Disruption of muscle membrane and phenotype divergence in two novel mouse models of dysferlin deficiency


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Limb girdle muscular dystrophy type 2B and Miyoshi myopathy are clinically distinct forms of muscular dystrophy that arise from defects in the dysferlin gene. Here, we report two novel lines of dysferlin-deficient mice obtained by (a) gene targeting and (b) identification of an inbred strain, A/J, bearing a retrotransposon insertion in the dysferlin gene. The mutations in these mice were located at the 3' and 5' ends of the dysferlin gene. Both lines of mice lacked dysferlin and developed a progressive muscular dystrophy with histopathological and ultrastructural features that closely resemble the human disease. Vital staining with Evans blue dye revealed loss of sarcolemmal integrity in both lines of mice, similar to that seen in mdx and caveolin-3 deficient mice. However, in contrast to the latter group of animals, the dysferlin-deficient mice have an intact dystrophin glycoprotein complex and normal levels of caveolin-3. Our findings indicate that muscle membrane disruption and myofiber degeneration in dysferlinopathy were directly mediated by the loss of dysferlin via a new pathogenic mechanism in muscular dystrophies. We also show that the mutation in the A/J mice arose between the late 1970s and the early 1980s, and had become fixed in the production breeding stocks. Therefore, all studies involving the A/J mice or mice derived from A/J, including recombinant inbred, recombinant congenic and chromosome substitution strains, should take into account the dysferlin defect in these strains. These new dysferlin-deficient mice should be useful for elucidating the pathogenic pathway in dysferlinopathy and for developing therapeutic strategies.

INTRODUCTION

Autosomal recessive forms of muscular dystrophies constitute a genetically and clinically heterogeneous group of disorders (1,2). Recently, two clinically distinct forms of muscular dystrophy, limb girdle muscular dystrophy type 2B (LGMD 2B) and Miyoshi myopathy (MM), were found to arise from defects in the dysferlin gene, DYSF (3,4). Although both disorders are characterized by early adult onset with progressive weakness and marked elevations of serum creatine kinase (CK) levels, they differ in the distribution of muscle weakness at onset. In LGMD 2B, weakness and atrophy of the proximal muscles predominate, whereas MM affects mainly the distal muscles.

Mutation analysis in MM and LGMD 2B patients reveals predominantly single nucleotide changes with no apparent mutational hotspots, and no obvious correlation between genotype and distribution of muscle weakness (5,6). Moreover, the same mutation in DYSF can cause either LGMD 2B or MM (hereafter referred as dysferlinopathy), even among members of the same kinship (3,7). These findings indicate that mutations in DYSF do not solely determine the disease phenotype, and additional factors (genetic or environmental) may contribute to the patterning of the muscle disease.
The 237 kDa dysferlin protein is ubiquitously expressed and is localized to the muscle cell membrane (8,9). Patients with dysferlinopathy have markedly reduced levels or a complete loss of dysferlin in their muscle and blood cells (8–10). Dysferlin is a member of the newly recognized family of proteins known as ‘ferlins’ (11–14). The biological function of these proteins is unknown but they share common motifs, notably multiple C2 domains and a single transmembrane segment at the carboxyl-terminus. C2 domains are known to bind calcium, phospholipids or proteins to trigger signaling events and membrane trafficking (15), and this has led to speculation that dysferlin is important for signaling pathways that mediate membrane repair or fusion in skeletal muscles (3,13). This hypothesis is partly supported by recent reports that the first C2 domain in dysferlin binds phospholipids in a calcium-dependent manner and that isolated dysferlin-deficient muscle fibers are defective in resealing disrupted membranes (16,17). However, very little is known about the signaling pathway or the mechanism involved in muscle membrane repair. In addition, the functions of the remaining C2 domains in dysferlin are also not known.

During the course of this study, the SJL mice were reported to be a naturally occurring mouse model for dysferlinopathy because they have a splice site mutation that removes part of the highly conserved C2E domain (18,19). Although SJL mice develop an active myopathy at around 6–8 months of age and have a marked reduction (~85%) in dysferlin levels (18,20), they also exhibit a number of other abnormalities not seen in patients, including extreme susceptibility to autoimmune diseases, lymphoma, viral infections and aggressive behavior (21–24). Currently, it is not clear if all of these abnormalities are linked to the dysferlin defect. Therefore, analysis of dysferlin function based solely on studies in the SJL mice could be misleading.

Identification of dysferlin deficiency in the A/J mice

The progressive muscular dystrophy phenotype of the A/J mice was discovered at The Jackson Laboratory (TJL) during routine pathological examination of adult skeletal muscle from a spontaneous craniofacial mutation (froggy) that arose on the A/J inbred background. Examination of littermates with and without the craniofacial defect revealed that the muscular dystrophy pathology, characterized by degeneration and regeneration of myofibers and centrally placed nuclei, was apparent in all mice from this colony. We expanded our survey to include the A/J mice from TJL production stocks as well as the investigator’s individual A/J lines and observed the same dystrophic muscle histopathology in all the animals examined.

