Contribution of Dysferlin Deficiency to Skeletal Muscle Pathology in Asymptomatic and Severe Dystroglycanopathy Models: Generation of a New Model for Fukuyama Congenital Muscular Dystrophy

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Abstract

Defects in dystroglycan glycosylation are associated with a group of muscular dystrophies, termed dystroglycanopathies, that include Fukuyama congenital muscular dystrophy (FCMD). It is widely believed that abnormal glycosylation of dystroglycan leads to disease-causing membrane fragility. We previously generated knock-in mice carrying a founder retrotrotransposon insertion in fukutin, the gene responsible for FCMD, but these mice did not develop muscular dystrophy, which hindered exploring therapeutic strategies. We hypothesized that dysferlin functions may contribute to muscle cell viability in the knock-in mice; however, pathological interactions between glycosylation abnormalities and dysferlin defects remain unexplored. To investigate contributions of dysferlin deficiency to the pathology of dystroglycanopathy, we have crossed dysferlin-deficient dysferlin+/+ mice to the fukutin-knock-in fukutinHp/+ and Large-deficient Largeemyd/myd mice, which are phenotypically distinct models of dystroglycanopathy. The fukutinHp/+ mice do not show a dystrophic phenotype; however, (dysferlin+/+; fukutinHp−) mice showed a deteriorated phenotype compared with (dysferlin+/+; fukutinHp+) mice. These data indicate that the absence of functional dysferlin in the asymptomatic fukutinHp− mice triggers disease manifestation and aggravates the dystrophic phenotype. A series of pathological analyses using double mutant mice for Large and dysferlin indicate that the protective effects of dysferlin appear diminished when the dystrophic pathology is severe and also may depend on the amount of dysferlin proteins. Together, our results show that dysferlin exerts protective effects on the fukutinHp+ FCMD mouse model, and the (dysferlin+/+; fukutinHp−) mice will be useful as a novel model for a recently proposed antisense oligonucleotide therapy for FCMD.

Introduction

Muscular dystrophies are a heterogeneous group of genetic disorders characterized by the progressive loss of muscle strength and integrity. Several lines of evidence have established that the structural linkage between the muscle extracellular matrix and the cytoskeleton is essential in preventing the progression of muscular dystrophy [1]. The dystrophin-glycoprotein complex (DGC) forms the structural linkage, and mutations in components of this complex cause several forms of muscular dystrophy, including Duchenne and limb-girdle muscular dystrophies (LGMDs) [2]. Within the DGC, α- and β-dystroglycans (DG) act as a molecular bridge between the extracellular matrix and the cytoskeleton. α-DG is a highly glycosylated extracellular subunit that functions as a receptor for extracellular matrix proteins such as laminins. O-mannosyl glycosylation and a novel phosphodiester-linked modification of O-mannose, termed post-phosphoryl modification, are necessary for α-DG to serve as a functional laminin receptor [3,4]. α-DG is anchored on the plasma membrane through non-covalent interaction with a transmembrane-type β-DG, which in turn binds to the dystrophin-actin cytoskeleton.

Fukuyama congenital muscular dystrophy (FCMD: MIM 253800) is an autosomal recessive disorder characterized by severe
muscular dystrophy, abnormal neuronal migration associated with mental retardation and, frequently, eye abnormalities [3]. We identified fukutin, the gene responsible for FCMD, and a 3-kb SINE-VNTR-Alu (SVA) retrotransposon insertion into the 3’ UTR of fukutin as the founder mutation in FCMD [6]. This insertion causes abnormal splicing that leads to the production of non-functional fukutin protein [7]. The introduction of antisense oligonucleotides that target the splice acceptor and splicing enhancers prevented the pathogenic abnormal splicing by SVA in the cells of FCMD patients as well as model mice that carry the retrotransposon insertion [7]. Point mutations in fukutin have been reported in patients both inside and outside Japan, and recent studies have revealed a broad clinical spectrum for fukutin-deficient muscular dystrophies [8]. In FCMD, α-DG is abnormally glycosylated, and its laminin-binding activity is decreased [3]. Several other forms of muscular dystrophy are caused by abnormal glycosylation of α-DG; collectively, these conditions are termed “dystroglycanopathies”. More than 10 genes have been identified as causative genes in dystroglycanopathies [9–14], some of which encode products that possess enzyme activities involved in synthesizing O-mannosyl sugar chains on α-DG [15–18]. Fukutin, LARGE, and Fukutin-related protein (FKRP) participate in forming the post-phosphoryl moiety [4,19]. Overall, dystroglycanopathy gene products appear to be involved in O-mannosyl chain synthesis and post-phosphoryl modification; mutations in these pathways commonly result in abnormal glycosylation of α-DG and reduced ligand-binding activity, disrupting the DG-mediated linkage between the extracellular matrix and the cytoskeleton [2].

