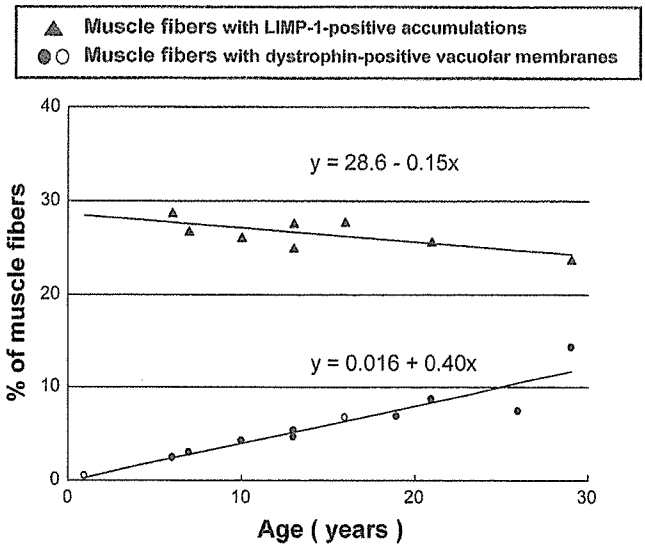

FIGURE 3. Relationship between age of the patients with Danon disease and number of muscle fibers with vacuoles highlighted with dystrophin or LIMP-1 on immunohistochemistry. The open circles show the only patient who had 2 muscle biopsies. The muscle fibers (circles) with intracytoplasmic vacuoles surrounded by dystrophin immuno-positive membrane (AVSFs) increased with age ($r = 0.936$). The muscle fibers (triangles) with overexpression of LIMP-1 showed a slight decrease with age ($r = 0.353$). r, Pearson's linear correlation coefficient.

autophagic materials encircled by membranes with basal lamina along the luminal side.

The proportion of muscle fibers with AVSFs increased with age in Danon disease and LAMP-2-deficient mice. In contrast, muscle fibers with LIMP-1-positive autolysosomal accumulations existed even in young patients and decreased slightly with age. It is most likely that the formation of these autolysosomal accumulations, which are clusters of autophagic vacuoles seen on electron microscopy, is a primary change in muscle fibers of Danon disease and related AVMs. The formation of peculiar membranes with sarcolemmal features around the autophagic vacuoles is hence a secondary phenomenon. Since muscle symptoms progress slowly in Danon disease, the development of muscle symptoms might be associated more closely with the formation of the unusual autophagic vacuoles rather than directly with the deficiency of LAMP-2.

LAMP-1 is the autosomal paralogous counterpart of LAMP-2 and both are thought to protect lysosomal membrane and cytoplasm from proteolytic enzymes within the lysosomes. LAMP-2 is tissue-specific but unlike LAMP 1, which is ubiquitously expressed, its expression is likely to be specifically regulated (26). Inhibition of LAMP-1 function results in failure of fusion of lysosomal and plasma membranes and therefore impaired exocytosis (27), a process usually by which cytoplasmic debris in the autophagosomes are extruded out from the cell through the sarcolemma (28). We therefore assume that LAMP-2 deficiency might likewise be related to dysregulation of exocytosis, leading to the development of the

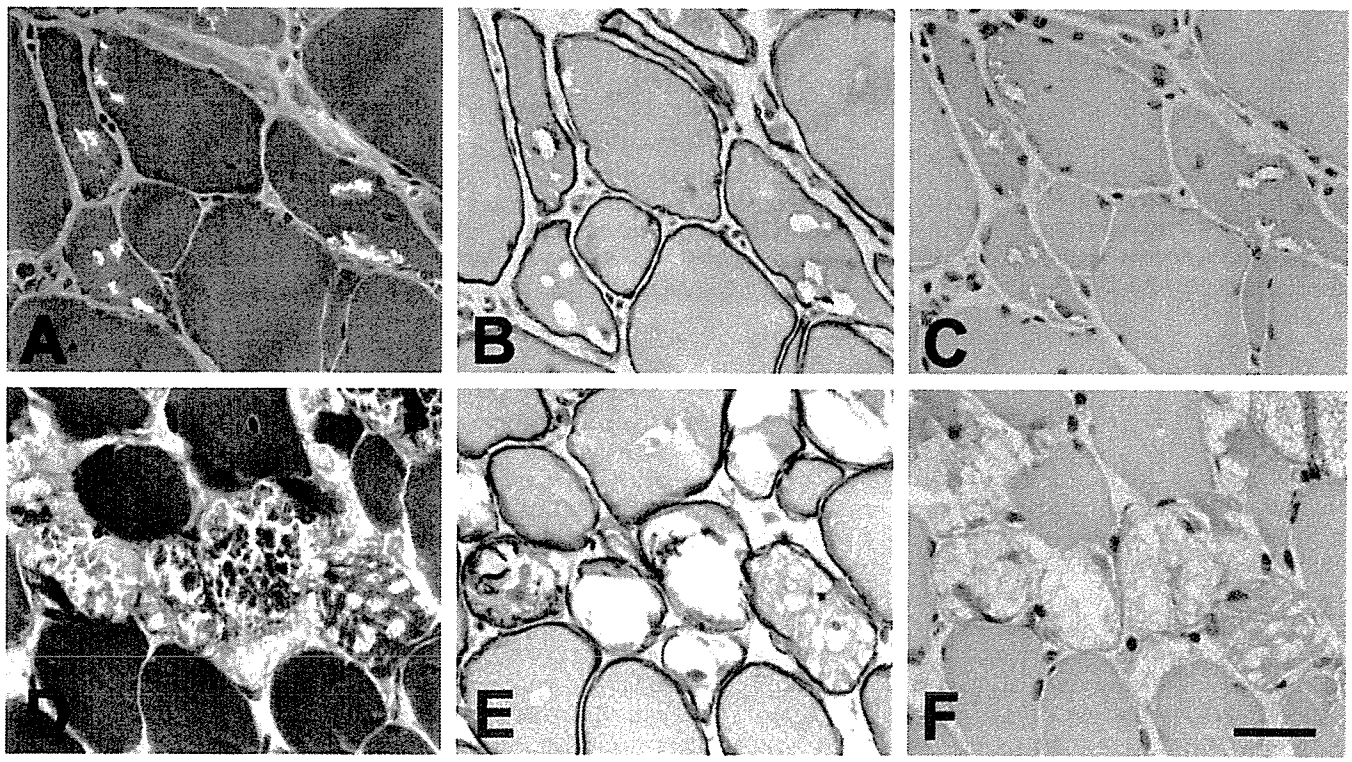

FIGURE 4. Transverse serial sections of muscle of patient with DMRV/HIBM **(A–C)** and with childhood AMD **(D–F)**. Only a few rimmed vacuoles in DMRV/HIBM showed presence of dystrophin. In AMD, dystrophin is present on some vacuolar materials. However, no vacuolar membranes have AChE activity in DMRV/HIBM and AMD. **(A, D)**: Gomori-trichrome stain; **(B, E)**: AChE stain; **(C, F)**: immunohistochemistry against dystrophin with DAB. Scale bar = 30 μm.