We first attempted to map the A/J muscular dystrophy mutation in the AXB and BXA recombinant inbred (RI) strains of mice because they were derived from the A/J (A) and C57BL/6J (B) progenitors (25) and had been genotyped for over 700 loci (26). However, no signs of dystrophic histopathology were evident in skeletal muscle sections from these lines.

RESULTS

Targeted disruption of dysferlin and generation of the Dysf<sup>−/−</sup> mice

To construct the targeting vector, we isolated a contig comprising five BAC clones that encompassed the murine dysferlin locus. Human and murine dysferlin share very similar amino acid sequences (~90% identity) and both are predicted to encode a large protein (>2000 amino acids) with six putative C2 domains and a single transmembrane segment at its C-terminus (19). To inactivate dysferlin gene expression, we replaced part of the highly conserved C2E domain with a neomycin gene cassette placed in the opposite transcriptional orientation (Fig. 1A). The targeting vector was introduced into the J1 ES cell line, and Southern blot analysis of 197 G418 and gancyclovir-resistant colonies revealed five homologous recombinant clones. Three of these clones were used to generate chimeras, which produced germ-line transmission. Dysferlin heterozygous mice (Dysf<sup>+/−</sup>) were interbred to obtain homozygous mutants (Dysf<sup>−/−</sup>) (Fig. 1B), and all the predicted genotypes were recovered in the expected Mendelian ratios, indicating there was no apparent developmental disadvantage for the Dysf<sup>−/−</sup> mice.

To determine if the targeted disruption produced a null allele, anti-dysferlin antibodies directed against four different epitopes of the protein (Materials and Methods) were used to examine the expression of dysferlin. No dysferlin protein was detected in the Dysf<sup>−/−</sup> muscle, whereas a prominent 230 kDa band was seen in the Dysf<sup>+/−</sup> and wild-type muscle (Fig. 1C–F). A weak dysferlin band was also detected in the SJL muscle, consistent with the previous report of a marked reduction of dysferlin levels in this strain of mouse (18) (Fig. 1C and D). It is noteworthy that dysferlin expression was not altered in the mdx mouse (a mouse model for Duchenne muscular dystrophy), indicating that loss of dystrophin does not affect dysferlin expression (Fig. 1C). The Dysf<sup>−/−</sup> mice have a normal appearance and size and were indistinguishable from the wild-type littermates. However at 8 months of age, some Dysf<sup>−/−</sup> mice developed hind limb clasping and were no longer able to spread their hind limbs and digits when suspended by their tails (data not shown). This abnormal behavior, which was also seen in the SJL mice (18), indicates that the hind limbs were weak.
retired breeders (two males and two females each, 6–12 months of age) in over 20 independent AXB and BXA RI lines. We also found no signs of dystrophic histopathology in two additional highly related inbred strains of mice (A/WySnJ and A/HeJ) that were separated from L.C. Strong’s progenitor A strain in 1927 and 1938, respectively (27). This suggested that the muscular dystrophy mutation in the A/J mice arose after the construction of the AXB and BXA RI lines in 1975 and had become fixed in the current A/J colony at TJL.

To map the muscular dystrophy locus in A/J, we performed a genome-wide scan with 57 N2 A/J/C2 (A/J/C2 B6) mice which included 32 affected and 25 unaffected mice on the basis of serum creatine levels. The A/J muscular dystrophy phenotype was non-recombinant in 57 meioses with D6Mit8 and the order of the nearest markers were, from centromere to telomere, D6Mit188—1.75 ± 1.74 cM—D6Mit8, A/J-dystrophy—5.26 ± 2.96 cM—D6Mit284. Dysferlin was chosen as a candidate gene for analysis because the SJL-Dysf mutation maps to a 1.2 cM region between D6Mit8 and D6Mit29 (18) and this was completely contained within our genetic interval. An allelism test between A/J and SJL/J was performed by generating (A/J × SJL/J) F1 hybrids. All F1 hybrid mice had histopathological evidence of muscle degeneration and regeneration (data not shown) and had significantly elevated serum CK levels suggesting that the two mutations were allelic. However, the A/J allele is a unique mutation and is not due to genetic contamination from the SJL strain because RT–PCR analysis across the region containing the SJL dysferlin mutation (18) amplified only the wild-type 500 bp in the A/J skeletal and cardiac muscle (data not shown).