Defects in DGC components or α-DG glycosylation disrupt the linkage between the extracellular matrix and the cytoskeleton, thus rendering the sarcolemma more susceptible to contraction-induced damage. This is thought to trigger an increase in intracellular Ca2+ concentration, eventually leading to necrosis and myofiber degeneration. Myofibers possess an intrinsic mechanism for repair of damaged membranes, and dysferlin plays a pivotal role in the skeletal muscle membrane repair pathway. In humans, dysferlin deficiency leads to LGMD2B, Miyoshi myopathy or a distal myopathy with anterior tibial onset [20]. Dysferlin-deficient mice show defective membrane repair and also develop muscular dystrophy [21]. Several proteins are known to interact with dysferlin [20], and it is expected that these proteins also participate in membrane repair. For example, mitsugumin 53 (MG53, also known as TRIM72) has been implicated in vesicle trafficking to the damage site during the membrane repair process [22].

We previously described a new FCMD mouse model that carries the retrotransposon insertion in the mouse fukutin ortholog [23]. These knock-in mice exhibit hypoglycosylated α-DG but do not develop muscular dystrophy. Therefore, these mice are not suitable for testing effectiveness of the antisense oligonucleotide therapy for FCMD. Although skeletal muscle-selective fukutin conditional knock-out mice, namely MCK-fukutin-cKO and Myf5-fukutin-cKO, show dystrophic phenotype [24], they are not applicable for the examination of the antisense oligonucleotide therapy because they do not possess the retrotransposon insertion. We previously reported that the small amount of normally glycosylated α-DG remaining in the skeletal muscle of the knock-in mice prevents muscular dystrophy [23]. However, it is not clear whether this residual glycosylation alone is sufficient to maintain skeletal muscle membrane integrity. We hypothesized that dysferlin functions compensate for presumed membrane fragility caused by a reduced interaction between α-DG and laminin. Furthermore, the exact contribution of dysferlin and dysferlin-interacting proteins to the pathology of dystroglycanopathy is not known. To investigate this question, we crossed dysferlin-deficient mice with two distinct dystroglycanopathy mouse models and analyzed the resultant phenotypes. In addition, if the double mutant mice carrying the retrotransposon insertion show worse dystrophic phenotype than those of dysferlin mutant mice, they can be the first model for the novel antisense oligonucleotide therapy for FCMD.

Materials and Methods

Animals

Dysferlin-deficient SJL/J mice, a strain with a large deletion in the Dysf gene [25], were purchased from Charles River Japan. The transgenic mouse carrying a neo cassette disruption of one fukutin allele (fukutin<sup>−/−</sup>) [26] and the transgenic knock-in homozygous mutant mouse carrying the retrotransposon insertion in the mouse fukutin ortholog (fukutin<sup>Hv/Hv</sup>) have been described previously [23]. Genotyping for the Dysf mutant allele and the fukutin mutant allele was performed as described previously [23,25]. All animal procedures were approved by the Animal Care and Use Committee of Kobe University Graduate School of Medicine (P120202-R2) in accordance with guidelines of Ministry of Education, Culture, Sports, Science and Technology (MEXT) and Japan Society for the Promotion of Science (JSPS). The animals were housed in cages (2–4 mice per cage) with wood-chip bedding in an environmentally controlled room (25°C, 12 h light-dark cycle) and provided food and water ad libitum at the animal facility of Kobe University Graduate School of Medicine. Well-trained and skilled researchers and experimental technicians, who have knowledge of methods to prevent unnecessary excessive pain, handled the animals and carried out the experiments. Euthanization was done by cervical dislocation. At sacrifice, the muscles were harvested and snap-frozen in liquid nitrogen (for biochemical analyses).

