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nature medicine

LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies

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Several congenital muscular dystrophies caused by defects in known or putative glycosyltransferases are commonly associated with hypoglycosylation of α-dystroglycan (α-DG) and a marked reduction of its receptor function. We have investigated changes in the processing and function of α-DG resulting from genetic manipulation of LARGE, the putative glycosyltransferase mutated both in Large^{myd} mice and in humans with congenital muscular dystrophy 1D (MDC1D). Here we show that overexpression of LARGE ameliorates the dystrophic phenotype of Large^{myd} mice and induces the synthesis of glycan-enriched α-DG with high affinity for extracellular ligands. Notably, LARGE circumvents the α-DG glycosylation defect in cells from individuals with genetically distinct types of congenital muscular dystrophy. Gene transfer of *LARGE* into the cells of individuals with congenital muscular dystrophies restores α-DG receptor function, whereby glycan-enriched α-DG coordinates the organization of laminin on the cell surface. Our findings indicate that modulation of LARGE expression or activity is a viable therapeutic strategy for glycosyltransferase-deficient congenital muscular dystrophies.

Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), and Walker-Warburg syndrome (WWS) are congenital muscular dystrophies with similar associated defects in brain development[1–3]. The genes involved in these diseases encode putative or known glycosyltransferases: mutations in *FCMD* (encoding fukutin) are responsible for FCMD, mutations in *POMGNT1* for MEB, and mutations in *POMT1* for a percentage of WWS[2,4,5]. WWS can also be caused by severe mutations in *FCMD*, in *POMGNT1* or in *FKRP*, the gene encoding fukutin-related protein, which is another putative glycosyltransferase responsible for MDC1C and limb-girdle muscular dystrophy 2I (LGMD2I)[6]. The common biochemical feature in these disorders is the abnormal glycosylation of α-DG, a ubiquitous external membrane protein, which suggests that α-DG may be a potential target of these enzymes[7–9].

Dystroglycan, which is present in skeletal muscle as part of the dystrophin-glycoprotein complex (DGC)[10], comprises two subunits, the extracellular α-DG and the transmembrane β-DG[11,12], derived from post-translational cleavage of a precursor polypeptide encoded by the *DAG1* gene. Both subunits undergo glycosylation, but whereas β-DG is consistently detected with a molecular mass (M_r) of 43 kDa, the mass of α-DG varies from 120 kDa to 200 kDa, owing to developmental and tissue-specific glycosylation of a 74-kDa core polypeptide[12]. We have previously shown that the inability of aberrantly glycosylated α-DG to bind extracellular matrix ligands such as laminin, agrin and neurexin causes muscle degeneration and abnormal neuronal migration in individuals with MEB and FCMD[13].

Animal models of these diseases are not available or are embryonically lethal[14], although the spontaneous mouse model Large^{myd} closely resembles the human diseases[13,15]. The convergence of clinical and biochemical phenotypes of persons with congenital muscular dystrophy and Large^{myd} mice suggests that the same glycosylation pathway of α-DG may be affected[13]. *LARGE*, the gene mutated in Large^{myd} mice and in persons with MDC1D[16,17], encodes a putative glycosyltransferase with two structurally distinct domains that are homologous to bacterial α-glycosyltransferase and mammalian β-1,3-*N*-acetylglucosaminyltransferase[16,18]. However, because the activity of glycosyltransferases of this class has not been shown to modify α-DG, it is possible that LARGE affects the glycosylation pathway of α-DG by modulating the activity of other enzymes.

Here we have evaluated the effect of LARGE on glycosylation of α-DG by genetic manipulation of *LARGE in vivo* and *in vitro*. In particular, we have investigated whether glycosylation in cells from individuals with congenital muscular dystrophy can be modulated or enhanced by expression of LARGE. Unexpectedly, we show that expression of

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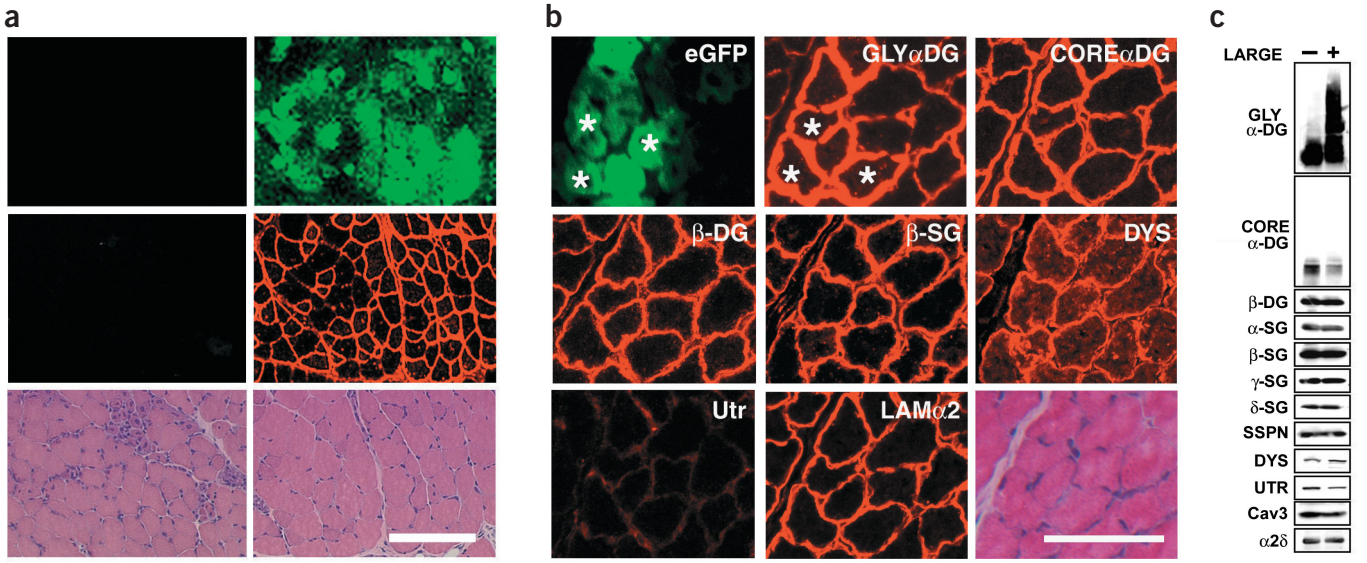
Published online 6 June 2004; doi:10.1038/nm1059