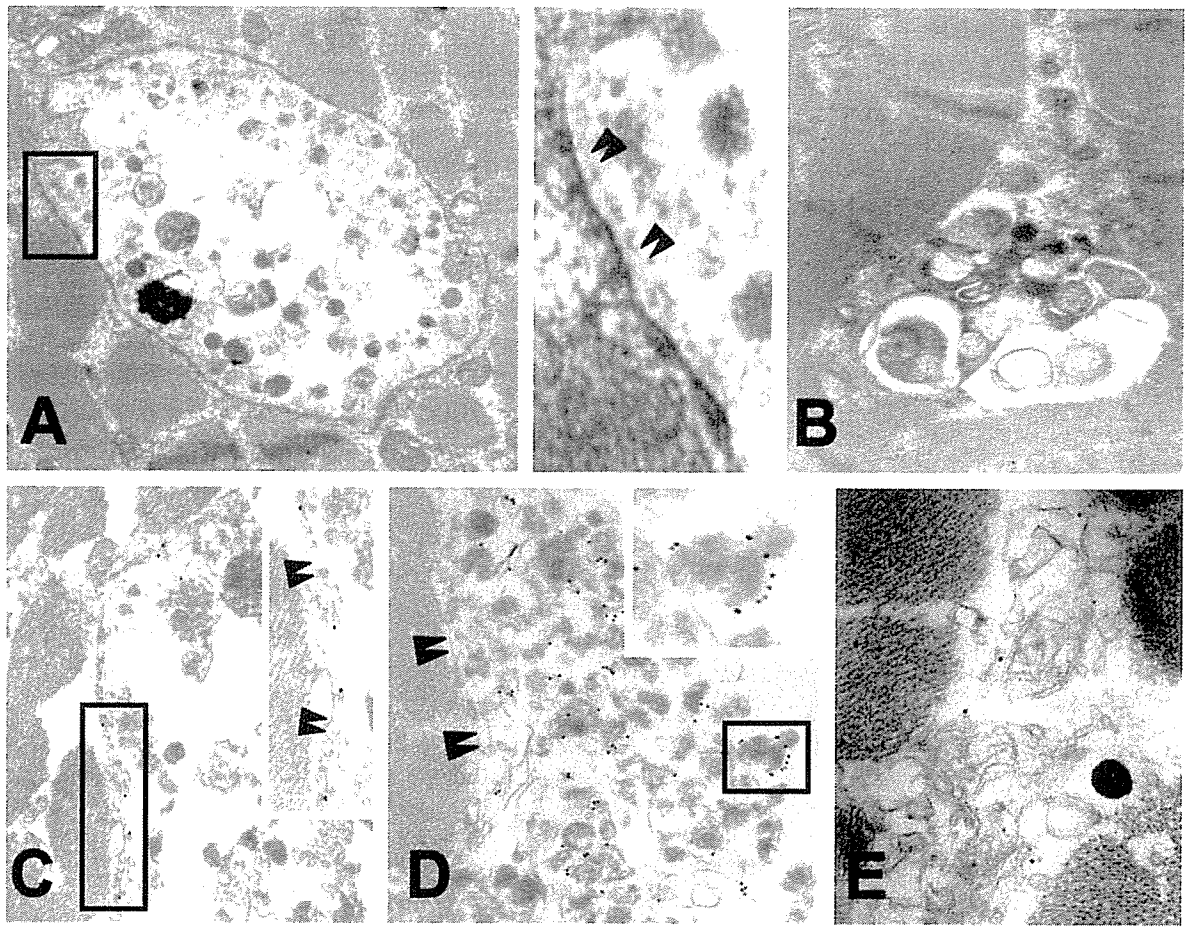

FIGURE 5. Electron micrograph in muscle from Danon disease patient. Scattered in the muscle fibers were clusters of autophagic vacuoles **(A)** containing cytoplasmic debris, electron dense material, and myeloid bodies. Some of these clusters were encircled by a membrane with basal lamina (paired arrowheads) on its luminal side, while other clusters are not limited by a membrane **(B)**. Electron immunohistochemistry after single labeling with dystrophin or LIMP-1 antibody shows localization of the proteins in autophagic vacuoles **(C–E)**. In the clusters with membranes, that is, the AVSF **(A)**, the immunogold particles show dystrophin **(C)** along the vacuolar membrane (paired arrowheads), and the immunogold particles show LIMP-1 **(D)** around autophagic material inside autophagic vacuoles. In contrast, in the clusters not surrounded by membranes, immunogold particles show LIMP-1 around autophagic materials with absence of dystrophin **(E)**. Original magnifications: **(A)** 15,000×; **(B)** 18,000×; **(C)** 20,000×; **(D)** 18,000×; **(E)** 30,000×.

unusual autophagic vacuoles with unique sarcolemmal features.

TfR and LDL-R are found in the membranes of recycling endosomes. In contrast, Rab5 and VAMP-7 are present in the membranes of early and late endosomes (29). We revealed the presence of all of these proteins in the fibers with autophagic vacuoles, indicating that in addition to the lysosomal system, the endosomal system is activated in Danon disease and related AVMs. Interestingly, VAMP-7 was increased in nonvacuolated fibers without autolysosomal accumulations, suggesting that maturation to late endosomes could prevent the formation of the unique vacuolar membranes.

Most of the vacuolar membranes were closed and were not connected to the sarcolemma in Danon disease. The autolysosomes containing cytoplasmic debris are therefore seen to be entrapped within the lumen of the vacuoles, and as such can be possibly considered to be extracellular space. Together with the observations that most AVSF did not accumulate in the subsarcolemmal region but were scattered in the cytoplasm, our findings suggest that the unique vacuolar membranes may be formed in situ in cytoplasm by a mechanism other than indentation of sarcolemma. One hypothesis is that the vacuolar membrane with basal lamina might be produced around clusters of autolysosomes (Fig. 6). The membranes surrounding the autophagic vacuoles might have originated from the lysosomal membrane or the isolation membrane that elongates and develops into the membrane of autophagosome (30), or is formed in situ and entirely de novo. If the vacuolar membranes are formed within the muscle fibers, it is compatible with the observation that the vacuolar membranes lack collagens IV and VI because collagens are thought to be produced mainly in the interstitium. Further studies are still necessary to understand the mechanism of the formation of these peculiar vacuolar membranes.

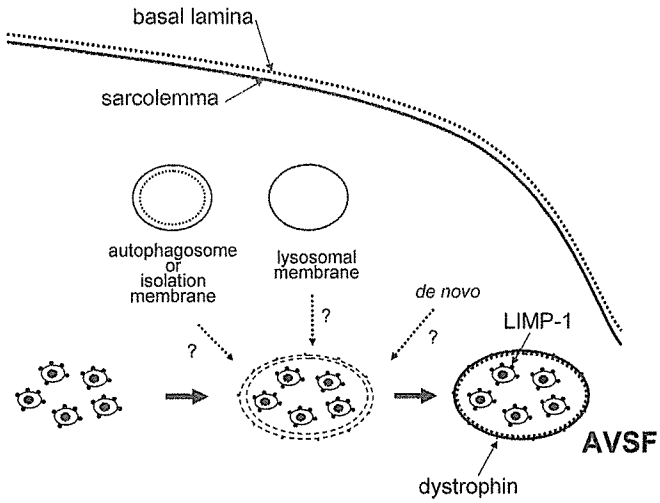

FIGURE 6. Schematic diagram of autophagic vacuoles in muscle fiber of patients with Danon disease. The membranes of the AVSFs were closed and were not connected to the sarcolemma. We suggest that the unique vacuolar membranes may be formed in situ in cytoplasm by a mechanism. One hypothesis is that the vacuolar membrane with basal lamina might be produced around clusters of autolysosomes, as illustrated.

In XMEA, vacuolar membranes have not been reported to have AChE activity (6). However, the strong similarity of the pathologic characteristics to Danon disease naturally raised the question of whether AChE activity is present in the vacuolar membranes in XMEA. Indeed, our Japanese patient with probable XMEA showed AChE activity in the vacuolar membranes. Therefore, Danon disease, XMEA, infantile AVM, and adult-onset AVM with multiorgan involvement share a common pathologic feature, namely, AVSF with AChE activity in the vacuolar membranes. Nevertheless, XMEA, infantile AVM, and adult-onset AVM are genetically different from Danon disease, as indicated by the presence of LAMP-2, which is absent in Danon disease. The observation that some features are not seen in Danon disease, like the presence of multilayered basal lamina and the deposition of C5b-9 over the surface of the muscle fiber, raise a possibility that some of these diseases might be allelic to XMEA, albeit different clinical phenotypes.

The autophagic vacuoles in AMD and rimmed vacuoles were reported to occasionally show presence of dystrophin. Nevertheless, these vacuoles are distinct from the AVSF seen in Danon disease and related AVMs, because the frequency of the vacuoles with sarcolemmal features is much less and most of sarcolemmal proteins are not consistently present in the vacuolar membranes of AMD and the rimmed vacuolar myopathies. Moreover, the activities of AChE and NSE were never found in the vacuolar membranes of these myopathies. In addition, on electron microscopy, the vacuolar membranes with basal lamina, such as those seen in Danon disease and related AVMs, were not found in AMD, DMRV/HIBM, or SIBM. According to the classification of De Bleecker et al, the AVSF may belong to type 1 vacuoles as the boundaries of type 1 vacuoles reacted for laminin, β-spectrin, and dystrophin (31). However, type 1 vacuoles were thought to be open to extracellular space and arise from invagination of the sarcolemma. Moreover, the membranes of AVSF have not only many sarcolemmal and extracellular proteins but also AChE activity, and may be formed in situ in cytoplasm as described above. Therefore, we think that the AVSF are a new, highly specific subtype of type 1 vacuoles.

Although the mechanism of their production still remains a mystery, overall, AVSF with AChE activity delineate Danon disease, XMEA, infantile AVM, and adult-onset AVM with multiorgan involvement from other AVMs. Most likely, this unique pathologic finding will probably be found in more diseases and therefore the list of AVMs in this group is likely to expand.

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REFERENCES

1. Nishino I, Fu J, Tanji K, et al. Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). Nature 2000;406:906–10
2. Danon MJ, Oh SJ, DiMauro S, et al. Lysosomal glycogen storage disease with normal acid maltase. Neurology 1981;31:51–57
3. Sugie K, Yamamoto A, Murayama K, et al. The clinicopathological features of genetically confirmed Danon disease. Neurology 2002;58: 1773–78
4. Nishino I, Yamamoto A, Sugie K, Nonaka I, Hirano M. Danon disease and related disorders. Acta Myologica 2001;20:120–24
5. Murakami N, Goto Y, Itoh M, et al. Sarcolemmal indentation in cardiomyopathy with mental retardation and vacuolar myopathy. Neuromuscul Disord 1995;5:149–55
6. Kalimo H, Savontaus ML, Lang H, et al. X-linked myopathy with excessive autophagy: A new hereditary muscle disease. Ann Neurol 1988;23: 258–65
7. Yamamoto A, Morisawa Y, Verloes A, et al. Infantile autophagic vacuolar myopathy is distinct from Danon disease. Neurology 2001;57:903–5
8. Kaneda D, Sugie K, Yamamoto A, et al. A novel form of autophagic vacuolar myopathy with late-onset and multiorgan involvement. Neurology 2003;61:128–31
9. Nishino I. Autophagic vacuolar myopathies. Curr Neurol Neurosci Rep 2003;3:64–69
10. Tanaka Y, Guhde G, Suter A, et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 2000;406: 902–6
11. Saftig P, Tanaka Y, Lullmann-Rauch R, von Figura K. Disease model: LAMP-2 enlightens Danon disease. Trends Mol Med 2001;7:37–39
12. Sugie K, Koori T, Yamamoto A, et al. Characterization of Danon disease in a male patient and his affected mother. Neuromuscul Disord 2003;13: 708–11
13. Nishino I, Noguchi S, Murayama K, et al. Distal myopathy with rimmed vacuoles is allelic to hereditary inclusion body myopathy. Neurology 2002;59:1689–93

