# Centronuclear myopathy in mice lacking a novel muscle-specific protein kinase transcriptionally regulated by MEF2

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**Myocyte enhancer factor 2 (MEF2) plays essential roles in transcriptional control of muscle development. However, signaling pathways acting downstream of MEF2 are largely unknown. Here, we performed a microarray analysis using *Mef2c*-null mouse embryos and identified a novel MEF2-regulated gene encoding a muscle-specific protein kinase, Srpk3, belonging to the serine arginine protein kinase (SRPK) family, which phosphorylates serine/arginine repeat-containing proteins. The *Srpk3* gene is specifically expressed in the heart and skeletal muscle from embryogenesis to adulthood and is controlled by a muscle-specific enhancer directly regulated by MEF2. *Srpk3*-null mice display a new entity of type 2 fiber-specific myopathy with a marked increase in centrally placed nuclei; while transgenic mice overexpressing *Srpk3* in skeletal muscle show severe myofiber degeneration and early lethality. We conclude that normal muscle growth and homeostasis require MEF2-dependent signaling by Srpk3.**

[*Keywords*: Myocyte enhancer factor 2; transcriptional regulation; serine arginine protein kinase (SRPK); Stk23/Srpk3; centronuclear myopathy]

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Skeletal muscle differentiation is cooperatively controlled by two families of transcription factors, the myogenic basic helix–loop–helix (bHLH) proteins and the myocyte enhancer factor 2 (MEF2) family of MADS domain proteins (Black and Olson 1998; Bailey et al. 2001; Pownall et al. 2002; Buckingham et al. 2003; Parker et al. 2003). Myogenic bHLH proteins, such as MyoD and myogenin, recognize a DNA sequence called an E box (CANNTG). Myogenic bHLH proteins associate and synergistically activate transcription with MEF2 factors, which bind to the A/T-rich DNA consensus [CTA-(A/T)$_4$-TA-G/A]. Additionally, myogenic bHLH proteins activate their own expression and the expression of MEF2, while MEF2 stimulates expression of myogenic bHLH protein genes and the *Mef2c* gene (Cserjesi and Olson 1991; Lassar et al. 1991; Edmondson et al. 1992; Cheng et al. 1993; Yee and Rigby 1993; Wang et al. 2001; Teboul et al. 2002; Dodou et al. 2003). Such auto- and cross-regulatory interactions establish a mutually reinforcing circuit to achieve myogenesis.

The essential role of MEF2 in muscle development was first shown in *Drosophila* in which a loss-of-function mutation in the single MEF2 ortholog *D-mef2* results in a complete block to differentiation of all muscle lineages: somatic, cardiac, and visceral (Bour et al. 1995; Lilly et al. 1995). In mice, the existence of four *Mef2* genes—*Mef2a*, *Mef2b*, *Mef2c*, and *Mef2d*—with overlapping expression patterns makes it more difficult to assess the roles of these factors individually (Black and Olson 1998). Mice homozygous for a *Mef2c*-null allele show embryonic lethality around embryonic day 9.5 (E9.5) caused by improper development of the heart (Lin et al. 1997). The mutant hearts do not undergo looping morphogenesis, the future right ventricle does not form, and

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a subset of cardiac muscle genes is not expressed. MEF2 has also been implicated in maintenance of the slow fiber phenotype of skeletal muscle, in the control of striated muscle energy metabolism, and in pathological remodeling of the adult heart in response to stress signaling (Black and Olson 1998; McKinsey et al. 2002).

In principle, MEF2 may regulate muscle-specific target genes directly, or it may act indirectly by controlling the expression of subordinate transcription factors or signaling molecules that act as intermediaries to connect MEF2 to downstream targets that themselves are not dependent on MEF2-binding sites in their *cis*-regulatory regions. Indeed, vast arrays of direct and indirect targets of MEF2 in skeletal muscle cells in culture were recently described (Blais et al. 2005).

In an effort to identify MEF2 target genes that serve as "downstream" effectors of MEF2 action during muscle development, we performed a microarray analysis using *Mef2c*-null embryos and identified a novel muscle-specific protein kinase Stk23/Srpk3, which is encoded by a direct MEF2 target gene. *Srpk3*-null mice display a new entity of centronuclear myopathy, while transgenic mice overexpressing *Srpk3* in skeletal muscle show severe myofiber degeneration. These findings reveal an essential role for serine arginine protein kinase (SRPK)-mediated signaling in muscle growth and homeostasis downstream of MEF2 transcription factors.

## Results

### Srpk3*: a novel muscle-specific protein kinase gene*

In an attempt to identify novel MEF2-regulated genes, we compared the gene expression profiles of hearts from wild-type and *Mef2c*-null mouse embryos by an RNA microarray analysis. Because *Mef2c*-null embryos die around E9.5 (Lin et al. 1997), we used hearts from wild-type and null embryos at E9.0 prior to overt cardiac demise. Among the genes that were dysregulated in the *Mef2c* mutants, we found the expression of *Stk23* to be significantly decreased in the *Mef2c*-null hearts. The down-regulation of *Stk23* expression in the *Mef2c*-null hearts was confirmed by RT–PCR (Fig. 1a). Residual expression of *Stk23* in the mutants may reflect the presence of other MEF2 factors that partially compensate for MEF2C.

*Stk23* was described in an analysis of human chromosomal DNA methylation as a potential protein-kinase-encoding gene (Grunau et al. 2000), but its expression profile and function have not been described. Structural comparison with various protein kinases clearly indicated that Stk23 possesses a bipartite kinase domain with high sequence similarity to the SRPK family kinases, Srpk1 and Srpk2 (Fig. 1b,c; Gui et al. 1994; Bedford et al. 1997; Kuroyanagi et al. 1998; Wang et al. 1998). As expected, Stk23 efficiently phosphorylated known SRPK

