

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

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Received July 21, 2008; Revised November 2, 2008; Accepted November 12, 2008

**Hypoglycosylation and reduced laminin-binding activity of  $\alpha$ -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  $\alpha$ -dystroglycan; however, no signs of muscular dystrophy were observed. More sensitive methods detected minor levels of intact  $\alpha$ -dystroglycan, and solid-phase assays determined laminin binding levels to be  $\sim$ 50% of normal. In contrast, intact  $\alpha$ -dystroglycan is undetectable in the dystrophic Large<sup>myd</sup> mouse, and laminin-binding activity is markedly reduced. These data indicate that a small amount of intact  $\alpha$ -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  $\alpha$ -dystroglycan. In addition, transfer of *LARGE* produced laminin-binding forms of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -dystroglycan. *POMGnT1* and the *POMT1/2* complexes are known to have glycosyltransferase activities that place *O*-mannosyl sugar chains on  $\alpha$ -dystroglycan (7,8). The exact functions of *fukutin*, *FKRP* and *LARGE* are still unknown.

$\alpha$ -Dystroglycan ( $\alpha$ -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  $\beta$ -DG (9).  $\alpha$ - and  $\beta$ -DGs are encoded by a single mRNA that is cleaved into two subunits during post-translational maturation. *O*-glycosylation of  $\alpha$ -DG is required for ligand-binding activity. Although the exact binding epitope for ligand is still unknown, one unique *O*-mannosyl glycan [Neu5Ac( $\alpha$ 2–3)Gal( $\beta$ 1–4)GlcNAc( $\beta$ 1–2)Man-Ser/Thr] (10) appears to be involved in ligand binding among extensive and heterogenous groups of *O*-linked sugar chains.  $\beta$ -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 retrotransposal 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,