14. Takemitsu M, Nonaka I, Sugita H. Dystrophin-related protein in skeletal muscles in neuromuscular disorders: Immunohistochemical study. Acta Neuropathol 1993;85:256–59
15. Metzinger L, Blake DJ, Squier MV, et al. Dystrobrevin deficiency at the sarcolemma of patients with muscular dystrophy. Hum Mol Genet 1997;6:1185–91
16. Ozawa E, Nishino I, Nonaka I. Sarcolemmopathy: Muscular dystrophies with cell membrane defects. Brain Pathol 2001;11:218–30
17. Tinsley JM, Blake DJ, Roche A, et al. Primary structure of dystrophin-related protein. Nature 1992;360:591–93
18. Collo G, Starr L, Quaranta V. A new isoform of the laminin receptor integrin α7β1 is developmentally regulated in skeletal muscle. J Biol Chem 1993;268:19019–24
19. Minetti C, Sotgia F, Bruno C, et al. Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy. Nat Genet 1998;18: 365–68
20. Matsuda C, Aoki M, Hayashi YK, Ho MF, Arahata K, Brown RH Jr. Dysferlin is a surface membrane-associated protein that is absent in Miyoshi myopathy. Neurology 1999;53:1119–22
21. Kuo HJ, Maslen CL, Keene DR, Glanville RW. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. J Biol Chem 1997;272:26522–29
22. Matthews-Bellinger JA, Salpeter MM. Fine structural distribution of acetylcholine receptors at developing mouse neuromuscular junctions. J Neurosci 1983;3:644–57
23. Ishikawa Y, Shimada Y. Acetylcholine receptors and cholinesterase in developing chick skeletal muscle fibers. Brain Res 1982;281:187–97
24. Fukuda M. Biogenesis of the lysosomal membrane. Subcell Biochem 1994;22:199–230
25. Winchester BG. Lysosomal membrane proteins. Eur J Paediat Neurol 2001;5:11–19
26. Kannan K, Divers SG, Lurie AA, Chervenak R, Fukuda M, Holcombe RF. Cell surface expression of lysosome-associated membrane protein-2 (lamp2) and CD63 as markers of in vivo platelet activation in malignancy. Eur J Haematol 1995;55:145–51
27. Reddy A, Caler EV, Andrews NW. Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell 2001;106:157–69
28. Engel AG. Acid maltase deficiency. In: Engel AG, Banqer BQ, eds. *Myology*, Vol. 2. New York, NY: McGraw-Hill, 1993:1533–53
29. Advani RJ, Yang B, Prekeris R, Lee KC, Klumperman J, Scheller RH. VAMP-7 mediates vesicular transport from endosomes to lysosomes. J Cell Biol 1999;146:765–76
30. Mizushima N, Yamamoto A, Hatano M, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J Cell Biol 2001;152:657–67
31. De Bleecker JL, Engel AG, Winkelmann JC. Localization of dystrophin and β-spectrin in vacuolar myopathies. Am J Pathol 1993;143:1200–1208
32. Sugiyama JE, Glass DJ, Yancopoulos GD, Hall ZW. Laminin-induced acetylcholine receptor clustering: An alternative pathway. J Cell Biol 1997;139:181–91

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

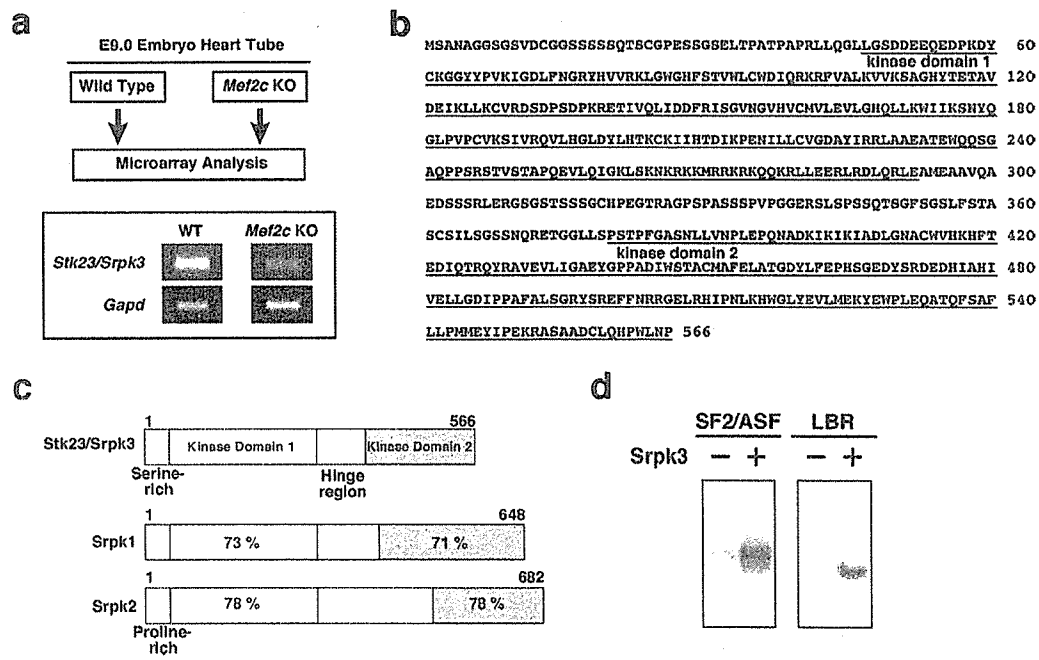

Figure 1. Identification of Stk23/Srpk3 as a novel SRPK. (*a*) Microarray analysis was performed using the hearts of E9.0 *Mef2c*-null and wild-type embryos. Down-regulation of the *Stk23/Srpk3* expression in the *Mef2c*-null hearts was confirmed by RT–PCR. (*Gapd*) Glyceraldehyde-3-phosphate dehydrogenase 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

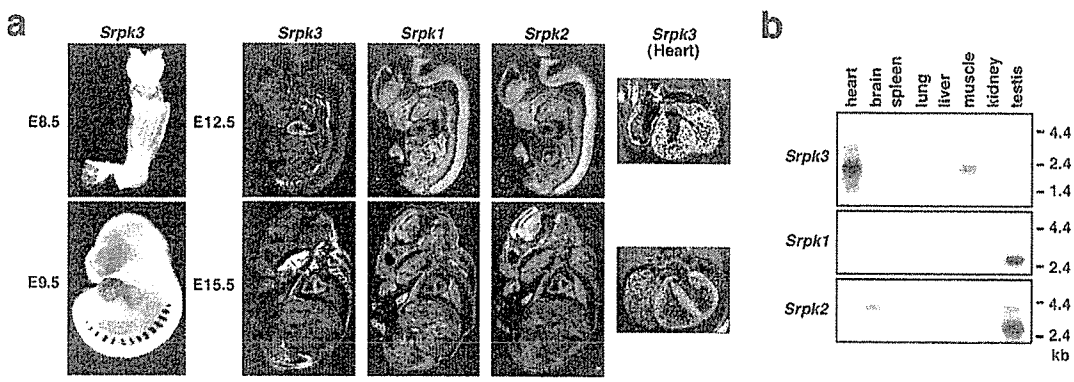

Figure 2. Muscle-specific expression of Srpk3. (*a*) Expression of the SRPK family in mouse embryos. Whole-mount and section in situ hybridization. *Srpk3* is expressed exclusively in the developing heart, somites, and embryonic skeletal muscle. *Srpk1* and *Srpk2* are widely expressed in embryonic tissues, 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.