To generate double mutant mice for dysferlin and fukutin deficiency, we crossed dysferlin-deficient SJL/J mice [25] (dysferlin<sup>Δ345/Δ345</sup>, SJL background) with two different lines of fukutin mutant mice. One is a transgenic mouse carrying a neo cassette disruption for a single fukutin allele (fukutin<sup>−/−</sup>; 129-C57BL/6 background) [26] (Fig. 1A, line A). The other is a transgenic knock-in homozygous mutant mouse carrying the retrotransposon insertion in the mouse fukutin ortholog [23] (fukutin<sup>Hv/Hv</sup>; 129-C57BL/6 background) (Fig. 1A, line B). Heterozygous F1 mice in both lines were intercrossed to obtain the following four genotypes (F2): (dysferlin<sup>Δ345/Δ345</sup>; fukutin<sup>+/−</sup>); (fukutin<sup>−/−</sup>; dysferlin<sup>Δ345/Δ345</sup>); (fukutin<sup>−/−</sup>; fukutin<sup>Hv/Hv</sup>); and (fukutin<sup>−/−</sup>; dysferlin<sup>Δ345/Δ345</sup>; fukutin<sup>Hv/Hv</sup>). We further crossed (dysferlin<sup>Δ345/Δ345</sup>; fukutin<sup>−/−</sup>) with (fukutin<sup>Hv/Hv</sup>; fukutin<sup>−/−</sup>) mice or (fukutin<sup>−/−</sup>; fukutin<sup>Hv/Hv</sup>) mice with (dysferlin<sup>Δ345/Δ345</sup>; fukutin<sup>Hv/Hv</sup>) mice (Fig. 1A, highlighted with gray) to produce four genotypes (F3): (dysferlin<sup>Δ345/Δ345</sup>; fukutin<sup>Hv/Hv</sup>); (fukutin<sup>−/−</sup>; dysferlin<sup>Δ345/Δ345</sup>; fukutin<sup>Hv/Hv</sup>); (fukutin<sup>Hv/Hv</sup>; fukutin<sup>−/−</sup>; dysferlin<sup>Δ345/Δ345</sup>); and (fukutin<sup>Hv/Hv</sup>; fukutin<sup>−/−</sup>; dysferlin<sup>Δ345/Δ345</sup>). To generate double mutant mice for dysferlin and Large deficiency, we crossed dysferlin-deficient SJL/J mice (C57BL/6 backcross 7) with Large-deficient Large<sup>myd/myd</sup> mice (Large<sup>myd/myd</sup>; C57BL/6 background) [27,28]. Heterozygous F1 mice were intercrossed and the following four genotypes were used for the analyses (F2): (dysferlin<sup>Δ345/Δ345</sup>; Large<sup>myd/myd</sup>); (fukutin<sup>Hv/Hv</sup>; Large<sup>myd/myd</sup>); (fukutin<sup>−/−</sup>; dysferlin<sup>Δ345/Δ345</sup>; Large<sup>myd/myd</sup>); and (fukutin<sup>−/−</sup>; fukutin<sup>Hv/Hv</sup>; dysferlin<sup>Δ345/Δ345</sup>; Large<sup>myd/myd</sup>). For more effective breeding, we crossed (dysferlin<sup>Δ345/Δ345</sup>; Large<sup>−/−</sup>; Large<sup>−/−</sup>) mice with (fukutin<sup>Hv/Hv</sup>; Large<sup>−/−</sup>) mice (Fig. 1B).
Figure 1. Generation of double-mutant mice exhibiting both abnormal α-DG glycosylation and reduced dysferlin expression. (A, B) Breeding strategy for the generation of double-mutant mice. sjl represents the dysferlin mutant allele, myd represents the Large mutant allele, and Hp represents the transgenic allele carrying the retrotransposon insertion in fukutin. Hp/+ represents a carrier with the insertion in fukutin. Hp/+ does not represent a compound heterozygote carrying the insertion and a neo-disrupted allele. For the dysferlin/fukutin double mutant line, we used mice carrying dysferlinHp/+ and fukutinHp/+ as the normal control (dysferlinHp/-, fukutinHp/+); dysferlinHp/- and fukutinHp/- as the dysferlin-mutant (dysferlinHp/-, fukutinHp/-); dysferlinHp/- and fukutinHp/- as the double-mutant (dysferlinHp/-, fukutinHp/-). For the dysferlin/Large double mutant line, we used mice carrying dysferlinHp/+ and LargeHp/+ as the normal control (dysferlinHp/-, LargeHp/+); dysferlinHp/- and LargeHp/+ as the dysferlin-mutant (dysferlinHp/-, LargeHp/+); dysferlinHp/- and LargeHp/+ as the Large-mutant (dysferlinHp/-, LargeHp/+); and dysferlinHp/- and LargeHp/+ as the double-mutant (dysferlinHp/-, LargeHp/+). (C, D) Abnormal α-DG glycosylation and reduced dysferlin protein expression. Solubilized skeletal muscle samples from each genotype were subjected to Western blot analysis for dysferlin protein expression (left panel). Tubulin was used as a loading control. The solubilized fractions were further enriched for DG by WGA-beads, and the DG-enriched fractions were subjected to Western blotting with the monoclonal IIH6 antibody, which recognizes glycosylated proteins and reduced dysferlin expression. Solubilized skeletal muscle samples from each genotype were subjected to Western blot analysis for dysferlin protein expression (left panel). Tubulin was used as a loading control. The solubilized fractions were further enriched for DG by WGA beads. Gels were transferred to polyvinylidene fluoride membranes. The proteins were stained with Ponceau S as a standard. Proteins were separated using 3–15% linear gradient SDS-gels. Gels were transferred to polyvinylidene fluoride membranes.

doi:10.1371/journal.pone.0106721.g001

the dysferlin/Large double mutant line and LargeHp/+ mouse colonies.

