retinal IPL and hair cells in the inner ear. Pikachurin expression was not detected in these sites, suggesting that pikachurin functions specifically in photoreceptor-bipolar synaptic apposition.

The human *PIKACHURIN* gene is located on chromosome 5, region p13.2-p13.1. Although human *PIKACHURIN* maps in the vicinity of early-onset autosomal dominant macular dystrophy (MCDR3), which was mapped to chromosome 5, region p13.1-p15.33 (RetNet, http://www.sph.uth.tmc.edu/Retnet/), *PIKACHURIN* mutations do not seem to be responsible for this disease when the phenotypes of the *Pikachurin* null mouse are taken into consideration. Notably, the *Pikachurin* null mouse showed an impairment of visual function detected by OKR (**Fig. 5i–k**). *Pikachurin* null mice showed normal visual function for large-angle stripes but significantly reduced visual function for small-angle stripes (unpaired *t* test, $P < 0.01$). This may suggest that a mutation of *PIKACHURIN* in humans leads not to an obvious clinical manifestation of eye disease but rather to impairment of spatial resolution in vision.

Functional interaction between pikachurin and dystroglycan

Our observations suggest the possibility of a functional interaction between pikachurin and dystroglycan (**Supplementary Fig. 6** online). We observed a reduction in the amplitude and delayed implicit time of the ERG b-wave (**Fig. 5a–f**) in the *Pikachurin*$^{-/-}$ mouse. Both in human and mouse, mutations of dystrophin, an intracellular component of the DGC, are known to cause an abnormality in the ERG b-wave. In humans, many individuals with DMD and BMD with mutations in dystrophin show abnormal dark-adapted ERG b-waves[17,28]. Studies of individuals with DMD deletions have shown that the location of the deleted sequence can affect the ERG phenotype[29]. Mutations in the central or 3′ region of the gene are associated with severe reductions of amplitude and prolongation of the implicit time in the b-wave, whereas mutations limited to the 5′ end of the gene appear to be associated with milder abnormalities and, in some cases, normal ERGs[29]. In mice, disruption of dystrophin (*mdx*^{Cv2} and *mdx*^{Cv4}) causes prolongation of the implicit time of the b-wave[18]. Our results suggest that functional disruption of the interaction between dystroglycan and pikachurin in the retina may produce abnormal dark-adapted ERG b-waves in individuals with DMD and BMD. In addition, lack of glycosylation of α-dystroglycan in glycosyltransferase-deficient mice (*Large*^{myd} and *Large*^{vls}) also shows an ERG phenotype that is similar to that of *Pikachurin* null mice[30]. The similarity of unique abnormalities of ERGs observed in the *Pikachurin* null, *mdx*^{Cv2}, *mdx*^{Cv4}, *Large*^{myd} and *Large*^{vls} mutants strongly suggest that there is a functional interaction between pikachurin and DGC components in the retinal ribbon synapses.

We also found a direct interaction of pikachurin with α-dystroglycan, an extracellular component of the DGC (**Fig. 6**). It has been reported that α-dystroglycan binds to laminins and perlecan in a glycosylation-dependent manner[35]. The inhibitory effect of IIH6 and divalent cation–dependent binding suggest that pikachurin binds to α-dystroglycan by a mechanism that is similar to other known ligands, such as laminins and perlecan. Supporting this idea, pikachurin colocalizes with β-dystroglycan in photoreceptor synaptic terminals (**Fig. 6a–c**).

On the basis of these data, pikachurin probably functionally interacts with DGC components to form proper synaptic connections between photoreceptors and bipolar cells in the retinal ribbon synapses.

Molecular mechanism of pikachurin in synapse formation

In NMJs, formation of the proper synaptic structure is regulated by several dystroglycan ligands, such as agrin, laminins and perlecan. These ligands interact with dystroglycan, localizing to the postsynaptic surface of NMJ, and induce the differentiation and maturation of postsynaptic structures through the clustering of appropriate postsynaptic components (**Supplementary Fig. 6**)[39,40]. In contrast to the postsynaptic localization of dystroglycan in NMJs, dystroglycan in the ribbon synapse localizes to the presynaptic membrane of photoreceptor synaptic terminals around the bipolar cell dendritic processes[12–14]. To the best of our knowledge, pikachurin is the first dystroglycan ligand that has been found to interact with the presynaptic dystroglycan (**Supplementary Fig. 6**). How does pikachurin control invagination by the bipolar dendritic tips of the photoreceptor presynaptic terminals? On the basis of our data and previous findings, we hypothesize two scenarios. The first scenario is that pikachurin is involved in forming the proper structure of photoreceptor terminals for invagination by the tips of bipolar dendrites. The interaction of pikachurin with dystroglycan on the surface of the presynapse may cause a structural change of the photoreceptor presynaptic terminals, forming the proper connection with the postsynaptic terminals of bipolar dendrites. This scenario leads to the hypothesis that fine structural conformation of the axon terminus is crucial for the initial specific and precise synaptic apposition of a dendrite to the axon terminus. After this, adhesive molecules function supportively for the successive development and maintenance of synaptic connections.

The second scenario is that pikachurin is an attractant that induces the bipolar dendritic tips into proximity with the photoreceptor ribbon synapse through interaction with an unknown factor (represented as a factor, X; **Supplementary Fig. 6**) on the postsynaptic terminals of bipolar cell dendrites. Pikachurin released from photoreceptor synapses may induce structural changes in bipolar dendritic tips, such as the clustering of postsynaptic components, via an interaction with the unknown factor expressed in the tips of the bipolar cell dendrites. This may result in the attraction and insertion of the bipolar dendritic tips to the invagination of photoreceptor synaptic terminals.

In this study, we demonstrated that a previously unknown dystroglycan-interacting protein, pikachurin, is important for the formation of the ribbon synapse, a specialized synaptic structure in the CNS. Dystroglycan is known to be expressed not only in muscular cells but also in various CNS neurons[41]. Our findings provide clues as to the mechanisms of dystroglycan and ECM molecules in the formation of fine CNS synaptic structures.

METHODS

Generation of *Pikachurin* mutant mouse. We obtained *Pikachurin* genomic clones from a screen of the 129/SvEv mouse genomic DNA library (Stratagene). We subcloned an 8.4-kb *SwaI-ScaI* fragment and an 8.1-kb *EcoRV-KpnI* fragment from the *Pikachurin* genomic clones into a modified pPNT vector[42], and transfected the linearized targeting construct into TC1 embryonic stem cell line[42]. The culture, electroporation and selection of TC1 were carried out as previously described[42]. Embryonic stem cells that were heterozygous for the targeted gene disruption were microinjected into C57BL/6 blastocysts to obtain chimeric mice.

We carried out immunohistochemistry, northern blot analysis, RT-PCR analysis, *in situ* hybridization, electron microscopy, ERG recordings, VEP recording, OKR analysis and pull down binding assays as described in the **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

S.S. and T. Furukawa designed the project. S.S., Y.O. and K. Katoh carried out the molecular, immunocytochemistry and electron microscopy experiments. S.S., M.K., K.M. and T.K. carried out the ERG experiments. S.S., A.T. and T. Furukawa produced the knockout mice. S.S. and N.K. performed the electron tomography analysis. J.U. carried out the immuno–electron microscopy experiments. S.S. and K.F. performed the OKR experiments. T.M. and H.S. carried out the VEP experiments. S.S., Y.O., M.K., K. Kobayashi and T.T. conducted the pull-down experiments. S.S., Y.O. and T. Furukawa wrote the manuscript. Y.T., T. Fujikado. and T. Furukawa supervised the project.

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Residual laminin-binding activity and enhanced dystroglycan glycosylation by LARGE in novel model mice to dystroglycanopathy

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Hypoglycosylation and reduced laminin-binding activity of α-dystroglycan are common characteristics of dystroglycanopathy, which is a group of congenital and limb-girdle muscular dystrophies. Fukuyama-type congenital muscular dystrophy (FCMD), caused by a mutation in the *fukutin* gene, is a severe form of dystroglycanopathy. A retrotransposal insertion in *fukutin* is seen in almost all cases of FCMD. To better understand the molecular pathogenesis of dystroglycanopathies and to explore therapeutic strategies, we generated knock-in mice carrying the retrotransposal insertion in the mouse *fukutin* ortholog. Knock-in mice exhibited hypoglycosylated α-dystroglycan; however, no signs of muscular dystrophy were observed. More sensitive methods detected minor levels of intact α-dystroglycan, and solid-phase assays determined laminin binding levels to be ~50% of normal. In contrast, intact α-dystroglycan is undetectable in the dystrophic Large^{myd} mouse, and laminin-binding activity is markedly reduced. These data indicate that a small amount of intact α-dystroglycan is sufficient to maintain muscle cell integrity in knock-in mice, suggesting that the treatment of dystroglycanopathies might not require the full recovery of glycosylation. To examine whether glycosylation defects can be restored *in vivo*, we performed mouse gene transfer experiments. Transfer of *fukutin* into knock-in mice restored glycosylation of α-dystroglycan. In addition, transfer of *LARGE* produced laminin-binding forms of α-dystroglycan in both knock-in mice and the *POMGnT1* mutant mouse, which is another model of dystroglycanopathy. Overall, these data suggest that even

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partial restoration of α-dystroglycan glycosylation and laminin-binding activity by replacing or augmenting glycosylation-related genes might effectively deter dystroglycanopathy progression and thus provide therapeutic benefits.

INTRODUCTION

Dystroglycanopathy is a group of congenital and limb-girdle muscular dystrophies that includes Walker–Warburg syndrome (WWS), muscle-eye-brain (MEB) disease, Fukuyama-type congenital muscular dystrophy (FCMD), congenital muscular dystrophy 1C/D (1,2) and limb-girdle muscular dystrophy (LGMD) 2I/K/M/N (3–6). Hypoglycosylation of α-dystroglycan is a hallmark of these disorders. So far, six genes (*POMT1*, *POMT2*, *POMGnT1*, *fukutin*, *FKRP* and *LARGE*) have been implicated in dystroglycanopathies and all are thought to be involved in glycosylation of α-dystroglycan. POMGnT1 and the POMT1/2 complexes are known to have glycosyltransferase activities that place *O*-mannosyl sugar chains on α-dystroglycan (7,8). The exact functions of fukutin, FKRP and LARGE are still unknown.