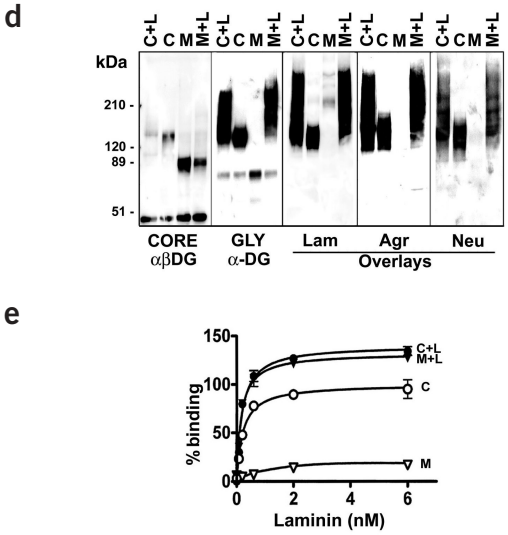


Figure 1 Expression of LARGE generates heavily glycosylated α-DG. (**a**) H&E staining (bottom row) and fluorescence localization of eGFP and glycosylated α-DG (top and middle rows, respectively) in cryosections from quadriceps muscles of Large^{myd} mice after injection of Ad5-LARGE-eGFP (right). Contralateral untreated muscles are also shown (left). Scale bar, 100 μm. (**b**) Analysis of wild-type skeletal muscle treated with Ad5-LARGE-eGFP. Shown are H&E staining and fluorescence localization of eGFP, glycosylated α-DG (GLYα-DG), α-DG core peptide (COREα-DG), β-DG, laminin α2 (Lamα2), β-sarcoglycan (β-SG), dystrophin (DYS) and utrophin (Utr). Asterisks indicate three transduced fibers. Scale bar, 100 μm. (**c**) SDS-PAGE of KCl-washed membranes from wild-type skeletal muscle untreated or treated with Ad5-LARGE-eGFP. Immunoblotting was done with antibodies to glycosylated α-DG, α-DG core peptide, β-DG, sarcoglycans (α-SG, β-SG, γ-SG, δ-SG), sarcospan (SSPN), dystrophin, utrophin and caveolin-3 (Cav-3). An antibody to the Ca^{2+} channel subunit α2δ was used as a loading control. (**d**) Immunoblot analysis of WGA-enriched homogenates from control (C) and Large^{myd} (M) mice untreated or treated with Ad5-LARGE-eGFP (+L). Antibodies to DG core protein (CORE-αβDG) and glycosylated α-DG were used. Bands at ~70 kDa in the GLYα-DG lane are endogenous immunoglobulins. Ligand overlay assays were done for laminin (Lam), agrin (Agr) and neurexin (Neu). (**e**) Solid-phase laminin-binding activity of α-DG from treated (+L) or untreated control (C) and Large^{myd} (M) skeletal muscle.

LARGE leads to a recovery of α-DG function as a receptor not only in Large^{myd} mouse muscle but also in cell cultures derived from individuals with FCMD, MEB and WWS.

RESULTS

LARGE prevents muscular dystrophy in Large^{myd} mice

To investigate the effect of LARGE on α-DG glycosylation, we generated an adenovirus expressing LARGE and enhanced green fluorescent protein (Ad5-LARGE-eGFP). Gene transfer was done in Large^{myd} and control littermate pups (2–4 days of age). Adenovirus-injected and non-injected contralateral leg muscles were examined for expression of DG by using antibodies to core and glycosylated α-DG. The transduction efficiency estimated by eGFP expression was 40–80% (**Fig. 1a**). All transduced muscle fibers reacted with antibodies to glycosylated α-DG. The expression of other DGC components did not change (**Fig. 1b**, **Supplementary Fig. 1** online and data not shown). Dystrophic histological features, such as internally placed nuclei, were reduced by more than 70% in the muscles of transduced Large^{myd} mice, and a significant reduction in fiber size variation was observed after treatment ($P = 0.007$; **Supplementary Fig. 2** online). Analysis of Large^{myd} mice treated with Ad5-LARGE-eGFP at older ages (12 d to 5 weeks) did not produce conclusive results, owing to an inflammatory response to the virus and rapid loss of expression of eGFP and glycosylated α-DG.

Notably, *LARGE* gene transfer in healthy muscle did not cause histological abnormalities (**Fig. 1b** and **Supplementary Fig. 2** online). Unexpectedly, we detected stronger expression of α-DG glycoepitopes at the sarcolemma of transduced control fibers, suggesting that *LARGE* gene transfer leads to the synthesis of α-DG species that are more enriched in glycans (**Fig. 1b**). Indeed, immunoblots of KCl-washed membranes from control skeletal muscle showed that after treatment with Ad5-LARGE-eGFP there were no changes in the DGC, aside from more heavily glycosylated α-DG (**Fig. 1c**).

Immunoblot analysis of wheat germ agglutinin (WGA)-enriched fractions from treated control and Large^{myd} muscles detected glycosylated α-DG as a broad band with an M_r of 150–300 kDa (**Fig. 1d**). Core peptide antibodies recognized α-DG with a high M_r only poorly, probably because of epitope masking by the additional carbohydrate moieties. Residual hypoglycosylated α-DG (90 kDa) from nontransduced fibers was also detected in treated Large^{myd} muscle[13]. Ligand overlay assays showed the rescue of α-DG receptor function in treated

Large^{myd} muscle and the persistent ligand-binding activity of heavily glycosylated α-DG (**Fig. 1d**).

Quantitative solid-phase laminin-binding assays of WGA-enriched fractions showed enhanced activity of nondenatured α-DG in Large^{myd} and control muscles after viral transduction (**Fig. 1e**). We obtained similar results in analogous experiments done on primary myoblast cultures from control and Large^{myd} mice. Notably, forced expression of LARGE induced synthesis of glycosylated α-DG in all transduced cell types: myotubes, myoblasts and fibroblasts (data not shown).

To determine whether functional benefit is conferred by *LARGE* gene transfer, we exercised Large^{myd} mice and control littermates that had been injected with Ad5-LARGE-eGFP in their hamstring muscles, where the transduction efficacy was higher. Uptake of Evans blue dye (EBD) showed that skeletal muscles of Large^{myd} mice were susceptible to exercise-induced sarcolemmal injury. Quantitative image analysis indicated that uptake of EBD in Large^{myd} muscles decreased, on average, from 11.3% to 1.4% after *LARGE* gene transfer. We did not observe EBD uptake in transduced areas of Large^{myd} muscle or control skeletal muscle (**Supplementary Fig. 2** online and not shown), indicating improved functional features in treated muscles and an absence of pathological effects from the overexpression of LARGE.

LARGE generates functional α-DG in FCMD myoblasts

Our findings in control and Large^{myd} muscle suggested that LARGE is an essential component of the glycosylation machinery of α-DG. Genetic and biochemical analysis of individuals with dystroglycanopathy indicates that residual glycosylation activity is present[13,19]. We therefore investigated whether the residual activity could be modulated or enhanced by expressing LARGE in cells from individuals with congenital muscular dystrophy. We first examined the expression and biochemical characteristics of α-DG in myoblasts obtained from three individuals with FCMD and one control subject.

The expression of functionally glycosylated α-DG increased throughout the differentiation of control myoblasts into myotubes; by contrast, highly reduced and patchy expression of glycosylated α-DG was observed in FCMD cultures (**Fig. 2a**), indicating a considerable reduction in fukutin activity in FCMD cells. Immunoblot analysis of WGA-enriched lysates of control myoblasts using antibodies to DG core peptide showed a smaller developmental α-DG isoform of 110–125 kDa that showed binding activity for laminin, neurexin and agrin (**Fig. 2b**). In FCMD myoblasts, α-DG was identified with antibodies to core DG as a broad band of 90–125 kDa, indicating the expression of variably glycosylated α-DG species (**Fig. 2b**).

The presence of residual glycosylated α-DG in FCMD myoblasts as judged by immunohistochemistry contrasts with what has been reported in FCMD muscle biopsies[13] and is similar to a phenomenon of transient re-expression of functional α-DG observed in individuals with a mild form of LGMD[20], suggesting that fukutin may be expressed differentially at earlier stages of muscle development. However, antibodies to glycoepitopes of α-DG did not detect the protein on immunoblots. Consistently, no binding of laminin to α-DG was observed, and ligand-binding activity for neurexin and agrin was considerably diminished and only present in a α-DG fraction with an M_r similar to that seen in control cultures (**Fig. 2b**).