Antibodies

Antibodies used in Western blotting and immunofluorescence were as follows: mouse monoclonal antibody 8D5 against β-DG (Novocastra); mouse monoclonal antibody IIIH6 against α-DG (Millipore); affinity-purified goat polyclonal antibody against the α-DG core protein (AP-074G-C) [23]; mouse monoclonal antibody NCL-Hamlet against dysferlin (Novocastra); rat monoclonal antibody against mouse F4/80 (BioLegend); rabbit polyclonal antibody against collagen I (AbD serotec); rabbit polyclonal antibody against albumin (DAKO); mouse monoclonal antibody against caveolin-3 (BD Transduction Laboratories); rabbit polyclonal antibody against caveolin-3 (Abcam); and rabbit polyclonal antibody against Trim72 (MG53) (Abcam).

Protein preparation and Western blotting

DG was enriched from solubilized skeletal muscle as described previously [23]. Briefly, skeletal muscles were solubilized in Tris-buffered saline (TBS) containing 1% Triton X-100 and protease inhibitors (Nacalai). The solubilized fraction was further enriched for DG by wheat germ agglutinin (WGA)-agarose beads (Vector Laboratories) at 4°C for 16 h, and then DG was eluted with SDS-PAGE loading buffer. For detection of dysferlin and dysferlin-interacting proteins, RIPA buffer (1% NP-40, 0.5% DOC, and 0.1% SDS in TBS with protease inhibitors) was used for protein extraction from skeletal muscle. For this experiment, we used fukutinHp/- mice and litter control fukutinHp/+ mice that were backcrossed to C57BL/6 mice more than 10 times. Protein concentration of the solubilized fractions was measured by Lowry methods, using BSA as a standard. Proteins were separated using 3–15% linear gradient SDS-gels. Gels were transferred to polyvinylidene fluoride membranes.
glycosylated α-DG. The ligand binding activity of α-DG in Large<sub>myd/myd</sub> mice is greatly reduced compared with that in fukutin<sub>Hp+/−</sub> mice [23]. Breeding strategies, genotypes, and abbreviations for these double mutant mice and their controls are shown in Figure 1A and 1B.

To confirm reduced protein expression of dysferlin and abnormal glycosylation of α-DG in these mice, we prepared solubilized fractions from skeletal muscle extracts and enriched for α-DG using wheat germ agglutinin (WGA)-agarose beads. Western blot analysis showed a dramatic reduction of dysferlin protein in skeletal muscle from (dysferlin<sub>gr/+</sub>, fukutin<sub>Hp+/−</sub>), (dysferlin<sub>gr/+</sub>, fukutin<sub>Hp+/−</sub>), (dysferlin<sub>gr/+</sub>, Large<sub>myd/myd</sub>), and (dysferlin<sub>gr/+</sub>, Large<sub>myd/myd</sub>) mice (Fig. 1C and D). We also confirmed a significant reduction of reactivity against the monoclonal antibody IIH6, which recognizes glycosylated epitopes on α-DG that are necessary for laminin binding activity, in (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>), (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>), (dysferlin<sub>gr/+</sub>; Large<sub>myd/myd</sub>), and (dysferlin<sub>gr/+</sub>; Large<sub>myd/myd</sub>) mice (Fig. 1C and D). Overall, these data confirmed the production of model mice with four biochemically distinct genotypes in each double mutant line.

More severe muscular dystrophy in (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) than in (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice

We examined the histopathology of (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice by hematoxylin and eosin (H&E) staining. The (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice showed no obvious pathological features of muscular dystrophy (Fig. 2). The (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice showed mild dystrophic changes such as the presence of necrotic fibers and centrally located nuclei (Fig. 2). The phenotypes of (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) and (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice are similar to those described previously for retrotransposon knock-in fukutin mutant mice and dysferlin-deficient SJL/J mice, respectively [23,25]. These results also indicate that disruption of one dysferlin or one fukutin allele does not affect the phenotype of fukutin<sub>Hp+/−</sub> or dysferlin<sub>gr/+</sub> single mutant mice, respectively. H&E staining showed that the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice showed further progression and more severe dystrophic features than did the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice in quadriceps (Quad), gastrocnemius (Gast), and tibialis anterior (TA) muscles (Fig. 2A and 3A). Comparison of the percentage of muscle fibers with centrally located nuclei confirmed a more severe dystrophic phenotype in the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice than that in the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice (Fig. 2B).