α-Dystroglycan (α-DG) is a receptor for laminin in the basement membrane and is anchored on the plasma membrane through non-covalent interaction with a transmembrane-type β-DG (9). α- and β-DGs are encoded by a single mRNA that is cleaved into two subunits during post-translational maturation. *O*-glycosylation of α-DG is required for ligand-binding activity. Although the exact binding epitope for ligand is still unknown, one unique *O*-mannosyl glycan [Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–2)Man-Ser/Thr] (10) appears to be involved in ligand binding among extensive and heterogenous groups of *O*-linked sugar chains. β-DG interacts with dystrophin, which in turn binds to actin filaments. The DG complex spans the plasma membrane, connecting the basement membrane to the actin cytoskeleton and presumably conferring mechanical stability to muscle cells during muscle contraction.

In Japan, FCMD is the most common congenital muscular dystrophy and, following Duchenne muscular dystrophy, is the second most common childhood muscular dystrophy. An autosomal recessive disorder, FCMD is characterized by severe muscular dystrophy, abnormal neuronal migration associated with mental retardation and epilepsy and, frequently, eye abnormalities (11). A recent study revealed aberrant neuromuscular junction formation and delayed muscle terminal maturation in FCMD, suggesting that a maturational delay of muscle fibers underlies the etiology of FCMD (12). Through positional cloning we identified *fukutin*, the gene responsible for FCMD (13). The predominant mutation in FCMD was identified as a 3 kb SINE-VNTR-*Alu* (SVA) retrotransposon insertion into the 3′-UTR of *fukutin*. In Japan, 70–80% of FCMD patients are homozygous for this retrotransposal insertion. Compound heterozygosity, exhibiting both a retrotransposonal mutation and a point mutation, is sometimes seen and generally exhibits more severe pathologies (13–15). Only a few cases with non-founder mutations (homozygous for point mutations) have been reported outside of Japan (5,16–19).

MEB disease is a severe autosomal recessive disease, similar to FCMD, characterized by congenital muscular dystrophy, ocular abnormalities and brain malformation. The gene responsible for MEB is *POMGnT1*, which encodes protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (7). In both FCMD and MEB disease, α-DG glycosylation and laminin-binding activity are severely disrupted (20). The Large^{myd} mouse, a spontaneous mutant, has been used as a model for dystroglycanopathy. As is the case with human dystroglycanopathies, α-DG in Large^{myd} mice is hypoglycosylated and shows reduced ligand-binding activity (20,21). Positional cloning in this model identified a disease-causing mutation in the *Large* gene (22), which encodes a protein with a transmembrane domain followed by a coiled-coil domain and two DxD-containing putative catalytic domains (23). *LARGE* mutations are also seen in human dystroglycanopathy (24). Although the exact function of the LARGE protein is not fully understood, it has been shown to produce hyperglycosylated α-DG in culture cells and mice (25,26). In addition, physical interaction between LARGE and α-DG is an essential step in acquiring ligand-binding activities of α-DG (25). Therefore, it is believed that LARGE plays a post-translational role in modulating both α-DG glycosylation and its functional expression.

To further investigate molecular pathogenesis and to explore therapeutic strategies for dystroglycanopathy, we generated several model mice for FCMD. We first generated mice with a targeted *fukutin* disruption, but this model showed embryonic lethality (27). We also generated chimeric fukutin mice by injecting homozygous targeted (*fukutin*$^{-/-}$) ES cells into blastocysts (28). Mice with high chimerism showed dystrophic skeletal muscle; however, the variability of chimerism among individuals, and with growth, limits this experimental approach. Therefore, we generated a transgenic knock-in mouse model carrying the retrotransposal insertion in *fukutin*. Our data revealed that even a small amount of intact α-DG is sufficient to maintain skeletal muscle function, and suggest that increasing the expression of glycosylation-related genes, which could be accomplished through various approaches, can be a therapeutic strategy for preventing or slowing progression of a broad range of dystroglycanopathies.

RESULTS

Generation of model mice for FCMD

To generate a transgenic knock-in mouse carrying the retrotransposal insertion, we replaced mouse *fukutin* exon 10 with a FCMD patient's exon 10, engineered to contain the retrotransposal insertion using a site-directed DNA integration technique. Exon 10 encodes amino acids from Tyr-392 to the C-terminal end and the 3′-UTR. We also generated another transgene containing a normal human exon 10. The terms Hn (human normal; Fig. 1A, no. 6) and Hp (human patient; Fig. 1A, no. 7) refer to transgenes containing the normal human exon 10 and the patient's exon 10, respectively.

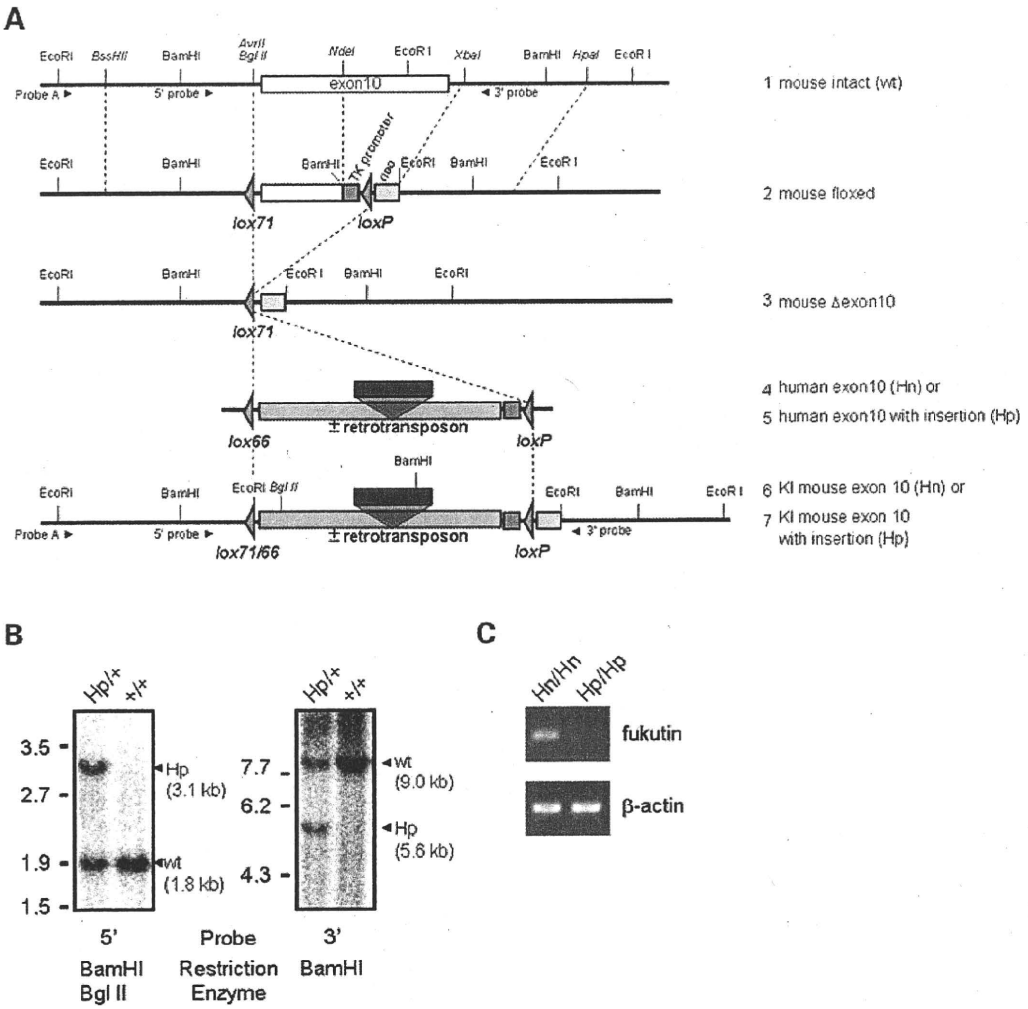

Figure 1. Generation of FCMD model mice that carry the retrotransposal insertion in the mouse *fukutin* gene. (**A**) Schematic representation of the targeting vector. Details are described in the Materials and Methods section. Human *fukutin* exon10 is shown in green, and the retrotransposon is shown in red. (**B**) Southern blot analysis of mouse genomic DNA. Insertion of the human exon10 with the retrotransposon yields new 3.1 kb BamHI/Bgl II and 5.6 kb fragments that hybridize, respectively, with the 5′ and 3′ probes shown in (A). (**C**) RT–PCR analysis. *fukutin* transcripts were amplified using RT-PCR. A β-actin internal control is shown (bottom panel).

Recombination was confirmed using Southern blot analysis of genomic DNA from ES cells (data not shown). Targeted ES cell clones were injected into blastocysts to obtain chimeric mice. Germline transmission of the knock-in allele was established via Southern blot analysis of mouse genomic DNA (Fig. 1B). Germline-competent heterozygous mice were in turn mated to generate homozygous mutants (Hn/Hn and Hp/Hp) (Fig. 2A, nos 3 and 4). RT–PCR showed a dramatic reduction of *fukutin* mRNA transcript levels in Hp/Hp mice (Fig. 1C). Through quantitative PCR, we determined that Hp/Hp mice express *fukutin* transcript at 5–10% of normal levels (data not shown). We consider Hp/Hp mice to be models for most FCMD cases that are homozygous for the retrotransposal insertion. Human patients who are compound heterozygous for the insertion and a nonsense *fukutin* mutation generally show more severe pathology than those who are homozygous for the insertion (14). Therefore, we crossed Hp/Hp mice with transgenic mice carrying a neo cassette disruption of one *fukutin* allele (fukutin$^{+/-}$) (27) to create a compound heterozygous line. The Hp/+ mice in this line represent retrotransposon carriers (Fig. 2A, no. 5) and the Hp/− mice represent compound heterozygotes (Fig. 2A, no. 6).

FCMD model mice exhibit hypoglycosylation of α-DG

To characterize the biochemical properties of α-DG in the knock-in mice, we prepared skeletal muscle samples enriched for α-DG with wheat germ agglutinin (WGA) beads, which is able to bind nearly all the DG in the muscle sample (20,29). These preparations were analyzed using western blot analysis with goat polyclonal antibodies against α-DG core protein (AP-074G-C) and the monoclonal antibody IIH6. IIH6 recognizes glycosylated epitopes on α-DG, and hypoglycosylation results in the absence of epitopes for the antibody (20).