After treatment with Ad5-LARGE-eGFP, all transduced cells in control and FCMD cultures expressed glycosylated α-DG (**Fig. 2c**). Cell viability, proliferation rate, and the time and degree of fusion did not vary after transduction with Ad5-LARGE-eGFP. Biochemical changes in α-DG glycosylation were similar to those observed *in vivo*: core-DG antibodies weakly detected α-DG with a higher M_r, and did not recognize heavily glycosylated forms (≥200 kDa; **Fig. 2d**). Notably, overlay assays showed that *LARGE*-transfected FCMD myoblasts gained laminin, agrin and neurexin binding to a highly glycosylated α-DG with an M_r similar to that seen in control cultures

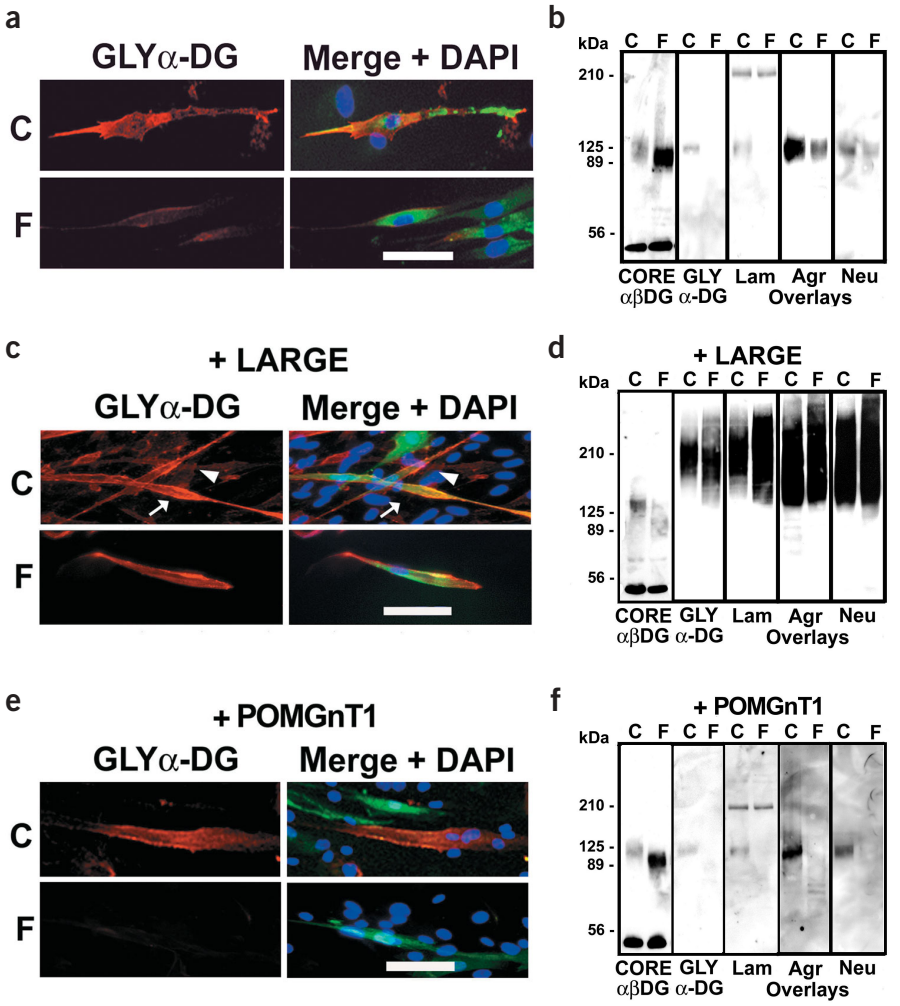


Figure 2 LARGE generates functional α-DG in FCMD myoblasts. (**a**–**f**) Analysis of untreated (**a**,**b**), or infected with Ad5-LARGE-eGFP (**c**,**d**) or Ad5-POMGnT1-eGFP (**e**,**f**) control (C) and FCMD (F) cultures. Immunofluorescence shows localization of desmin (**a**) or eGFP (**c**,**e**). Arrows indicate myotubes and arrowheads indicate myoblasts. Scale bars, 10 μm. WGA-enriched lysates of treated cultures were also analyzed by SDS-PAGE followed by immunoblot analysis with antibodies to DG core protein (CORE-αβDG) and glycosylated α-DG (**b**,**d**,**f**). Laminin (Lam), neurexin (Neu) and agrin (Agr) were used for ligand overlay assays. Bands at ~210 kDa in **b** and **f** are endogenous laminin. Data are representative of three individuals with FCMD.