To identify the putative mutation in the Dysf gene, we designed PCR primers to amplify ~7 kb of the dysferlin cDNA from the A/J and control A/HeJ skeletal muscle in 10 overlapping fragments. No mutations were identified in any of the A/J cDNA fragments compared with control (data not shown). To search for a mutation in the genomic DNA of the A/J mice, a 5’ RT–PCR product corresponding to nucleotides 89–580 of the mouse Dysf mRNA (accession no. AF188290) was used as a probe on a Southern blot containing DNA from A/J, A/HeJ and A/WySnJ. Altered Eco RI and HindIII restriction patterns unique to A/J genomic DNA were identified (data not shown). A search of the Celera mouse genome database for genomic fragments sequenced...
from A/J, DBA/2J and 129X1/SvJ corresponding to sequences from the Southern blot probe (exons 1–5) revealed a unique ETn retrotransposon insertion within intron 4 in genomic fragments derived from A/J (Fig. 2A). Both the 5′ and 3′ junctions of the ETn insertion are present in the database and include a perfect 6 bp duplication at the insertion site (Celera accession no. GA_x1PFAP6U11E and GA_x1PFAP640Q8, respectively). The ETn insertion is not present in the DBA/2J or 129X1/SvJ sequences from Celera or in the public C57BL/6J mouse genomic sequence (http://www.ensembl.org/Mus_musculus/). PCR amplification of genomic DNA across the insertion site produced a 207 bp fragment in the A/HeJ, A/WySnJ and SJL/J samples but consistently failed in the A/J samples due to the large size (5–6 kb) of the ETn insert (Fig. 2B). The ETn sequence was confirmed to be unique to A/J genomic DNA by specific amplification of the 3′ LTR/intron 4 junction and no amplification product was detected in the A/HeJ, A/WySnJ or SJL/J samples (Fig. 2B).

To evaluate the effect of ETn insertion on dysferlin expression, we carried out northern and western blot analyses. Using a 3′ murine dysferlin cDNA clone (corresponding to amino acid 1993–2070) as probe, the ~7.0 kb dysferlin transcript was detected in the A/WySnJ and C57BL6/J muscle RNA but not in A/J (Fig. 2C). Consistent with this finding, western blot analysis using different anti-dysferlin antibodies showed no detectable dysferlin protein in the A/J mice (Fig. 2D and E), indicating that the retrotransposon insertion had created a null allele.

**Dysferlin-deficient mice (Dysf<sup>−/−</sup> and A/J) develop a progressive muscular dystrophy**

To determine if loss of dysferlin causes muscular dystrophy, we examined hematoxylin and eosin (H&E)-stained frozen sections of various muscles from the Dysf<sup>−/−</sup> mice and age-matched wild-type littermates. The earliest pathological features were seen in 2-month-old Dysf<sup>−/−</sup> mice, which consisted of scattered degenerating and regenerating fibers with centrally placed nuclei (Fig. 3Aa). These early dystrophic changes were found primarily in the proximal muscles (quadriceps femoris and triceps brachii), whereas distal muscles (gastrocnemius, soleus and tibialis anterior) were unaffected (data not shown). The muscle pathology progressed as a function of age. By 5 months of age, there were pronounced changes in the histopathology of the proximal muscles, characterized by increased numbers of necrotic and regenerating fibers, infiltration of mononuclear cells into intact fibers, phagocytosis and marked variation of fiber size (Fig. 3Ab). In addition, hypertrophic fibers, fiber splitting and fat replacement were also evident. Importantly, mild myopathic features were seen in the distal muscles at this stage (data not shown), which indicates a gradual spreading of the muscle disease with age. By 6 months of age, there was active myopathy of differing severity in all the skeletal muscles examined. The quadriceps femoris and triceps brachii were the most severely affected muscles, whereas gastrocnemius, soleus and tibialis anterior showed mild histopathology, even at late stages of the disease (Fig. 3Bg and h). Unexpectedly, we found that the abdominal muscles were as severely affected as the proximal muscles (Fig. 3Bi). In contrast, diaphragm, biceps brachii, masseter and pectoralis muscles were only mildly affected (data not shown). Perivascular infiltrates, endomyosial fibrosis and inflammation were seen in 8-month-old Dysf<sup>−/−</sup> mice and these became more widespread with age (Fig. 3Ac). Wild-type and Dysf<sup>+/−</sup> mice showed no evidence of muscular dystrophy at any age.

In parallel studies of the SJL mice, we found that the age of disease onset, the sites of muscle histopathology and the progression of the muscle disease were similar to the Dysf<sup>−/−</sup> mice (data not shown). In contrast to the Dysf<sup>−/−</sup> and SJL mice, onset of dystrophic features in the A/J mice appeared later at around 4–5 months of age, characterized by mild histopathology and a slower progression of muscle disease (Fig. 3Ad–f). The notable exception is the abdominal muscles in the A/J mice, which showed a similar histopathology as the age-matched Dysf<sup>−/−</sup> mice (Fig. 3Bj). Despite these differences, the distribution of muscle disease in the A/J mice was the same as the SJL and Dysf<sup>−/−</sup> mice, with proximal muscles more severely affected than the distal muscles (Fig. 3Bj and k).