To compare the pathological severity in (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) and (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) skeletal muscle more precisely, we counted the percentage of muscle fibers (TA) with centrally located nuclei at different ages (Fig. 3A and B). In 8-week-old mice, we observed a few fibers with centrally located nuclei and necrotic fibers in both the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) and the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice, but no significant differences were seen between the two (data not shown). At 15 weeks and 30 weeks of age, the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice show significantly more fibers with centrally located nuclei than do the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice (Fig. 3B). The proportion of fibers with centrally located nuclei in the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice increased with age. These results indicate more frequent cycles of muscle cell degeneration and regeneration in the (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) mice. We next compared infiltration of macrophage and connective tissue as indicators of disease severity. Immunofluorescence analysis using the monoclonal F4/80 antibody, a marker for macrophages, indicated that macrophage infiltration was increased in (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) skeletal muscle compared with (dysferlin<sub>gr/+</sub>; fukutin<sub>Hp+/−</sub>) skeletal
latent membrane fragility, which is protected partially by dysferlin.

Figure 2. Histological analysis of skeletal muscle from dysferlin/fukutin double mutant mice. (A) Quadriceps (Quad) and gastrocnemius (Gast) muscle tissues from the four mouse genotypes at 15 weeks were analyzed by H&E staining. Bar, 50 μm. (B) Myofibers with centrally located nuclei were counted and quantitatively compared between (dysferlin\textsuperscript{gld/gld}, fukutin\textsuperscript{Hp/-}) and (dysferlin\textsuperscript{gld/gld}, fukutin\textsuperscript{Hp/+}) mice (*, p<0.05). Data shown are mean ± s.e.m. for each group (n is indicated in the graph). The (dysferlin\textsuperscript{gld/gld}, fukutin\textsuperscript{Hp/-}), (dysferlin\textsuperscript{gld/gld}, fukutin\textsuperscript{Hp/+}), (dysferlin\textsuperscript{gld/gld}, fukutin\textsuperscript{Hp/-}), and (dysferlin\textsuperscript{gld/gld}, fukutin\textsuperscript{Hp/+}) mice are abbreviated as (sjl/+ : Hp/+), (sjl/- : Hp/-), (sjl/sjl : Hp/+), and (sjl/sjl : Hp/-), respectively.

doi:10.1371/journal.pone.0106721.g002

Characterization of muscular dystrophic changes in (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/myd}) mice

We subsequently analyzed the histopathology of (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/myd}) mice. Large\textsuperscript{myd/myd} mice show severe muscular dystrophic phenotypes such as infiltration of connective and fat tissues and marked variation in fiber size [28]. Almost all α-DG is hypoglycosylated in Large\textsuperscript{myd/myd} mice [23]. We confirmed that the pathology of (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/myd}) mice was more severe than that in (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/+}) mice (Fig. 6). To examine whether the dysferlin functions have protective roles in Large\textsuperscript{myd/myd} skeletal muscle, we compared the pathology in (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/myd}) and (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/+}) mice. The (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/myd}) mice showed necrotic and centrally nucleated fibers, indicating frequent cycles of muscle degeneration and regeneration (Fig. 6C). In addition, some animals showed signs of advanced muscular dystrophic changes such as variations in fiber size and connective tissue infiltration (Fig. 6D). The (dysferlin\textsuperscript{gld/gld}, Large\textsuperscript{myd/+}) mice exhibited severe pathology, including marked variation in fiber size and large areas with infiltration (Fig. 6E and F). We evaluated these pathologies quantitatively by measuring the areas of macrophage or connective tissue infiltration and the population of albumin-positive muscle fibers in (dysferlin\textsuperscript{gld/gld}, fukutin\textsuperscript{Hp/-}) skeletal muscle (Fig. S1A and B). Immunofluorescence analysis also showed no obvious change in dysferlin expression pattern between fukutin\textsuperscript{Hp/-} and fukutin\textsuperscript{Hp/+} skeletal muscle (Fig. S1C).
muscle fibers (Fig. 6I, J, and K). Both the macrophage-infiltrated area and the population of albumin-positive muscle fibers tended to be larger in (dysferlin<sup>sjl/sjl</sup>; Large<sup>myd/myd</sup>) than in (dysferlin<sup>sjl/+</sup>; Large<sup>myd/myd</sup>); however, we did not observe statistically significant differences between the two groups. Furthermore, quantification of collagen I immunofluorescence showed no significant difference in connective tissue infiltration between (dysferlin<sup>sjl/sjl</sup>; Large<sup>myd/myd</sup>) and (dysferlin<sup>sjl/+</sup>; Large<sup>myd/myd</sup>) skeletal muscles. These results suggest that dysferlin function produces limited protective effects against the progression of severe muscular dystrophy in Large<sup>myd/myd</sup> mice. Interestingly, however, when compared with the (dysferlin<sup>sjl/+</sup>; Large<sup>myd/myd</sup>) mice, the (dysferlin<sup>sjl/sjl</sup>; Large<sup>myd/myd</sup>) mice showed significant increases in F4/80, collagen I and intracellular albumin staining (Fig. 6I, J, and K). The amount of dysferlin protein in total lysates from (dysferlin<sup>sjl/sjl</sup>; Large<sup>myd/myd</sup>) and (dysferlin<sup>sjl/+</sup>; Large<sup>myd/myd</sup>) skeletal muscles was estimated to