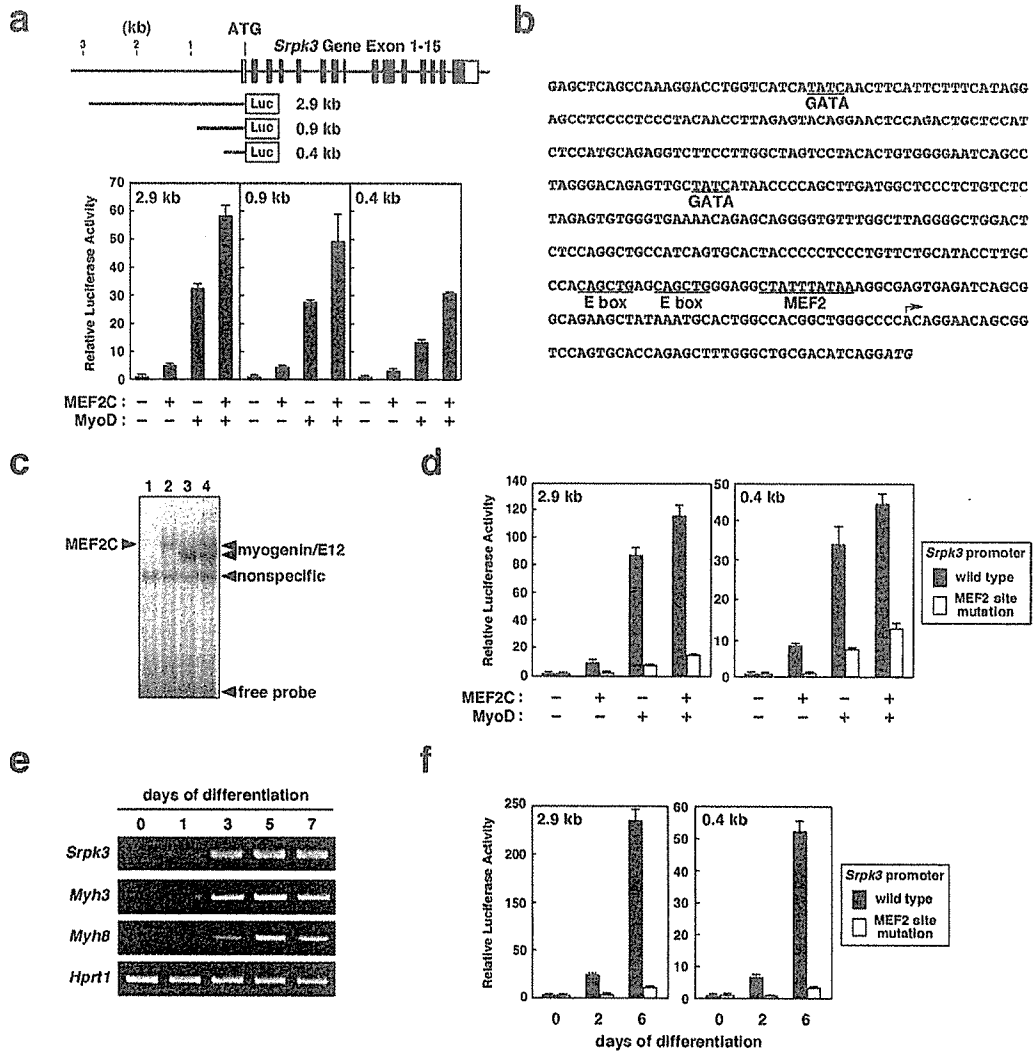

Figure 3. MEF2-dependent muscle-specific transcription of Srpk3. (*a*) The structure of the mouse *Srpk3* gene with schematics of the luciferase constructs and the results of luciferase assays are shown. Filled and open boxes indicate the exons for protein-coding and noncoding regions, respectively. 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-

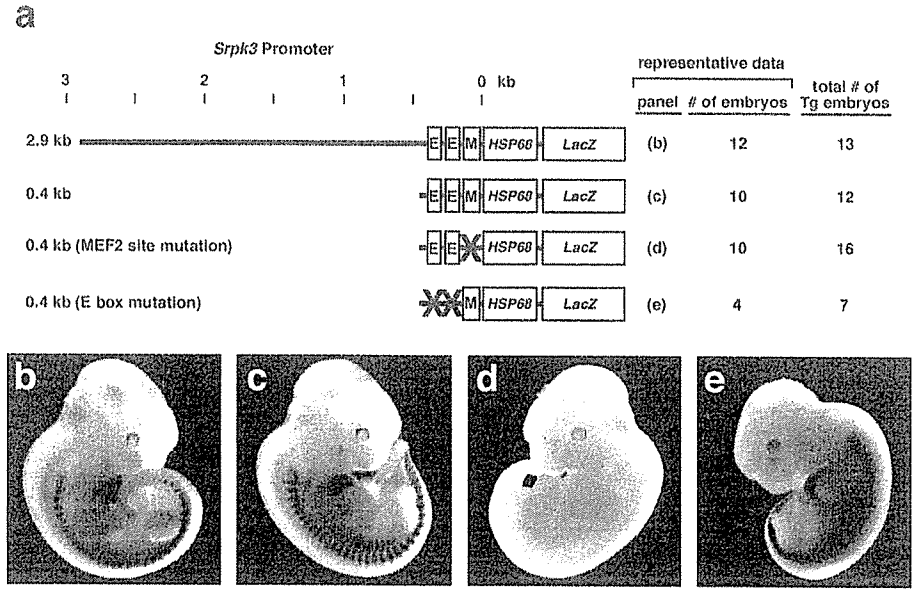

Figure 4. MEF2-dependent transcriptional control of Srpk3 in mouse embryos. (*a*) Schematic representation of *Srpk3-HSP68-LacZ* reporter constructs and results of F0 transgenic mouse analysis. The number of embryos that showed representative expression patterns and total transgenic embryos are shown 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-

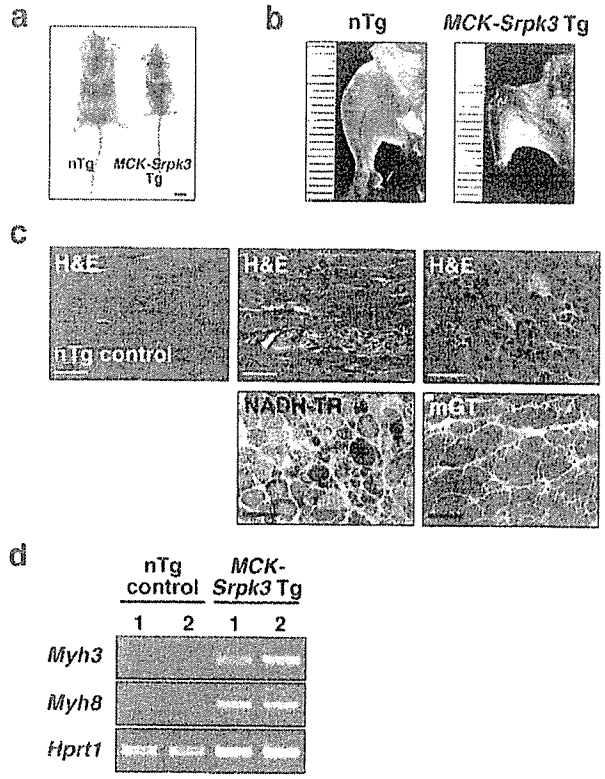

Figure 5. Muscle defects in *MCK–Srpk3* transgenic mice. (*a*,*b*) *Muscle creatine kinase* (*MCK*)*–Srpk3* transgenic (Tg) mice showed significant growth retardation and muscle defects compared with nontransgenic (nTg) littermates at 1 mo of age. Bar in panel *a* represents 1 cm. (*c*) Histological 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

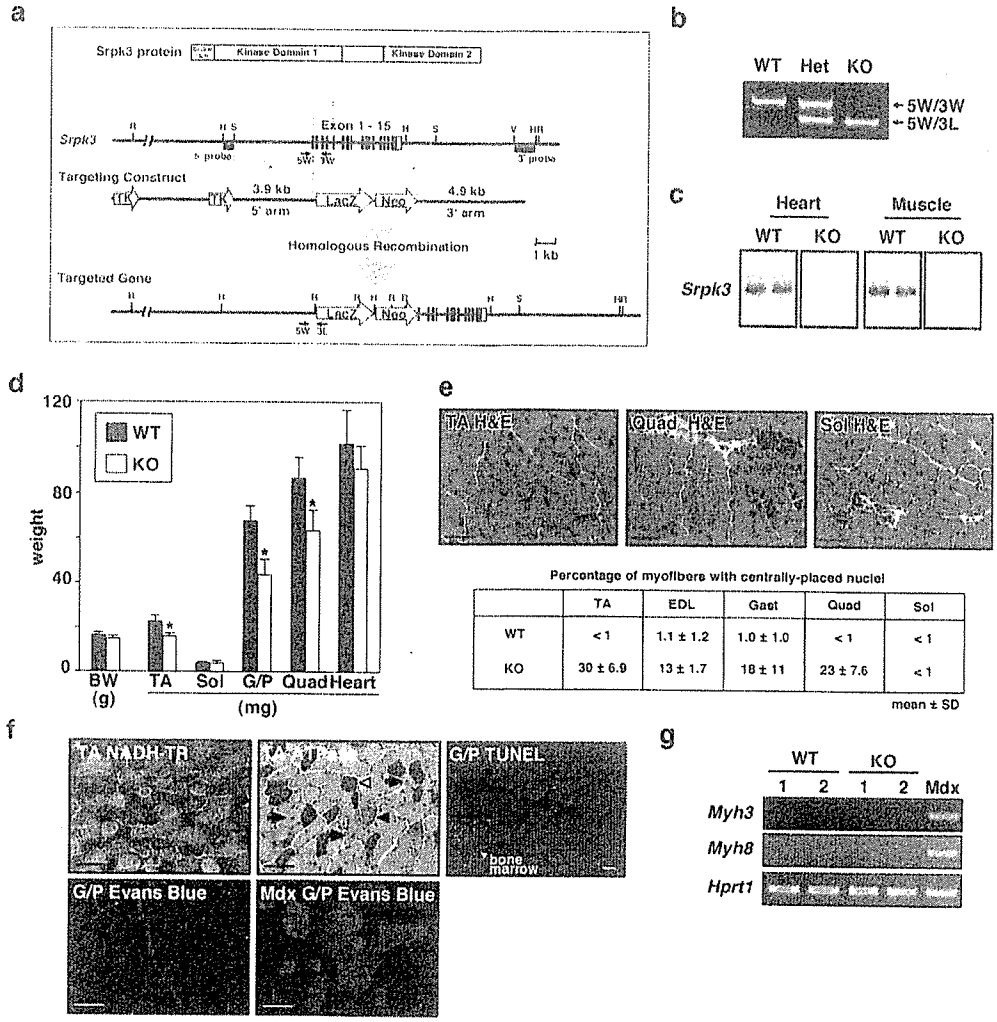

Figure 6. Centronuclear myopathy of *Srpk3*-null mice (*a*) Gene targeting strategy. The mouse *Srpk3* gene is shown with the targeting vector and the targeted allele. Parts of the exons 1 and 5, and the entire exons 2, 3, and 4 were replaced with the *LacZ–neomycin resistance* gene (*Neo*) cassette. 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-