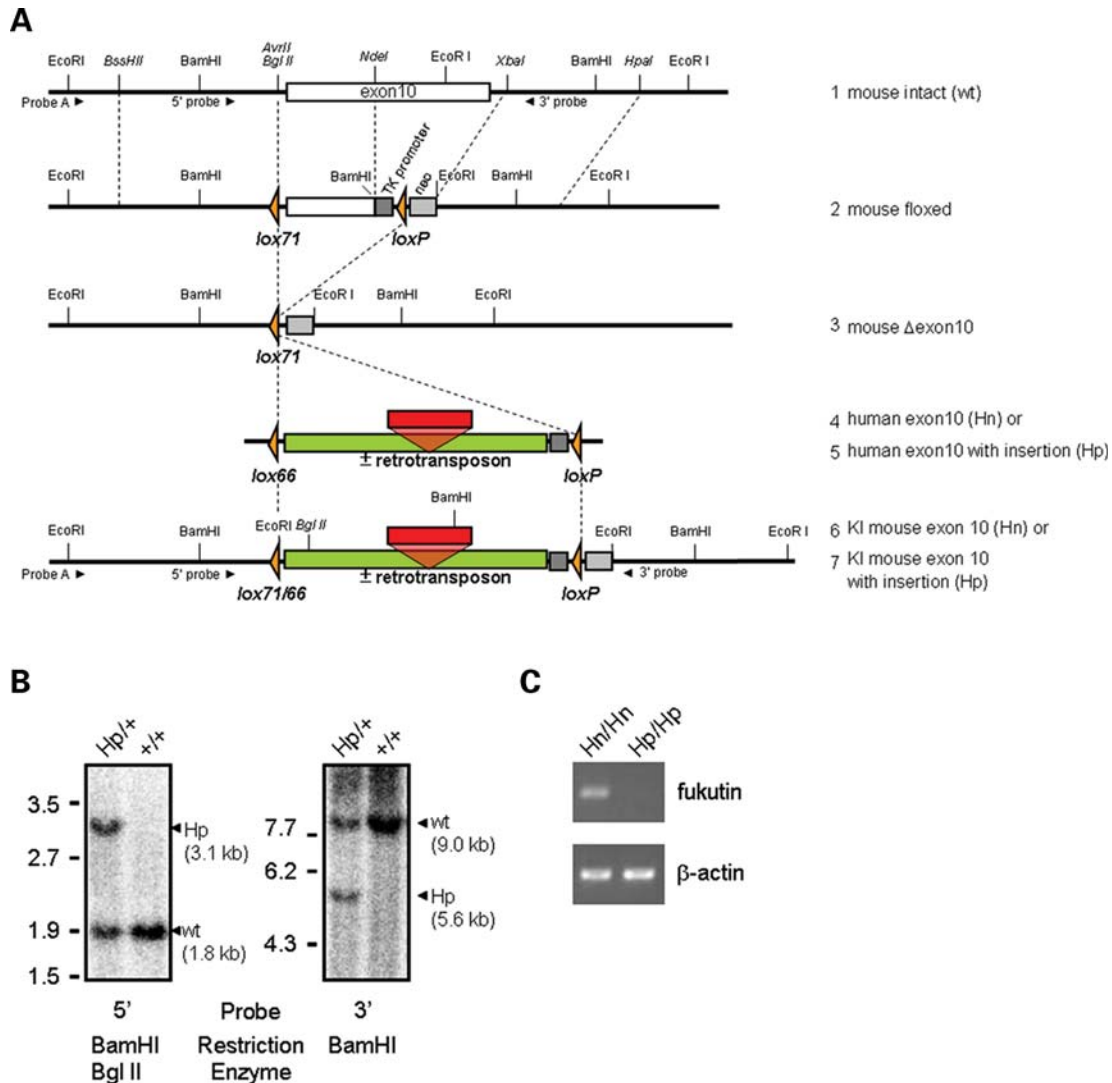
ocular abnormalities and brain malformation. The gene responsible for MEB is *POMGnT1*, which encodes protein *O*-linked mannose  $\beta$ 1,2-*N*-acetylglucosaminyltransferase 1 (7). In both FCMD and MEB disease,  $\alpha$ -DG glycosylation and laminin-binding activity are severely disrupted (20). The *Large*<sup>myd</sup> mouse, a spontaneous mutant, has been used as a model for dystroglycanopathy. As is the case with human dystroglycanopathies,  $\alpha$ -DG in *Large*<sup>myd</sup> 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  $\alpha$ -DG in culture cells and mice (25,26). In addition, physical interaction between *LARGE* and  $\alpha$ -DG is an essential step in acquiring ligand-binding activities of  $\alpha$ -DG (25). Therefore, it is believed that *LARGE* plays a post-translational role in modulating both  $\alpha$ -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*<sup>-/-</sup>) 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  $\alpha$ -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.