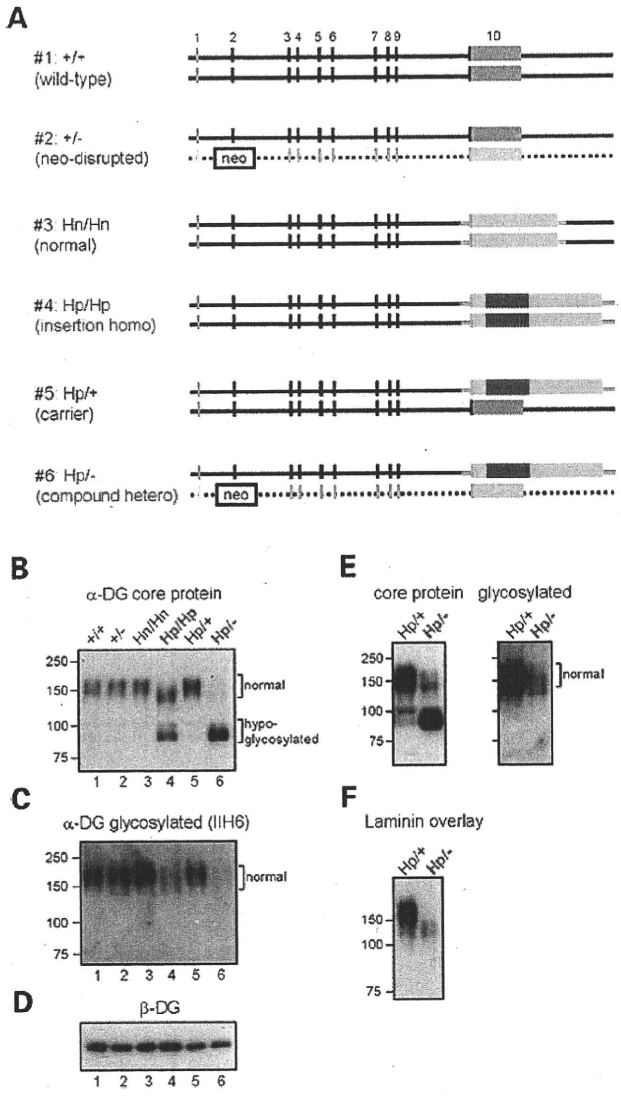

Figure 2. FCMD models exhibit hypoglycosylation and laminin-binding activity. (**A**) Schematic representation of the control and mutant *fukutin* genes in model mice. 1, wild-type mice (+/+); 2, mice carrying a neo-disrupted *fukutin* allele (+/−); 3, mice homozygous for the Hn allele (Hn/Hn); 4, mice homozygous for the Hp allele (Hp/Hp); 5, mice with a Hp allele and an intact mouse *fukutin* allele (Hp/+); and 6, mice with a Hp allele and a neo-disrupted allele (Hp/−). Exons are indicated with filled boxes. Portions derived from human *fukutin* exon 10 are shown in orange and green (3′-UTR). The retrotransposal insertion is shown in red. (**B**–**F**) Biochemical characterization of FCMD model mice. WGA beads were added to solubilized skeletal muscle samples to enrich DG from each model mouse. FCMD models are shown in red (Hp/Hp and Hp/−). WGA preparations were analyzed by western blot using antibodies against core protein (B) and glycosylated α-DG (C). The western blot for β-DG shows comparable amounts of DG proteins in each lane (D). Overexposure of blots analyzing core protein and glycosylated α-DG detected the presence of intact α-DG proteins in Hp/− mice (E). The portions of normal-sized and hypoglycosylated α-DGs are indicated at the right side of the blots. A laminin overlay assay was performed using samples from Hp/− mice and the litter control Hp/+ mice (F).

Western blot analysis of α-DG core protein revealed the presence of ∼150 kDa α-DG proteins in the control group (+/+, +/−, Hn/Hn and Hp/+ mice) (Fig. 2B, lanes 1–3 and 5). A slight reduction in molecular weight was observed in Hp/Hp mice (Fig. 2B, lane 4, upper band). In Hp/− mice, we observed a much-reduced intensity of the ∼150 kDa bands (Fig. 2B, lane 6). In addition, lower molecular weight (∼90 kDa) bands were detected in Hp/Hp and Hp/− mice (Fig. 2B, lanes 4 and 6). Western blotting with IIH6 detected ∼150 kDa bands in the control groups (+/+, +/−, Hn/Hn and Hp/+) (Fig. 2C, lanes 1–3 and 5). IIH6 reactivity at ∼150 kDa in Hp/Hp and Hp/− mice was reduced relative to controls (Fig. 2C, lanes 4 and 6). α-DG proteins with reduced molecular weight (∼90 kDa) were not recognized by IIH6, indicating that they are hypoglycosylated. Western blot analysis of β-DG confirmed comparable levels of DG proteins among the samples (Fig. 2D). Hp/− mice consistently contained more hypoglycosylated α-DG than Hp/Hp mice; therefore, we used Hp/− mice as models for FCMD and their Hp/+ littermates as controls. Longer exposure of blots from Hp/− mice detected an α-DG species recognized by IIH6 with the molecular weight of ∼150 kDa (Fig. 2E), suggesting that a small amount of intact α-DG also is present. Analysis of laminin-binding activity in Hp/− mice and Hp/+ littermates using a laminin overlay assay (Fig. 2F) showed reduced laminin-binding activity in Hp/− mice.

A small amount of intact α-DG prevents muscular dystrophy

We examined hematoxylin and eosin (H&E) stained sections of the quadriceps, gastrocnemius, tibialis anterior, soleus, iliopsoas and diaphragm muscles in Hp/+ and Hp/− mice. H&E staining revealed no clear difference between Hp/+ and Hp/− mice. Histopathological features of muscular dystrophy, such as centrally located nuclei, tissue fibrosis and fatty infiltration were not observed in 10-week-old FCMD models Hp/− (Fig. 3A) and Hp/Hp mice (data not shown). Although FCMD onset in humans occurs at or near birth, we also examined older mice to determine whether onset in Hp/− mice was delayed. Even in older mice (>1 year old), we observed no signs of muscular dystrophy (Fig. 3B). There was no obvious change in the expression level of laminin α2 chain, which is the major ligand of α-DG in the skeletal muscle (Supplementary Material, Fig. S1).

Both hypoglycosylated and IIH6-positive intact α-DG proteins were detected in Hp/Hp and Hp/− mouse brains (Supplementary Material, Fig. S2). As is the case with skeletal muscle, Hp/− mice contained more hypoglycosylated α-DG. Apparent brain histological abnormality was hardly detected in Hp/− mice; only a few mice showed a very small ectopic cluster of neurons migrating into the marginal zone. We also analyzed α-DG in heart, liver, and lung from Hp/− mice, and found that the levels of hypoglycosylation and laminin-binding activity vary between the tissues (less affected in heart and liver) (Supplementary Material, Figs S2 and S3).

To analyze potential weakness in muscle cell membrane integrity, which may not be detectable in housed mice by H&E staining, Hp/− mice were subjected to treadmill exercise followed by the measurement of Evans blue dye (EBD) incorporation into muscle fibers. EBD is a membrane-impermeant molecule that binds to serum albumin and is physically restricted from fibers unless the skeletal muscle membrane is damaged (30). Even after exercising to exhaustion, Hp/− mice showed no EBD uptake in muscle cells (data not shown).

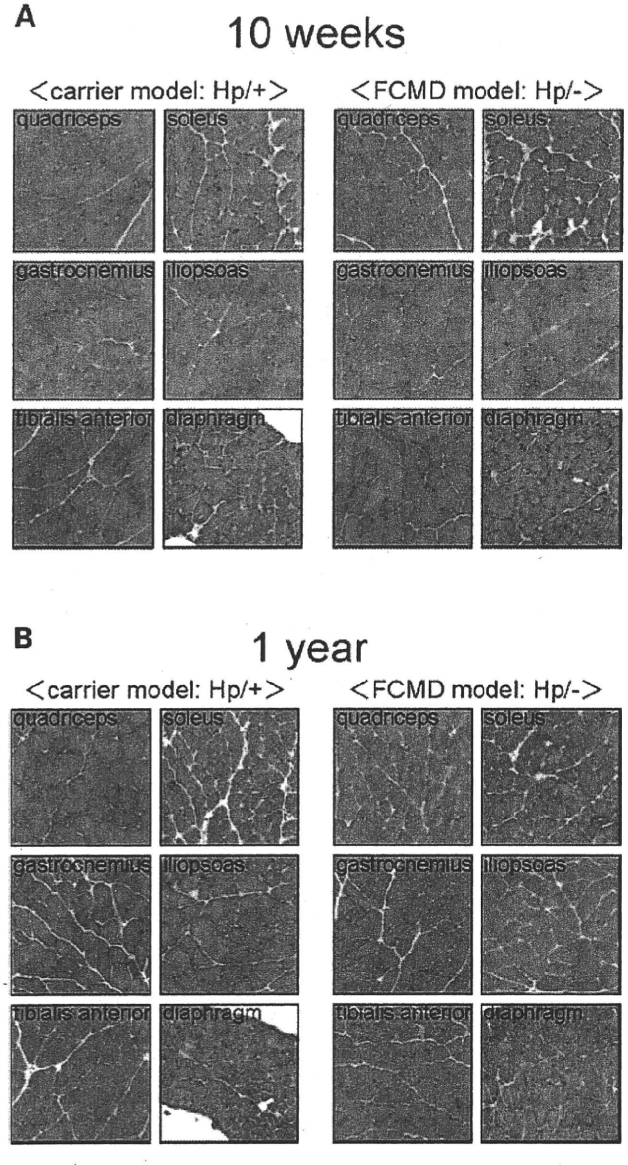

Figure 3. FCMD mice do not develop a muscular dystrophy phenotype. Various skeletal muscle tissues from Hp/− and littermate control Hp/+ mice at 10 weeks (**A**) and >1 year (**B**) of age were analyzed by H&E staining. No features of muscular dystrophy or other variation from controls were observed in Hp/− mice.