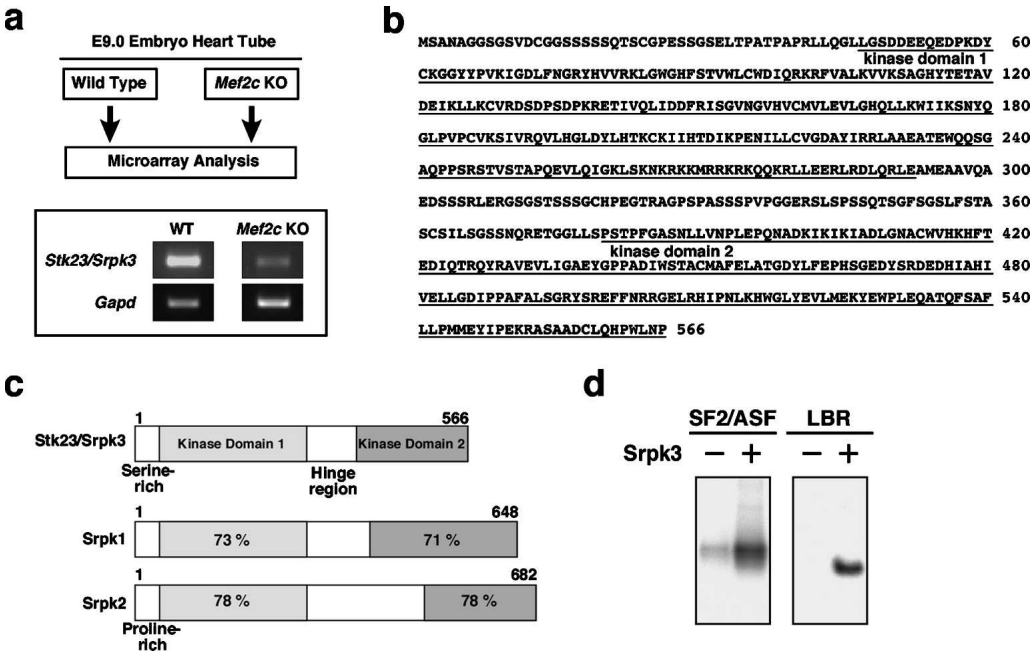


**Figure 1.** Identification of Stk23/Srpk3 as a novel SRPK. (*a*) Microarray analysis was performed using the hearts of E9.0 *Mef2c*-null and wild-type embryos. Down-regulation of the *Stk23/Srpk3* expression in the *Mef2c*-null hearts was confirmed by RT–PCR. (*Gapd*) Glyceraldehyde-3-phosphate dehydrogenase expression as a control. (*b*) Amino acid structure of mouse Stk23/Srpk3. Srpk3 is a 566-amino-acid protein that contains a bipartite kinase domain (underlined), N terminus, and a hinge region. (*c*) Schematic representation of mouse SRPK family kinases. Srpk3 shows high sequence similarity to Srpk1 and Srpk2 in the kinase domain, but not in the N terminus and hinge region. The percentages in the boxes are identities of Srpk1 and Srpk2 to Srpk3 at an amino acid level. Srpk3 and Srpk2 have serine- and proline-rich sequences in the N terminus, respectively. Amino acid numbers are also shown. (*d*) Phosphorylation assays using SR domain proteins. Srpk3 phosphorylated SF2/ASF and the N-terminal region of Lamin B Receptor (LBR) in vitro.

substrates, the splicing factor SF2/ASF, and Lamin B Receptor in in vitro kinase assays (Fig. 1d; Nikolakaki et al. 1997; Koizumi et al. 1999; Yeakley et al. 1999; Takano et al. 2002). Therefore, we propose a functionally descriptive nomenclature, Srpk3, for Stk23.

In situ hybridization with mouse embryos showed *Srpk3* to be specifically expressed in the developing heart, somites, and skeletal muscles, in contrast to broad expression of Srpk1 and Srpk2 (Fig. 2a). Expression in the heart was first detected in the heart tube, immediately following expression of MEF2C, and expression in the somite myotomes was observed by E9.5. Within the embryonic heart, *Srpk3* is expressed throughout the atrial and ventricular chambers (Fig. 2a). *Srpk3* is highly expressed in the heart and skeletal muscle in adult mice (Fig. 2b). Muscle-specific expression was also observed in human fetal and adult tissues (Supplementary Fig. 1).

### *Regulation of the Srpk3 promoter by MEF2*

To examine if *Srpk3* is transcriptionally regulated by MEF2, we performed reporter analyses in vitro and in vivo. MEF2C and MyoD significantly activated the expression of a luciferase reporter controlled by a 2.9-kb DNA fragment encompassing the 5′ *Srpk3* promoter region, and serial deletion analysis revealed that a 0.4-kb fragment was sufficient for the activation by MEF2C and MyoD (Fig. 3a). Indeed, the 0.4-kb fragment contains a MEF2-binding site and two E boxes (Fig. 3b). Although there are also two GATA sites in the 0.4-kb fragment, GATA proteins did not enhance *Srpk3* promoter activity in luciferase assays (data not shown). An oligonucleotide probe, containing the MEF2 site and E boxes in the *Srpk3* promoter, was bound by MEF2C as well as by myogenin and its ubiquitous bHLH dimerization partner, E12 (Fig. 3c). Mutation of the MEF2 site in the context of the 2.9- or 0.4-kb fragment significantly decreased the response to MEF2C and MyoD in luciferase assays (Fig. 3d), suggesting that MEF2 is an obligate activator of the *Srpk3* promoter.

### *Transcriptional control of Srpk3 during myogenic differentiation in vitro*

We next examined the expression of *Srpk3* mRNA during differentiation of C2C12 myoblasts, which are triggered to differentiate and form myotubes upon transfer to medium with low serum (Lu et al. 2000). *Srpk3* mRNA was not expressed in C2C12 myoblasts but was markedly induced together with embryonic and perinatal myosin genes, *Myh3* and *Myh8*, during myogenesis (Fig. 3e).

Consistently, expression of a luciferase reporter controlled by the *Srpk3* promoter was markedly enhanced during differentiation of C2C12 cells (Fig. 3f). Mutation of the MEF2 site almost abolished the promoter activity, suggesting that *Srpk3* expression is activated upon myogenic differentiation directly by MEF2 proteins.

### *Transcriptional control of Srpk3 in vivo*

We further examined the transcriptional regulation of *Srpk3* expression in transgenic mice. The 2.9-kb and 0.4-kb *Srpk3* regulatory regions fused to the *HSP68* basal promoter (Fig. 4a) directed the expression of a *LacZ* reporter in the embryonic heart and somites (Fig. 4b,c), recapitulating the muscle-specific expression pattern of the endogenous gene. The 0.4-kb fragment was also sufficient to direct muscle-specific expression without the *HSP68* minimal promoter (data not shown). Analyses of embryo sections confirmed that *LacZ* expression was observed throughout cardiac muscle walls, including the outflow tract, and in the somite myotomes (data not shown).

Consistent with the luciferase reporter analyses, the mutation of the MEF2-binding site in the context of the 0.4-kb fragment completely abolished *LacZ* expression in the heart and somites. Ten out of 16 transgenic embryos harboring the transgene with the mutant MEF2 site showed no LacZ staining (Fig. 4d), and the remaining showed weak ectopic expression that was not specific to