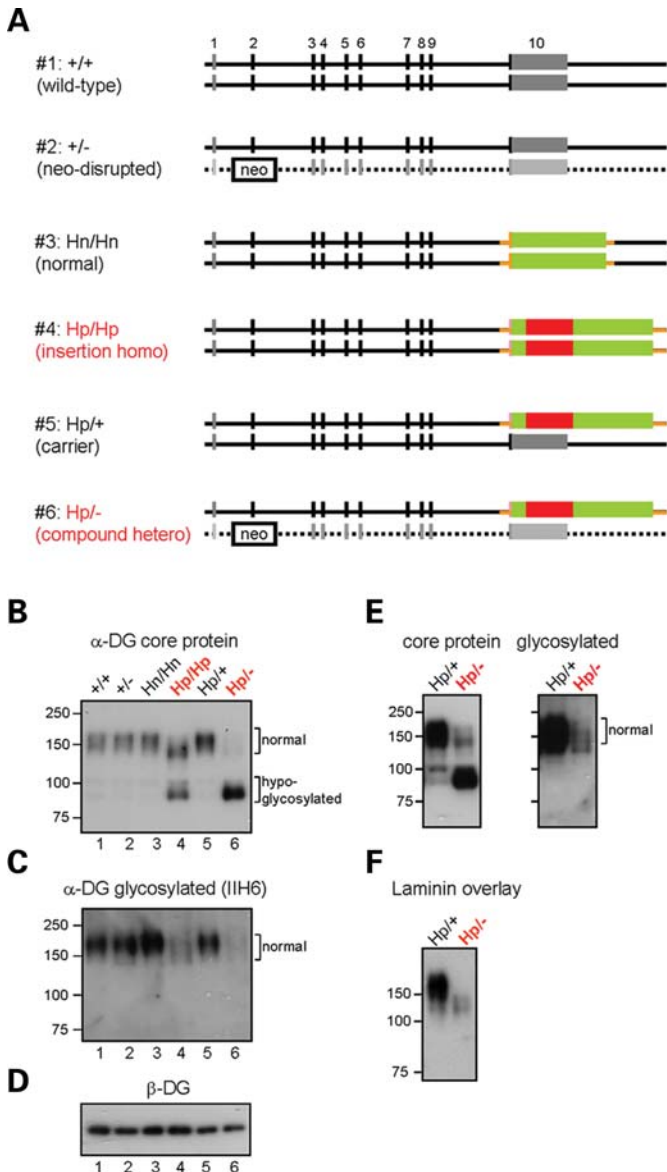
**Figure 1.** Generation of FCMD model mice that carry the retrotransposal insertion in the mouse *fukutin* gene. (A) Schematic representation of the targeting vector. Details are described in the Materials and Methods section. Human *fukutin* exon10 is shown in green, and the retrotransposon is shown 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  $\beta$ -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*<sup>+/-</sup>) (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 $\alpha$ -DG

To characterize the biochemical properties of  $\alpha$ -DG in the knock-in mice, we prepared skeletal muscle samples enriched for  $\alpha$ -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  $\alpha$ -DG core protein (AP-074G-C) and the monoclonal antibody IIH6. IIH6 recognizes glycosylated epitopes on  $\alpha$ -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

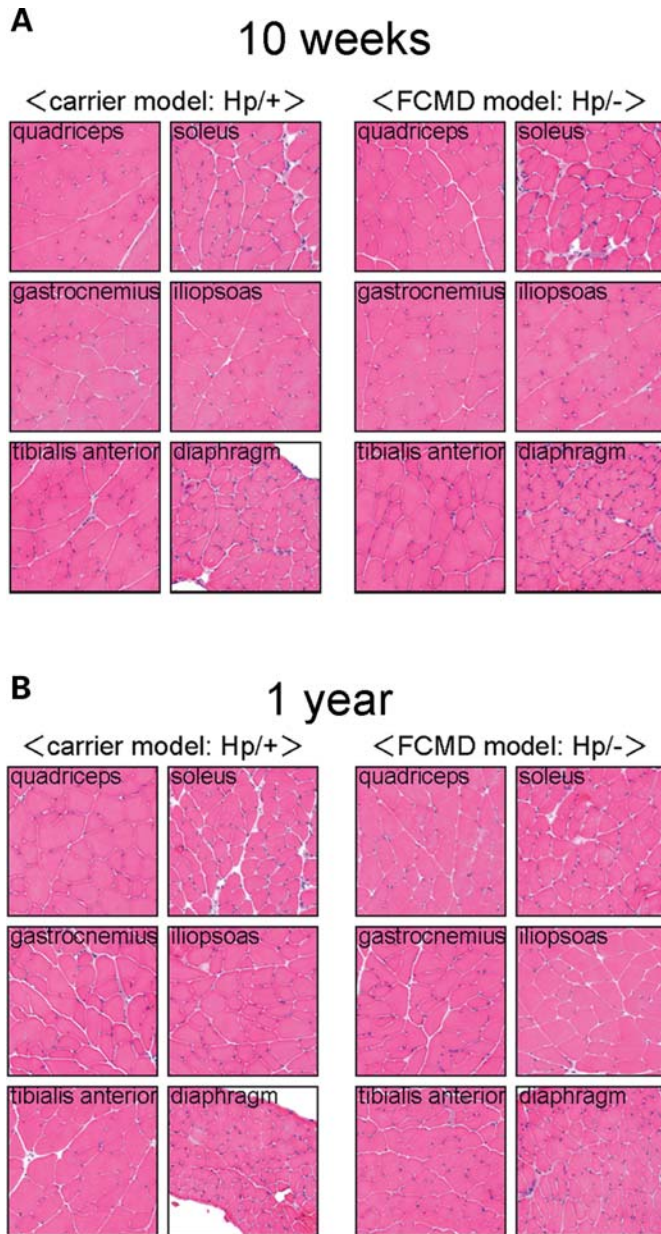
mice (Fig. 2B, lane 4, upper band). In Hp/- mice, we observed a much-reduced intensity of the ~150 kDa bands (Fig. 2B, lane 6). In addition, lower molecular weight (~90 kDa) bands were detected in Hp/Hp and Hp/- mice (Fig. 2B, lanes 4 and 6). Western blotting with IIH6 detected ~150 kDa bands in the 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).