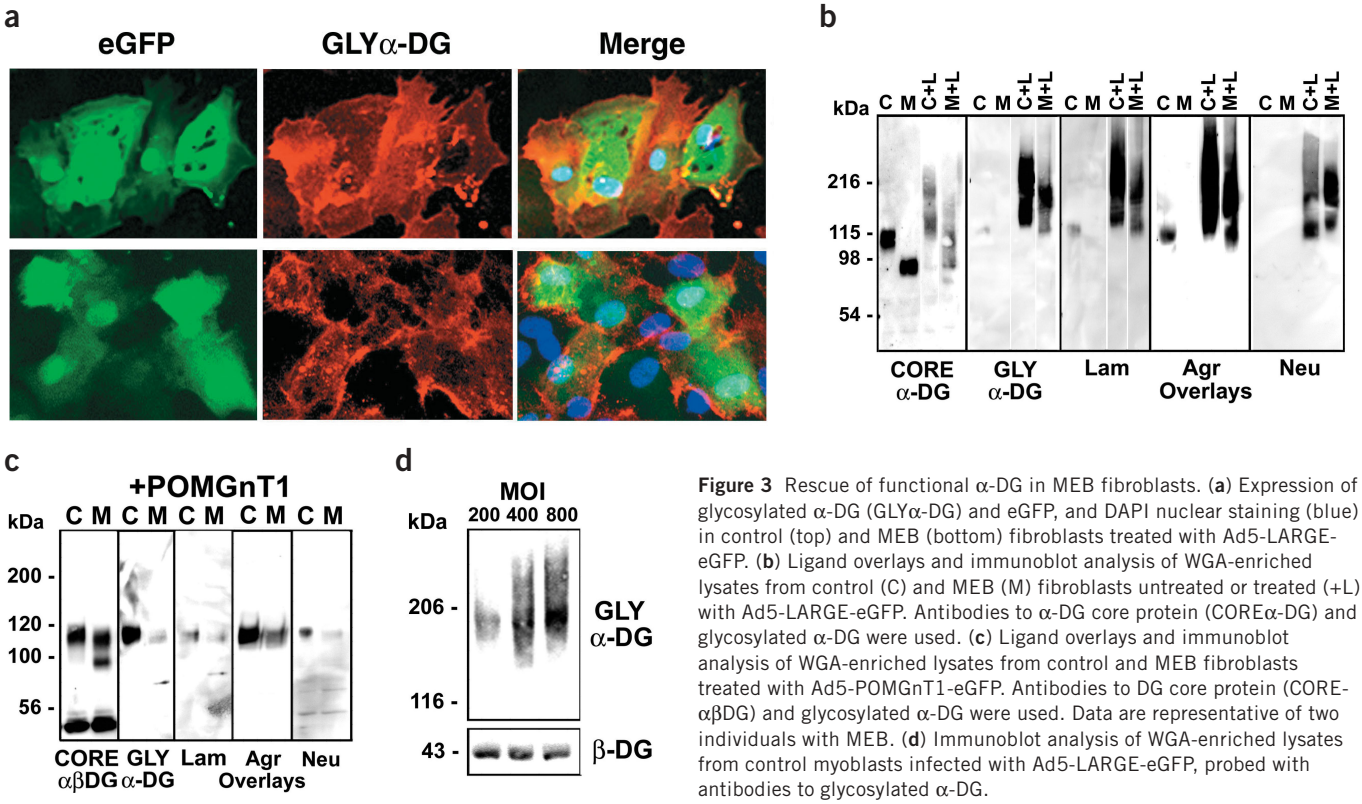


Figure 3 Rescue of functional α-DG in MEB fibroblasts. (**a**) Expression of glycosylated α-DG (GLYα-DG) and eGFP, and DAPI nuclear staining (blue) in control (top) and MEB (bottom) fibroblasts treated with Ad5-LARGE-eGFP. (**b**) Ligand overlays and immunoblot analysis of WGA-enriched lysates from control (C) and MEB (M) fibroblasts untreated or treated (+L) with Ad5-LARGE-eGFP. Antibodies to α-DG core protein (COREα-DG) and glycosylated α-DG were used. (**c**) Ligand overlays and immunoblot analysis of WGA-enriched lysates from control and MEB fibroblasts treated with Ad5-POMGnT1-eGFP. Antibodies to DG core protein (CORE-αβDG) and glycosylated α-DG were used. Data are representative of two individuals with MEB. (**d**) Immunoblot analysis of WGA-enriched lysates from control myoblasts infected with Ad5-LARGE-eGFP, probed with antibodies to glycosylated α-DG.

(**Fig. 2d**). Analysis at different stages of myoblast differentiation did not detect substantial differences (data not shown).

Given that the retrotransposon insertions in the *FCMD* gene in individuals with FCMD lead to a marked reduction in, but not absence of, fukutin[21], we reasoned that the observed synthesis of functionally glycosylated α-DG species in FCMD myoblasts following overexpression of LARGE might be due to either enhancement of the residual activity of fukutin or activation of compensatory glycosylation pathways. To test whether the observed changes were a specific consequence of LARGE overexpression, we treated our cultures with an analogous adenoviral vector expressing eGFP and full-length human protein *O*-mannosyl β-1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1). Although infection resulted in more than a 34-fold increase in POMGnT1 enzyme activity, as assessed with the substrate Man(α1-)*O*-benzyl[22], we did not observe notable variations in α-DG (**Fig. 2e,f**).

LARGE functionally rescues α-DG in MEB fibroblasts

The rare oligosaccharide NeuAcα2,3 Galβ1,4 GlcNAcβ1,2 Manα–O-Ser/Thr is important in the interaction of α-DG with its ligands[23,24]. POMGnT1 has been shown to be active in this pathway, catalyzing the second step of the synthesis of the tetrasaccharide[4]. To investigate the mechanism of LARGE-dependent glycosylation, we transferred *LARGE* into fibroblasts from two siblings affected with MEB. Expression of glycosylated α-DG was detected in control and MEB fibroblasts by immunofluorescence analysis only after treatment with Ad5-LARGE-eGFP (**Fig. 3a**). Immunoblotting with antibodies to DG core peptide detected α-DG species of 115–125 kDa in control and 90 kDa in MEB fibroblasts, whereas glycosylated α-DG with laminin- and agrin-binding activity was detected only in control cells (**Fig. 3b**). Treatment with Ad5-LARGE-eGFP generated glycosylated α-DG expression in every transduced control and MEB cell, and produced highly glycosylated, functional α-DG (**Fig. 3b**).

The low but significant levels of POMGnT1 detected in individuals with MEB are consistent with a proportion of proper glycosylation occurring in the presence of mutated POMGnT1 (refs 13,25). To assess whether LARGE modulates the partially functional enzyme, we evaluated POMGnT1 activity in control and MEB fibroblasts infected with Ad5-LARGE-eGFP or Ad5-eGFP. The 5.9% residual activity of POMGnT1 found in MEB fibroblasts did not vary after *LARGE* gene transfer (data not shown). In MEB fibroblasts, *POMGNT1* gene transfer resulted in expression of α-DG with a M_r comparable to that seen in control cells and restored ligand-binding activity (**Fig. 3c**). However, overexpression of POMGnT1 did not cause changes as marked as those caused by LARGE overexpression.

The relative abundance of *LARGE* transcripts in tissues where α-DG is more heavily glycosylated[18], coupled with the finding that high concentrations of LARGE generate heavily glycosylated α-DG species, suggest that levels of LARGE may be a limiting factor in α-DG glycosylation. Indeed, treating human control cells with Ad5-LARGE-eGFP at increasing multiplicity of infection (MOI) resulted in the synthesis of α-DG forms with an increasing M_r (**Fig. 3d**). These results show that induction of LARGE can activate compensatory mechanisms for the glycosylation of α-DG in a dose-dependent fashion.

LARGE generates functional α-DG in WWS cells

To further investigate whether the extensive glycosylation of α-DG after *LARGE* gene transfer is due to the synthesis of novel glycan chains that are normally absent or less represented in skeletal muscle, we transferred *LARGE* into myoblast and fibroblast cells from four individuals with WWS. Myoblasts from one of the individuals were mutated in the gene encoding protein *O*-mannosyltransferase 1 (POMT1), which catalyzes the first step of the *O*-mannose linked glycan motif of α-DG[26].

As also described for WWS skeletal muscle[9], glycosylated α-DG was not seen at any stage in WWS cell cultures, and immunoblot analysis

detected hypoglycosylated α-DG (**Fig. 4a** and data not shown). After treatment with Ad5-LARGE-eGFP, but not Ad5-POMGnT1-eGFP, staining for glycosylated α-DG was observed in transduced cells (**Fig. 4a** and **Supplementary Fig. 3** online). In all of the WWS cell lines examined, treatment with Ad5-LARGE-eGFP led to the synthesis of α-DG species with a higher range of M_r values than were seen in control cultures, although residual hypoglycosylated α-DG was still found (**Fig. 4b**). Notably, hyperglycosylation correlated with functional rescue of α-DG as a receptor for its extracellular ligands (**Fig. 4b**). These results indicate that induction of LARGE leads to functional glycosylation of α-DG on alternative *O*-linked residues.

Because the sialyl *N*-acetyllactosamine moiety involved in the interaction of α-DG with its ligands is a common constituent of many *N*-glycans, we subjected WGA-enriched fractions from treated control and WWS cells to enzymatic deglycosylation by *N*-glycanase. Enzyme activity was verified by complete *N*-deglycosylation of β-DG. The M_r of α-DG did not change after enzymatic treatment, confirming the predominance of *O*-linked oligosaccharides (**Fig. 4c**). Reactivity for glycosylated α-DG also disappeared after alkaline *O*-deglycosylation (data not shown). Our attempts at enzymatic deglycosylation with exoglycosidases for sugars that are putatively added by LARGE did not release any sugars. Collectively, these data suggest either that LARGE modulates the activities of other as yet undetermined enzymes, thereby affecting an alternative glycosylation pathway of α-DG, or that LARGE activity may differ from that predicted by homology of its catalytic domains.