**Loss of sarcolemmal integrity in the dysferlin-deficient mice**

Evans blue dye (EBD) is widely used to demonstrate myofiber permeability in various mouse models of muscular dystrophies (28,29). EBD is a small molecular mass tracer that binds tightly to serum albumin. The EBD–albumin conjugate will readily infiltrate muscle fibers with damaged cell membranes but is unable to penetrate healthy muscles. To evaluate the sarcolemmal integrity of the dysferlin-deficient muscle, EBD was injected intraperitoneally into the mutant and wild-type mice to assess uptake of the dye. In the controls and Dysf<sup>−/−</sup> mice, no EBD staining was detected macroscopically or microscopically (Fig. 4A and B). In contrast, the EBD staining was seen in the Dysf<sup>−/−</sup>, SJL and to a lesser extent in A/J mice, and they appeared mainly in quadriceps femoris, triceps brachii, adductor leg muscles and abdominal muscles. In particular, uptake of EBD in the adductor and abdominal muscles produced a striking banding pattern that was not seen in the wild-type muscles (Fig. 4C). Interestingly, this type of banding pattern was also observed in the diaphragm muscles of mice lacking γ-sarcoglycan (30) or caveolin-3 (31). When viewed under a fluorescence microscope, EBD-stained fibers displayed a red fluorescence (Fig. 4D) and these fibers were usually found to be necrotic or hypertrophic by H&E staining.

We also determined the serum CK levels to evaluate sarcolemmal integrity. The serum CK levels of 4-week-old Dysf<sup>−/−</sup>, A/J and SJL mice were 4–6-fold higher than controls (Fig. 4E), providing further evidence of muscle membrane damage in the dysferlin-deficient mice.

**Expression of dystrophin and its associated proteins in the dysferlin-deficient muscle**

Mutations in dystrophin and its associated proteins are linked to several forms of muscular dystrophies (2). To determine if loss of dysferlin alters the expression of dystrophin and its associated proteins, we examined these proteins in the
dysferlin-deficient muscles by immunofluorescence and western blot analyses. Contrary to previous reports (17,32), we found that dysferlin was localized to the sarcolemma in the wild-type muscles and there was no evidence of the dysferlin expression in cytoplasmic vesicles (Fig. 5A). We obtained the same result using different anti-dysferlin antibodies (NCL-Hamlet, Sal-I and C-19) and our findings were consistent with reports from other laboratories (8,9,33–35).

Despite the absence of dysferlin from the sarcolemma of the Dysf/2, A/J and SJL mice, the expression and distribution of dystrophin and β-sarcoglycan were unaffected (Fig. 5A). Similarly, the expression and distribution of sarcospan, α-, δ- and γ-sarcoglycans were also not altered by the loss of dysferlin (data not shown). Consistent with these findings, immunoblot analysis showed that the levels of dystrophin, β-dystroglycan, dystrobrevin, α-sarcoglycan, telethonin and nNOS were not altered in the dysferlin-deficient mice (Fig. 5B).

Caveolin-3 and calpain-3 expression in the dysferlin-deficient muscle
Caveolin-3 is a muscle-specific membrane protein that is important for the formation of caveolae membranes by acting as a scaffold to organize and concentrate caveolin-interacting lipids and proteins (36). Caveolin-3 was reported to interact with dysferlin on the basis of co-immunoprecipitation studies and the finding of reduced caveolin-3 expression in some dysferlinopathy patients by immunofluorescence and western blot analyses (37,38). We found no evidence of altered expression or distribution of caveolin-3 in the Dysf/2, SJL and A/J muscles (Fig. 5A). Similarly, immunoblot analysis showed normal levels of caveolin-3 protein in the dysferlin-deficient muscles compared with controls (Fig. 5B). Calpain-3, a muscle-specific calcium-activated protease, has also been suggested to associate with dysferlin because its levels were reduced in half of the LGMD 2B patients studied (39). However, our immunoblot analysis showed normal levels of calpain-3 in the Dysf/2, SJL and A/J mice compared with controls (Fig. 5B).

Ultrastructural analysis of the dysferlin-deficient muscle
To characterize further the muscle membrane abnormalities highlighted by the EBD staining, we examined the ultrastructural features of the dysferlin-deficient muscle from the Dysf/2, A/J and SJL mice. The most prominent and consistent feature found in all three lines of mice was the marked thickening and focal duplication of the basal lamina (Fig. 6B and C). In addition, there were isolated areas of membrane discontinuity (Fig. 6C) and accumulation of vesicles near the sarcolemma (Fig. 6B). The contractile apparatus, thin and thick filaments and neuromuscular junctions appeared normal in the dysferlin-deficient mice.
Behavioral differences among different lines of the dysferlin-deficient mice

In our colony of SJL mice, we consistently noticed severe bite wounds on female mice that were mated with a SJL male. In addition, male littermates had to be separated at around 8 weeks of age to prevent serious injuries from fighting. This extreme aggressive behavior of the SJL mouse is well known (24) but it is striking that neither the Dysf^{−/−} nor A/J mice displayed similar aggressive traits. In fact, the A/J