Reduction of laminin-binding activity due to hypoglycosylation of α-DG is thought to be the main cause of dystroglycanopathy. Therefore, we hypothesized that the minimal levels of intact α-DG species observed in Hp/− mice are sufficient to maintain linkage to laminin and prevent disease progression. To test this hypothesis, we compared the laminin-binding activity in Hp/− mice with that in Large^{myd} (myd/myd) mice, which represent another dystroglycanopathy model with a muscular dystrophy phenotype (21). H&E analysis confirmed signs of muscular dystrophy (centrally located nuclei and fiber size variation) in myd/myd mice, but not in Hp/− mice (Fig. 4A and B). In contrast with Hp/− mice,

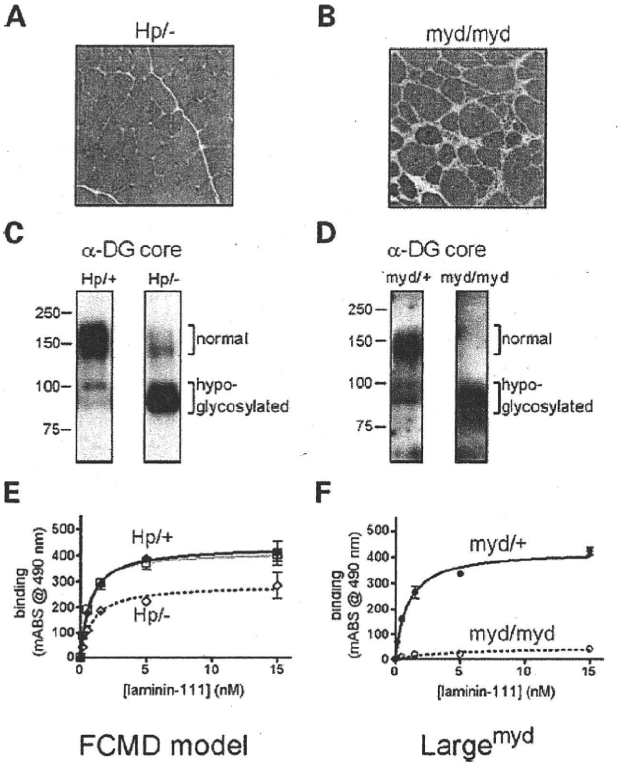

Figure 4. Laminin-binding activity is maintained in Hp/− mice but barely detected in Large^{myd} mice. H&E staining of quadriceps tissue from Hp/− (**A**) and Large^{myd} (myd/myd) (**B**) mice are shown. WGA preparations from the Hp/− (**C**) and the myd/myd (**D**) skeletal muscle were also analyzed by western blot using an antibody against α-DG core protein. Laminin-binding activity in Hp/− (**E**) and myd/myd (**F**) mice were measured using solid-phase binding assays and compared to the littermate controls (Hp/+ and myd/+). Open squares (gray line) in panel E indicate laminin-binding activity in wild-type mice.

western blot analysis of α-DG core protein in myd/myd mice revealed no intact size (~150 kDa) of α-DG species (Fig. 4C and D), indicating that almost all α-DG is hypoglycosylated in myd/myd mice. The laminin-binding activity of α-DG in Hp/− and myd/myd mice was measured using a quantitative solid-phase laminin-binding assay and compared with litter controls (Hp/+ and myd/+ mice, respectively) (Fig. 4E and F). Laminin-binding activity was ~50% of normal in Hp/− mice but less than 5% of normal in myd/myd mice. The solid-phase binding analysis shows no obvious difference between wild-type and Hp/+. These data demonstrate that levels of glycosylation (indicated by IIH6 immunoreactivity and the presence of ~150 kDa α-DG) influence laminin-binding activity and indicate that only a small amount of IIH6-reactive α-DG is required to maintain skeletal muscle function.

Fukutin gene transfer restores glycosylation of α-DG in knock-in mice

Our data strongly suggest that even partial restoration of α-DG glycosylation is effective in reducing disease severity in

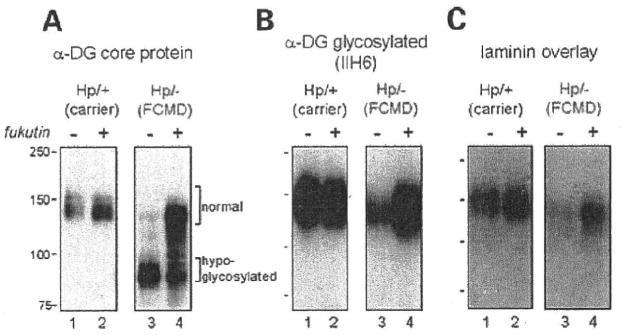

Figure 5. *Fukutin* gene transfer rescues the glycosylation abnormality in Hp/− mice. Hp/+ or Hp/− pups were injected with adenovirus encoding wild-type human *fukutin* in one leg (+) and with saline in the contralateral leg (−). Calf muscle was analyzed using western blot with antibodies against core α-DG protein (**A**) and glycosylated α-DG (**B**) and using a laminin overlay assay (**C**). Transfer of *fukutin* produced increases in α-DG molecular weight, IIH6 reactivity and laminin binding activity in Hp/− mice.

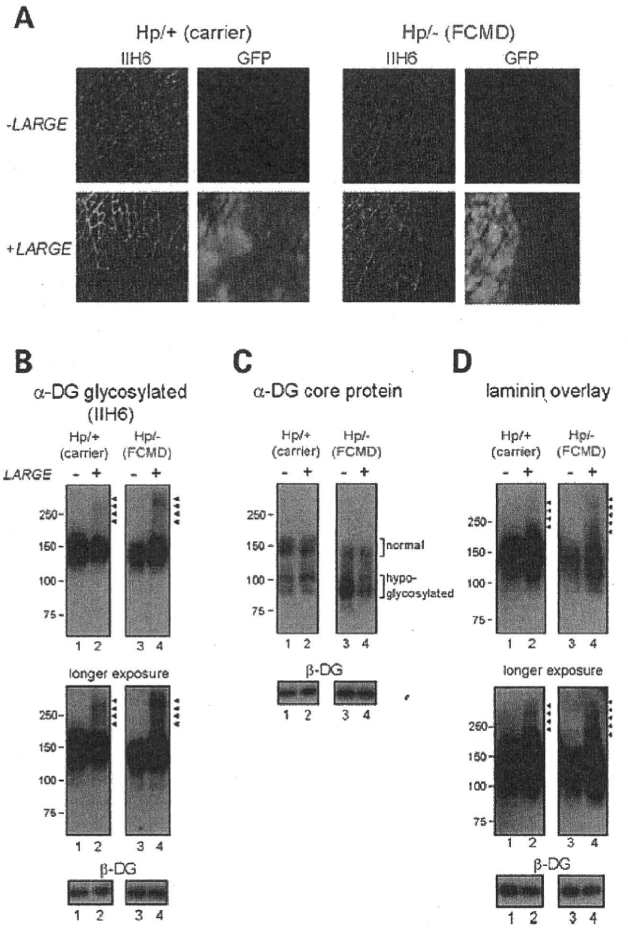

Figure 6. *LARGE* gene transfer produces functionally glycosylated α-DG in Hp/− mice. Hp/+ or Hp/− pups were injected with an adenovirus encoding *LARGE* in one leg (+) and with saline in the contralateral leg (−). Calf muscle was analyzed using IIH6 immunofluorescence (**A**). GFP fluorescence represents muscle fibers successfully transduced by the adenoviral vectors. WGA preparations were analyzed using western blots with antibodies against glycosylated α-DG (**B**), α-DG core protein (**C**) and using a laminin overlay assay (**D**). The western blot for β-DG shows comparable amounts of DG proteins in each lane. Images with longer-exposures better indicate the presence of hyperglycosylated α-DG (arrowheads). These results show that the transfer of *LARGE* increases IIH6 reactivity and laminin-binding activity in Hp/− mice.

dystroglycanopathy. To examine whether glycosylation defects can be recovered *in vivo*, a recombinant fukutin adenovirus was injected into the hind limb muscle of 3-day-old Hp/− and litter control Hp/+ mice. Following 4 weeks of injections, α-DG enriched samples were prepared using WGA beads and analyzed for glycosylation and laminin-binding activity. Western blot analysis with anti-α-DG core protein antibodies revealed that *fukutin* gene transfer into Hp/− mice reduced hypoglycosylated α-DG (~90 kDa) and increased levels of the normal-sized α-DG species (~150 kDa) (Fig. 5A, lanes 3 and 4). IIH6 reactivity and laminin-binding activity also increased following *fukutin* gene transfer into Hp/− mice (Fig. 5B and C, lanes 3 and 4). No obvious changes were observed in Hp/+ mice after the gene transfer (Fig. 5C, lanes 1 and 2). These results demonstrate that *fukutin* gene transfer can correct biochemical abnormalities of α-DG in *fukutin*-deficient skeletal muscle, and support that fukutin protein is involved in glycosylation of α-DG.

Large gene transfer produces laminin-binding forms of α-DG in dystroglycanopathy models

Hypoglycosylation leading to dystroglycanopathies is caused by mutations in six known genes (*fukutin*, *POMGnT1*, *POMT1*, *POMT2*, *FKRP* and *LARGE*) and other, unidentified genes. In an effort to bypass the need for identification of disease-causing genes in developing therapies (e.g. gene transfer), we further explored a unique feature of LARGE. LARGE has been demonstrated to induce α-DG hyperglycosylation, which is detected by IIH6 as a broad band detected at 150–300 kDa via SDS gel electrophoresis. This band shows increased ligand-binding activity in samples from genetically distinct diseases showing defective α-DG glycosylation (FCMD, MEB and WWS) (26).

We examined whether adenoviral *LARGE* gene transfer into Hp/− skeletal muscle induces hyperglycosylation and increases laminin-binding activity of α-DG. Immunofluorescence analysis of untreated control muscles revealed weaker IIH6 reactivity in Hp/− than in Hp/+ (Fig. 6A, *−LARGE*). Muscle sections subjected to gene transfer showed increased α-DG glycosylation in transduced areas, as indicated by eGFP expression in both Hp/− and Hp/+ mice (Fig. 6A, *+LARGE*). We also examined adenovirus-injected and non-injected contralateral leg muscles using western blot analysis with antibodies against α-DG core protein and IIH6. These experiments showed that the *LARGE* gene transfer increased IIH6 reactivity at ~150 kDa in the Hp/− muscle and produced a broad band with a molecular weight of 150–250 kDa in both Hp/− and Hp/+ muscles (Fig. 6B). Anti-α-DG core protein antibodies poorly recognized a higher molecular weight α-DG species (Fig. 6C), which is consistent with previous reports (26). Following the *LARGE* gene transfer, levels of hypoglycosylated α-DG species decreased (Fig. 6C, lanes 3 and 4). These data indicate that

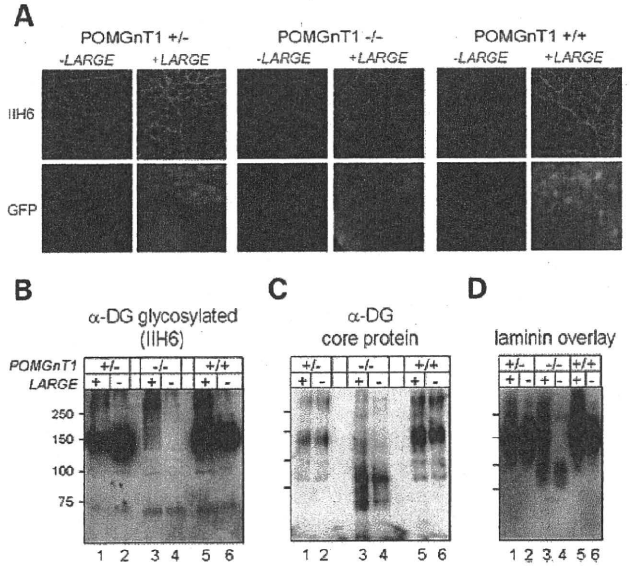

Figure 7. *LARGE* gene transfer produces functionally glycosylated α-DG in MEB disease model mice. POMGnT1$^{+/+}$, POMGnT1$^{+/-}$ or POMGnT1$^{-/-}$ pups were injected with adenovirus encoding *LARGE* in one leg (+*LARGE*) and with saline in the contralateral leg (−*LARGE*). Calf muscle was analyzed using IIH6 immunofluorescence (**A**). GFP fluorescence represents muscle fibers successfully transduced by the adenoviral vectors. WGA preparations were analyzed using western blots with antibodies against glycosylated α-DG (**B**) and α-DG core protein (**C**) and using a laminin overlay assay (**D**). These results show that transfer of *LARGE* increases IIH6 reactivity and laminin-binding activity in POMGnT1$^{-/-}$ mice, the model for MEB disease.