α-DG-laminin affinity facilitates laminin clustering

The perturbation of the basement membrane described in FCMD and WWS muscle is an important pathogenetic event common to many congenital muscular dystrophies[27,28]. We examined the ability of glycan-enriched α-DG to coordinate the assembly of extracellular matrix proteins by analyzing the formation of exogenous laminin-1 clusters on differentiating myoblasts. DG-mediated laminin clustering is a dynamic process in which small dot-shaped clusters merge into complex structures[29,30].

Very little endogenous laminin-1 was on the surface of control cells before treatment (data not shown). Within 2 h of incubation with laminin-1, only a few cells showed dot-like clusters. By 16 h, more cells were found to be positive for laminin clusters and a few myoblasts bearing linear and small plaque-like clusters were observed (**Fig. 5a,b**). In FCMD and WWS cultures, a small amount of exogenous laminin bound to the cell surface but did not organize into complex structures (**Fig. 5a**).

A marked change in clustering was observed after *LARGE* viral treatment: complex plaques formed within 2 h in all cultures. In control and FCMD cultures, plaques were the predominant type of cluster and the distribution of cells with dot, linear and plaque-like clusters remained stable over 16 h. In WWS myoblasts, the punctate pattern of laminin progressed more slowly into organized arrays (**Fig. 5b**). Notably, laminin always colocalized with glycosylated α-DG. Similar results were obtained in Large^{myd} myoblasts after *LARGE* gene transfer (data not shown). The laminin binding activity in control myoblast cultures was very low and we were unable to measure substantial differences among control, FCMD and WWS samples by quantitative solid-phase assays. Forced expression of LARGE markedly increased maximum laminin binding and affinity in control and FCMD cells, and to a lesser degree in WWS cells (**Fig. 5c**).

We conclude that very low binding activity is adequate for α-DG to act as a receptor for extracellular matrix proteins; however, the efficiency of assembling and remodeling the extracellular matrix increases with a higher affinity of α-DG for its ligands. As the receptor activity of α-DG is restored by inducing LARGE expression, it seems likely that the mechanical properties of diseased muscle fibers will be improved by this induction.

DISCUSSION

The pathogenetic mechanisms underlying glycosyltransferase-deficient muscular dystrophies are becoming increasingly clear. Our study provides evidence that LARGE has a regulatory role in α-DG

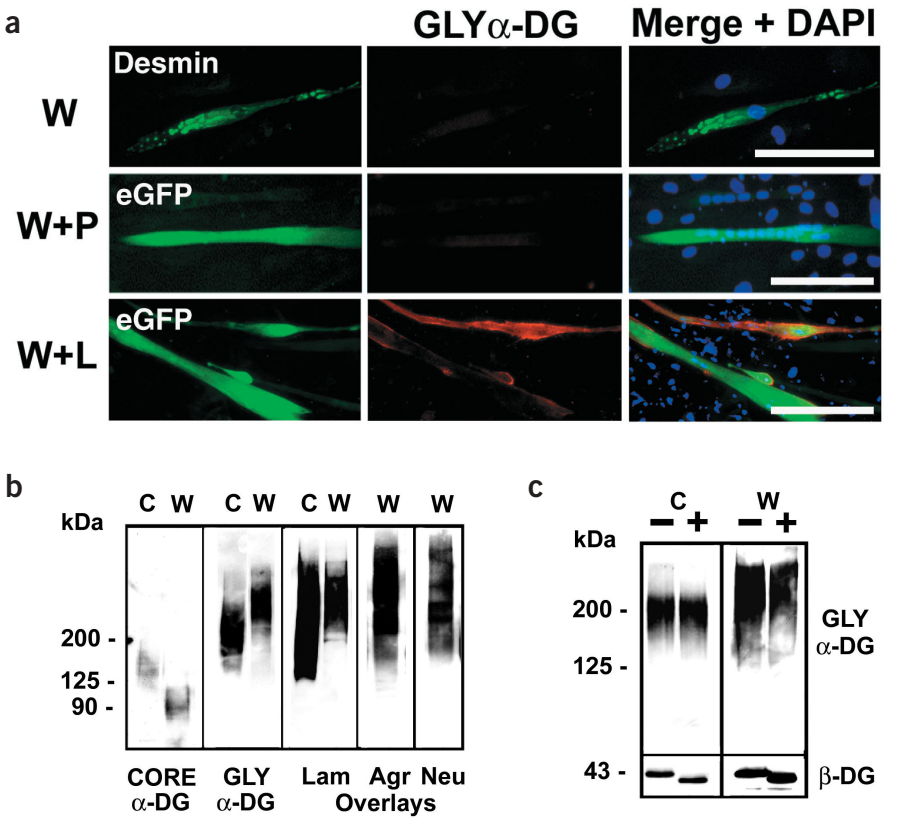


Figure 4 LARGE induces the synthesis of functional α-DG in WWS myoblasts and myotubes. (**a**) Expression of eGFP, desmin (green, upper row) and glycosylated α-DG (GLYα-DG), and nuclear labeling (DAPI, blue) in WWS myoblasts either untreated (W) or treated with Ad5-LARGE-eGFP (W+L) or Ad5-POMGnT1-eGFP (W+P). Scale bar, 20 μm. (**b**) Biochemical analysis of WGA-enriched fractions from control (C) and WWS myoblasts (W) treated with Ad5-LARGE-eGFP. Immunoblotting was done with antibodies to glycosylated α-DG and α-DG core protein (COREα-DG). Ligand overlay assays were done for laminin (Lam), agrin (Agr) and neurexin (Neu). (**c**) *N*-glycanase deglycosylation of WGA-enriched fractions from control (C) and WWS myoblasts (W) treated with Ad5-LARGE-eGFP. Immunoblotting was done with antibodies to β-DG and glycosylated α-DG. Untreated samples (–) are shown. Data are representative of four individuals with WWS.