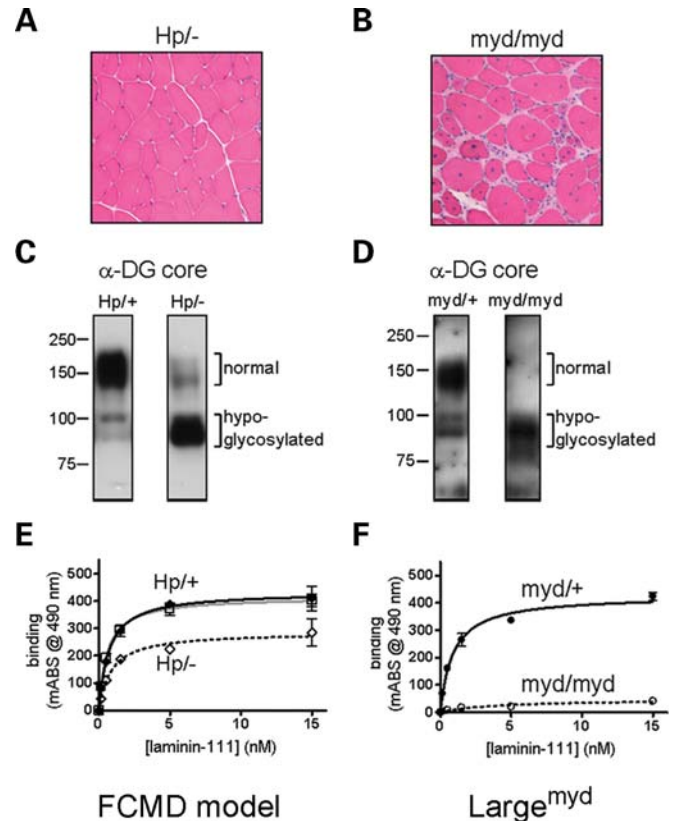
Both hypoglycosylated and IIH6-positive intact α-DG proteins were detected in Hp/Hp and Hp/- mouse brains (Supplementary Material, Fig. S2). As is the case with skeletal muscle, Hp/- mice contained more hypoglycosylated α-DG. Apparent brain histological abnormality was hardly detected in Hp/- mice; only 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  $\alpha$ -DG is thought to be the main cause of dystroglycanopathy. Therefore, we hypothesized that the minimal levels of intact  $\alpha$ -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,