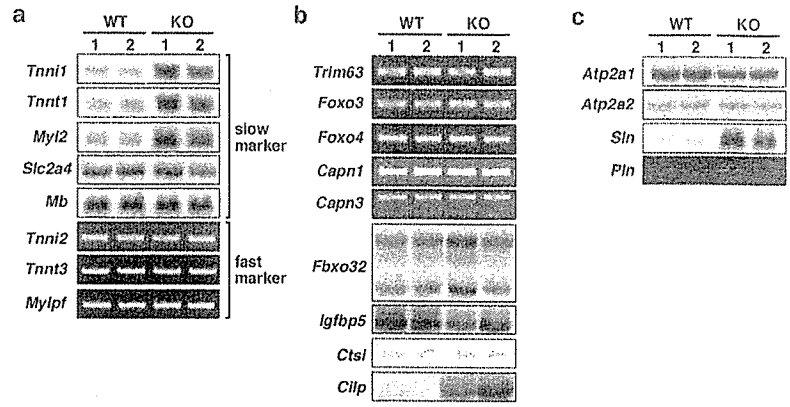

Figure 7. Expression profiles of muscle genes in *Srpk3*-null mice Expression of various muscle genes in the tibialis anterior muscle of *Srpk3*-null mice (KO) and wild-type control (WT). Ethidium bromide staining of RT–PCR products or phosphorimages of Northern blot hybridization are shown. (*a*) Slow- 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 [γ-^{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).

Acknowledgments

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References

Anderson, J.P., Dodou, E., Heidt, A.B., De Val, S.J., Jaehnig, E.J., Greene, S.B., Olson, E.N., and Black, B.L. 2004. HRC is a direct transcriptional target of MEF2 during cardiac, skeletal, and arterial smooth muscle development in vivo. *Mol. Cell. Biol.* **24:** 3757–3768.

Bailey, P., Holowacz, T., and Lassar, A.B. 2001. The origin of skeletal muscle stem cells in the embryo and the adult. *Curr. Opin. Cell Biol.* **13:** 679–689.

Bedford, M.T, Chan, D.C., and Leder, P. 1997. FBP WW domains and the Abl SH3 domain bind to a specific class of proline-rich ligands. *EMBO J.* **16:** 2376–2383.

Belbin, T., Singh, B., Barber, I., Socci, N., Wenig, B., Smith, R., Prystowsky, M., and Childs, G. 2002. Molecular classification of head and neck squamous cell carcinoma using cDNA microarrays. *Cancer Res.* **62:** 1184–1190.

Black, B.L. and Olson, E.N. 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu. Rev. Cell Dev. Biol.* **14:** 167–196.

Blais, A., Tsikitis, M., Acosta-Alvear, D., Sharan, R., Kluger, Y., and Dynlacht, B.D. 2005. An initial blueprint for myogenic differentiation. *Genes & Dev.* **19:** 553–569.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H., Abmayr, S.M., and Nguyen, H.T. 1995. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes & Dev.* **9:** 730–741.

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D., and Relaix, F. 2003. The formation of skeletal muscle: From somite to limb. *J. Anat.* **202:** 59–68.

Buj-Bello, A., Laugel, V., Messaddeq, N., Zahreddine, H., Laporte, J., Pellissier, J.F., and Mandel, J.L. 2002. The lipid phosphatase myotubularin is essential for skeletal muscle maintenance but not for myogenesis in mice. *Proc. Natl. Acad. Sci.* **99:** 15060–15065.

Carpenter, S. and Karpati, G., 2001. *Pathology of skeletal muscle.* Oxford University Press, New York.

Cheng, T.C., Wallace, M.C., Merlie, J.P., and Olson, E.N. 1993. Separable regulatory elements governing myogenin transcription in mouse embryogenesis. *Science* **261:** 215–218.

Cserjesi, P. and Olson, E.N. 1991. Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. *Mol. Cell. Biol.* **11:** 4854–4862.

Daub, H., Blencke, S., Habenberger, P., Kurtenbach, A., Dennenmoser, J., Wissing, J., Ullrich, A., and Cotton, M. 2002. Identification of SRPK1 and SRPK2 as the major cellular protein kinases phosphorylating hepatitis B virus core protein. *J. Virol.* **76:** 8124–8137.

Ding, J.H., Xu, X., Yang, D., Chu, P.H., Dalton, N.D., Ye, Z., Yeakley, J.M., Cheng, H., Xiao, R.P., Ross, J., et al. 2004. Dilated cardiomyopathy caused by tissue-specific ablation of SC35 in the heart. *EMBO J.* **23:** 885–896.

Dodou, E., Xu, S.M., and Black, B.L. 2003. Mef2c is activated directly by myogenic basic helix–loop–helix proteins during skeletal muscle development in vivo. *Mech. Dev.* **120:** 1021–1032.

Edmondson, D.G., Cheng, T.-C., Cserjesi, P., Chakraborty, T., and Olson, E.N. 1992. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. *Mol. Cell. Biol.* **12:** 3665–3677.

Emery, A.E. 2002. The muscular dystrophies. *Lancet* **359:** 687–695.

Garry, D.J., Meeson, A., Elterman, J., Zhao, Y., Yang, P., Bassel-Duby, R., and Williams, R.S. 2000. Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF. *Proc. Natl. Acad. Sci.* **97:** 5416–5421.

Grunau, C., Hindermann, W., and Rosenthal, A. 2000. Large-scale methylation analysis of human genomic DNA reveals tissue-specific differences between the methylation profiles of genes and pseudogenes. *Hum. Mol. Genet.* **9:** 2651–2663.

Gui, J.F., Lane, W.S., and Fu, X.D. 1994. A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature* **369:** 678–682.

Han, J., Jiang, Y., Li, Z., Kravchenko, V.V., and Ulevitch, R.J. 1997. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* **386:** 296–299.

Jeannet, P.Y., Bassez, G., Eymard, B., Laforet, P., Urtizberea, J.A., Rouche, A., Guicheney, P., Fardeau, M., and Romero,

N.B. 2004. Clinical and histologic findings in autosomal centronuclear myopathy. *Neurology* **62:** 1484–1490.

Johnson, K., Farley, D., Hu, S.I., and Terkeltaub, R. 2003. One of two chondrocyte-expressed isoforms of cartilage intermediate-layer protein functions as an insulin-like growth factor 1 antagonist. *Arthritis Rheum.* **48:** 1302–1314.

Kanagawa, M., Saito, F., Kunz, S., Yoshida-Moriguchi, T., Barresi, R., Kobayashi, Y.M., Muschler, J., Dumanski, J.P., Michele, D.E., Oldstone, M.B., et al. 2004. Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell* **117:** 953–964.

Kasler, H.G., Victoria, J., Duramad, O., and Winoto, A. 2000. ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. *Mol. Cell. Biol.* **20:** 8382–8389.

Kato, Y., Kravchenko, V.V., Tapping, R.I., Han, J., Ulevitch, R.J., and Lee, J.D. 1997. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* **16:** 7054–7066.

Koizumi, J., Okamoto, Y., Onogi, H., Mayeda, A., Krainer, A.R., and Hagiwara, M. 1999. The subcellular localization of SF2/ASF is regulated by direct interaction with SR protein kinases (SRPKs). *J. Biol. Chem.* **274:** 11125–11131.

Kuisk, I.R., Li, H., Tran, D., and Capetanaki, Y. 1996. A single MEF2 site governs desmin transcription in both heart and skeletal muscle during mouse embryogenesis. *Dev. Biol.* **174:** 1–13.

Kuroyanagi, N., Onogi, H., Wakabayashi, T., and Hagiwara, M. 1998. Novel SR-protein-specific kinase, SRPK2, disassembles nuclear speckles. *Biochem. Biophys. Res. Commun.* **242:** 357–364.

Kuroyanagi, H., Kimura, T., Wada, K., Hisamoto, N., Matsumoto, K., and Hagiwara, M. 2000. SPK-1, a *C. elegans* SR protein kinase homologue, is essential for embryogenesis and required for germline development. *Mech. Dev.* **99:** 51–64.

Laporte, J., Hu, L.J., Kretz, C., Mandel, J.L., Kioschis, P., Coy, J.F., Klauck, S.M., Poustka, A., and Dahl, N. 1996. A gene mutated in X-linked myotubular myopathy defines a new putative tyrosine phosphatase family conserved in yeast. *Nat. Genet.* **13:** 175–182.

Laporte, J., Bedez, F., Bolino, A., and Mandel, J.L. 2003. Myotubularins, a large disease-associated family of cooperating catalytically active and inactive phosphoinositides phosphatases. *Hum. Mol. Genet.* **12 Spec No 2:** R285–R292.

Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66:** 305–315.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A., and Olson, E.N. 1995. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science* **267:** 688–693.

Lin, Q., Schwarz, J., Bucana, C., and Olson, E.N. 1997. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276:** 1404–1407.