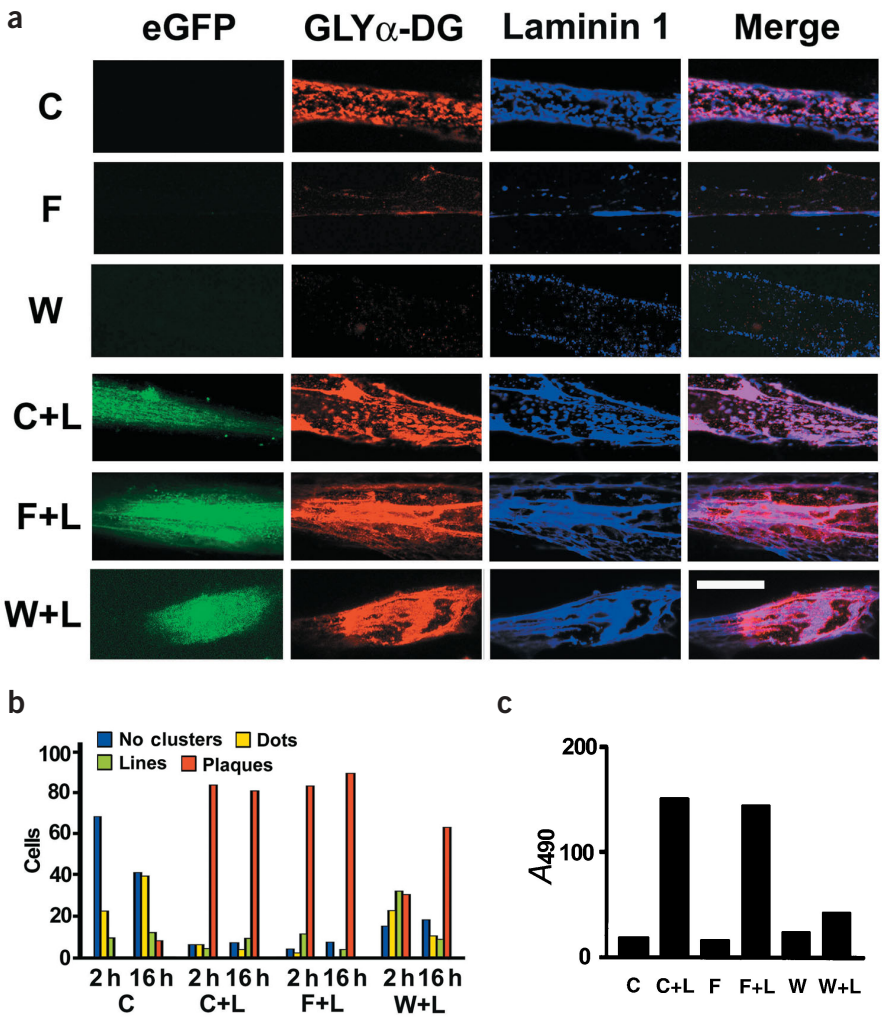


Figure 5 Laminin clustering correlates with high α-DG–laminin affinity. (**a**) Immunofluorescence localization of glycosylated α-DG (GLYα-DG), laminin-1 and eGFP after 16 h of incubation with laminin-1. Scale bar, 20 μm. Shown are untreated and Ad5-LARGE-eGFP–treated (+L) control (C), FCMD (F) and WWS (W) myoblasts. (**b**) Kinetic analysis of laminin-1 cluster formation after 2 and 16 h of incubation with laminin-1. About 100 cells were evaluated for each group. (**c**) Solid-phase assay of maximum binding between α-DG and laminin at 0.6 nM laminin-1 in control (C), FCMD (F) and WWS (W) WGA-enriched glycoprotein fractions from untreated and Ad5-LARGE-eGFP–treated (+L) myoblasts.

glycosylation. We have shown that *LARGE* gene transfer restores α-DG function in Large^{myd} skeletal muscle and ameliorates muscular dystrophy, indicating that adjusting the glycosylation status of α-DG can improve the muscle phenotype.

Another mechanism for preventing muscular dystrophy, involving upregulation of utrophin through the transgenic expression of an endogenous glycosyltransferase, has been explored in the *mdx* mouse model of Duchenne muscular dystrophy. Ectopic expression of the cytotoxic T cell (CT) GalNAc transferase, which is normally present at the neuromuscular junction, leads to extrasynaptic localization of the utrophin-glycoprotein complex and consequently improves the dystrophic phenotype in the transgenic *mdx*-CT mouse[31,32]. α-DG was glycosylated with the CT carbohydrate in *mdx*-CT muscle. CT GalNAc transferase has not, however, been shown to modify the ligand-binding activity of α-DG. In addition, overexpression of this enzyme in transgenic mice causes a marked reduction in the diameter of myofibers and alterations in the neuromuscular junction architecture[31].

By contrast, forced expression of LARGE in wild-type muscle does not result in morphological or pathological changes. In addition, Large^{myd} mice and individuals with dystroglycanopathy have normal expression of the DGC at the sarcolemma, showing that correct localization of the DGC is necessary but not sufficient for the protection and stability of the myofibers. *LARGE* gene transfer did not change the expression of any of the DGC components and ameliorated the pathological phenotype by restoring the function of the DGC via glycosylation of α-DG.

Unexpectedly, we found that the effect of LARGE was similar in all of the cell types and tissues that we analyzed, suggesting that induction of LARGE may be effective in the treatment not only of MDC1D but also of FCMD, MEB, WWS, LGMD2I (R.B. and K.P.C., unpublished data) and other glycosyltransferase-deficient muscular dystrophies. Because the rescue effect of LARGE is similar in cells from individuals with distinct diseases, it seems unlikely that LARGE is directly or indirectly activating the mutant enzyme in each disorder. In addition, there is evidence that a direct interaction between the amino-terminal domain of α-DG and LARGE is essential for the functional post-translational modification of α-DG (M.K. and P.K.C., unpublished data).

Our findings point toward the existence of dual, concentration-dependent functions of LARGE. At physiological concentration, LARGE may regulate the *O*-mannosylation pathway of α-DG, whereas forced expression of LARGE may activate alternative pathways for the *O*-glycosylation of α-DG that possibly generate a type of repeating polymer of variable lengths, such as glycosaminoglycan-like or core 1 or core 2 structures. This alternative glycan mimics the *O*-mannose glycan in its ability to bind α-DG ligands and can compensate for the defective tetrasaccharide (**Fig. 6**).

The finding that an endogenous protein can modulate the glycosylation of α-DG in genetically distinct diseases without undesirable effects arising from its upregulation makes LARGE an attractive target for the design of therapies intended to manipulate α-DG glycosylation. Several reported muscle pathologies associated with defective glycosylation of α-DG and unlinked to known genes[33] might particularly benefit from such therapeutic approaches. Glycotherapies and treatments aimed at modulating the expression or the activity of LARGE may be the basis of an adequate therapeutic option for the whole group of glycosyltransferase-deficient muscular dystrophies.

METHODS

Antibodies. VIA4-1 and IIH6 are monoclonal antibodies to fully glycosylated species of α-DG[10]. We used the antibodies interchangeably and refer to them as GLYα-DG in the figure legends; however, most of the data shown represent staining obtained with IIH6. GT20ADG (COREα-DG) is from goat

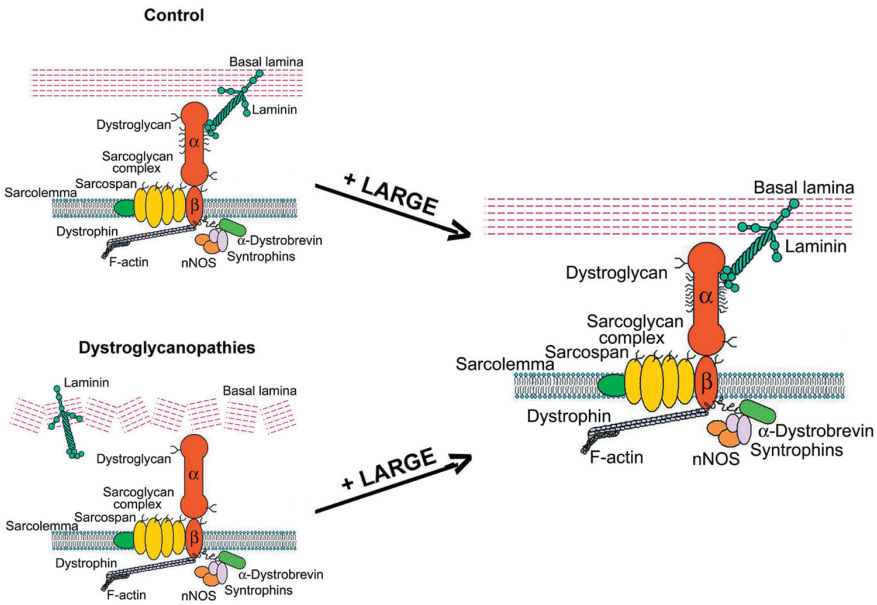


Figure 6 Effect of LARGE on α-DG glycosylation. Representation of the effect of overexpressing LARGE in skeletal muscle from control and affected individuals. See text for details.