Figure 3. Histological analysis of H&E-stained muscles from the Dysf^{−/−} and A/J mice. (A) Comparative analysis of quadriceps femoris muscles from 2-month-, 5-month- and 14-month-old Dysf^{−/−} (a, b, c) and A/J (d, e, f) mice. Both lines of mice developed a progressive muscular dystrophy but differ in their severity and progression. (a) Early dystrophic features in 2-month-old Dysf^{−/−} mice consist of scattered necrotic fibers (center, pale staining) and some fibers with centrally placed nuclei. (b) Muscles from 5-month-old mice show pronounced histopathological changes that include increased necrosis and regenerating fibers, mononuclear cell infiltration, phagocytosis, hypertrophic fibers, fiber splitting and fat replacement (bottom, white areas). (c) As the disease progressed, there was extensive endomysial fibrosis, fat replacement and perivascular infiltrates. (d–f) Quadriceps muscles from the A/J mice show a less severe muscle histopathology compared with the age-matched Dysf^{−/−} mice. The earliest dystrophic feature was seen in 5-month-old A/J mice, comprising a few muscle fibers with centrally placed nuclei (e). The muscle histopathology of 14-month-old A/J mice (f) resembles that of a 9-month-old Dysf^{−/−} mice (g), indicating a slower progression of the muscle disease in A/J compared with Dysf^{−/−}. (B) Analysis of the distribution of muscle histopathology in 9-month-old Dysf^{−/−} (g, h, i) and 11-month-old A/J (j, k, l) mice. Both lines of mice showed a similar distribution of muscle disease, with quadriceps muscles (proximal) more severely affected than the gastrocnemius (distal). Unexpectedly, the histopathology of the abdominal muscles in the A/J mice is similar to the Dysf^{−/−} mice. Scale bar: (c, i, l) 100 μm, all other figures is 50 μm.
Mouse models of human muscular dystrophies have provided valuable insights in our understanding of the pathogenic mechanism in muscle diseases (42). In this study, we report two novel lines of the dysferlin-deficient mice as models for dysferlinopathy. The two lines of mice were obtained by (a) targeted gene inactivation and (b) identification of a spontaneous mutation in the dysferlin gene in the A/J mice. Both lines of mice developed a progressive muscular dystrophy and recapitulated the clinical and histopathological features of the human disease, including elevated serum CK levels, myofiber degeneration and regeneration, internal myonuclei, fiber size variation, endomysial fibrosis, fat replacement and muscle inflammation.

The A/J and Dysf<sup>−/−</sup> mice developed proximal muscle degeneration at onset despite differences in their genetic background and the site of their mutation. The retrotransposon insertion in the A/J mice occurred near the 5′ end (intron 4) of the dysferlin gene, whereas the targeted disruption was at the 3′ end (exon 45). Hence, similar to patients with dysferlin deficiency, there was no apparent correlation between the site of mutation and the distribution of muscle disease in animal models of dysferlinopathy. These findings support the hypothesis that additional factors may be involved in the patterning of muscle degeneration in dysferlinopathy (3,7).

The two lines of the dysferlin-deficient mice also showed phenotype divergence, similar to the clinical heterogeneity seen in patients. In particular, the A/J mice displayed a later age of onset and a slower progression of the muscle disease compared with the Dysf<sup>−/−</sup> mice. Because both lines of mice lacked dysferlin, differences in the type of genetic lesion or the site of the mutation are unlikely to account for the milder phenotype in A/J. The variant muscle disease phenotype between the A/J and Dysf<sup>−/−</sup> mice was most likely influenced by differences in their genetic background. Therefore, the A/J mice should be a valuable resource to map and identify genes that can delay the onset or progression of the muscle disease. Identification of these modifier genes may provide additional targets for therapeutic intervention.

Loss of sarcolemma integrity is a characteristic feature of several forms of muscular dystrophies and plays a major role in the pathogenesis of muscle cell degeneration and necrosis (29). We investigated whether dysferlinopathy is associated with membrane defects by vital staining with EBD, and assessment of serum CK levels. We found elevated serum CK levels and intracellular uptake of EBD in the dysferlin-deficient muscle, indicating there was loss of sarcolemmal integrity in dysferlin-deficient muscles. Error bars indicate the standard deviation and n refers to the number of mice analyzed in each set.

**DISCUSSION**

Mouse models of human muscular dystrophies have provided valuable insights in our understanding of the pathogenic mechanism in muscle diseases (42). In this study, we report two novel lines of the dysferlin-deficient mice as models for dysferlinopathy. The two lines of mice were obtained by (a) targeted gene inactivation and (b) identification of a spontaneous mutation in the dysferlin gene in the A/J mice. Both lines of mice developed a progressive muscular dystrophy and recapitulated the clinical and histopathological features of the human disease, including elevated serum CK levels, myofiber degeneration and regeneration, internal myonuclei, fiber size variation, endomysial fibrosis, fat replacement and muscle inflammation.