Lorenzo, P., Bayliss, M.T., and Heinegard, D. 1998. A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *J. Biol. Chem.* **273:** 23463–23468.

Lu, J., McKinsey, T.A., Zhang, C.L., and Olson, E.N. 2000. Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol. Cell* **6:** 233–244.

Lu, J.R., Bassel-Duby, R., Hawkins, A., Chang, P., Valdez, R., Wu, H., Gan, L., Shelton, J.M., Richardson, J.A., and Olson, E.N. 2002. Control of facial muscle development by MyoR and capsulin. *Science* **298:** 2378–2381.

MacLennan, D.H., Asahi, M., and Tupling, A.R. 2003. The regulation of SERCA-type pumps by phospholamban and sarcolipin. *Ann. NY Acad. Sci.* **986:** 472–480.

Maniatis, T. and Tasic, B. 2002. Alternative pre-mRNA splicing and proteome expansion in metazoans. *Nature* **418:** 236–243.

McCullagh, K.J., Calabria, E., Pallafacchina, G., Ciciliot, S., Serrano, A.L., Argentini, C., Kalhovde, J.M., Lomo, T., and Schiaffino, S. 2004. NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. *Proc. Natl. Acad. Sci.* **101:** 10590–10595.

McKinnell, I.W. and Rudnicki, M.A. 2004. Molecular mechanisms of muscle atrophy. *Cell* **119:** 907–910.

McKinsey, T.A., Zhang, C.L., Lu, J., and Olson, E.N. 2000. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* **408:** 106–111.

McKinsey, T.A., Zhang, C.L., and Olson, E.N. 2002. Signaling chromatin to make muscle. *Curr. Opin. Cell Biol.* **14:** 763–772.

Nakagawa, O., Nakagawa, M., Richardson, J.A., Olson, E.N., and Srivastava, D. 1999. HRT1, HRT2, HRT3: A new subclass of bHLH transcription factors marking specific cardiac, somitic and pharyngeal arch segments. *Dev. Biol.* **216:** 72–84.

Nakagawa, O., McFadden, D.G., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D., and Olson, E.N. 2000. Members of the HRT family of bHLH proteins act as transcriptional repressors downstream of Notch signaling. *Proc. Natl. Acad. Sci.* **97:** 13655–13660.

Nikolakaki, E., Meier, J., Simos, G., Georgatos, S.D., and Giannakouros, T. 1997. Mitotic phosphorylation of the Lamin B Receptor by a serine/arginine kinase and p34(cdc2). *J. Biol. Chem.* **272:** 6208–6213.

Parker, M.H., Seale, P., and Rudnicki, M.A. 2003. Looking back to the embryo: Defining transcriptional networks in adult myogenesis. *Nat. Rev. Genet.* **4:** 497–507.

Pele, M., Tiret, L., Kessler, J.L., Blot, S., and Panthier, J.J. 2005. A SINE exonic insertion in the PTPLA gene leads to multiple splicing defects and segregates with the autosomal recessive centronuclear myopathy in dog. *Hum. Mol. Genet.* **14:** 1417–1427.

Phan, D., Rasmussen, T.L., Nakagawa, O., McAnally, J., Gottlieb, P.D., Tucker, P.W., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. 2005. *Bop*, a regulator of right ventricular heart development, is a direct transcriptional target of MEF2C in the anterior heart field. *Development* **132:** 2669–2678.

Pownall, M.E., Gustafsson, M.K., and Emerson Jr., C.P. 2002. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu. Rev. Cell Dev. Biol.* **18:** 747–783.

Seki, S., Kawaguchi, Y., Chiba, K., Mikami, Y., Kizawa, H., Oya, T., Mio, F., Mori, M., Miyamoto, Y., Masuda, I., et al. 2005. A functional SNP in *CILP*, encoding cartilage intermediate layer protein, is associated with susceptibility to lumbar disc disease. *Nat. Genet.* **37:** 607–612.

Serrano, A.L., Murgia, M., Pallafacchina, G., Calabria, E., Coniglio, P., Lomo, T., and Schiaffino, S. 2001. Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proc. Natl. Acad. Sci.* **98:** 13108–13113.

Siebel, C.W., Feng, L., Guthrie, C., and Fu, X.D. 1999. Conservation in budding yeast of a kinase specific for SR splicing

factors. *Proc. Natl. Acad. Sci.* **96:** 5440–5445.
Sternberg, E.A., Spizz, G., Perry, W.M., Vizard, D., Weil, T., and Olson, E.N. 1988. Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine kinase gene. *Mol. Cell. Biol.* **8:** 2896–2909.
Takano, M., Takeuchi, M., Ito, H., Furukawa, K., Sugimoto, K., Omata, S., and Horigome, T. 2002. The binding of lamin B receptor to chromatin is regulated by phosphorylation in the RS region. *Eur. J. Biochem.* **269:** 943–953.
Tang, Z., Yanagida, M., and Lin, R.J. 1998. Fission yeast mitotic regulator Dsk1 is an SR protein-specific kinase. *J. Biol. Chem.* **273:** 5963–5969.
Teboul, L., Hadchouel, J., Daubas, P., Summerbell, D., Buckingham, M., and Rigby, P.W. 2002. The early epaxial enhancer is essential for the initial expression of the skeletal muscle determination gene Myf5 but not for subsequent, multiple phases of somitic myogenesis. *Development* **129:** 4571–4580.
Tupling, A.R., Asahi, M., and MacLennan, D.H. 2002. Sarcolipin overexpression in rat slow twitch muscle inhibits sarcoplasmic reticulum Ca^{2+} uptake and impairs contractile function. *J. Biol. Chem.* **277:** 44740–44746.
Vega, R.B., Harrison, B.C., Meadows, E., Roberts, C.R., Papst, P.J., Olson, E.N., and McKinsey, T.A. 2004. Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Mol. Cell. Biol.* **24:** 8374–8385.
Wang, H.Y., Lin, W., Dyck, J.A., Yeakley, J.M., Songyang, Z., Cantley, L.C., and Fu, X.D. 1998. SRPK2: A differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. *J. Cell Biol.* **140:** 737–750.
Wang, D.Z., Valdez, M.R., McAnally, J., Richardson, J., and Olson, E.N. 2001. The Mef2c gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. *Development* **128:** 4623–4633.
Wilson, K.L. 2000. The nuclear envelope, muscular dystrophy and gene expression. *Trends Cell Biol.* **10:** 125–129.
Woods, A.E. and Ellis, R.C. 1996. *Laboratory histopathology, a complete reference.* Churchill-Livingston Press, Oxford.
Wu, H., Naya, F.J., McKinsey, T.A., Mercer, B., Shelton, J.M., Chin, E.R., Simard, A.R., Michel, R.N., Bassel-Duby, R., Olson, E.N., et al. 2000. MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. *EMBO J.* **19:** 1963–1973.
Xu, X., Yang, D., Ding, J.H., Wang, W., Chu, P.H., Dalton, N.D., Wang, H.Y., Bermingham Jr., J.R., Ye, Z., Liu, F., et al. 2005. ASF/SF2-regulated CaMKIIδ alternative splicing temporally reprograms excitation–contraction coupling in cardiac muscle. *Cell* **120:** 59–72.
Yang, C.C., Ornatsky, O.I., McDermott, J.C., Cruz, T.F., and Prody, C.A. 1998. Interaction of myocyte enhancer factor 2 (MEF2) with a mitogen-activated protein kinase, ERK5/BMK1. *Nucleic Acids Res.* **26:** 4771–4777.
Ye, Q., Callebaut, I., Pezhman, A., Courvalin, J.C., and Worman, H.J. 1997. Domain-specific interactions of human HP1-type chromodomain proteins and inner nuclear membrane protein LBR. *J. Biol. Chem.* **272:** 14983–14989.
Yeakley, J.M., Tronchere, H., Olesen, J., Dyck, J.A., Wang, H.Y., and Fu, X.D. 1999. Phosphorylation regulates in vivo interaction and molecular targeting of serine/arginine-rich pre-mRNA splicing factors. *J. Cell Biol.* **145:** 447–455.
Yee, S.P. and Rigby, P.W. 1993. The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes & Dev.* **7:** 1277–1289.

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Expression profiling of muscles from Fukuyama-type congenital muscular dystrophy and laminin-α2 deficient congenital muscular dystrophy; is congenital muscular dystrophy a primary fibrotic disease? ☆

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Abstract

Fukuyama-type congenital muscular dystrophy (FCMD) and laminin-α2 deficient congenital muscular dystrophy (MDC1A) are congenital muscular dystrophies (CMDs) and they both are categorized into the same clinical entity of muscular dystrophy as Duchenne muscular dystrophy (DMD). All three disorders share a common etiologic defect in the dystrophin–glycoprotein complex, which connects muscle structural proteins with the extracellular basement membrane. To investigate the pathophysiology of these CMDs, we generated microarray gene expression profiles of skeletal muscle from patients in various clinical stages. Despite diverse pathological changes, the correlation coefficient of overall gene expression among these samples was considerably high. We performed a multi-dimensional statistical analysis, the Distillation, to extract determinant genes that distinguish CMD muscle from normal controls. Up-regulated genes were primarily extracellular matrix (ECM) components, whereas down-regulated genes included structural components of mature muscle. These observations reflect active interstitial fibrosis with less active regeneration of muscle cell components in the CMDs, characteristics that are clearly distinct from those of DMD. Although the severity of fibrosis varied among the specimens tested, ECM gene expression was consistently high without substantial changes through the clinical course. Further, in situ hybridization showed more prominent ECM gene expression on muscle cells than on interstitial tissue cells, suggesting that ECM components are induced by regeneration process rather than by 'dystrophy.' These data imply that the etiology of FCMD and MDC1A differs from that of the chronic phase of classical muscular dystrophy, and the major pathophysiologic change in CMDs might instead result from primary active fibrosis.