Figure 3. Pathological comparisons between (dysferlin<sup>sjl/+</sup>; fukutin<sup>Hp/+</sup>) and (dysferlin<sup>sjl/sjl</sup> and fukutin<sup>Hp/+</sup>) mice. (A) H&E staining of TA muscle from (dysferlin<sup>sjl/+</sup>; fukutin<sup>Hp/+</sup>), (dysferlin<sup>sjl/+</sup>; fukutin<sup>Hp/-</sup>), (dysferlin<sup>sjl/sjl</sup>; fukutin<sup>Hp/+</sup>) and (dysferlin<sup>sjl/sjl</sup>; fukutin<sup>Hp/-</sup>) mice at 8, 15 and 30 weeks. Bar, 50 μm. (B) Myofibers with centrally located nuclei were counted and quantitatively compared between (dysferlin<sup>sjl/+</sup>; fukutin<sup>Hp/+</sup>) and (dysferlin<sup>sjl/sjl</sup>; fukutin<sup>Hp/-</sup>) mice at 15 and 30 weeks (*, p < 0.05). Data shown are mean ± s.e.m. for each group (n is indicated in the graph). The (dysferlin<sup>sjl/+</sup>; fukutin<sup>Hp/+</sup>), (dysferlin<sup>sjl/+</sup>; fukutin<sup>Hp/-</sup>), (dysferlin<sup>sjl/sjl</sup>; fukutin<sup>Hp/+</sup>), and (dysferlin<sup>sjl/sjl</sup>; fukutin<sup>Hp/-</sup>) mice are abbreviated as (sjl/+; Hp/+), (sjl/+; Hp/-), (sjl/sjl; Hp/+), and (sjl/sjl; Hp/-), respectively.

doi:10.1371/journal.pone.0106721.g003
be \( \sim 20\% \) and \( \sim 60\% \) of that from \( \text{dysferlin}^{+/+}: \text{Large}^{myd/myd} \) muscle, respectively (Fig. 6L). These results suggest that the dramatic reduction in the amount/activity of dysferlin protein may be associated with a worse phenotype in the \( \text{dysferlin}^{sjl/sjl}: \text{fukutin}^{Hp/2} \) mice. Overall, our results suggest that the protective effects of dysferlin on dystroglycanopathy phenotype appear to be diminished when the dystrophic pathology is severe and progressive and also may depend on the amount of dysferlin proteins.

Figure 4. Macrophage and connective tissue infiltration in dysferlin/fukutin double mutant mice. (A) Macrophage infiltration was determined by immunofluorescence analysis using the F4/80 antibody (red). The sarcolemma and nuclei were stained by laminin (green) and DAPI (blue), respectively. TA muscle sections from 30-week-old mice were used. Bar, 50 \( \mu \)m. (B) F4/80-positive immunofluorescence signals were quantified using Image J software. (C) Connective tissue infiltration was determined by Masson-Trichrome staining. TA muscle sections from 30-week-old mice were used. Bar, 50 \( \mu \)m. (D) Quantitative analysis of connective tissue infiltration, determined by immunofluorescence analysis using anti-collagen I antibody. The collagen I-positive area was quantified using Image J software. For quantitative analysis (B and D), data shown are mean \pm s.e.m. for each group (\( n \) is indicated in the graph; *, \( p < 0.05 \)). The \( \text{dysferlin}^{sjl/+: fukutin}^{Hp/+} \), \( \text{dysferlin}^{sjl/+: fukutin}^{Hp/-} \), \( \text{dysferlin}^{sjl/sjl: fukutin}^{Hp/+} \), and \( \text{dysferlin}^{sjl/sjl: fukutin}^{Hp/-} \) mice are abbreviated as (sjl/+: Hp/+), (sjl/+: Hp/-), (sjl/sjl: Hp/+), and (sjl/sjl: Hp/-), respectively.