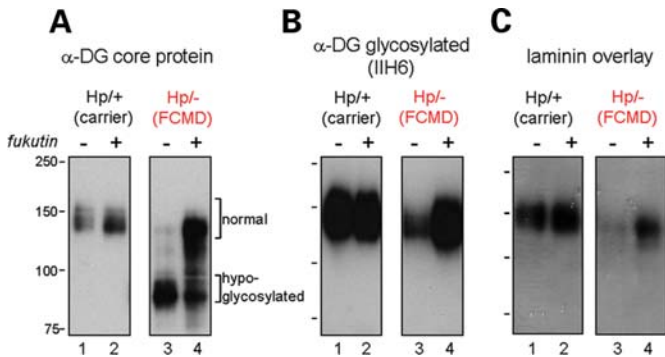


**Figure 4.** Laminin-binding activity is maintained in  $Hp/-$  mice but barely detected in  $Large^{myd}$  mice. H&E staining of quadriceps tissue from  $Hp/-$  (A) and  $Large^{myd}$  ( $myd/myd$ ) (B) mice are shown. WGA preparations from the  $Hp/-$  (C) and the  $myd/myd$  (D) skeletal muscle were also analyzed by western blot using an antibody against  $\alpha$ -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  $\alpha$ -DG core protein in  $myd/myd$  mice revealed no intact size ( $\sim 150$  kDa) of  $\alpha$ -DG species (Fig. 4C and D), indicating that almost all  $\alpha$ -DG is hypoglycosylated in  $myd/myd$  mice. The laminin-binding activity of  $\alpha$ -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  $\sim 50\%$  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  $\sim 150$  kDa  $\alpha$ -DG) influence laminin-binding activity and indicate that only a small amount of IIH6-reactive  $\alpha$ -DG is required to maintain skeletal muscle function.

#### Fukutin gene transfer restores glycosylation of $\alpha$ -DG in knock-in mice

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



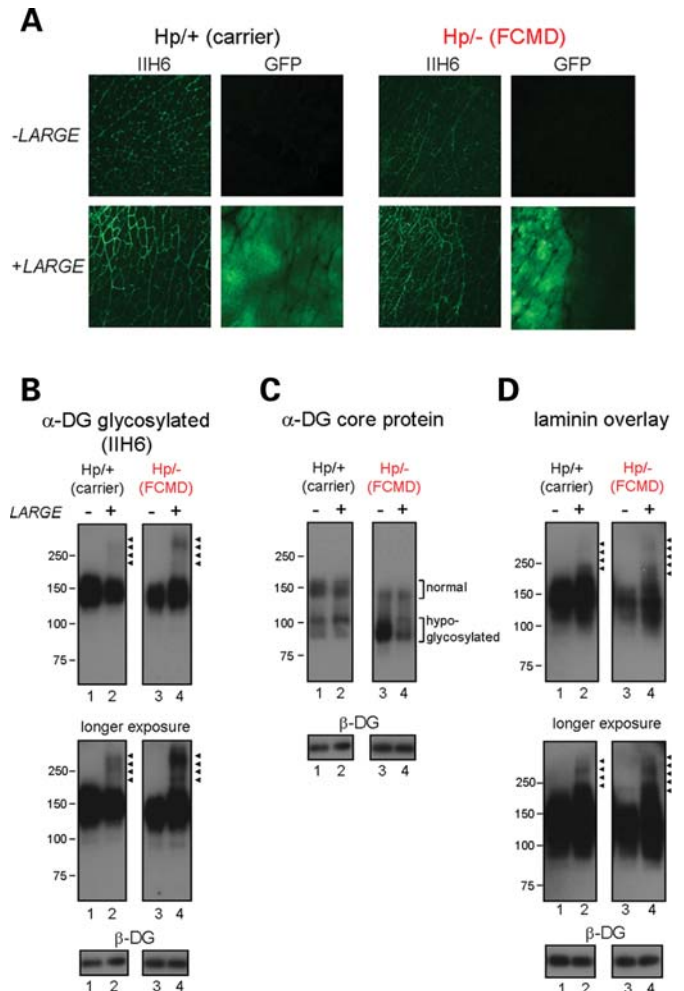
**Figure 5.** *Fukutin* gene transfer rescues the glycosylation abnormality in *Hp*<sup>-</sup> mice. *Hp*<sup>+</sup> or *Hp*<sup>-</sup> pups were injected with adenovirus encoding wild-type human *fukutin* in one leg (+) and with saline in the contralateral leg (-). Calf muscle was analyzed using western blot with antibodies against core  $\alpha$ -DG protein (A) and glycosylated  $\alpha$ -DG (B) and using a laminin overlay assay (C). Transfer of *fukutin* produced increases in  $\alpha$ -DG molecular weight, IIH6 reactivity and laminin binding activity in *Hp*<sup>-</sup> 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*<sup>-</sup> and litter control *Hp*<sup>+</sup> mice. Following 4 weeks of injections,  $\alpha$ -DG enriched samples were prepared using WGA beads and analyzed for glycosylation and laminin-binding activity. Western blot analysis with anti- $\alpha$ -DG core protein antibodies revealed that *fukutin* gene transfer into *Hp*<sup>-</sup> mice reduced hypoglycosylated  $\alpha$ -DG (~90 kDa) and increased levels of the normalized  $\alpha$ -DG species (~150 kDa) (Fig. 5A, lanes 3 and 4). IIH6 reactivity and laminin-binding activity also increased following *fukutin* gene transfer into *Hp*<sup>-</sup> mice (Fig. 5B and C, lanes 3 and 4). No obvious changes were observed in *Hp*<sup>+</sup> mice after the gene transfer (Fig. 5C, lanes 1 and 2). These results demonstrate that *fukutin* gene transfer can correct biochemical abnormalities of  $\alpha$ -DG in *fukutin*-deficient skeletal muscle, and support that fukutin protein is involved in glycosylation of  $\alpha$ -DG.