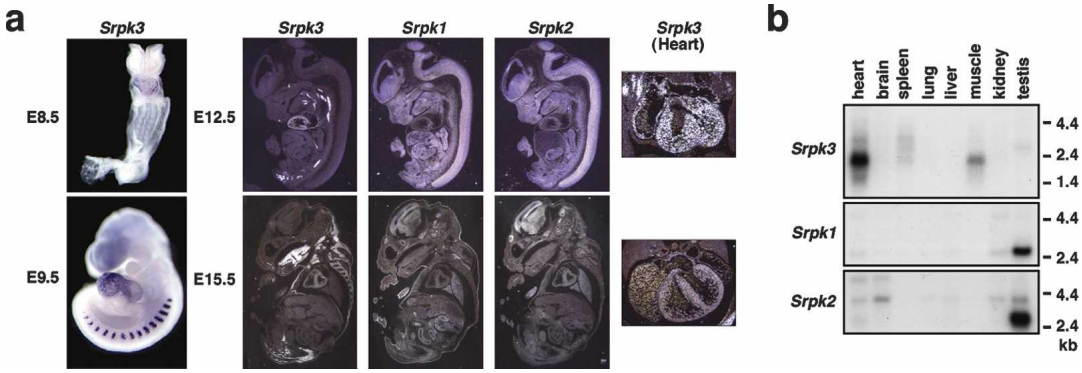


**Figure 2.** Muscle-specific expression of Srpk3. (*a*) Expression of the SRPK family in mouse embryos. Whole-mount and section in situ hybridization. *Srpk3* is expressed exclusively in the developing heart, somites, and embryonic skeletal muscle. *Srpk1* and *Srpk2* are widely expressed in embryonic tissues, with enrichment in the neural tube and brain. Enlargements of the heart from each stage are shown to the *right*. (*b*) Expression of the SRPK family in adult mice. *Srpk3* is specifically expressed in the heart and skeletal muscle, with a faint expression in the spleen. Testis-enriched expression of *Srpk1* and *Srpk2* is also shown *below*. With longer exposure, lower levels of *Srpk1/-2* expression were observed ubiquitously.

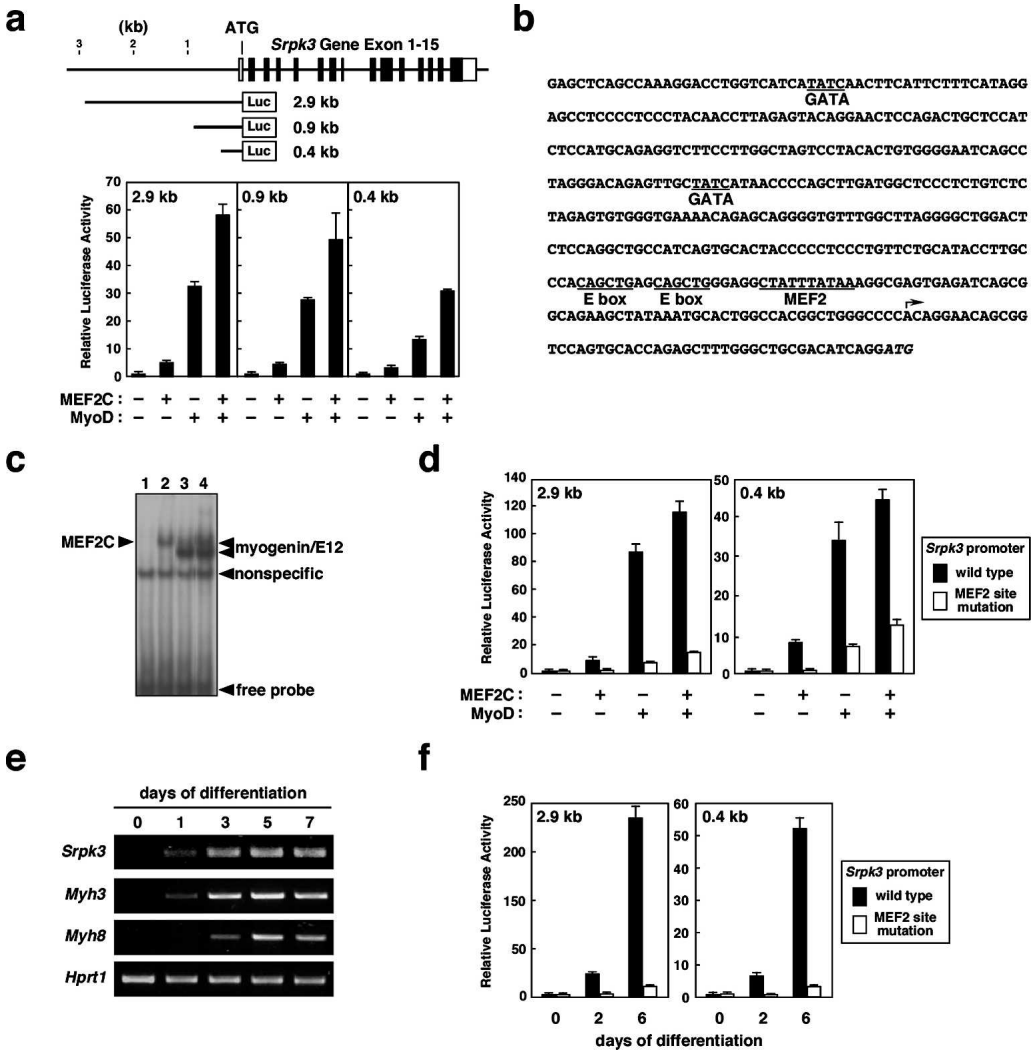


**Figure 3.** MEF2-dependent muscle-specific transcription of Srpk3. (*a*) The structure of the mouse *Srpk3* gene with schematics of the luciferase constructs and the results of luciferase assays are shown. Filled and open boxes indicate the exons for protein-coding and noncoding regions, respectively. MEF2C and MyoD strongly activate luciferase reporter expression controlled by the *Srpk3* enhancer/promoter in C2C12 myoblasts. The 0.4-kb fragment is sufficient for the response. (*b*) Sequence of the 0.4-kb minimal muscle enhancer/promoter of *Srpk3*. The MEF2-binding site, E boxes, and GATA-binding sites are underlined. The putative transcriptional start site estimated by the most 5′-end of Srpk3 cDNA clones is indicated by an arrow. The translational start site (*ATG*) is italicized. (*c*) MEF2C and the myogenin/E12 complex bind to the fragments encompassing the MEF2 site and E boxes in the minimal muscle enhancer of *Srpk3*. Electrophoretic mobility shift assay. (Lane *1*) Control. (Lane *2*) MEF2C. (Lane *3*) Myogenin and E12. (Lane *4*) MEF2C, myogenin, and E12. (*d*) Mutation of the MEF2-binding site in the context of the 2.9- or 0.4-kb *Srpk3* fragments significantly impairs the response to MEF2C and MyoD in luciferase assays. (*e*) *Srpk3* expression is activated by 1 d after stimulation during myogenic conversion of C2C12 cells. Expression patterns of embryonic and perinatal myosins, *Myh3* and *Myh8*, respectively, are also shown. (*Hprt1*) Hypoxanthine guanine phosphoribosyl transferase 1 as a control. (*f*) Activity of the *Srpk3* luciferase reporters is markedly stimulated during myogenic conversion of C2C12 cells, while mutation of the MEF2-binding site almost abolishes it.

the heart and somites. In contrast, mutation of the two E boxes did not result in a significant decrease of *LacZ* expression in the heart and somites (Fig. 4e). We conclude that *Srpk3* is a direct transcriptional target of MEF2 proteins in vivo.