Keywords: Fukuyama-type congenital muscular dystrophy; Laminin-α2 deficient congenital muscular dystrophy; DNA microarray

☆ *Abbreviations:* FCMD, Fukuyama-type congenital muscular dystrophy; MDC1A, laminin-α2 deficient congenital muscular dystrophy; CMD, congenital muscular dystrophy; DMD, Duchenne muscular dystrophy; α-DG, α-dystroglycan; ECM, extracellular matrix.

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Fukuyama-type congenital muscular dystrophy (FCMD; MIM 253800) is an autosomal recessive disorder and the second most common muscular dystrophy, following DMD, in Japan [1]. Clinical manifestations of FCMD include severe muscle wasting from early infancy with malformation of the brain and eyes. We previously isolated the responsible gene for FCMD, termed *fukutin* [2,3]. Fukutin presumably modulates the glycosylation of α-dystroglycan (α-DG), one of the major components of the dystrophin–glycoprotein complex, since α-dystroglycan is hypoglycosylated in FCMD muscle [4,5]. Fukutin-mediated glycosylation is crucial for binding of α-DG to ECM proteins such as laminin, agrin, and perlecan, which are important for maintaining muscle cell integrity [5]. Hypoglycosylation of α-DG is likely to attenuate the stable connection between skeletal muscle sarcoplasmic membrane and the basement membrane. This architectural and functional instability of muscle fibers is presumed to induce severe muscular dystrophy in FCMD [4,6].

Accumulating evidence indicates that hypoglycosylation of α-DG underlies a number of muscular dystrophies. Hypoglycosylated α-DG provokes the post-translational disruption of dystroglycan-ligand interactions in skeletal muscle, leading to the severe phenotypes of congenital muscular dystrophies [7]. Mutations in glycosyltransferases have been linked to several muscular dystrophies, including POMGnT1 (protein *O*-mannose β-1, 2-*N*-acetylglucosaminyltransferase 1) with muscle–eye–brain disease; POMT1/2 (protein *O*-mannosyltransferase 1/2) with Walker–Warburg syndrome; FKRP (fukutin-related protein) with congenital muscular dystrophy type 1C (MDC1C). In addition, mutations in the putative glycosyltransferase LARGE correlate with myodystrophy in mice and human congenital muscular dystrophy 1D (MDC1D) [8–13].

Another congenital muscular dystrophy, laminin-α2 deficient congenital muscular dystrophy (MDC1A), is one of the most common childhood congenital muscular dystrophies in the European population [14]. MDC1A is an autosomal recessive disorder that is caused by mutation of the *LAMA2* gene [15]. Clinical muscular features of MDC1A are grossly identical to the FCMD phenotype and characterized as severe neonatal hypotonia. Laminin-α2 is the main component of the basement membrane and plays an essential role in its formation, serving as a signaling molecule to interact with other ECM or sarcoplasmic membrane components [16]. α-DG is a primary receptor for laminin-α2 on the sarcoplasmic membrane [17,18]. Thus, attenuated connections between laminin and α-DG might account for muscular dystrophy in MDC1A.

Although DMD and these CMDs are categorized into the same clinical entity, 'muscular dystrophies,' manifestations of CMDs are known to differ from those of DMD in several ways. First, patients with CMDs show congenital anomalies in muscle pathology, implying developmental defects in CMD muscle [19]. Second, these CMDs show severe pathological changes in the early infantile period with almost fixed characteristics thereafter, unlike the later onset and progressive features of DMD [1]. Finally, the pathophysiology of CMD muscle is characterized by few active dystrophic and regenerating fibers, correlating with moderate increases in serum CK levels [1]. In contrast, muscle specimens from DMD patients show severe necrotic changes and active regeneration, both of which are evidenced by extremely high serum CK levels.

Dystrophic features in CMD have been defined by the observation of 'opaque fibers' in biopsied specimens, which are suggested to indicate pre-necrotic fibers like those often seen in DMD muscle. Opaque fibers in CMD biopsied muscle specimens are unexpectedly prominent, considering the moderate serum CK levels. However, we suspect that the opaque fibers are artificially generated by the biopsy itself, due to the technical tension placed on fragile CMD muscle membranes (unpublished data). Therefore, the pathological characteristics of these CMDs are not likely the same as those of late-stage DMD. The pathophysiological and molecular mechanisms of these CMDs deserve further investigation.

Although gene expression profiling of DMD skeletal muscle has been described previously [20–22], neither FCMD nor MDC1A has been characterized. To investigate the molecular mechanisms of CMDs relative to DMD, we profiled gene expression in FCMD and MDC1A muscle using a custom cDNA microarray. Profiles were analyzed by a multi-dimensional statistical analysis, the Distillation, to extract determinant genes, revealing a more detailed molecular mechanism for these CMDs.

Materials and methods

Patient materials. All clinical materials were collected for diagnostic purposes. Four muscle specimens (biceps branchii) from FCMD patients (F1–F4) and one from MDC1A (M1) were used in the analysis (Fig. 1). Genetic screening identified a homozygous retrotransposal insertion into the 3′ untranslated region of *fukutin* in all patients [3]. For MDC1A, the patient showed a typical disease onset, and the specimen was negative for anti-laminin-α2 staining. We used pooled muscle RNA (Origene Technologies) as an internal standard for microarray experiments. For age-matched non-dystrophic controls, muscle RNA from two children was used (C1 and C2, 1 year old, and 7 years old, respectively). These two samples were selected based on normal laboratory findings, normal plasma CK levels, and no histological findings of muscular dystrophy.

Histological analysis. Serial sections from the biopsied cryospecimen used for microarray experiments were stained with hematoxylin and eosin by standard methods. Scion Image Beta 3b (Scion) was used for the two-dimensional morphometric analysis of muscle specimen. For each cryospecimen, ten pictures were taken randomly. We measured the area of muscle tissue, adipose tissue, and the remaining interstitial tissues, and then measured the average cross-sectioned muscle fiber diameters. Statistical analysis was performed using Student's t test ($p < 0.005$).

RNA isolation and expression profiling. Generation of cDNA microarrays of skeletal muscle transcripts has been reported previously [22]. Using a similar method, we constructed a cDNA chip containing 5600 genes expressed in skeletal muscle. RNA isolation, hybridization, and detection methods also have been previously reported. Microarray experiments were carried out using a competitive hybridization method with two labeled targets: one for muscle RNAs from FCMD, MDC1A or normal controls, and another for pooled muscle RNA (Origene), which