### Large gene transfer produces laminin-binding forms of $\alpha$ -DG in dystroglycanopathy models

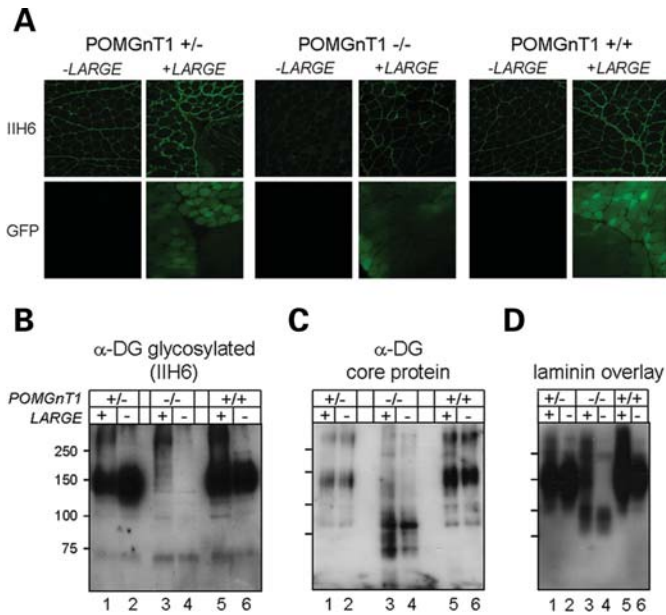
Hypoglycosylation leading to dystroglycanopathies is caused by mutations in six known genes (*fukutin*, *POMGnT1*, *POMT1*, *POMT2*, *FKRP* and *LARGE*) and other, unidentified genes. In an effort to bypass the need for identification of disease-causing genes in developing therapies (e.g. gene transfer), we further explored a unique feature of *LARGE*. *LARGE* has been demonstrated to induce  $\alpha$ -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  $\alpha$ -DG glycosylation (FCMD, MEB and WWS) (26).