antiserum raised against the whole DGC and purified against a hypoglycosylated full-length α-DG–human IgGFc fusion protein[13,34]. CORE-αβDG is purified from sheep polyclonal antiserum raised against the whole DGC and recognizes both α- and β-DG[12]. Polyclonal antibodies to DGC components were used as described[35]. Rabbit polyclonal antibody to laminin-1 and mouse monoclonal antibody to desmin were from Sigma, and mouse monoclonal antibodies to agrin and caveolin-3 were from Chemicon and Transduction Laboratories, respectively.

Mice. We obtained myodystrophy mice (Large^{myd}) and control littermate mice (Large$^{+/+}$ or Large$^{myd/+}$) by mating heterozygous pairs provided by Jackson Laboratories. All mice were maintained at the University of Iowa Animal Care Unit in accordance with animal usage guidelines.

Adenovirus generation and infection. E1-deficient recombinant adenoviruses, Ad5-LARGE-eGFP and Ad5-POMGnT1-eGFP, which contain eGFP plus the complete open reading frame of human *LARGE* or human *POMGNT1* in separate expression cassettes, respectively, were generated by the University of Iowa Gene Transfer Vector Core. Transcription of *LARGE* or *POMGNT1* was initiated by the cytomegalovirus promoter (CMV), and transcription of eGFP was initiated by the Rous sarcoma virus promoter (RSV). Translation of the transcripts yielded LARGE or POMGnT1 and eGFP as separate proteins. *In vivo* gene transfer was done on Large^{myd} and control littermate pups, aged 2-4 d, as described[36]. Injected and non-injected contralateral leg muscles were examined after 4 weeks. Cell cultures were infected with viral vector for 12 h at an MOI of 200–800. At this concentration, cell cytotoxicity was <15%. We examined cultures 3–10 d after treatment.

Immunohistochemical analysis. Cryosections (7 μm) were processed for immunofluorescence as described[35]. Cultured cells were fixed in 4% paraformaldehyde before being incubated with primary antibodies. Slides were observed with an MRC-600 laser scanning confocal microscope (Bio-Rad). Digitized images were captured under identical conditions.

Glycoprotein enrichment and biochemical analysis. Frozen samples and cultured cells were processed as described[13]. Immunoblots and ligand overlay assays were done on polyvinylidene difluoride membranes as described[13]. Blots were developed by horseradish peroxidase (HRP) enhanced chemiluminescence (Pierce). *N*-deglycosylation was done by using *N*-glycanase (ProZyme) according to the manufacturer's instructions. We did the solid-phase binding assay as described[13].

Treadmill exercise. Mice were exercised by an Omnipacer Treadmill (Model LC4/M-MGA/AT; Accuscan Instruments) at a 15° downward angle with increasing speed up to 19 m/min for 30 min. Large^{myd} and littermate mice, aged 5–7 weeks, injected with *LARGE* adenovirus in the right hamstrings were tested ($n = 4$). All mice were injected with EBD intraperitoneally 5 h before exercise. Mice were killed 24 h after exercise and sections of right and left hamstrings were compared for EBD uptake and expression of glycosylated α-DG. Quantification of EBD-positive areas in sections of skeletal muscle was done by using ImageJ software (National Institutes of Health; NIH). The percentage of positively stained areas was calculated by dividing the area stained by the total area of the analyzed skeletal muscle section.

Human cells. The MEB fibroblasts were from two compound heterozygote siblings with a G1908→A transversion in exon 21 (Arg605His) in one allele and a single-base-pair insertion in exon 11 in the other allele (1106insT, causing a frameshift and premature termination at codon 338) of the *POMGNT1* gene[25]. The FCMD myoblasts were from three Japanese individuals: one was homozygous for the retrotransposon insertion in *FCMD*[37]; the other two were heterozygous for the insertion and either Cys250Gly or Leu353Stop (plus polymorphism Arg203Glu). The WWS myoblasts were from a Japanese boy homozygous for Leu421del in *POMT1* and from a Hispanic male aged 6 months[38]. The WWS fibroblasts were from a 3-year-old female and a 4-year-old male with clinical diagnosis of WWS. Analysis of the full-length *POMT1* and *POMGNT1* complementary DNA in these individuals did not identify mutations. The regions of DG cDNA corresponding to the laminin-binding domains and glycosylated domains were normal. We obtained and tested all tissues in agreement with the Human Subjects Institutional Review Board of the University of Iowa; informed consent was obtained from all subjects.

Cell cultures. Cells were maintained at 37 °C and 5% CO_2 in DMEM medium plus 20% fetal bovine serum, 0.18 μg/ml of insulin and 0.5% penicillin-streptomycin (Gibco). The myoblast/fibroblast ratio estimated by desmin staining was 1/7 in control and FCMD cultures, and 1/5 in WWS cultures. Myoblast fusion was done by decreasing the serum concentration to 2% (differentiation medium). For the laminin clustering assay, myoblasts were switched to fresh differentiation medium containing 7.5 nM mouse EHS laminin-1 and incubated for 2, 5 or 16 h. Confocal immunofluorescence images were compiled from a 5-μm *z*-series extending from the dorsal surface of the cells in 0.5-μm steps. Morphological evaluation of clusters was done as described[39].

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

ACKNOWLEDGMENTS

We thank C.C. Chen and all members of the Campbell laboratory for discussions and technical support; J. Lilien for discussions; J. Vajsar for the MEB fibroblasts; M. Oldstone for the DGFc5 construct; and T. Südhof for the neurexin fusion protein cDNA. We also thank C. Lovig and the University of Iowa Hybridoma Facility, and the University of Iowa Gene Transfer Vector Core, which is supported in part by the Carver Foundation and the NIH. Samples used in this study were provided in part by the National Institute of Child Health and Human Development Brain Bank HD83284. This work was supported by the Muscular Dystrophy Association (R.B., S.A.M., K.P.C.) and the NIH (S.A.M.). D.E.M. was supported by a Cardiovascular Interdisciplinary Research Fellowship and a University of Iowa Biosciences Initiative Fellowship. K.P.C. is an investigator of the Howard Hughes Medical Institute.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 26 November 2003; accepted 18 May 2004
Published online at http://www.nature.com/naturemedicine/