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The excess vesicles seen in the dysferlin-deficient muscle resemble the mutant phenotype of fer<sup>−1</sup> in Caenorhabditis elegans, a dysferlin ortholog expressed in the large vesicles of spermatin. In fer<sup>−1</sup> mutants, the large vesicles failed to fuse with the plasma membrane and are retained in the cytoplasm, indicating that fer<sup>−1</sup> is required to mediate the fusion
process (11). By analogy, the thickened basal lamina and the excess vesicles seen in the dysferlin-deficient muscle may reflect unsuccessful attempts by the mutant muscle cell to reseal its disrupted membranes. On the basis of these observations and its homology to fer-1, dysferlin may function in muscle membrane repair by using some of its C2 domains as sensors to monitor intracellular calcium concentration and to mediate the fusion of cytoplasmic vesicles to disrupted membrane sites for resealing when there is an abnormal influx of calcium ions. Such a role for dysferlin is supported by recent calcium flux data and the finding of membrane patches at the wounded sites of the wild-type muscle fibers (6,38), whereas the rest of the mutations are at the 3′ splice junction (4,45,46). Incidentally, the mutation in the SJL mouse is also located at the 3′ splice junction of exon 45 (19). All of the earlier mentioned mutations lead to either a marked reduction or a complete loss of dysferlin. It is noteworthy that residual amounts of dysferlin (<50%) are not sufficient to prevent onset or alter the severity of the muscle disease in humans (8,45) or in mouse (18).

Although the SJL and Dysf<sup>−/−</sup> mice have mutations in the same exon and show similar muscle histopathology, they also exhibit marked phenotypic differences. The most striking difference between these two lines of mice is the aggressive behavior of the SJL mice, which required these animals to be housed individually. In contrast, the Dysf<sup>−/−</sup> and A/J mice show low intra-strain aggression (41). The SJL mice are also extremely susceptible to autoimmune diseases and have been extensively used as a model to study tumor immunobiology because of the high incidence of B-cell lymphomas in this strain (22,24). Currently, it is not clear if all of these diverse phenotypes are linked to dysferlin deficiency but it is striking that these phenotypes were not seen in the Dysf<sup>−/−</sup> or A/J mice. Because SJL is an inbred strain, there could be additional genetic lesions in this strain to account for the diverse phenotypes. This hypothesis is supported by the finding that in addition to the dysferlin defect, the SJL mice are also homozygous for the retinal degeneration 1 mutation (Pde6br<sup>rd1</sup>), which causes blindness by the age of weaning (48). It is also possible that the divergent phenotypes in the SJL mice are not due to additional mutations in the

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**Figure 5.** Immunofluorescence and immunoblot analyses of sarcolemmal proteins in the dysferlin-deficient skeletal muscle. (A) Cryosections of quadriceps muscle from the wild-type (+/+), homozygous mutant (Dysf<sup>−/−</sup>), A/J and SJL mice were stained with antibodies against dysferlin (DYSF), dystrophin (DYS), β-sarcoglycan (β-SG) and caveolin-3 (Cav-3). Dysferlin was absent from the sarcolemma of Dysf<sup>−/−</sup>. A/J and SJL mice compared with the wild-type mice but the expression and distribution of dystrophin, β-sarcoglycan and caveolin-3 were not affected. (B) Immunoblot analysis of quadriceps muscle homogenates from wild-type (+/+), homozygous mutant (−/−) A/J and SJL, using antibodies against dystrophin (DYS), β-sarcoglycan (β-DG), dystrobrevin (DBV), α-sarcoglycan (α-SG), caveolin-3 (Cav-3), calpain-3 (Calp-3), telethonin (Tel) and neuronal nitric oxide synthase (n-NOS). The expression of these proteins was not altered in the dysferlin-deficient muscle.
strain but due to a unique combination of alleles in its genetic background. Table 1 summarizes the salient features of the three lines of the dysferlin-deficient mice.

The A/J strain of mouse is one of the most widely used strains in cancer and immunology research (27). It is extensively used as a model to study lung cancers because of the high incidence of spontaneous and chemically induced lung adenomas in this strain (49). More importantly, the A/J and C57BL/6J inbred strains are differentially susceptible to more than 30 traits and diseases, which include aging, behavior, lung cancer, infectious diseases, immunological responses and complex diseases such as diabetes and obesity (50,51). Because of the wealth of phenotypic differences between these two strains, they have been used to construct (i) RI strains, AXB and BXA (25), (ii) recombinant congenic strains, AcB and BcA (50) and (iii) chromosome substitution strains, B6.A (52,53), as tools to study the genetic basis of inter-strain differences in spontaneous or experimentally induced diseases. In addition to the earlier mentioned lines, the A/J mice have also been used to establish the SMXA RI strain from reciprocal crosses between the SM/J and A/J parental strains (54).