We examined whether adenoviral *LARGE* gene transfer into *Hp*<sup>-</sup> skeletal muscle induces hyperglycosylation and increases laminin-binding activity of  $\alpha$ -DG. Immunofluorescence analysis of untreated control muscles revealed weaker IIH6 reactivity in *Hp*<sup>-</sup> than in *Hp*<sup>+</sup> (Fig. 6A, -*LARGE*). Muscle sections subjected to gene transfer showed increased



**Figure 6.** *LARGE* gene transfer produces functionally glycosylated  $\alpha$ -DG in *Hp*<sup>-</sup> mice. *Hp*<sup>+</sup> or *Hp*<sup>-</sup> 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  $\alpha$ -DG (B),  $\alpha$ -DG core protein (C) and using a laminin overlay assay (D). The western blot for  $\beta$ -DG shows comparable amounts of DG proteins in each lane. Images with longer-exposures better indicate the presence of hyperglycosylated  $\alpha$ -DG (arrowheads). These results show that the transfer of *LARGE* increases IIH6 reactivity and laminin-binding activity in *Hp*<sup>-</sup> mice.

$\alpha$ -DG glycosylation in transduced areas, as indicated by eGFP expression in both *Hp*<sup>-</sup> and *Hp*<sup>+</sup> mice (Fig. 6A, +*LARGE*). We also examined adenovirus-injected and non-injected contralateral leg muscles using western blot analysis with antibodies against  $\alpha$ -DG core protein and IIH6. These experiments showed that the *LARGE* gene transfer increased IIH6 reactivity at ~150 kDa in the *Hp*<sup>-</sup> muscle and produced a broad band with a molecular weight of 150–250 kDa in both *Hp*<sup>-</sup> and *Hp*<sup>+</sup> muscles (Fig. 6B). Anti- $\alpha$ -DG core protein antibodies poorly recognized a higher molecular weight  $\alpha$ -DG species (Fig. 6C), which is consistent with previous reports (26). Following the *LARGE* gene transfer, levels of hypoglycosylated  $\alpha$ -DG species decreased (Fig. 6C, lanes 3 and 4). These data indicate that



**Figure 7.** *LARGE* gene transfer produces functionally glycosylated  $\alpha$ -DG in MEB disease model mice. POMGnT1<sup>+/+</sup>, POMGnT1<sup>+/-</sup> or POMGnT1<sup>-/-</sup> 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  $\alpha$ -DG (B) and  $\alpha$ -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<sup>-/-</sup> mice, the model for MEB disease.

*LARGE*-induced glycosylation occurs on hypoglycosylated  $\alpha$ -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  $\alpha$ -DG in *fukutin*-deficient skeletal muscle.

We further investigated whether *LARGE* gene transfer induced hyperglycosylation and produced laminin-binding forms of  $\alpha$ -DG species in another dystroglycanopathy model, the *POMGnT1*-disrupted mouse (POMGnT1<sup>-/-</sup>) (Miyagoe-Suzuki *et al.*, manuscript in preparation). Western blot analysis using  $\alpha$ -DG molecular weight to 60–90 kDa in POMGnT1<sup>-/-</sup> 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  $\alpha$ -DG in POMGnT1<sup>-/-</sup> mice. Accordingly, laminin-binding activity was significantly reduced in POMGnT1<sup>-/-</sup> mice compared with POMGnT1<sup>+/-</sup> or POMGnT1<sup>+/+</sup> littermates (Fig. 7D, lanes 2, 4 and 6). The minor laminin binding protein (~80–100 kDa, lane 4) detected only in POMGnT1<sup>-/-</sup> 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<sup>-/-</sup> (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  $\alpha$ -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<sup>-/-</sup> skeletal muscle showed only hyperglycosylated IIH6-positive species, while the POMGnT1<sup>+/+</sup> and the POMGnT1<sup>+/-</sup> muscles showed both hyperglycosylated and the original 150 kDa IIH6-positive species. Overlay assays showed that the laminin-binding epitope was produced on hyperglycosylated  $\alpha$ -DG (Fig. 7D). These data support the idea that *LARGE* is an effective target for increasing or restoring laminin-binding activity of  $\alpha$ -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  $\alpha$ -DG, as is seen in FCMD patients (20,31), so we consider them to be novel models for FCMD.