### *Muscle defects in Srpk3 transgenic mice*

SRPKs are known to regulate mRNA splicing and the assembly of nuclear lamina proteins, by phosphorylating SR splicing factors and Lamin B Receptor (Gui et al. 1994; Bedford et al. 1997; Nikolakaki et al. 1997; Kuroyanagi et al. 1998; Wang et al. 1998; Koizumi et al. 1999; Yeakley et al. 1999; Takano et al. 2002). RNAi experiments showed that SRPK is essential for germline development in *Caenorhabditis elegans* (Kuroyanagi et al. 2000), but in vivo functions of the SRPK family have not been examined in mammals.

To analyze the effects of excessive SRPK activity in striated muscles, we generated transgenic mice overex-

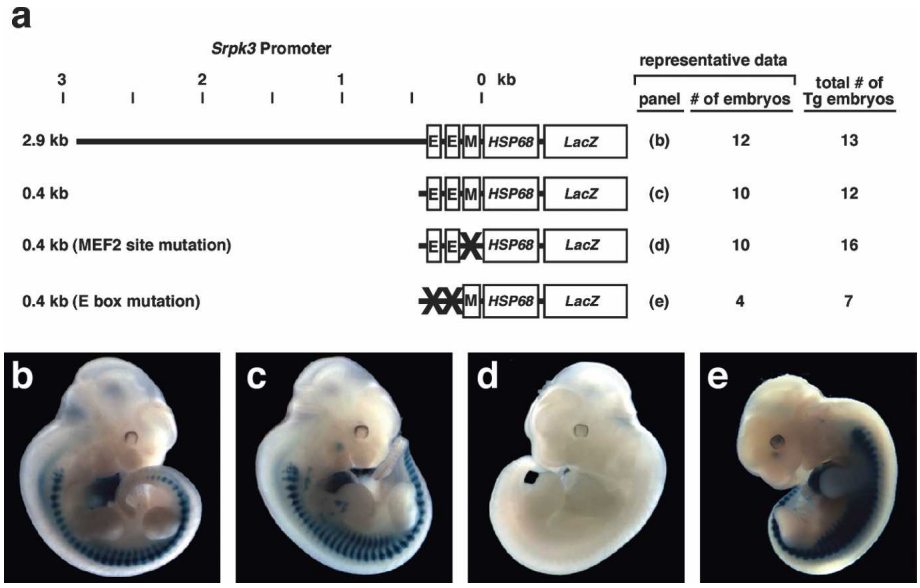


**Figure 4.** MEF2-dependent transcriptional control of Srpk3 in mouse embryos. (*a*) Schematic representation of *Srpk3-HSP68-LacZ* reporter constructs and results of F0 transgenic mouse analysis. The number of embryos that showed representative expression patterns and total transgenic embryos are shown on the *right*. Representative results are shown in panels *b–e*. The 2.9- and 0.4-kb fragments drove *LacZ* expression in the heart and somites in E11.5 embryos (*b*,*c*, respectively). The MEF2 site mutation (*d*), but not the E-box mutations (*e*), abolished the *LacZ* activity both in the heart and somites.

pressing Srpk3 in skeletal muscle, using the *muscle creatine kinase* (*MCK*) gene promoter and enhancer (Sternberg et al. 1988). Multiple F0 transgenic mice died prematurely with severe muscle wasting and growth retardation. Two F0 mice survived to sexual maturity and were fertile, but the F1 transgenic mice derived from these founders died at 2–8 wk of age, which prevented us from establishing transgenic mouse lines. However, those transgenic mice shared muscle defects with myofiber disarray and degeneration (Fig. 5a–c). These mice also showed myocyte regeneration characterized by an increase in centrally placed nuclei (Fig. 5c) and activation of embryonic gene markers (Fig. 5d). Although these characteristics are similar to those of muscular dystrophy, Evans Blue dye injection did not show abnormalities of sarcolemmal integrity (data not shown), and the expression of dystrophin and related sarcolemmal proteins was intact (Supplementary Fig. 2), suggesting that excess SRPK activity causes muscle degeneration by a different mechanism.

### *Centronuclear myopathy in Srpk3-null mice*

To further elucidate the significance of Srpk3 in vivo, we generated *Srpk3*-null mice (Fig. 6a–c). The mutation we introduced into the gene deleted parts of exons 1 and 5 and all of exons 2, 3, and 4, which encode the N-terminal portion of Kinase Domain 1 (Fig. 6a). Northern analysis showed the complete absence of *Srpk3* transcripts in the heart and skeletal muscle of mutant mice (Fig. 6c).

Since the *Srpk3* gene is located on the X chromosome, wild-type and hemizygously null male mice were examined in this study. *Srpk3*-null mice were viable to adulthood, but displayed apparent defects in skeletal muscle growth. The mass of various skeletal muscle groups was significantly smaller than that of wild-type littermates at 1 mo (Fig. 6d) and 3 mo of age (data not shown). Histological analysis revealed a marked increase in centrally placed nuclei and a disorganized intermyofibrillar network, occasionally with ring-fiber-like structure or spheroid bodies (Fig. 6e,f; data not shown).

The pathological characteristics of *Srpk3*-null muscle were different from those of *MCK–Srpk3* transgenic mice. Although an increase in centrally placed nuclei is frequently indicative of muscle regeneration in response to disease or injury (Garry et al. 2000; Carpenter and Karpati 2001; Emery 2002), *Srpk3*-null mice did not show up-regulation of embryonic/perinatal muscle markers, which is typically seen during muscle regeneration (Fig. 6g). There were no signs of inflammation, neutrophil infiltration, or fibrosis in *Srpk3*-null mice (Fig. 6e), nor was there an increase in apoptotic cell death detectable by TUNEL staining (Fig. 6f). The serum creatine kinase activity indicative of sarcolemmal leakage also did not significantly increase in *Srpk3*-null mice (wild type, 409 ± 192; null, 486 ± 291; IU/L, $n = 10$), and Evans Blue dye injection did not show abnormalities of sarcolemmal integrity (Fig. 6f).

The pathological characteristics of the skeletal muscle in *Srpk3*-null mice, especially an increase of centronucleated myofibers without apparent myocyte death, are reminiscent of human centronuclear myopathy (Carpenter and Karpati 2001; Jeannet et al. 2004). However, centrally placed nuclei were observed only in type 2 fi-