LARGE-induced glycosylation occurs on hypoglycosylated α-DG species. The IIH6-positive broad-molecular-weight band was able to bind laminin in both Hp/− and Hp/+ skeletal muscle samples (Fig. 6D, lanes 2 and 4). These data indicate that LARGE can increase laminin-binding forms of α-DG in *fukutin*-deficient skeletal muscle.

We further investigated whether *LARGE* gene transfer induced hyperglycosylation and produced laminin-binding forms of α-DG species in another dystroglycanopathy model, the *POMGnT1*-disrupted mouse (POMGnT1$^{-/-}$) (Miyagoe-Suzuki *et al.*, manuscript in preparation). Western blot analysis using α-DG core protein antibodies showed a reduction of α-DG molecular weight to 60–90 kDa in POMGnT1$^{-/-}$ mice (Fig. 7C, lane 4). Little IIH6 reactivity was detected via immunofluorescence (Fig. 7A) and western blot (Fig. 7B, lane 4) analysis. These data indicate hypoglycosylation of α-DG in POMGnT1$^{-/-}$ mice. Accordingly, laminin-binding activity was significantly reduced in POMGnT1$^{-/-}$ mice compared with POMGnT1$^{+/-}$ or POMGnT1$^{+/+}$ littermates (Fig. 7D, lanes 2, 4 and 6). The minor laminin binding protein (~80-100 kDa, lane 4) detected only in POMGnT1$^{-/-}$ is unidentified; however, similar laminin binding was also observed in POMGnT1-deficient MEB patients (20). A solid-phase binding assay also showed minor levels of laminin-binding activity in POMGnT1$^{-/-}$ (Supplementary Material, Fig. S4). For all genotypes, adenoviral *LARGE* gene transfer increased IIH6 reactivity in transduced areas indicated by eGFP expression (Fig. 7A, +/+, +/−, and −/−). Western blot analysis using IIH6 showed that *LARGE* gene transfer also induced hyperglycosylation of α-DG in all genotypes, as indicated by broad bands with molecular weights from 150 to >250 kDa (Fig. 7B). After the gene transfer, the POMGnT1$^{-/-}$ skeletal muscle showed only hyperglycosylated IIH6-positive species, while the POMGnT1$^{+/+}$ and the POMGnT1$^{+/-}$ muscles showed both hyperglycosylated and the original 150 kDa IIH6-positive species. Overlay assays showed that the laminin-binding epitope was produced on hyperglycosylated α-DG (Fig. 7D). These data support the idea that LARGE is an effective target for increasing or restoring laminin-binding activity of α-DG in dystroglycanopathy.

DISCUSSION

We have used several approaches to generate FCMD model animals. Fukutin-null mice result in embryonic lethality (27). Fukutin-chimera mice derived from ES cells targeted for both *fukutin* alleles (28) develop muscular dystrophy, but are inappropriate therapeutic study models because (i) they show wide variation in disease severity, and (ii) muscle cell fusion events during growth and regeneration can alter the population of fukutin-null cells. Therefore, we decided to introduce the disease-causing retrotransposon into the mouse *fukutin* gene to mimic the most prevalent form of human FCMD. In these knock-in Hp/Hp and Hp/− mice, we detected hypoglycosylated α-DG, as is seen in FCMD patients (20,31), so we consider them to be novel models for FCMD.

Spontaneous Large^{myd} and Large^{vls} mice (21,32) and genetically engineered POMGnT1-deficient mice (33) have been reported as dystroglycanopathy models. Because these models mimic null mutations such as nonsense and frameshift mutations, they do not necessarily represent human diseases caused by missense mutations. Our knock-in mice with the retrotransposal *fukutin* insertion are the first dystroglycanopathy model that carries a human disease-causing mutation. Such models are needed to explain the molecular pathogenesis of diseases, to determine the function of responsible genes and to screen drugs that correct specific defects (34).

Although these mice genetically and biochemically represent features of *fukutin*-deficient muscular dystrophies, histological analysis has revealed no signs of muscular dystrophy. In typical cases of FCMD, normal-sized α-DG with IIH6-reactivity is barely detected, and laminin-binding activity is dramatically reduced (20). Comparing Hp/− mice with Large^{myd} mice led us to reason that the remaining intact α-DG and laminin-binding activity in Hp/− mice might be sufficient to prevent disease progression. In the future, it would be important to elucidate the threshold level of glycosylation required to avoid a phenotype by using a model system that can control glycosylation levels *in vivo*. In Hp/− mice, residual laminin-binding is detected from DG species with slightly lower molecular weight (<150 kDa) (Fig. 2F), whereas this is not the case for human patients even with retained laminin binding (35). The difference suggests that mice may have additional laminin-binding epitopes, which are less susceptible to fukutin defects. Alternatively, other factors may compensate for

reduced laminin-binding to α-DG. For example, it has been suggested that integrin α7, another laminin receptor in skeletal muscle, may account for the difference in clinical severity between mice and humans with dystrophin- or the DGC-defects (36,37). Clarifying the factors involved would be necessary for a better understanding of pathomechanism, which could promote identification of novel therapeutic targets.

Also important is the finding that even a small amount of IIH6-immunoreactivity of α-DG is sufficient to maintain skeletal muscle function. This concept is supported by milder cases of human patients with fukutin mutations (35). Murakami *et al.* have described reduced but detectable IIH6-reactivity and intact α-DG in patients who are compound heterozygous for the *fukutin* retrotransposon insertion and a missense mutation (R179T or Q358P). These individuals showed minimal dystrophic features and normal intelligence. Laminin-binding activity is also retained in all cases. These findings provide further evidence that the disease severity of fukutin-deficient muscular dystrophy is related to the ratio of normal glycosylation to hypoglycosylation.

Such correlation has been observed in other dystroglycanopathies. LGMD2I patients at the severe end of the clinical spectrum tend to show the greatest reduction in α-DG glycosylation, while those at the milder end tend to have relatively well-preserved α-DG glycosylation (38). Most known missense mutations in *POMGnT1* disrupt POMGnT enzyme activity, causing hypoglycosylation of α-DG and a severe congenital muscular dystrophy phenotype (39,40). Clement *et al.* (6) have reported a patient with a milder LGMD phenotype who carries a novel homozygous missense mutation in *POMGnT1*. Studies of this patient's fibroblasts showed an altered kinetic profile but intact enzyme activity, explaining the relatively mild phenotype. Furthermore, a recent systematic and large-scale study of genotype–phenotype correlation in dystroglycanopathy revealed a wide spectrum of clinical severity in specific disease-causing genes (18). A broad correlation between the amount of depleted glycosylated epitope and phenotypic severity was described, though not systematically quantified. A more recent study reported a few cases with less correlation between clinical course and α-DG immunolabeling (41). We propose that, in addition to immunolabeling, combination of western blotting and laminin binding assays will be necessary for further advances in both clinical and basic biomedical research.

The present study strongly suggests that full recovery of α-DG glycosylation is not always necessary; partial restoration of α-DG glycosylation might be enough to prevent or slow disease progression. The simplest way to restore α-DG glycosylation in dystroglycanopathies would be by replacing a defective gene with the normal version. In many cases, though, the disease-causing gene is not known. A recent study revealed that most patients with a dystroglycanopathy harbor mutations in novel genes (18). To increase amounts of glycosylated α-DG with laminin-binding activity regardless of the responsible gene, we took advantage of the observation that overexpression of LARGE can produce hyperglycosylated α-DG with increased laminin-binding activity in cells from genetically distinct dystroglycanopathies (26). LARGE-induced hyperglycosylation of α-DG has also been observed in both CHO glycosylation mutants showing defective transfer of sialic acid, galactose or fucose to glycoconjugates and in a mutant that is unable to synthesize *O*-mannose glycan (42). Such a 'super-effect' of LARGE on α-DG glycosylation has been observed *in vitro*, but no *in vivo* study has been reported except in Large^{myd} mice (26). Gene transfer of *LARGE* into Large^{myd} mice essentially replaces the defective gene with the normal version of the gene. Our results provide the first *in vivo* evidence that *LARGE* gene transfer can bypass the glycosylation defects of α-DG in models other than the Large^{myd} mice. These results support the idea that glycotherapies aimed at modulating LARGE may be a therapeutic option for many α-DG glycosylation-deficient muscular dystrophies.

Overall, our biochemical, histological and gene transfer experiments using novel model mice with disease-causing mutations support the efficacy of glycotherapy in dystroglycanopathies. The models developed here will be powerful in understanding the pathomechanism of FCMD and other related diseases.

MATERIALS AND METHODS

Generation of model mice

A targeting vector containing the retrotransposal insertion of human FCMD patients was generated using a site-directed DNA integration technique (43). Briefly, lox71 and TK-loxP-neo pA fragments (44) were inserted 5′ and 3′ to exon 10 of mouse *fukutin* (Fig. 1A, no. 2). To excise a floxed part of exon 10 (Fig. 1A, no. 3 Δexon10), Cre was expressed in mouse embryonic stem (ES) cells. Meanwhile, lox66 and TK-loxP fragments were inserted 5′ and 3′ to exon 10 of human *fukutin*, with or without a retrotransposal insertion (Fig. 1A, nos 4 and 5). Each construct was co-transfected with a Cre-expressing vector into ES cells that constitutively express the Δexon10 construct, to obtain recombinant knock-in alleles (Fig. 1A, nos 6 and 7). The transgenic alleles containing normal human exon 10 and mutant exon 10 were named Hn (representing 'human normal') and Hp (representing 'human patient'), respectively. Targeted ES cell clones were injected into blastocysts, and germline-competent heterozygous mice were in turn mated to generate homozygous mutants.