Spontaneous *Large*<sup>myd</sup> and *Large*<sup>vis</sup> 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  $\alpha$ -DG with IIH6-reactivity is barely detected, and laminin-binding activity is dramatically reduced (20). Comparing Hp/- mice with *Large*<sup>myd</sup> mice led us to reason that the remaining intact  $\alpha$ -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  $\alpha$ -DG. For example, it has been suggested that integrin  $\alpha$ 7, 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  $\alpha$ -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  $\alpha$ -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  $\alpha$ -DG glycosylation, while those at the milder end tend to have relatively well-preserved  $\alpha$ -DG glycosylation (38). Most known missense mutations in *POMGnT1* disrupt POMGnT enzyme activity, causing hypoglycosylation of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -DG glycosylation is not always necessary; partial restoration of  $\alpha$ -DG glycosylation might be enough to prevent or slow disease progression. The simplest way to restore  $\alpha$ -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  $\alpha$ -DG with laminin-binding activity regardless of the responsible gene, we took advantage of the observation that overexpression of LARGE can produce hyperglycosylated  $\alpha$ -DG with increased laminin-binding activity in cells from genetically distinct dystroglycanopathies (26). LARGE-induced hyperglycosylation of  $\alpha$ -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  $\alpha$ -DG glycosylation has been observed *in vitro*, but no *in vivo* study has been reported except in Large<sup>myd</sup> mice (26). Gene transfer of *LARGE* into Large<sup>myd</sup> 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  $\alpha$ -DG in models other than the Large<sup>myd</sup> mice. These results support the idea that glycotherapies aimed at modulating LARGE may be a therapeutic option for many  $\alpha$ -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  $\Delta$ exon10), 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  $\Delta$ exon10 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, GAAACTCTGC-CATGACACCTC; HNC440R, ACCAGCTTAAATGCCCA-GAAG; Wild R2, GAAGCCAACCTGTGTACCACAC. 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 TTGGACAAGTT.

C57BL/6 mice were obtained from Japan SLC, Inc., and Large<sup>myd</sup> 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  $\beta$ -DG (Novacastra); mouse monoclonal antibody IIH6 against  $\alpha$ -DG (Upstate); and polyclonal anti-laminin (Sigma). We generated goat polyclonal antibodies against  $\alpha$ -DG core protein using GST fusion proteins containing the N- or C-terminal domains of mouse  $\alpha$ -DG. Antisera (074G) were affinity-purified using an  $\alpha$ -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  $\mu$ l 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  $\mu$ l 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  $\mu$ m) 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<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 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<sub>2</sub>O<sub>2</sub>, then reactions were stopped with 2 N H<sub>2</sub>SO<sub>4</sub> 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 *Sma*I, 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  $1 \times 10^8$ – $1 \times 10^9$  particles in 10  $\mu$ l 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.

## ACKNOWLEDGEMENTS

We would like to thank past and present members of the Clinical Genetics laboratory for fruitful discussions and scientific contributions. We also thank Norihiro Miyazawa and Takashi Wadatsu for technical supports and Dr Jennifer Logan for help in editing the manuscript.

*Conflict of Interest statement.* None declared.

## FUNDING

This work was supported by the Ministry of Health, Labor and Welfare of Japan (Research on Psychiatric and Neurological Diseases and Mental Health H20-016, and The Research Grant for Nervous and Mental Disorders 17A-10 to T.T.); Japan Society for the Promotion of Science (a Grant-in-Aid for Young Scientists (B) 19790232 to M.K.); Mizutani Foundation for Glycoscience (to K.K.); and the Ministry of Education, Culture, Sports, Science, and Technology of Japan (the 21st Century COE program 14013037). Funding to pay the Open Access charge was provided by Research on Psychiatric and Neurological Diseases and Mental Health H20-016.

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