotypes may reflect species-specific differences in MEF2C function or the lack of complete takeout of mef2c protein by morpholino knockdown

FIG. 5. *myomesin 1* and *2* are direct targets of MEF2C. (A) Alignment of mouse and human *myomesin 1* and *2* promoters using ClustalW identifies conserved MEF2 binding sites in close proximity to previously identified transcription start sites (arrows) (the MEF2 site [red], E box [green], and TATA box [blue] are labeled; asterisks mark conserved nucleotides). (B) MEF2C transactivates the *myomesin* promoters. Cotransfection of increasing amounts (0, 5, 10, 25, 50, and 100 ng) of a MEF2C expression plasmid with wild-type (WT) *myomesin 1* or *2* luciferase reporter constructs in COS cells results in significant activation of the reporter, which is not seen with the reporters containing a mutated MEF2 site (ΔMEF2) in the promoter. Mutation of the E box (ΔE-box) in the *myomesin 1* promoter does not abolish responsiveness to MEF2. Error bars indicate standard deviations. (C) Gel mobility shift assays were performed with labeled probes corresponding to the *myomesin 1*, *myomesin 2*, or *MCK* MEF2 consensus binding site and extracts of COS cells overexpressing Myc-MEF2C. The DNA-protein complex was supershifted in the presence of an anti-Myc antibody and was competed by excess unlabeled probe competitor. (D) Transgenic reporter mice harboring a *lacZ* cassette behind the wild-type *myomesin 1* promoter show β-galactosidase expression in somites, heart, and facial and limb muscle (E11.5). LacZ expression is abolished in transgenic mice when the conserved MEF2 site is mutated (ΔMEF2). Numbers indicate the fraction of F_0 transgenic embryos showing *lacZ* expression in the somites and heart.

in zebrafish. Consistent with the zebrafish study, muscles lacking *Mef2c* undergo early steps of muscle differentiation but do not mature properly. Interestingly, Hinits and Hughes reported that muscles from *mef2c/d* double knockdown zebrafish display a phenotype similar to that of mice lacking titin's M-line region (24). We show that *Mef2c* SKM KO muscles display M-line defects and that MEF2C directly regulates myomesin expression, which could explain the zebrafish phenotype.

Influence of genetic modifiers on the functions of *Mef2c* in skeletal muscle. Recently, we reported that mice with a skeletal muscle deletion of *Mef2c* in a 129/SvEv mixed genetic background were viable and showed a reduction in slow fibers (47). In contrast, the skeletal muscle deletion of *Mef2c* in a C57BL/6 mixed background shown here results in perinatal lethality with complete penetrance. These findings suggest that the activity of *Mef2c* in skeletal muscle development is sensitive to genetic modifiers, which are capable of modulating sarcomere structure and function. Notably, the cyto-architectural and mitochondrial defects observed in cardiac muscle of *Mef2a*-deficient mice are also highly sensitive to genetic modifiers (38).

MEF2 factors as multifunctional regulators of muscle gene expression. Considered together with other studies, it is now apparent that MEF2 factors play important roles in numerous steps of muscle development, including the control of myoblast differentiation and fusion (34, 42), maintenance of myofiber integrity as shown in the present study, and regulation of mitochondrial biogenesis and fiber type specification (47, 58). In each of these settings, MEF2 regulates distinct sets of downstream target genes, which likely reflects its ability to associate combinatorially with other coactivators and corepressors and to respond to upstream signaling pathways that vary in response to developmental, physiological and pathological cues. Understanding the molecular basis of target gene recognition and activation by different MEF2 isoforms in these various processes represents a fascinating and important problem for the future.

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