Genotyping of each transgene was performed using PCR with the following primers: FCMDKIF1, GAAACTCTGCCATGACACCTC: HNC440R, ACCAGCTTAAATGCCCAGAAG: Wild R2, GAAGCCAACTGTGTACCACAC. The FCMDKIF1 and HNC440R, and FCMDKIF1 and Wild R2 primer pairs yielded bands of ~800 bp (knock-in allele) and ~1100 bp (wild-type allele), respectively. Genotyping of a *fukutin* allele disruption by a neo replacement (*fukutin* null) was described previously (45). The primers for *fukutin* RT–PCR are AGGGAATGGGCTGGTAGACT and GTGCCATT TTGGGACAAGTT.

C57BL/6 mice were obtained from Japan SLC, Inc., and Large^{myd} mice were obtained from The Jackson Laboratory. Mice were maintained in accordance with the animal care guidelines of Otsuka Pharmaceutical Co. Ltd. and Osaka University.

Antibodies

Antibodies used in western blots and immunofluorescence were as follows: mouse monoclonal antibody 8D5 against β-DG (Novacastra); mouse monoclonal antibody IIH6 against α-DG (Upstate); and polyclonal anti-laminin (Sigma). We generated goat polyclonal antibodies against α-DG core protein using GST fusion proteins containing the N- or C-terminal domains of mouse α-DG. Antisera (074G) were affinity-purified using an α-DG-Fc fusion protein expressed in HEK293 cells. The purified antibody was named AP-074G-C.

Dystroglycan preparation and western blotting

DG was enriched from solubilized skeletal muscle as previously described (20,29). Briefly, 100 mg of muscle was solubilized in 1 ml of Tris-buffered saline (TBS) containing 1% Triton X-100 and protease inhibitors (Funakoshi). The solubilized fraction was incubated with 30 μl of WGA–agarose beads (Vector Labs) at 4°C for 16 h. Beads were washed three times in 1 ml TBS containing 0.1% Triton X-100 and protease inhibitors. The beads were then either directly boiled for 5 min in SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer (western blot and laminin overlay) or eluted with 300 μl TBS containing 0.1% Triton X-100, protease inhibitors and 300 mM *N*-acetylglucosamine (solid-phase binding assay). Proteins were separated using 7.5% or 10% SDS–PAGE. Gels were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Blots were probed with DG antibodies and then developed with horseradish peroxidase (HRP)-enhanced chemiluminescence (Supersignal West Pico, Pierce; or ECL Plus, GE Healthcare).

Immunofluorescence and histological analysis

Cryosections (7 μm) were prepared and analyzed using immunofluorescence or H&E staining. Sections were stained for 2 min in hematoxylin, 1 min in eosin and then dehydrated with ethanol and xylenes. For immunofluorescence staining with IIH6, sections were treated with cold ethanol/acetone (1:1) for 1 min, blocked with 5% goat serum in MOM Mouse Ig Blocking Reagent (Vector Laboratories) at room temperature for 1 h and then incubated with primary antibodies diluted in MOM Diluent (Vector Laboratories) overnight at 4°C. The slides were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-mouse IgM antibody (Molecular Probes) at room temperature for 30 min. For GFP detection, sections were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS three times and then mounted. Permount® (Fisher Scientific) and TISSU MOUNT® (Shiraimatsu Kikai) were used for H&E staining and immunofluorescence, respectively. Sections were observed under fluorescence microscopy (Leica DMR, Leica Microsystems). For EBD uptake, mice were exercised on a treadmill (MK-680S, Muromachi Kikai) as described (34).

Laminin-binding assay

Laminin-binding activity was examined as previously reported (20) with slight modifications. Laminin overlay assays were performed on PVDF membranes using mouse Engelbreth–Holm–Swarm (EHS) laminin (Sigma). Briefly, PVDF membranes were blocked in laminin-binding buffer (LBB: 10 mM triethanolamine, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) containing 5% non-fat dry milk followed by incubation with 7.5 nM laminin at 4°C for 12 h in LBB with 3% BSA. Membranes were washed and incubated with anti-laminin (Sigma) at 4°C for 3 h followed by anti-rabbit IgG–HRP at room temperature for 45 min. Blots were developed by enhanced chemiluminescence (Supersignal West Pico, Pierce).

For the solid-phase binding assay, WGA eluates were diluted 1:50 in TBS and coated on polystyrene ELISA microplates (Costar) for 16 h at 4°C. Plates were washed in LBB and blocked for 2 h in 3% BSA in LBB. Mouse EHS laminin was diluted in LBB and applied for 1 h. Wells were washed with 3% BSA in LBB, incubated for 1 h with 1:10,000 anti-laminin (Sigma) followed by anti-rabbit HRP. Plates were developed with *o*-phenylenediamine dihydrochloride and H_2O_2, then reactions were stopped with 2 N H_2SO_4 and values obtained on a microplate reader. The data were fit to the equation $A=B_{max}x/(K_d+x)$, where K_d is the dissociation constant, A is absorbance and B_{max} is maximal binding.

Adenoviral gene transfer

The complete open reading frame of mouse *fukutin* was cloned into the EcoRI site of the pKSCX-EGFP vector (46). The pKSCX-EGFP vector contains IRES-EGFP so that both the *fukutin* and *GFP* genes are expressed bicistronically under the CAG promoter. This expression cassette was digested with SwaI, and then its blunt-ended fragment was ligated into the adenoviral cosmid vector. The recombinant adenoviral vector encoding *fukutin* was generated using the method of Tashiro *et al.* (46).

Generation of the recombinant adenoviral vector encoding *LARGE* has been previously described (26). Amplified adenoviruses were purified using VIVAPURE ADENOPACK 100 (VIVASCIENCE).

In vivo gene transfer was performed with Hp/− and control littermate Hp/+ pups, age 2–4 d. Adenoviruses were injected percutaneously into the calf and hamstring with 1×10^8–1×10^9 particles in 10 μl of saline solution. Mock injections used saline solution only. Four weeks after injection, experimental and control contralateral leg muscles were subjected to immunofluorescence and biochemical analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

Fukutin Gene Retrotransposal Insertion in a Non-Japanese Fukuyama Congenital Muscular Dystrophy (FCMD) Patient

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Fukuyama-type congenital muscular dystrophy (FCMD) is an autosomal recessive disorder, characterized by severe muscular dystrophy associated with brain malformation. FCMD is the second most common form of muscular dystrophy and one of the most common autosomal recessive diseases among the Japanese population; however, no typical FCMD cases have been reported in any other population. In this study, we report on the first identification of a Chinese FCMD patient; our findings are supported by clinical, histological, and magnetic resonance imaging (MRI) evidence, as well as *fukutin* gene mutational analyses. The patient presented with neonatal hypotonia, seizures, and delayed motor and speech development. Additional testing revealed cerebral and cerebellar gyrus abnormalities with white matter signal intensity changes, elevated serum creatine kinase (CK) levels, and dystrophic skeletal muscle with α-dystroglycan hypoglycosylation, and normal β-dystroglycan and merosin expression. Genetic analysis of the *fukutin* gene showed one copy with a Japanese founder 3-kilobase (kb) retrotransposal insertion in the 3′-non-coding region and the other copy with a known c.139C>T mutation. This is the first FCMD case reported in the Chinese population and the first case in which the 3-kb insertion has been found outside of the Japanese population. This report emphasizes the importance of considering the *fukutin* founder mutation for diagnostic purposes outside of Japan.

Key words: 3-kb retrotransposal insertion; FCMD; Chinese

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INTRODUCTION

Fukuyama-type congenital muscular dystrophy (FCMD; OMIM 253800) is an autosomal recessive disorder, characterized by severe muscular dystrophy associated with brain malformation. It is the second most common form of muscular dystrophy and one of the most common autosomal recessive diseases among the Japanese population. Clinical features vary, but typically include generalized hypotonia and weakness in early infancy, followed by marked muscle atrophy, multiple joint contractures, and psychomotor developmental delay in childhood. Classically, the highest achievable motor function in patients is sliding while sitting on the buttocks. Intellectual, cognitive, and communicative functions are severely delayed. The course is slowly progressive and inexorable. The average age at death is 16 years, and no effective treatment currently exists [Fukuyama et al., 1981]. The gene responsible for FCMD, *fukutin*, was identified at 9q31 by linkage analysis and positional cloning [Toda et al., 1993; Kobayashi et al., 1998a, 2001]. FCMD is the first known human disease caused by an ancient retrotransposal integration [Kobayashi et al., 1998a]. Most Japanese FCMD patients have a 3-kb retrotransposal insertion (founder) mutation in the 3'-non-coding region of *fukutin*. Loss-of-function *fukutin* point mutations have also been identified, but no FCMD patients have been reported

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with non-founder (point) mutations in both alleles [Kondo-Iida et al., 1999]. Until now, no FCMD patients who carry the 3-kb insertion have been identified among non-Japanese people [Silan et al., 2003; Fukuyama, 2008]. Here, we describe a Chinese FCMD patient possessing a c.139C>T *fukutin* gene point mutation in one allele and the founder 3-kb retrotransposal *fukutin* gene insertion in the other.

CLINICAL REPORT

The male patient, who was 5 years 9 months of age, had displayed motor abnormalities (floppy infant) since birth. He was born at full-term, delivered by Cesarean to a healthy G_1P_1 young mother. His parents were non-consanguineous Chinese and had no family history of neuromuscular disease. His father and mother were born in Henan Province and Shanxi Province, respectively. The patient's CK levels were 11,600 IU/L when he was 1 year old and had decreased to 648 IU/L at 5 years 9 months of age. He achieved head control when 2 years old and sat unsupported at 4 years old, but was unable to slide on his buttocks. He developed both knee and ankle joint contractures after the first year. Progressive contractures over both knees and elbow joints were present. He could speak very few words. Physical examination, the patient displayed brilliant eyes, round cheeks, and a myopathic face (Fig. 1). He had eye closure weakness, a high arched palate, and generalized muscle weakness, including weak neck muscles. Fundus examination was normal. Prominent muscle atrophy was present, especially over the shoulder and pelvic girdle muscles. Calf muscles were hypertrophied and deep tendon reflexes were absent. Febrile convulsion occurred once, at the age of 5 years 6 months. An electromyogram revealed myopathic changes, but an electrocardiogram was normal. A brain magnetic resonance imaging (MRI) showed sporadic periventricular hyperintensity areas on a T2-weighted image, frontal lobe micropolygyria, cerebellar cysts, and cerebellar and brain stem hypoplasia (Fig. 2). The patient's IQ (Wechsler Preschool and Primary Scale of Intelligence-Revised) was 52.