Given the widespread use of the A/J strain of mice, some or all of the earlier mentioned strains derived from A/J may carry the dysferlin defect. We have determined that the AXB and BXA RI strains, which were generated in 1975, do not carry the A/J mutation. In contrast, PCR analysis of DNA from chromosome substituted strain B6.A-chromosome 6, revealed that it carries the A/J mutation (data not shown). Because B6.A was constructed in the early 1990s, these findings suggest that the mutation in the A/J strain must have occurred between the late 1970s and the early 1980s, and had become fixed in the production breeding stocks. These results also suggest that the A/J mouse may no longer be suitable as parental controls for the AXB and BXA RI strains because the latter do not have the A/J mutation. The AcB and BcA recombinant congenic strains are predicted to carry the A/J mutation because they were constructed in the mid-1990s. Therefore, all studies involving the A/J mice or mice derived from A/J should take into account the dysferlin defect in these strains, especially for studies evaluating locomotor activity and coordination in these mice (55).

In summary, we have obtained and characterized two independent lines of the dysferlin-deficient mice with features that closely resemble the human disease of dysferlinopathy. These mice, along with SJL, should be useful models for investigating the pathogenic pathway underlying dysferlinopathy and for developing new therapeutic strategies. In addition, comparative genetic analysis of the different lines of the dysferlin-deficient mice should help to validate phenotypes that are linked to dysferlin deficiency and to identify modifier genes that can delay the onset or the progression of the muscle disease.

**MATERIALS AND METHODS**

**Construction of targeting vector**

The murine *dysferlin* gene was isolated from a 129/SvJ BAC library (Research Genetics) by hybridization screening with the human dysferlin cDNA probes corresponding to different regions of *DYSF*. Five overlapping BAC clones that encompassed the dysferlin gene were established by restriction enzyme mapping, partial sequencing and Southern blot analysis. An 11 kb EcoRI fragment corresponding to exons 42–46 of the human *dysferlin* gene was subcloned from a 3‘ BAC clone. From this subclone, a 2.6 kb StuI/KpnI fragment proximal to exon 45 and a 6.0 kb SalI/EcoRI fragment distal to exon 45 were cloned into a positive–negative selection vector pKO (Stratagene) (Fig. 1).

**Generation of the Dyst^−/−_ mice**

The *NotrI* linearized targeting vector was introduced into the J1 ES cell line by electroporation and colonies surviving G418 and gancyclovir selection were isolated, expanded and screened by Southern blot analysis. The ES cells from three correctly targeted clones were microinjected into the C57BL/6J blastocysts and transferred into pseudopregnant recipients. Chimeric males were mated to C57BL/6J females to generate heterozygous offspring on a mixed 129SvJ and C57BL/6J background. Germ-line transmission was assessed by coat color and confirmed by Southern blot analysis of tail DNA (Fig. 1). Heterozygous mice were interbred to produce homozygous mice on a mixed background. All the animals in this study were handled and treated in accordance with the protocol approved by the Massachusetts General Hospital subcommittee on research animal care.
**Northern blot and western blot analyses**

Total RNA was isolated from skeletal muscle using the RNAeasy kit (Qiagen) according to manufacturer’s instructions. An amount of 15 μg of total RNA was fractionated on a 1.2% agarose gel containing 5% formaldehyde, blotted onto Hybond-N membrane (Amersham) and hybridized with a mouse dysferlin cDNA probe corresponding to amino acid 1993–2070.

For protein analysis, mouse tissues were homogenized in 20 volumes of tissue extraction buffer, T-PER (Pierce, Rockford, IL, USA), and boiled for 5 min. After centrifugation at 14 000 g for 10 min, the protein content of the supernatant was determined using a Coomassie protein assay reagent (Pierce). Immunoblotting was performed as previously described (10).

**Antibodies**

The monoclonal antibody, NCL-Hamlet, (Novacastra Laboratories, Newcastle Upon Tyne, UK) and affinity-purified rabbit polyclonal antibodies (Sal-1) against dysferlin have been described previously (8,9). In addition, anti-dysferlin goat polyclonal antibodies, C-19, was purchased from Santa Cruz Biotechnology (San Diego, CA, USA) and rabbit polyclonal antibodies, HM38, were raised against synthetic peptides corresponding to the carboxyl-terminus of human dysferlin (amino acid 2071–2080). Monoclonal antibodies against dystrophin (NCL-DYS), α-sarcoglycan (NCL-a-SARC), β-sarcoglycan (NCL-b-SARC), β-dystroglycan (NCL-b-DG) and calpain-3 (NCL-CALP-12A) were purchased from Novacastra Laboratories for western blot analysis. Rabbit polyclonal antibodies against telethonin, and neuronal nitric oxide were purchased from Santa Cruz Biotechnology Inc and caveolin-3 from Transduction Laboratories (Lexington, KY, USA).

**Histology and immunofluorescence analysis**

Histological and immunohistochemical analyses of skeletal muscles were performed on unfixed tissues frozen in liquid nitrogen-cooled isopentane. Transverse sections of 8–10 μm thickness of different muscles were stained with hematoxylin and eosin (H&E) for histopathological analysis. Immunofluorescence analyses were performed using specific rabbit polyclonal antibodies as described previously (56).