doi:10.1371/journal.pone.0106721.g004
Discussion

Here we have characterized the contribution of dysferlin-deficiency to the pathology of dystroglycanopathy using double mutant mice for dysferlin and α-DG glycosylation. To date, several dystroglycanopathy model mice have been established. *Largemyd* mice [28] and knock-in mice carrying the FKRP P448L mutation [32] show no detectable amounts of functionally glycosylated α-DG, no laminin binding activity, and progressive muscular dystrophy. On the other hand, other dystroglycanopathy mouse models do not show a muscular dystrophy phenotype [23]. We previously reported that a small amount of intact α-DG in *fukutin* Hp−/− mice is sufficient to maintain muscle cell integrity, thus preventing muscular dystrophy [23]. These results and others suggest that the presence of functionally glycosylated α-DG can decrease disease severity [33,34]. In the present study, however, we showed that although *dysferlin* sjl+/Hp−/+ mice did not exhibit a muscular dystrophy phenotype, *dysferlin* sjl+/Hp−/- mice developed a more exacerbated phenotype than did the *dysferlin* single-mutant *dysferlin* sjl+/Hp−/+ mice. It has been widely accepted that α-DG glycosylation plays an important role in preventing disease-causing membrane fragility by maintaining a tight association between the basement membrane and the muscle cell membrane, and its defects produce muscle membrane that is susceptible to damage [24,29]. The synergically exacerbated phenotype of the *dysferlin* sjl+/Hp−/- mice suggests latent membrane fragility in *fukutin*-deficient *fukutin* Hp−/− skeletal muscle. Indeed, the increased number of intracellular albumin-positive fibers in the *dysferlin* sjl+/Hp−/- mice also supports this hypothesis. It is assumed in the *fukutin* Hp−/− myofiber that interaction between the basement membrane and the cell membrane may be weakened, and therefore disease-causative membrane damage could occur during...
Figure 6. Histopathological analysis of skeletal muscle from dysferlin/Large double mutant mice. (A–H) H&E staining of TA muscle from (dysferlin sjl+/ : myd/m+) A, (dysferlin sjl/sjl : myd/m+), B, (dysferlin sjl+/ : myd/m+), C and D, (dysferlin sjl/sjl : myd/myd), E and F, and (dysferlin sjl/sjl : myd/m+), G and H) mice at 15 weeks. Bar, 50 μm. (I) Quantitative analysis of macrophage infiltration, determined by immunofluorescence analysis using F4/80 antibody. (J) Quantitative analysis of connective tissue infiltration determined by immunofluorescence analysis using Dysferlin in Dystroglycanopathy Pathology.
muscle contractions. However, such presumable membrane fragility may be protected in part by the dysferlin functions.

It is known that dysferlin plays a role in membrane repair pathway and several proteins are known to interact with dysferlin, suggesting that dysferlin forms a protein complex during the membrane repair process. MG53 has been shown to interact with dysferlin and participate in membrane repair, and genetic disruption of MG53 in mice results in muscular dystrophy [22]. Cavocolin-3 is known to interact with dysferlin and MG53 [31,35]. In the present study, however, we did not observe compensatory upregulation of these proteins in dysferlin-/- mice, suggesting that dysferlin functions other than membrane repair may play protective roles in the fukutinHp/- mice. Recently, accumulating evidence has suggested new dysferlin roles other than membrane repair, such as T-tubule formation, maintenance, and stabilizing stress-induced Ca\(^{2+}\) signaling [36,37]. In addition, it has been reported that dysferlin deficiency leads to increased expression of complement factors and that complement-mediated muscle injury is associated with the pathogenesis of dysferlin-deficient muscular dystrophy [38]. Therefore, it is possible that such impairments independently or synergically contribute to the pathology of the double mutant mice.

Our results showed, rather unexpectedly, that the double-mutant (dysferlin\(^{+/+}\), Large\(^{+/+}\)/myd\(^{−/−}\) mice did not exhibit significant deterioration of muscle pathology compared with the single-mutant (dysferlin\(^{+/+}\), Large\(^{+/+}\)/myd\(^{−/−}\) mice. These data suggest that the protective effects of dysferlin in Large\(^{+/+}\)/myd\(^{−/−}\) mice were slightly or much reduced compared with those in fukutinHp/- mice. Since Large\(^{+/+}\)/myd\(^{−/−}\) mice showed severe and rapid progressive pathology while fukutinHp/- mice were asymptomatic, our data suggest that the protective effect of dysferlin may be less when disease pathology is advanced and/or severe. It has been reported that a double mutant of dysferlin and dystrophin produced a more exacerbated phenotype than did either single mutant [39]. In our colony, Large\(^{+/+}\)/myd\(^{−/−}\) mice show much more severe and rapid progressive pathology than do dystrophin-deficient mdx mice, supporting our hypothesis of a limited protective effect of dysferlin in dystrophic pathology. Interestingly, the (dysferlin\(^{+/+}\), Large\(^{+/+}\)/myd\(^{−/−}\) mice, however, showed a significantly worse phenotype that did the (dysferlin\(^{+/+}\), Large\(^{+/+}\)/myd\(^{−/−}\) mice. In addition, there is a tendency toward a worse phenotype in the order of dysferlin amount, i.e., (dysferlin\(^{+/−}\), Large\(^{+/+}\)/myd\(^{−/−}\), (dysferlin\(^{+/−}\), Large\(^{+/+}\)/myd\(^{−/−}\), and (dysferlin\(^{+/−}\), Large\(^{+/+}\)/myd\(^{−/−}\)). These data support the possibility that the protective effect of dysferlin is present even in the severe dystrophic Large\(^{+/+}\)/myd\(^{−/−}\) mice. We conclude that dysferlin has the potential to protect muscular dystrophy progression; however, its effect may depend on disease severity and the amount/activity of dysferlin proteins.