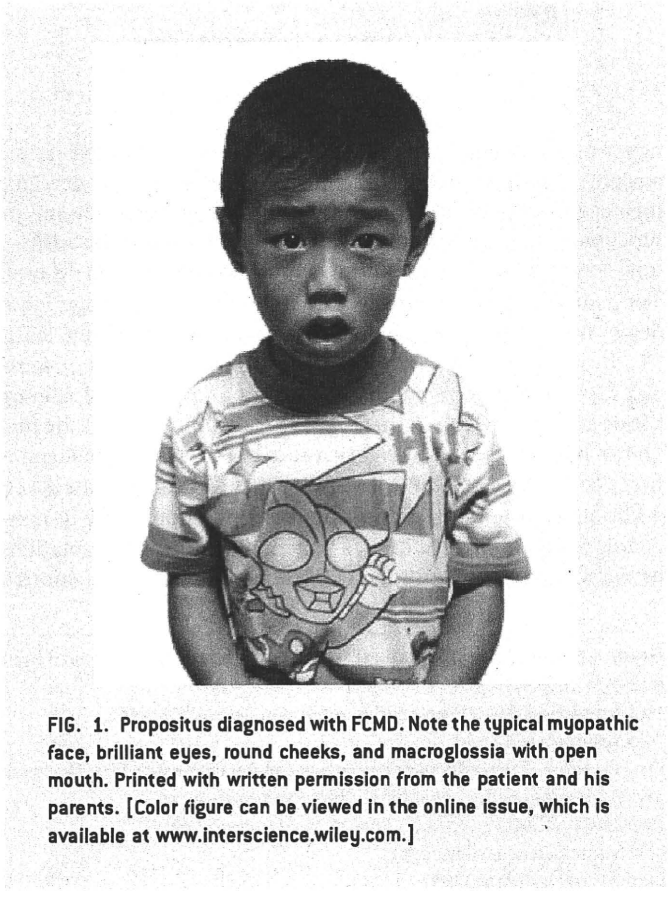

FIG. 1. Propositus diagnosed with FCMD. Note the typical myopathic face, brilliant eyes, round cheeks, and macroglossia with open mouth. Printed with written permission from the patient and his parents. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MATERIALS AND METHODS

Immunohistochemistry

Muscle biopsy specimens, obtained after parental informed consent, were frozen and stored in liquid nitrogen-cooled isopentane. Using commercially available monoclonal antibodies, α-dystroglycan (IIH6; Upstate Biotech, Lake Placid, NY), β-dystroglycan (8D5; Novocastra, Newcastle upon Tyne, UK) and merosin (mAb1922; Chemicon International, Temecula, CA) expression patterns were analyzed on 8-μm-thick cryosections.

Sequence Analysis and PCR

Peripheral blood genomic DNA from the patient and his parents was phenol–chloroform extracted and precipitated with isopropanol. Primers were designed from the *fukutin* genomic sequence to amplify each exon and surrounding intronic sequences (primer sequences available upon request). After detecting only one mutation by direct DNA sequencing, we performed a previously reported three primer-PCR method [Watanabe et al., 2005]. Additionally, we designed a primer pair to detect genomic sequence downstream of the 3-kb insertion (forward, position ins2795–2814, 5′-ATT-AAGGGCGGTGCAAGATG-3′; reverse, position c. 4469–4488, 5′-GAGAGAAGGAGGCAAACTGG-3′). Cycling conditions were identical to those of the three primer-PCR method. PCR products were analyzed by electrophoresis on 2% agarose gels. Study protocols were approved by the Ethical Committee of Peking University.

Haplotype Analysis

The patient and his parents were genotyped with polymorphic microsatellite markers cen-D9S306-D9S2105-(FCMD)-D9S2170-D9S2171-D9S2107-D9S172-tel, as described previously [Kobayashi et al., 1998b].

RESULTS

Hematyoxylin and eosin (H&E) staining of muscle biopsies showed few muscle fibers and indicated a terminal dystrophic stage. Dystrophic features were evident, including fiber size variability, fibrosis, and fat replacement (Fig. 3). Immunohistochemical analysis showed greatly reduced α-dystroglycan staining but normal sarcolemmal β-dystroglycan and merosin immunostaining (Fig. 3). We screened all *fukutin* exons and flanking introns in the patient and his parents by polymerase chain reaction (PCR)

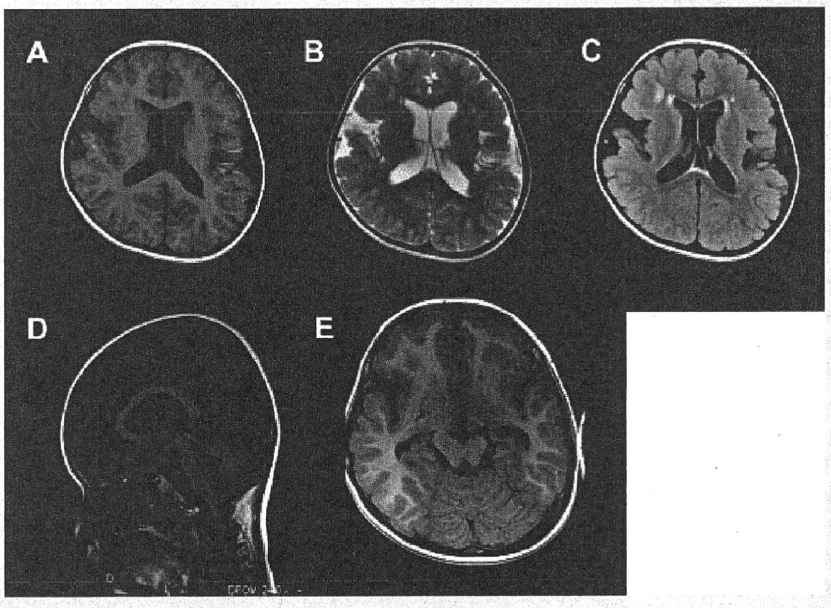

FIG. 2. A–C: Brain MRI showed sporadic periventricular hyperintensity in a T2-weighted image, frontal lobe polymicrogyria; (D) cerebellar, brain stem hypoplasia, and (E) cerebellar cyst.

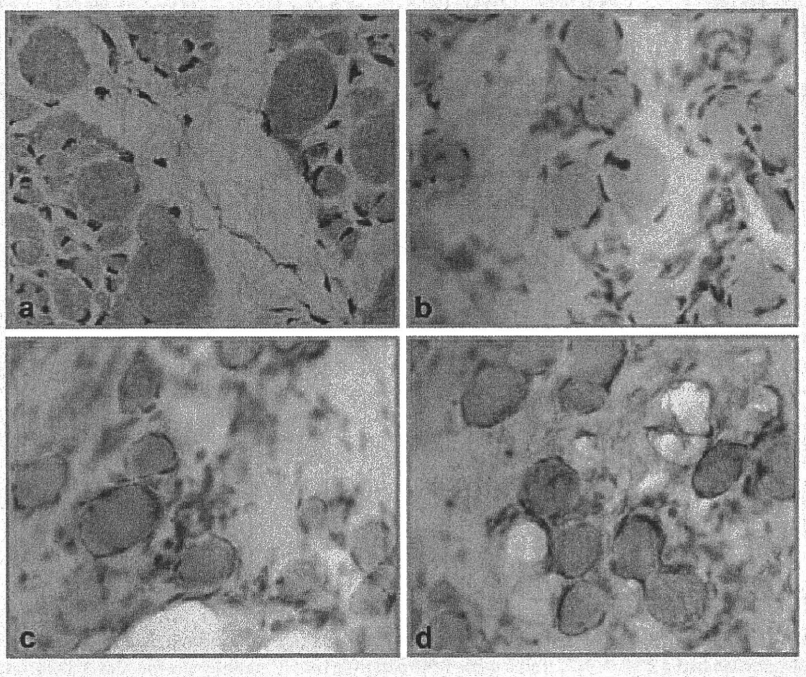

FIG. 3. Hematyoxylin and eosin staining of skeletal muscle showed dystrophic changes, including fiber size variability, fibrosis, and fat replacement (a). Serial sections were immunostained with (b) anti-α-dystroglycan, (c) β-dystroglycan, and (d) merosin antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

direct sequencing. We detected a paternally derived heterozygous C-to-T transition at base 139 in exon 3 of the *fukutin* gene, resulting in premature termination (c. 139C>T, p. R47X; Fig. 4A); this mutation has been previously described [Kobayashi et al., 1998a]. We next looked for additional mutations in the second allele. We used a modified rapid PCR-based diagnostic method [Watanabe et al., 2005] to indirectly confirm the presence of the 3-kb retrotransposal *fukutin* gene insertion. We used three primers (LAT7ura, LAT7-2, and ins385–359) in one reaction mixture to detect the normal and insertion alleles simultaneously, and two other primers to detect flanking genomic sequences. As expected, our results indicated that the patient had the Japanese founder insertion. His mother was heterozygous for this mutation (Fig. 4B). The family was then genotyped using polymorphic microsatellite markers cen-D9S306-D9S2105-(FCMD)-D9S2170-D9S2171-D9S2107-D9S172-tel, as described previously [Kobayashi et al., 1998b]. The patient had the same two haplotypes as Japanese patients; the paternally derived mutation had the 130-201-157-183 haplotype and maternally derived mutation had the 138-192-147-183 haplotype [Kondo-Iida et al., 1999]. Therefore, the maternal chromosome containing the retrotransposon was concordant with the founder haplotype represented as 138-192-147-183.

DISCUSSION

The propositus was clinically diagnosed with congenital muscular dystrophy based on neonatal hypotonia, muscle weakness, joint contractures, high serum CK levels, and electromyogram abnormalities. Histopathology showed muscle wasting, fibrosis, and evidence of α-dystroglycanopathy. Brain and eye involvement suggested a congenital muscular dystrophy syndrome with central nervous system dysplasia and ocular anomaly, all of which are consistent with FCMD, muscle–eye–brain disease (MEB) and Walker–Warburg syndrome (WWS). WWS patients have hydrocephalus and severe retinal and cerebellar malformations and typically die earlier than FCMD or MEB patients [Beltrán-Valero de Bernabé et al., 2002]. We excluded WWS for diagnosis because our patient's clinical manifestations were milder than is typical for WWS. Although FCMD is only relatively common among Japanese people and there have been no reports describing Chinese FCMD patients [Jong et al., 2000], after observing the patient's mild eye involvement, we decided to examine the *fukutin* gene.