**Vital staining with EBD and serum CK assay**

To evaluate muscle cell membrane integrity, EBD (10 mg/ml in phosphate-buffered saline) was injected intraperitoneally into mice (0.1 ml/10 g body weight) as described previously (28). The mice were killed 12–16 h after injection, and their muscles were sectioned and examined under a fluorescence microscope for uptake of dye.

For CK measurements, blood samples were collected from the retro-orbital sinus using heparinized capillary tubes from 4-week-old mice. Plasma samples obtained by centrifugation at 2000 g were analyzed individually using a Beckman Synchro 5 Chemistry Analyzer using reagents provided by the manufacturer. For purposes of phenotyping mice in the A/J×(A/J × B6) N2 cross, a CK threshold of 200 IU/l was used to designate affected mice.

**Mice**

Stocks of the A/J, A/HeJ, A/WySnJ, B6.A-Tyr<sup>+</sup>, C57BL/6J, SJL/J, AXB and BXA RI mice were bred and maintained in standardized conditions in the Research Animal Facility at TJL. They were maintained on NIH 6% fat chow and acidified water, with 12:12 h dark:light cycle and in conventional facilities that are monitored regularly to maintain a specific

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**Table 1. Summary of the salient features of the three lines of the dysferlin-deficient mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Genetic defect</th>
<th>Effect of the mutation</th>
<th>Age of onset</th>
<th>Site of onset</th>
<th>Distinctive features</th>
</tr>
</thead>
</table>
| Dysf<sup>−/−</sup> | Deletion of exon 45 | Loss of dysferlin protein | ~2-month-old | Proximal muscles | (a) Progressive muscular dystrophy with elevated serum CK levels  
(b) Not aggressive  
(c) Highly susceptible to lung tumorigenesis  
(d) Widely used to construct RI, recombinant congenic and chromosome substitution strains |
| A/J | ETn insertion in intron 4 | Loss of dysferlin protein | ~5-month-old | Proximal muscles | (a) Similar to (a) above but show a later age of onset and less severe muscle histopathology than the Dysf<sup>−/−</sup> and SJL mice  
(b) Not aggressive  
(c) Highly susceptible to lung tumorigenesis  
(d) Highly susceptible to autoimmune diseases, viral infections and B-cell lymphomas  
(e) Also carry a mutation in the retinal degeneration 1 gene (Pde6<sup>brd1</sup>) |
| SJL | Splice site mutation at exon 45 | Marked reduction (~85%) in dysferlin levels | ~2 month-old | Proximal muscles | (a) Muscle histopathology is similar to the Dysf<sup>−/−</sup> mice  
(b) Extremely aggressive  
(c) Highly susceptible to autoimmune diseases, viral infections and B-cell lymphomas  
(d) Also carry a mutation in the retinal degeneration 1 gene (Pde6<sup>brd1</sup>) |

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pathogen free environment. Procedures used in the experiments were approved by the Institutional Animal Care and Use Committee.

Genetic mapping

DNA isolated from the 57 N2 A/J × (A/J × B6) cross was subjected to a genome-wide scan in which ~80 robust simple sequence length polymorphic markers known to differ between B6 and A/J were used. Once a probable map position was identified on chromosome 6, additional markers were tested around loci that showed significant skewing of alleles to confirm linkage. For PCR amplification, 25 ng DNA was used in a 15 μl volume containing 50 mM KCl, 10 mM Tris–Cl, pH 8.3, 2.0 mM MgCl₂, 0.2 μM oligonucleotides, 200 μM each dNTP and 0.02 U AmpliTaq DNA polymerase. The reactions which were initially denatured for 2 min at 95°C were subjected to 35 cycles of 10 s at 95°C, 15 s at 55°C, 30 s at 72°C and a 2 min extension at 72°C. PCR products were separated by electrophoresis on a 4% MetaPhor (FMC, ME) agarose gel and visualized under UV light after staining with ethidium bromide.

RT–PCR analysis

Total RNA was isolated from the skeletal muscle of the A/J, A/HeJ, A/WySnJ and SJL/J mice by the Trizol method (Gibco BRL Life Technologies, Rockville, MD, USA) according to manufacturer's instructions. For RT–PCR analysis of dysferlin, 5 μg of total RNA from each strain was treated with DNase and reverse transcribed using random hexamers and oligo-dT as previously described (57). Resulting PCR products were separated on 1% agarose gels and the bands were excised and gel-purified for cloning and sequencing using the QIAEX II procedure (Qiagen).

Primers for mutation detection

The primers for detecting the mutation in the A/J mice were: dysf-F (A/J-F), TTCTCTCTTGTGCTGTCATG; dysf-R (A/J-R), CTTCACTGGAAGATGTGCTC; ETn-or (3’ LTR), GCCTTGATCAGAGTAACTGTC and ETn-R2 (5’ LTR), AGCAAGATCTTCTCTGTG.

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