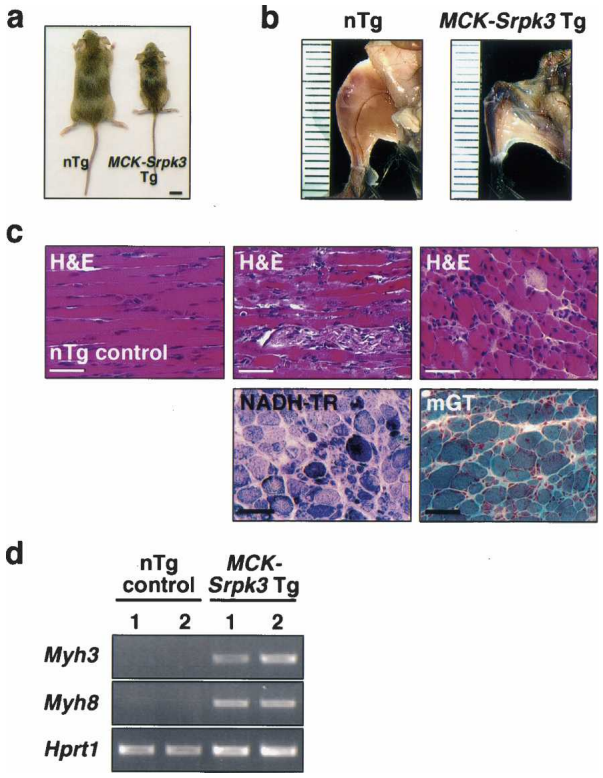


**Figure 5.** Muscle defects in *MCK–Srpk3* transgenic mice. (*a*,*b*) *Muscle creatine kinase* (*MCK*)–*Srpk3* transgenic (Tg) mice showed significant growth retardation and muscle defects compared with nontransgenic (nTg) littermates at 1 mo of age. Bar in panel *a* represents 1 cm. (*c*) Histological analysis of *MCK–Srpk3* Tg mice showed severe muscle degeneration with myofiber disarray, increase in centrally placed nuclei, and fibrosis. Hematoxylin & eosin (H&E), NADH-tetrazolium reductase (NADH-TR), and modified Gomori Trichrome (mGT) staining of gastrocnemius muscle. H&E staining of nTg control is also shown. Bar, 100 µm. (*d*) Muscle regeneration markers, *Myh3* and *Myh8*, are up-regulated in the skeletal muscle of *MCK–Srpk3* Tg mice. Similar results were obtained using F1 mice derived from two F0 founders of *MCK–Srpk3* transgenic mice.

bers, but not in type 1 fibers, in *Srpk3*-null mice (Fig. 6f), in contrast to type 1 fiber specific abnormalities associated with human centronuclear myopathy (Laporte et al. 1996; Carpenter and Karpati 2001; Jeannet et al. 2004). These results suggested that *Srpk3*-null mice displayed a new entity of centronuclear myopathy.

### *Alteration of muscle gene expression in* Srpk3*-null mice*

To further characterize muscle abnormalities of *Srpk3*-null mice, we examined the expression of various muscle genes in type 2 fiber-enriched tibialis anterior muscles. A subset of type 1/slow fiber-enriched genes showed increased expression in *Srpk3*-null mice; however, the lack of activation of two representative slow-twitch fiber markers (Serrano et al. 2001; McCullagh et al. 2004), *Slc2a4/Glut4* and *Myoglobin* (*Mb*), suggests that the altered gene expression patterns are not a simple reflection of fiber type switching (Fig. 7a). Consistently, expression of type 2/fast fiber-enriched genes (Fig. 7a) as well as the proportions of type 1 and 2 fibers were not altered in *Srpk3*-null mice (wild type: type 1, 14.6%; type 2, 85.4%, $n = 700$; *Srpk3* null: type 1, 17.9%; type 2, 82.1%, $n = 800$).

Although there was a significant decrease of muscle mass in *Srpk3*-null mice, most atrophy-related genes (McKinnell and Rudnicki 2004) did not show alteration of expression in *Srpk3*-null mice. However, we observed a significant increase in expression of the *cartilage intermediate layer protein* (*Cilp*) gene (Fig. 7b), which encodes a secreted inhibitor of transforming growth factors and insulin-like growth factors (Lorenzo et al. 1998; Johnson et al. 2003; Seki et al. 2005). Given the importance of growth factor signaling in muscle diseases (McKinnell and Rudnicki 2004), up-regulation of *Cilp* expression may contribute, at least in part, to myopathy and muscle growth defects in *Srpk3*-null mice.

Calcium homeostasis plays critical roles in the regulation of skeletal muscle growth and contractility, and abnormalities in calcium handling have been implicated in various muscle diseases (MacLennan et al. 2003). Interestingly, mRNA expression of *Sarcolipin* (*Sln*) markedly increased in *Srpk3*-null mice (Fig. 7). Sln shares structural and functional similarity with Phospholamban, and they physically associate with and inhibit sarco(endo)plasmic reticulum calcium ATPase (Tupling et al. 2002; MacLennan et al. 2003). Forced expression of Sln in skeletal muscle represses sarcoplasmic reticulum calcium uptake and impairs contractile function (Tupling et al. 2002). Despite its important function, the expression patterns of *Sln* in muscle diseases have not been previously studied. We also observed that *Sln* expression increased in dystrophic Mdx mice as well as in cardiotoxin muscle injury models (Garry et al. 2000; Carpenter and Karpati 2001; Emery 2002; data not shown). How *Sln* expression is regulated in those conditions is unknown, but up-regulation of *Sln* expression may be a common characteristic of muscle diseases.

## Discussion

MEF2 is a key regulator of cardiac and skeletal muscle development as well as remodeling of adult muscles in response to physiologic and pathologic signals (Black and Olson 1998; McKinsey et al. 2002). While MEF2 has been shown to regulate a wide range of muscle structural genes, few other target genes that might mediate its actions in muscle have been identified (Kuisk et al. 1996; Anderson et al. 2004; Phan et al. 2005). The results of the present study identify Srpk3 as a novel muscle-specific protein kinase, which is directly regulated by MEF2 and is essential for normal growth and homeostasis of skeletal muscle.

### *Abnormalities in skeletal muscle resulting from dysregulation of Srpk3*

Striated muscles are highly sensitive to the level of Srpk3 expression. Overexpression of Srpk3 in skeletal