FCMD is the second most common form of childhood muscular dystrophy and one of the most common autosomal recessive disorders in Japan. Specifically, 1 in 188 people carry the founder insertion [Watanabe et al., 2005] and most Japanese FCMD cases are

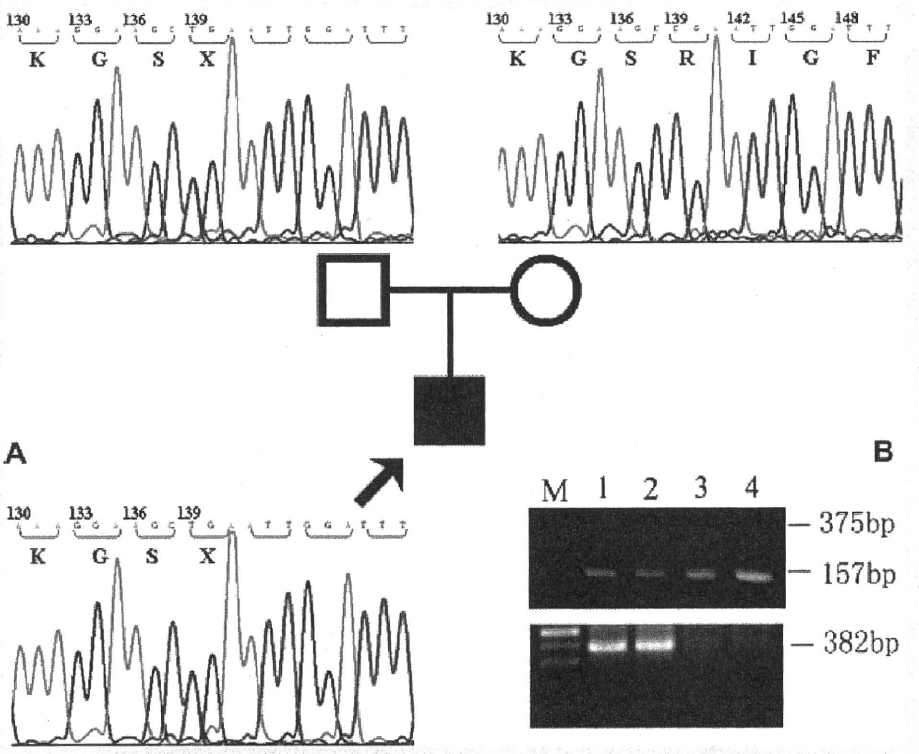

FIG. 4. Mutational analysis of the *fukutin* gene. Our patient was heterozygous for (A) a previously described c.139C>T point mutation and (B) a founder 3-kb retrotransposal insertion in the 3′ UTR. B: PCR pedigree of the patient's family, bearing the founder 3-kb retrotransposal insertion allele. Lanes 1 (propositus) and 2 (his mother) showed 375 and 157 bp PCR products (upper) and a 382 bp PCR product (lower). The 375 and 382 bp bands arose from the founder insertion. Lanes 3 (father) and 4 (normal control) showed only the non-insertion 157-bp PCR product (upper). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the result of founder mutations. The strikingly high FCMD prevalence among Japanese people appears to result from the initial founder effect, which expanded in relative isolation. Most Japanese FCMD-bearing chromosomes arose from a single ancestral Japanese founder approximately 2,000–2,500 years ago [Toda et al., 1996; Kobayashi et al., 1998b]. Compound heterozygous Japanese FCMD cases are rarer. In contrast to its relatively common occurrence in the Japanese population, the 3-kb retrotransposal insertion has never been reported in a Chinese individual. Moreover, a large northeast Asian population study found that the founder mutation was not detected in 766 mainland Chinese individuals, demonstrating the rarity of this mutation in non-Japanese ethnic groups. Instead, FCMD and related clinical syndromes (e.g., WWS and MEB) typically result from bi-allelic coding region point mutations. Non-Japanese individuals who are homozygous for truncating mutations in *fukutin* were reported to show more severe, WWS-like phenotypes. In contrast, patients with compound heterozygous truncating mutations near the 3′ terminus of the *fukutin* coding region seemed to display milder phenotypes [Beltrán-Valero de Bernabé et al., 2003; Silan et al., 2003; Godfrey et al., 2007; Manzini et al., 2008].

Kondo-Iida et al. [1999] found a higher frequency of severe phenotypes, including WWS-like manifestations such as hydrocephalus and microphthalmia, among compound heterozygous propositi than among founder insertion homozygotes. In their study, clinical FCMD was classified into three groups, according to the patients' maximum motor abilities: (a) typical—patients sat unassisted or slid on the buttocks (levels 2–4); (b) mild—patients could stand or walk with or without support (levels 5–8); and (c) severe—patients were only able to sit with support or had no head control (levels 0–1). Our patient displays a typical FCMD phenotype, according to Kondo-Iida's classification system.

Chromosomes carrying the founder insertion in the *fukutin* 3′ UTR may produce subnormal mature fukutin levels and generate a relatively milder FCMD phenotype. Non-founder coding region mutations lead to major structural changes in the fukutin protein; thus, they are likely to produce more severe effects. The most common point mutation reported among Japanese is c. 139C>T/p. R47X. Compound heterozygous Japanese patients carrying this mutation and the founder insertion showed severe FCMD phenotypes, including significant hydrocephalus [Kondo-Iida et al., 1999]. Our patient possesses the same *fukutin* mutations previously described in these Japanese case studies, and we can definitively diagnose our case as FCMD. However, his clinical phenotype is milder than what has been described in Japanese patients carrying identical compound heterozygous mutations. We posit that this difference could be due to considerable variable expressivity or phenotypic heterogeneity. The normal function of the *fukutin* gene remains unknown. We have previously demonstrated that fukutin forms a complex with *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) and may modulate its enzymatic activity [Xiong et al., 2006]. The loss-of-function mutations in the gene encoding POMGnT1 cause MEB [Yoshida et al., 2001]. It is possible that our patient has slightly higher POMGnT1 enzymatic activity than Japanese patients with the same mutations.

We further performed a haplotype analysis of the family and found that both of the chromosomes of the propositus had the same haplotypes as Japanese patients, the maternal chromosome containing the retrotransposon was concordant with the founder haplotype represented as 138-192-147-183, indicating that both mutant alleles were derived from the same founder as Japanese patients. We presume there may be more FCMD patients and founder mutation carriers in China. This suggests that segments of the Chinese and Japanese populations may have a recent common ancestor. Here, we describe the first *fukutin* founder mutation in a Chinese individual with no known Japanese ancestry. Using a rapid diagnostic method, we identified the first founder insertion outside of Japan. Our procedure is more rapid and convenient than previously reported methods [Kato et al., 2004]. In addition to the immediate diagnostic implications for the family, our findings emphasize the importance of considering the *fukutin* founder mutation for diagnostic purposes outside Japan.

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Post-translational Maturation of Dystroglycan Is Necessary for Pikachurin Binding and Ribbon Synaptic Localization*[S]

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Pikachurin, the most recently identified ligand of dystroglycan, plays a crucial role in the formation of the photoreceptor ribbon synapse. It is known that glycosylation of dystroglycan is necessary for its ligand binding activity, and hypoglycosylation is associated with a group of muscular dystrophies that often involve eye abnormalities. Because little is known about the interaction between pikachurin and dystroglycan and its impact on molecular pathogenesis, here we characterize the interaction using deletion constructs and mouse models of muscular dystrophies with glycosylation defects (*Large*^{myd} and *POMGnT1*-deficient mice). Pikachurin-dystroglycan binding is calcium-dependent and relatively less sensitive to inhibition by heparin and high NaCl concentration, as compared with other dystroglycan ligand proteins. Using deletion constructs of the laminin globular domains in the pikachurin C terminus, we show that a certain steric structure formed by the second and the third laminin globular domains is necessary for the pikachurin-dystroglycan interaction. Binding assays using dystroglycan deletion constructs and tissue samples from *Large*-deficient (*Large*^{myd}) mice show that Large-dependent modification of dystroglycan is necessary for pikachurin binding. In addition, the ability of pikachurin to bind to dystroglycan prepared from *POMGnT1*-deficient mice is severely reduced, suggesting that modification of the GlcNAc-β1,2-branch on *O*-mannose is also necessary for the interaction. Immunofluorescence analysis reveals a disruption of pikachurin localization in the photoreceptor ribbon synapse of these model animals. Together, our data demonstrate that post-translational modification on *O*-mannose, which is mediated by Large and POMGnT1, is essential for pikachurin binding and proper localization, and suggest that their disruption underlies the molecular pathogenesis of eye abnormalities in a group of muscular dystrophies.

Dystroglycan (DG),[2] a cell surface receptor for several extracellular matrix proteins, plays important roles in various tissues (1–7). DG consists of an extracellular, heavily glycosylated α subunit (α-DG) and a transmembrane β subunit (β-DG). α-DG and β-DG are encoded by a single gene and post-translationally cleaved to generate the two subunits. α-DG is a receptor for extracellular proteins such as laminin-111, laminin-211, agrin, perlecan, and neurexin. β-DG binds to α-DG in the extracellular space, anchoring α-DG at the cell surface. Inside the cell, β-DG binds to dystrophin, which in turn is linked to the actin cytoskeleton. Thus, α/β-DG functions as a molecular axis, connecting the extracellular matrix with the cytoskeleton across the plasma membrane.

DG ligand proteins commonly contain laminin globular (LG) domains, which mediate binding to α-DG. *O*-Mannosylation of α-DG is required for its interaction with ligands; however, the precise ligand-binding sites and epitope are not known. A unique *O*-mannosyl tetrasaccharide (Neu5Ac-α2,3-Gal-β1, 4-GlcNAc-β1,2-Man) was first identified on peripheral nerve α-DG (8). The initial Man transfer to Ser/Thr residues on the α-DG peptide backbone is catalyzed by the POMT1-POMT2 complex (9). Both *POMT1* and *POMT2* were originally identified as responsible genes in Walker-Warburg syndrome (10, 11). *POMGnT1*, a causative gene for muscle-eye-brain disease, encodes a glycosyltransferase that transfers GlcNAc to *O*-Man on α-DG (12). Because mutations in these enzymes cause abnormal glycosylation of α-DG and reduce its ligand binding activity, it is recognized that the GlcNAc-β1,2-branch on *O*-Man is essential to α-DG function as a matrix receptor.

Additional proteins, including fukutin, FKRP, and LARGE, are also involved in synthesizing the glycans on α-DG that are required for ligand binding activity. Recently, a GalNAc-β1, 3-GlcNAc-β1,4-branch and a phosphodiester-linked modification on *O*-Man were identified (13). α-DG from cells with mutations in *fukutin* or *Large* shows defective post-phosphoryl modification on *O*-Man, suggesting that this phosphoryl branch serves a laminin-binding moiety. *fukutin* was originally identified as the responsible gene for Fukuyama-type congenital muscular dystrophy (14), and the *fukutin* homologue *FKRP* was identified through sequence homology (15). Mutation of

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[2] The abbreviations used are: DG, dystroglycan; ERG, electroretinogram; LG, laminin globular.