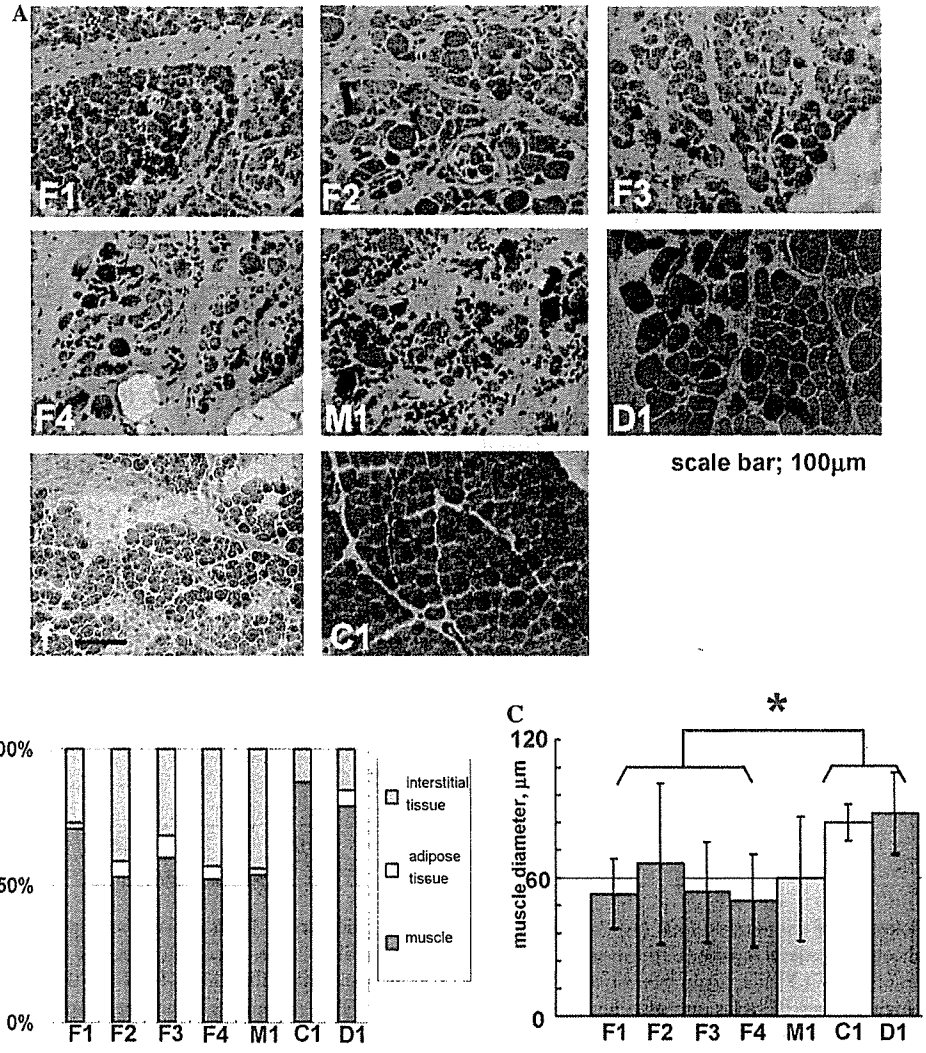

Fig. 1. Morphometric analysis of FCMD and MDC1A skeletal muscle. (A) HE stain of biopsied skeletal muscle used for further analysis (F1–F4 and M1). For comparison, DMD muscle and skeletal muscle from normal fetus (f, 21 weeks) are shown. Note that fetal tissue is quite similar to F1–F4. Fibrosis exists in F1 patient, though necrotic lesions are not prominent in F2–F4 and in M1. Extremely thin muscle fibers with high nuclear to cytoplasmic contents are seen. FCMD-1 (F1, 20 days), FCMD-2 (F2, 7 months), FCMD-3 (F3, 1 year 4 months), FCMD-4 (F4, 1 year 6 months), MDC1A-1 (M1, 1 year 8 months), DMD (D1, 1 year), and normal control (C1, 1 year). Scale bar = 100μm. (B) Components of each skeletal muscle (muscle components, interstitial tissue, and adipose tissue) were calculated for FCMD-1, FCMD-2, FCMD-3, FCMD-4, MDC1A-1, normal control, and DMD-1. Note that interstitial components are prominent in F1–F4 and M1. (C) Diameter of skeletal muscle fiber. The mean diameters of F1–F4 and M1 are significantly smaller than those of the age-matched control (C1) and DMD muscle (D1) ($^*p < 0.005$). The variance of the diameter also is high in FCMD and MD muscle fibers.

served as an internal control for per-chip normalization. Hybridization was conducted at least twice. Hybridization intensities of each spot and background intensities were calculated and normalized using ScanArray 5000 with Quant Array software (Perkin-Elmer Life Science).

Statistical analysis. Analysis of microarray data was performed using Microsoft Excel (Microsoft) and Genespring software version 6.2 (Silicon Genetics). Data used for further analysis were calculated using a previously reported method [22]. To avoid 'false positive' signals, we excluded genes from the analysis for which average normal expression level constraints are under 500. Next, per spot and per chip, intensity dependent (Lowess) normalization was performed to minimize the variation in each microarray experiment. The average expression signal compared to pooled muscle RNA of each gene signal was estimated as the fold ratio. Two thousand three hundred and forty-five genes were selected for further analysis. The correlation coefficient for each signal between FCMD, MDC1A, and normal controls was calculated using Microsoft Excel for 688 genes whose expression level is reproducible (p-value $t < 0.05$).

To extract genes that characterize the expression profile of FCMD and MDC1A skeletal muscle compared to normal muscle, we adopted an original statistical analysis method, the Distillation [23]. Using this analysis, we could find genes whose expression pattern significantly distinguishes a certain disease from others. This analysis also sorts paired genes whose mutual expression patterns can discern one disease or normal control from another.

Quantitative real-time PCR. To confirm the microarray data, we performed real-time quantitative PCR. We produced single-strand cDNA with random primers, and quantitative real-time RT-PCR using SYBR-green was performed using the ABI Prism 7900 sequence detection system (Applied Biosynthesis). Primer sequences were as follows.

Gapdh: forward 5′-CATCTTCCAGGAGCGAGATC, reverse 5′-TGCAAATGAGCCCCAGCCTT; *TNNT2:* forward 5′-ATCAATGTTCTCCGAAACAGGATCAA, reverse 5′-GAGGAGCAGATCTTTGGTGAAGGA; *CGI-38*: forward 5′-CTCGGGTCATCAACTATGAGGAGTTCAA, reverse 5′-CTACAGCACCCCCTGTTTTTGCTTTAGT; *sarcoglycan* β forward 5′-AGCCTATTGTTTTTCAGCAAGGGACAAC, reverse

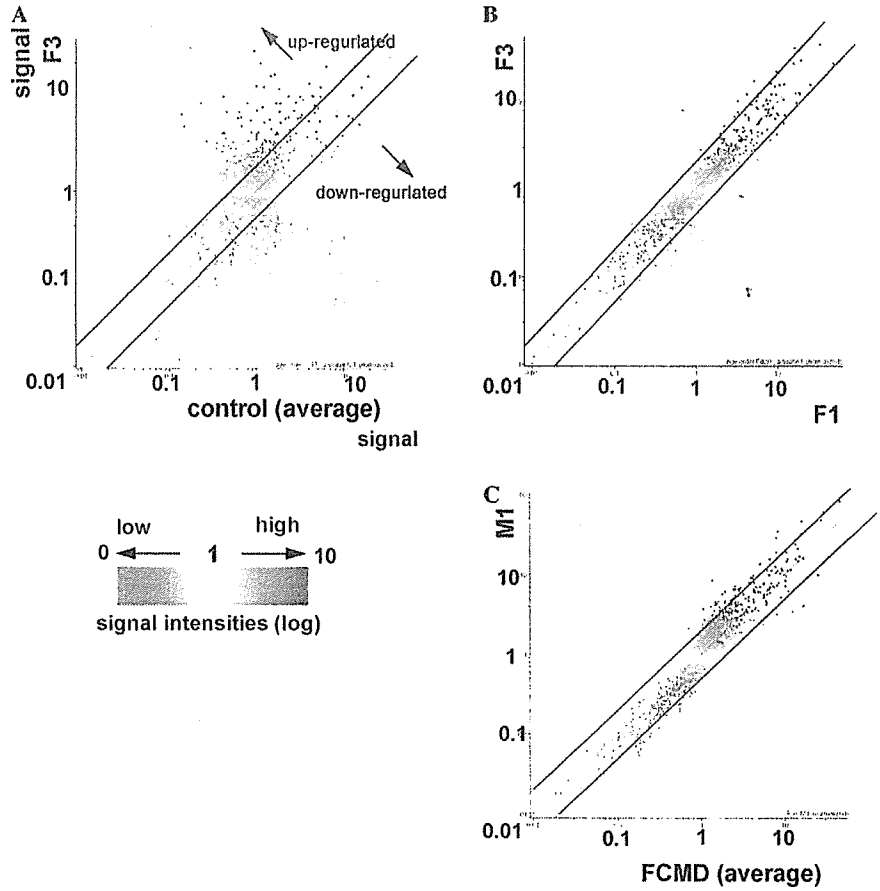

Fig. 2. Correlation co-efficiencies of microarray data. (A) Scatter graph of a cDNA microarray. Each axis shows expression signal intensities. *X* Axis, signal intensities of normal control skeletal muscle (C, the average signal of normal controls); *Y* axis, signal intensities of F3. (B) *X* axis, signal intensities of F1; *Y* axis, signal intensities of F3. (C) *Y* axis, signal intensities of M1; *X* axis, average signal intensities of F1–F4. Note that the correlation co-efficiency of microarray data is quite high among FCMDs and also high between FCMD and MDC1A samples. Blue lines show 2-, 1-, and 0.5-fold increases, respectively.

Table 1
Correlation coefficient of data from each microarray experiment

Sample No.	Age	C1	C2	F1	F2	F3	F4	M1
		1y	7y	20d	7m	1y4m	1y6m	8m
C1	1y	1	0.97	0.6	0.42	0.53	0.32	0.42
C2	7y	0.95	1	0.6	0.44	0.55	0.33	0.45
F1	20d	0.65	0.67	1	0.84	0.87	0.77	0.82
F2	7m	0.47	0.53	0.9	1	0.88	0.85	0.83
F3	1y4m	0.57	0.64	0.93	0.93	1	0.78	0.86
F4	1y6m	0.38	0.45	0.84	0.91	0.9	1	0.76
M1	8m	0.49	0.56	0.89	0.9	0.92	0.87	1

5′-TTTTCACTCCACTTGGCAAATGAAACTC; *MYH2:* forward 5′-AGTTCCGCAAGGTGCAGCACGAGCT, reverse 5′-CCACCTAAAGGGCTGTTGCAAAGGC.

Statistical analysis was performed with Student's *t* test, using duplicate experiments of two patients for each disease and normal control.

In situ hybridization. In situ hybridization was performed according to standard methods. Sense and anti-sense sequence templates (*collagen3a, collagen15a1, Thrombospondin-4,* and *osteoblast-specific factor-2*) containing T7 or SP6 promoters were produced through PCR, and digoxigenin-labeled RNA probes were generated by transcribing with T7 or SP6 RNA polymerase. Sliced cryosections (8 μm) of skeletal muscle from FCMD (1 year of age) and a normal control (1 year of age) were fixed with 4% paraformaldehyde for 30 min. After treatment with proteinase K (0.5 μg/mg) for 5 min, samples were overlaid with hybridization solution containing digoxigenin-labeled probe. Hybridization signals were detected according to manufacturer's instructions.

Results

FCMD and MDC1A skeletal muscle are histologically distinct from DMD

We performed histological examination to document the characteristics of skeletal muscle samples used for