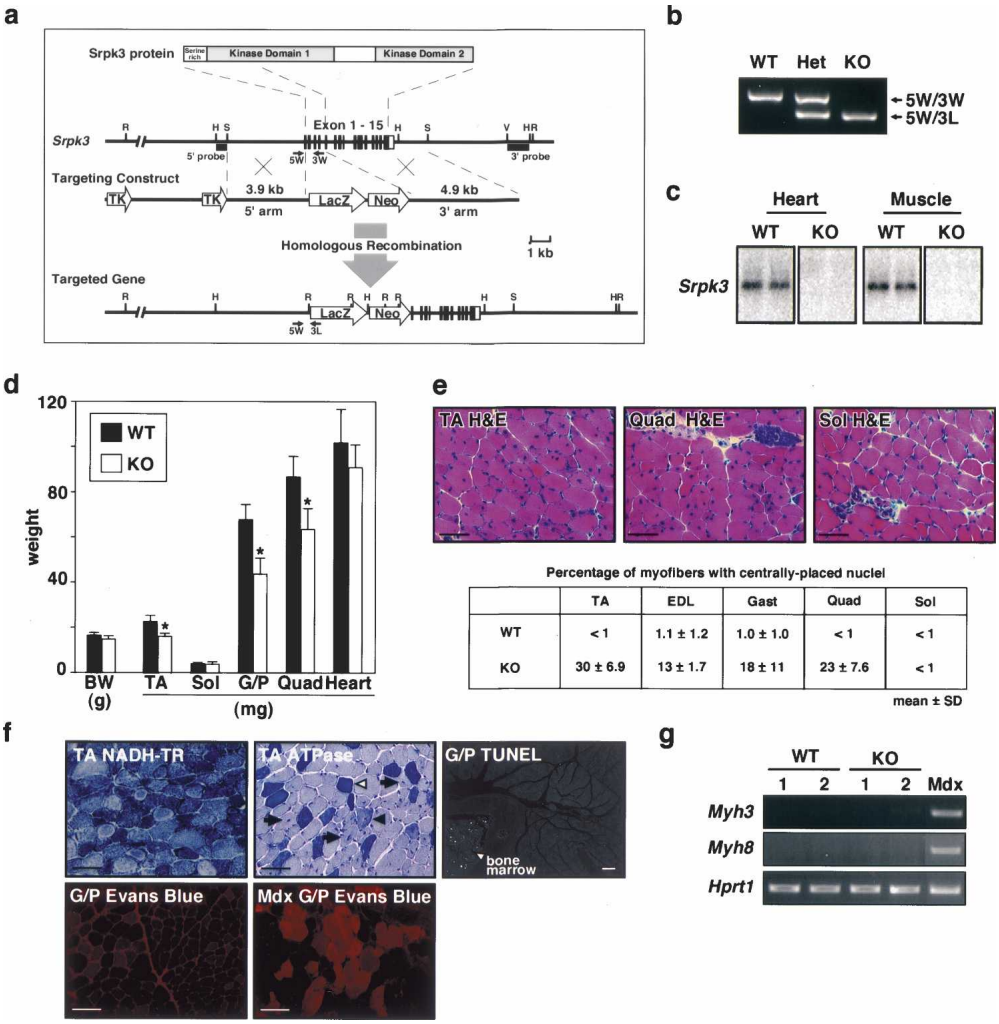


**Figure 6.** Centronuclear myopathy of *Srpk3*-null mice (*a*) Gene targeting strategy. The mouse *Srpk3* gene is shown with the targeting vector and the targeted allele. Parts of the exons 1 and 5, and the entire exons 2, 3, and 4 were replaced with the *LacZ–neomycin resistance* gene (*Neo*) cassette. Genotypes were determined by Southern blot analysis and PCR. The positions of the 5′ and 3′ Southern probes and PCR primers (5W, 3W, 3L) are shown. (TK) Thymidine kinase; (R) EcoRI; (H) HindIII; (S) SpeI; (V) EcoRV. (*b*) A representative result of PCR genotyping. (WT) Wild-type mouse; (Het) female heterozygous mouse; (KO) male hemizygously null mouse. (*c*) The *Srpk3* mRNA expression is not detectable in the heart and skeletal muscle of *Srpk3*-null mice by Northern blot analysis. (*d*) The weight of various skeletal muscle groups significantly decreased in *Srpk3*-null mice. (BW) Body weight. Mean ± SD; $n = 6$; (*) $p < 0.01$. (*E*) H&E staining of various muscle groups of *Srpk3*-null mice. Bars, 100 µm. Percentages of centrally placed nuclei are also shown ($n = 400$–600). (*f*) The NADH-TR, ATPase and TUNEL staining, and the result of Evans Blue injection of *Srpk3*-null mice. ATPase activity stains type 1 fibers dark blue (open triangle), type 2B fibers blue (filled triangle), and type 2A fibers light blue (arrows). Cells in the bone marrow, but not skeletal myocytes, showed positive signals in TUNEL staining. Evans Blue injection showed no apparent abnormality of sarcolemmal integrity in *Srpk3*-null mice, in contrast to Mdx dystrophic mice shown as a positive control. Bar, 100 µm. Muscle groups: (EDL) extensor digitorum longus; (G/P) gastrocnemius and plantaris; (Quad) quadriceps; (Sol) soleus; (TA) tibialis anterior. (*g*) Expression of muscle regeneration markers, *Myh3* and *Myh8*, is not activated in *Srpk3*-null mice, in contrast to Mdx mice. RT–PCR analysis.

muscle causes severe myofiber abnormalities, while overexpression in the heart results in dilated cardiomyopathy characterized by chamber dilatation, reduced contractility, and disease marker expression (M. Arnold, M. Nakagawa, H. Hamada, O. Nakagawa, J.A. Richardson, and E.N. Olson, unpubl.). Conversely, targeted deletion of the *Srpk3* gene results in a unique form of skeletal myopathy with a marked increase in centrally placed nuclei. The histological characteristics of these mice share similarity with those in human centronuclear myopathy, a congenital myopathy characterized by the presence of numerous centronuclear myofibers (Laporte et al. 1996; Carpenter and Karpati 2001; Jeannet et al. 2004).

Central placement of myonuclei occurs in various pathological conditions and commonly accompanies the regeneration process following myofiber degeneration and death (Garry et al. 2000; Carpenter and Karpati 2001; Emery 2002). In response to muscle injury, quiescent muscle progenitor cells, called satellite cells, are acti-

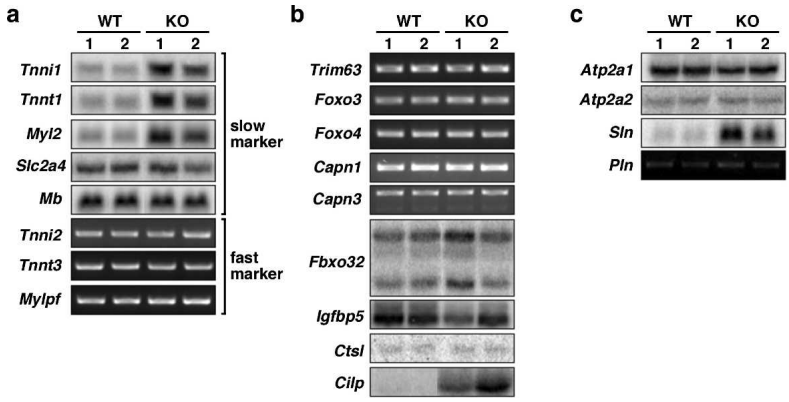


**Figure 7.** Expression profiles of muscle genes in *Srpk3*-null mice Expression of various muscle genes in the tibialis anterior muscle of *Srpk3*-null mice (KO) and wild-type control (WT). Ethidium bromide staining of RT–PCR products or phosphorimages of Northern blot hybridization are shown. (*a*) Slow- and fast-twitch myofiber markers. (*b*) Atrophy-related proteins. (*c*) Molecules related to calcium handling. At least three mice for each genotype were examined, and two representative results are shown. Gene names: (Atp2a1) ATPase, $Ca^{++}$ transporting, cardiac muscle, fast twitch 1 or Serca1; (Atp2a2) ATPase, $Ca^{++}$ transporting, cardiac muscle, slow twitch 2 or Serca2; (Capn1/-3) Calpain 1/-3; (Cilp), cartilage intermediate layer protein; (Ctsl) Cathepsin L; (Fbxo32) F-box-only protein 32, MAFbx or Atrogin-1; (Foxo3/-4) Forkhead box o3/o4; (Igfbp5) insulin-like growth factor-binding protein 5; (Mb) myoglobin; (Myl2) myosin, light polypeptide 2, regulatory, cardiac, slow; (Mylpf) myosin light chain, phosphorylatable, fast skeletal muscle; (Pln) Phospholamban; (Slc2a4) solute carrier family 2 (facilitated glucose transporter), member 4 or Glut4; (Sln) sarcolipin; (Tnni1) Troponin I, skeletal, slow 1; (Tnni2) Troponin I, skeletal, fast 2; (Tnnt1) Troponin T1, skeletal, slow; (Tnnt3) Troponin T3, skeletal, fast; (Trim63) tripartite motif-containing 63 or MuRF1.