Recently, we showed that the retrotransposal insertion in the 3’-UTR region of fukutin causes abnormal mRNA splicing, which is induced by a strong splice acceptor site in SVA and a rare alternative donor site in the last exon, to produce an aberrantly spliced fukutin protein [7]. The introduction of antisense oligonucleotides that target the splice acceptor, the predicted exon splicing enhancer, and the intronic splicing enhancer prevented the pathogenic exon trapping by SVA in the cells of FCMD patients as well as model mice (fukutinHp/Hp and fukutinHp/+). This therapeutic strategy can potentially be applied to almost all FCMD patients in Japan, and can therefore be the first radical clinical treatment for dystroglycanopathies. However, there was no animal model to test the effectiveness of the antisense oligonucleotide therapy. Since fukutinHp/- mice do not exhibit any signs of muscular dystrophy [23], they are not a great model for examining therapeutic effects of this strategy. Skeletal muscle-selective fukutin cKO mice, MCK-fukutin-cKO and MyD-fukutin-cKO, showed dysphoric pathology [24], but they do not possess the retrotransposal insertion, and thus they are not applicable for testing the antisense oligonucleotide therapy. Our present study demonstrates more severe dystrophic phenotype of (dysferlin\(^{+/+}\), fukutinHp/-) mice compared with (dysferlin\(^{+/+}\), fukutinHp/+), (dysferlin\(^{+/+}\), fukutinHp/+), and (dysferlin\(^{+/+}\), fukutinHp/+). Since the (dysferlin\(^{+/+}\), fukutinHp/-) mice possess the retrotransposal insertion and show dystrophic phenotype, they will be used as the first model for evaluation of the antisense oligonucleotide therapy for FCMD. There is a possibility that the absence of dysferlin could add hurdles on how to interpret the results of the antisense oligonucleotide treatments; however, our quantitative assessments established in this study could overcome this issue. For example, macrophage infiltration (Fig 4B), connective tissue infiltration (Fig 4D), and membrane fragility in quadriceps muscles (Fig 5B) were significantly increased only in the (dysferlin\(^{+/+}\), fukutinHp/-) mice. These parameters in the (dysferlin\(^{+/+}\), fukutinHp/+), (dysferlin\(^{+/+}\), fukutinHp/-), and (dysferlin\(^{+/+}\), fukutinHp/-) mice were not changed compared with those in the (dysferlin\(^{+/+}\), fukutinHp/-) mice and therefore can be used for quantitative evaluation for therapeutic effects of the antisense oligonucleotide treatments. We hope that generation of this novel FCMD model and establishment of the quantitative evaluation for disease severity will accelerate the future translational researches to overcome FCMD.

**Supporting Information**

**Figure S1 Expression of dysferlin and dysferlin-interacting proteins in fukutinHp/- mice.** (A) Western blot analysis of dysferlin, caveolin-3, and MG53 in skeletal muscle extracts from fukutin-deficient fukutinHp/- (Hp/-), and control fukutinHp/+ (Hp/+). A representative two individual samples for each mouse line are shown in the blots. (B) Quantification of protein expression (panel A) was shown in graphs. Data shown are the average with standard deviations (n = 4 for dysferlin, n = 3 for caveolin-3 and MG53). (C) Immunofluorescence analysis of dysferlin in fukutinHp/- (Hp/-) and fukutinHp/+ (Hp/+). Bar, 50 μm.

**Acknowledgments**

We would like to thank past and present members of the Dr. Toda’s laboratory for fruitful discussions and scientific contributions. We also thank Dr. Jennifer Logan for help in editing the manuscript.
Author Contributions
Conceived and designed the experiments: MK ZL TT. Performed the experiments: MK ZL CI KM. Analyzed the data: MK CI. Contributed reagents/materials/analysis tools: CM KM. Contributed to the writing of the manuscript: MK TT.

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