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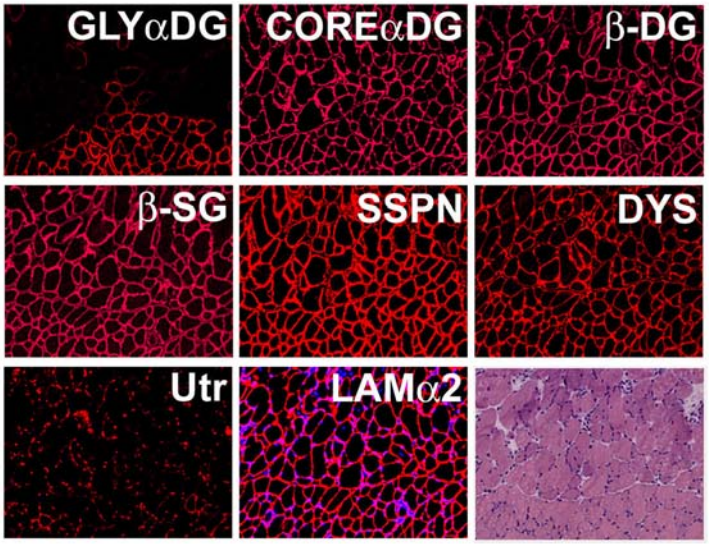
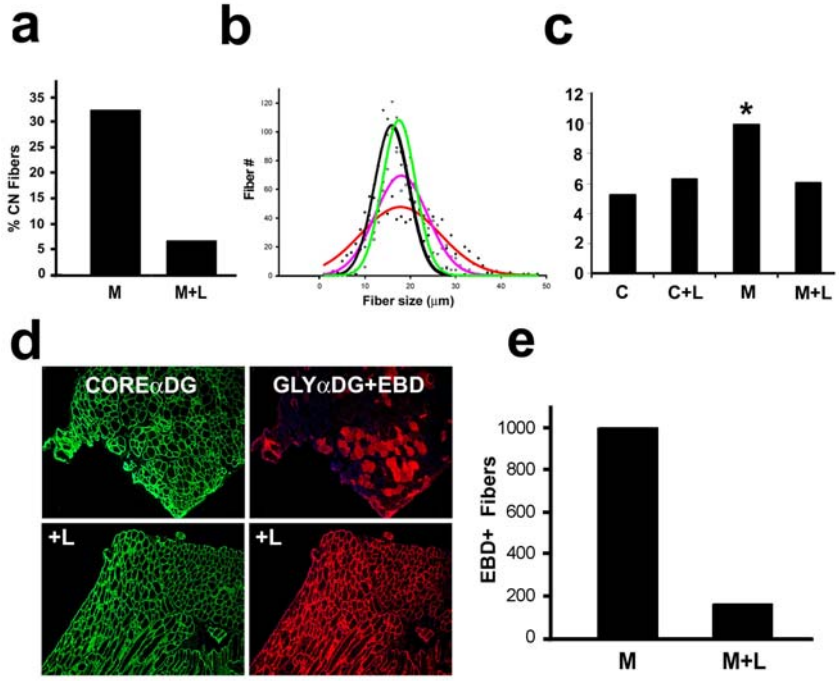
Supplemental Data for:
Barresi, et al., Nature Medicine 10, pp. 696-703

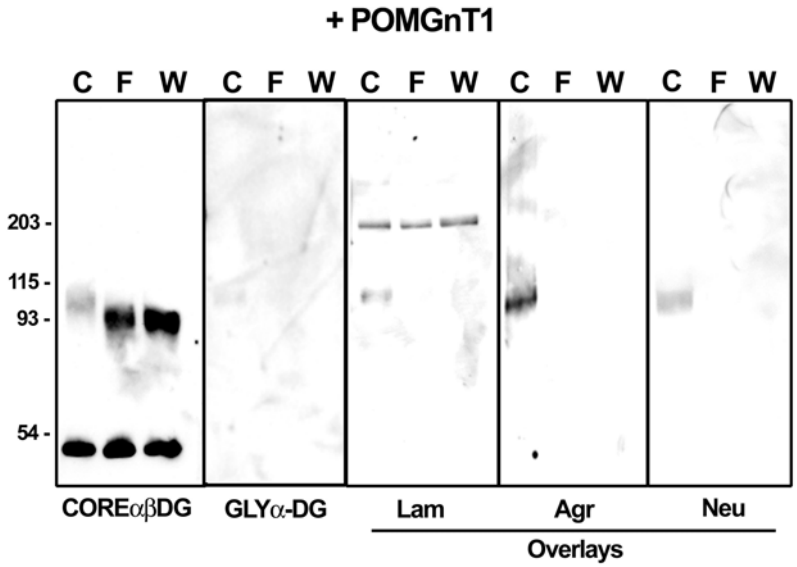
Supplemental Experimental Procedures



Supplementary Figure 1 DGC expression in Large^{myd} skeletal muscle after LARGE gene transfer. Analysis of Large^{myd} skeletal muscle treated with Ad5-LARGE/eGFP. Hematoxylin-eosin staining and fluorescence localization of glycosylated α-DG (GLYα-DG), α-DG core peptide (COREα-DG), β-dystroglycan (β-DG), laminin α2 (Lamα2), α-sarcoglycan (α-SG), β-sarcoglycan (β-SG), dystrophin (DYS), and utrophin (Utr). Nuclei were stained with DAPI. Transduced areas express glycosylated α-DG. Staining for other DGC components do not vary between transduced and non-transduced fibers.



Supplementary Figure 2 Improved morphology and function of Large^{myd} transduced skeletal muscle. (a) Percentage of centrally nucleated fibers (CN) in Large^{myd} untreated (M) and treated (M+L) skeletal muscle. *n*=900. (b) Fiber size variability in control (circles) and Large^{myd} (triangles) skeletal muscle before, and after treatment with Ad5-LARGE/eGFP (open circles and triangles). Curves fit with gaussian equation for normal distribution are represented in black (control), green (control + LARGE), red (Large^{myd}), and magenta (Large^{myd} + LARGE). *n* = 1000 fibers from four 5-7 weeks old mice. (c) Statistical analysis of muscle fiber diameter variance (ANOVA) performed on the standard deviations between each group. Significant difference in fiber size variation was observed between Large^{myd} (M) and the other groups ($P = 0.007$). Variation in fiber size between Large^{myd} treated (M+L), control (C), and control treated (C+L) muscles was not significant ($P > 0.05$). *n* = 2-4 muscles from 5-7 weeks old mice with a total of 300-500 fibers assayed per muscle. (d) Hamstring muscles from exercised Large^{myd} mice untreated and treated (+L) with LARGE adenovirus. Staining in green denotes α-DG core peptide, and staining in red denotes EBD uptake and glycosylated α-DG. (e) Number of EBD positive fibers in untreated (M) and contralateral treated (M+L) hamstrings from four 5-7 weeks old Large^{myd} mice.



Supplementary Figure 3 Forced expression of POMGnT1 does not improve the functional glycosylation of α-DG. SDS-PAGE analysis of WGA-enriched lysates from control (C), FCMD (F) and WWS (W) myoblasts untreated and infected with Ad5-POMGnT1/eGFP (+POMGnT1). Immunoblots were performed with anti-dystroglycan core protein (CORE-αβDG) and glycosylated α-DG (GLYα-DG) antibodies. Laminin (Lam), neurexin fusion protein (Neu), and agrin (Agr) were used for ligand overlay assays. Bands at ~210 kDa in laminin overlay represent endogenous laminin. Data shown are representative of two WWS patients. Similar results were obtained by infecting fibroblasts from other two WWS patients with Ad5-POMGnT1/eGFP.