vated and differentiate into new myofibers with centrally placed nuclei as are also seen in newly formed muscle fibers during embryonic development. However, in contrast to muscular dystrophy models, *Srpk3*-null mice did not show histological signs of myocyte death or reactivation of early developmental markers, which typically accompanies regeneration. Instead, the skeletal muscle of *Srpk3*-null mice may retain partially immature characteristics, although myofiber differentiation in these mice apparently progresses beyond the stages with embryonic and perinatal myosin expression. The conclusion that centronuclear myopathy in *Srpk3* mutant mice reflects a developmental abnormality is also supported by the absence of signs of muscle degeneration and regeneration, such as muscle cell death, myofiber disarray, or neutrophil infiltration, at early ages (data not shown).

The only gene shown to be involved in human centronuclear myopathy is *myotubularin 1* (*MTM1*), which encodes a ubiquitously expressed dual-specificity phosphatase (Laporte et al. 1996, 2003; Buj-Bello et al. 2002). Mutations of *MTM1* cause an X-linked form of centronuclear myopathy, characterized by an immature myotube-like appearance of skeletal muscle (Carpenter and Karpati 2001; Laporte et al. 2003). Null mutation of the *Mtm1* gene also causes centronuclear myopathy in mice (Buj-Bello et al. 2002). Muscle growth defects in *Mtm1*-null mice are more severe than those of *Srpk3*-null mice, and centrally placed nuclei are observed predominantly in type 1 fibers. However, these two models share similar features, such as the proportions of centronuclear myofibers and the lack of inflammation, apoptosis, and sarcolemmal disruption. The Myotubularin substrates that are related to skeletal muscle abnormalities remain unidentified. Perhaps there is cross-talk of signaling pathways downstream of Srpk3 and Myotubularin. The phenotype of *Srpk3* null mice also raises the question whether mutations in the *SRPK3* gene might be responsible for human myopathies that have not yet been ascribed to a specific gene.

### *Cellular functions of SRPK in skeletal muscle*

The SRPK family of protein kinases is highly conserved among species; Sky1p and Dsk1 in yeast, SPK-1 in *C. elegans*, SRPK in *Drosophila*, and Srpk1 and Srpk2 in mice and humans (Gui et al. 1994; Bedford et al. 1997; Kuroyanagi et al. 1998, 2000; Tang et al. 1998; Wang et al. 1998; Siebel et al. 1999). SRPKs phosphorylate serine/arginine (SR)-rich domain proteins and modulate their protein–protein interactions and intracellular localization. For example, unphosphorylated SR splicing factors associate with SRPK in the cytoplasm and, once phosphorylated, dissociate from SRPK and are translocated to the nucleus (Koizumi et al. 1999; Yeakley et al. 1999).

In this regard, it is interesting to note that abnormal mRNA splicing has been implicated in a variety of muscle diseases (Maniatis and Tasic 2002), and cardiac-specific deletion of the SR splicing factor genes, *Sfrs1* and *Sfrs2*, causes dilated cardiomyopathy in mice (Ding et al. 2004; Xu et al. 2005). Additionally, splicing defects of the tyrosine phosphatase-like gene, *Ptpla*, lead to centronuclear myopathy in dogs (Pele et al. 2005). SRPKs also phosphorylate Lamin B Receptor, an integral protein in the inner nuclear membrane or nuclear lamina (Nikolakaki et al. 1997; Ye et al. 1997; Takano et al. 2002). Mutations of the genes encoding the nuclear lamina proteins, Emerin or Lamin A/C, cause Emery-Dreifuss type muscular dystrophy (Wilson 2000). Thus, it is tempting to speculate that defects of mRNA splicing or nuclear lamina assembly contribute to the muscle abnormalities in *Srpk3*-null and *Srpk3* transgenic mice.

It should also be pointed out that proteins without typical SR-rich domains have recently been shown to serve as SRPK substrates (Daub et al. 2002). Although *Srpk3* expression is equivalent in type 1- and type 2-enriched muscles (data not shown), there is apparent fiber type specificity of the muscle pathology in *Srpk3*-null mice, suggesting the involvement of Srpk3 in muscle functions that are important in type 2 fibers, such as

glycolytic metabolism. The abnormal muscle gene expression profiles of *Srpk3*-null mice suggest roles of Srpk3 in growth factor signaling and calcium homeostasis. Identification of muscle-enriched SRPK substrates will provide further insights into functions of Srpk3 in muscle development and disease.

### *MEF2 as a nexus of kinase signaling pathways*

A variety of kinase signaling pathways have been shown to augment MEF2 activity (McKinsey et al. 2002). Signaling by p38 MAP kinase, for example, results in phosphorylation of the transcription activation domain of MEF2 and consequent enhancement of transcriptional activity (Han et al. 1997), while the MAP kinase ERK5 interacts directly with MEF2 and serves as a transcriptional coactivator (Kato et al. 1997; Yang et al. 1998; Kasler et al. 2000). In addition, signaling by calcium/calmodulin-dependent kinase and protein kinases C and D induces MEF2 activity by releasing the repressive influence imposed by class II histone deacetylases (McKinsey et al. 2000; Vega et al. 2004). The results of this study, which show that MEF2 is an obligate activator of *Srpk3* expression, point to MEF2 as a nexus between upstream and downstream kinase signaling pathways that control muscle development and function.

## Materials and methods

### Mef2c-*null embryos, microarray analysis, and cloning of Srpk3 cDNA*

Since *Mef2c*-null embryos die around E9.5 (Lin et al. 1997), the hearts from the wild-type and *Mef2c*-null embryos were collected prior to overt cardiac demise at E9.0. Microarray analysis was performed using 1 μg of total RNA as previously described (Belbin et al. 2002). The data were queried for difference of signal intensity between two samples, reproducibility in dye swap arrays, and signal intensity compared with local background. A clone that showed decreased signals in the hybridization with *Mef2c*-null embryo-derived RNA probes was identical to a part of mouse *Stk23*/*Srpk3* cDNA sequence (NM_019684). Full-length *Srpk3* cDNA fragments were isolated by screening of an embryonic heart cDNA library (Stratagene), using partial cDNA fragments obtained from the EST resources as a probe. Full-length cDNA fragments of human *SRPK3* were also obtained from the EST resources.

### *Cell culture*

C2C12 myoblasts were maintained in the growth medium containing 10% fetal calf serum, and the plasmid transfection was performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Myogenic differentiation of C2C12 cells was triggered by the transfer to the differentiation medium containing 2% horse serum (Lu et al. 2000). For the luciferase assays during the course of differentiation, C2C12 cells were transfected with the plasmids in growth medium, and myogenic differentiation was stimulated 2 d after the transfection.

### *Srpk3 transgenic mice*

Transgenic mice overexpressing Flag-tagged Srpk3 were generated using the *MCK* promoter/enhancer (Sternberg et al. 1988). Genotyping was performed by Southern blot analysis, and the skeletal muscle-specific expression of Flag-Srpk3 was confirmed by Western blot analysis.

### Srpk3-*null mice*

A BAC clone containing the *Srpk3* gene was obtained by screening of a 129s6/SvEvTAC mouse genomic BAC library (BACPAC Resources). The targeting vector was linearized and electroporated into mouse embryonic stem cells of 129Sv origin. Correctly targeted embryonic stem cell clones, as identified by Southern blotting using both 5′ and 3′ probes, were injected into blastocysts isolated from C57BL/J mice. Chimeras obtained from these blastocyst injections were bred to obtain heterozygous mice that carry the targeted *Srpk3* locus in their germline. Genotyping was performed by Southern blot analysis and PCR (primers: 5W, 5′-AGGTCTTCCTTGGCTAGTCCTACACTGTGG-3′; 3W, 5′-TAGTCCTTAGGGTCTTCCTGTTCCTCATC-3′; and 3L, 5′-CCATGGTGGATCCTGAGACTGGGGAATTC-3′). *Srpk3*-null mice in the pure 129s6/SvEvTAC background and the mixed 129s6/SvEvTAC-C57BL6/J background showed identical skeletal muscle pathology.

### *In situ hybridization*

Whole-mount and radioactive section in situ hybridization was performed as described (Nakagawa et al. 1999), using *Srpk1/-2/-3* RNA probes. For each gene, identical results were obtained using two probes that were prepared using different cDNA fragments.

### *Northern blot analysis*

Northern blot analysis of the SRPK family genes was performed on mouse and human poly(A)$^+$ RNA blots (Clontech), as described (Nakagawa et al. 1999). Northern blot analysis of muscle genes was performed using total RNA of the tibialis anterior muscle of *Srpk3*-null and wild-type mice. cDNA fragments for probe preparation were prepared by RT–PCR or were obtained from the EST resources. Detailed information of the cDNA probes is available upon request.

### *RT–PCR*

RT–PCR was performed using Superscript II reverse transcriptase (Invitrogen) and Advantage2 DNA polymerase (Clontech). Primer sequences are available upon request.

### *Luciferase and LacZ reporter analyses*

Three different *Srpk3* genomic DNA fragments, which had the identical 3′-ends at the translational start site, were ligated into a luciferase reporter plasmid, pGL3 basic (Promega). The genomic DNA fragments were also ligated into a promoter-less *LacZ* reporter plasmid or a *LacZ* reporter plasmid containing the *HSP68* minimal promoter sequence (Wang et al. 2001). Mutations were introduced by PCR into a MEF2-binding site (wild-type, 5′-GGCTATTTATAAAG-3′; mutant, 5′-GGCTAGGGCTAAAG-3′) and E boxes (wild-type, 5′-CAGCTG-3′; mutant, 5′-ACGCGT-3′) in the *Srpk3* regulatory region of the reporter plasmids. Luciferase assays were performed in C2C12 cells, and *LacZ* reporter assays of F0 transgenic mouse embryos were per-

formed as previously described (Nakagawa et al. 2000; Wang et al. 2001).

*Electrophoretic mobility shift assay*

The cell lysates containing MEF2C, myogenin, and/or E12 were prepared from COS1 cells transfected with combinations of expression plasmids. In vitro binding analysis was performed as previously described (Wang et al. 2001), using oligonucleotide fragments that contained a MEF2-binding site and E boxes (underlined) in the *Srpk3* promoter (5′-CTTGCCCACAGCTGAG CAGCTGGGAGGCTATTTATAAAGGCGAG-3′). The oligonucleotide fragments with a mutated MEF2-binding site (5′-GGCTAGGGCTAAAG-3′) and those with mutated E boxes (5′-ACGCGT-3′) did not show binding by MEF2C and the myogenin/E12 complex, respectively (data not shown).

*In vitro phosphorylation assays*

Myc-tagged proteins of full-length SF2/ASF (Koizumi et al. 1999) and the N terminus of Lamin B Receptor (Ye et al. 1997) were prepared by in vitro transcription and translation using TNT reticulocyte lysate system (Promega). Myc-Srpk3 and the control sample prepared using the empty plasmid vector were also prepared by the TNT reaction. Proteins were immunoprecipitated using rabbit polyclonal anti-Myc antibody (Santa Cruz), and in vitro kinase reaction was performed with [$\gamma$-$^{32}$P]ATP at 30°C.

*Histological analysis of skeletal muscle*

Histological staining of skeletal muscles, myofiber typing by the ATPase staining, TUNEL staining, and the Evans Blue dye injection were performed as previously described (Woods and Ellis 1996; Wu et al. 2000; Lu et al. 2002; Kanagawa et al. 2004).

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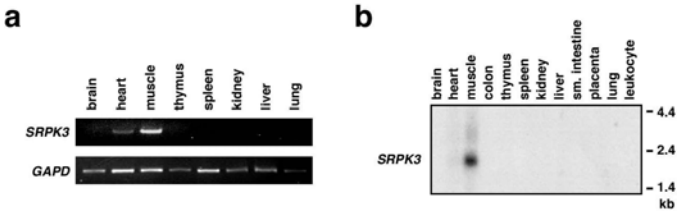
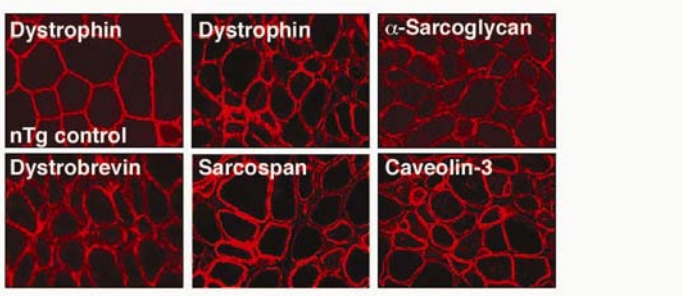



***Supplementary Figure 1 Expression of human SRPK3 in fetal and adult organs.***

(a) Human *SRPK3* expression is observed in the heart and skeletal muscle in human fetus. Glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) expression is shown as a control. (b) The *SRPK3* expression was abundant in adult skeletal muscle. The expression in the adult heart was lower, but detectable with longer exposure of Northern blot.




***Supplementary Figure 2 Expression of sarcolemmal proteins in Srpk3 transgenic mice.***

Expression of Dystrophin, Dystrobrevin, α-Sarcoglycan, Sarcospan, and Caveolin-3 does not show abnormality in *MCK-Srpk3* transgenic mice. The Dystrophin expression in the nontransgenic (nTg) littermate is also shown as a control.