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High Throughput Screening for Compounds That Alter Muscle Cell Glycosylation Identifies New Role for *N*-Glycans in Regulating Sarcolemmal Protein Abundance and Laminin Binding*[S]

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Background: Genetic alteration of muscle cell glycosylation in muscular dystrophy models has ameliorated disease.
Results: A high throughput screen identified a small molecule, lobeline, which altered muscle cell glycosylation and improved laminin binding.
Conclusion: Lobeline increased abundance of sarcolemmal glycoproteins and increased laminin binding in an *N*-glycan-dependent manner.
Significance: A novel approach revealed an unexpected role for *N*-glycans in muscle cell function.

Duchenne muscular dystrophy is an X-linked disorder characterized by loss of dystrophin, a cytoskeletal protein that connects the actin cytoskeleton in skeletal muscle cells to extracellular matrix. Dystrophin binds to the cytoplasmic domain of the transmembrane glycoprotein β-dystroglycan (β-DG), which associates with cell surface α-dystroglycan (α-DG) that binds laminin in the extracellular matrix. β-DG can also associate with utrophin, and this differential association correlates with specific glycosylation changes on α-DG. Genetic modification of α-DG glycosylation can promote utrophin binding and rescue dystrophic phenotypes in mouse dystrophy models. We used high throughput screening with the plant lectin *Wisteria floribunda* agglutinin (WFA) to identify compounds that altered muscle cell surface glycosylation, with the goal of finding compounds that increase abundance of α-DG and associated sarcolemmal glycoproteins, increase utrophin usage, and increase laminin binding. We identified one compound, lobeline, from the Prestwick library of Food and Drug Administration-approved compounds that fulfilled these criteria, increasing WFA binding to C2C12 cells and to primary muscle cells from wild type and *mdx* mice. WFA binding and enhancement by lobeline required complex *N*-glycans but not *O*-mannose glycans that bind laminin. However, inhibiting complex *N*-glycan processing reduced laminin binding to muscle cell glycoproteins, although *O*-mannosylation was intact. Glycan analysis demonstrated a general increase in *N*-glycans on lobeline-treated cells rather than specific alterations in cell surface glycosylation, consistent with increased abundance of multiple sarcolemmal glycoproteins. This demonstrates the feasibility of high throughput screening with plant lectins to identify compounds that alter muscle cell glycosylation and identifies a novel role for *N*-glycans in regulating muscle cell function.

Duchenne muscular dystrophy (DMD)[3] is an X-linked recessive disorder resulting from loss of expression of functional dystrophin, a cytoplasmic protein that links the actin cytoskeleton to the extracellular matrix via a complex of plasma membrane glycoproteins termed the dystrophin-glycoprotein complex (DGC) (1, 2). Although dystrophin is the primary linker protein between actin and β-dystroglycan (β-DG), a component of the DGC at the muscle cell sarcolemma, utrophin fulfills this function at the neuromuscular junctions of muscle cells, creating the utrophin-glycoprotein complex (UGC) (3, 4). Thus, a critical goal in development of novel therapies for DMD is identification of strategies to enhance utrophin expression and substitute utrophin for dystrophin as the cytoskeletal linker

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[S] This article contains supplemental Tables 1–3 and Figs. 1 and 2.
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[3] The abbreviations used are: DMD, Duchenne muscular dystrophy; DGC, dystrophin glycoprotein complex; UGC, utrophin glycoprotein complex; DG, dystroglycan; WGA, wheat germ agglutinin; WFA, *W. floribunda* agglutinin; PHA, phytohemagglutinin; POMT, protein *O*-mannosyltransferase; DMNJ, deoxymannojirimycin; SG, sarcoglycan; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

molecule for β-DG at the sarcolemma (5). Factors that regulate selective association of dystrophin *versus* utrophin with the glycoprotein complex are not well understood; however, it has been demonstrated in several cellular and animal models that altering glycosylation of DG is sufficient to promote utilization of utrophin over dystrophin (6–10). Similarly, enhanced utrophin association with β-DG (*e.g.* in *mdx* mice that lack dystrophin) also correlates with altered glycosylation of DG (6, 10). Thus, understanding factors that regulate DG glycosylation and identification of novel approaches to alter muscle cell glycosylation may reveal new therapeutic strategies for enhancing muscle survival and function in DMD patients.

DG is composed of two subunits, β-DG, which is a transmembrane protein with a cytoplasmic domain that interacts with dystrophin/utrophin, and α-DG, a soluble subunit that non-covalently associates with β-DG on the exterior face of the plasma membrane (1, 11). α-DG in muscle cells can be differentially glycosylated; α-DG that primarily binds the plant lectin wheat germ agglutinin (WGA) is most abundant at the sarcolemma and preferentially associates with dystrophin, whereas α-DG that primarily binds the plant lectin *Wisteria floribunda* agglutinin (WFA) is most abundant at the neuromuscular junction and preferentially associates with utrophin (6). WGA preferentially recognizes the saccharides *N*-acetylglucosamine and sialic acid, although the specific glycans on DG that bind WGA are not known. WFA preferentially recognizes the saccharide *N*-acetylgalactosamine (GalNAc), and although the precise glycans that bind WFA on DG at the neuromuscular junction are also not known, Martin and colleagues (6) found that overexpression of a specific glycosyltransferase can modify DG to promote WFA binding. This glycosyltransferase, Galgt2, (also known as CTGalNAcT), adds a GalNAc residue to the SAα2,3Galβ1,4GlcNAc trisaccharide, to create the tetrasaccharide SAα2,3Gal(β1,3GalNAc)β1,4GlcNAc, a structure also known as the Sda blood group antigen. Overexpression of the Galgt2 enzyme in *mdx* mice lacking dystrophin, as well as in mouse models of congenital muscular dystrophy 1A and limb girdle muscular dystrophy 2D (6, 8, 9), resulted in increased expression of the Sda tetrasaccharide, detected with specific antibodies, as well as increased reactivity of cells with WFA and increased association of utrophin with DG. Overexpression of the Galgt2 enzyme also increased the abundance of other components of the muscle cell glycoprotein complex, including α- and β-DG, as well as α-, β-, and γ-sarcoglycans that associate with and stabilize DG. Galgt2 overexpression ameliorated the dystrophic phenotype in these mouse models, possibly by promoting utrophin association with and/or stabilization of sarcolemmal DG, although the precise mechanisms of muscle cell rescue remain unknown.

Several aspects of α-DG glycosylation have been studied extensively; α-DG bears multiple *O*-mannose glycans that are modified by the product of the *Large* gene, and these unusual glycans on α-DG are essential for binding to laminin in the extracellular matrix (12–14). The *O*-mannose glycans as well as multiple mucin-type *O*-glycans are found on the linear mucin domain of α-DG that joins the globular N-terminal and C-terminal domains. α-DG also has three *N*-glycosylation sites, one on the N-terminal globular domain and two on the C-terminal globular domain, and β-DG has one *N*-glycosylation site as well (15). Proper glycosylation of the DG precursor polypeptide is critical for proteolytic cleavage into the α- and β-subunits (16). Modification of glycans on α-DG in cells overexpressing Galgt2 has been described, although the types of glycans on α-DG that bear the Sda tetrasaccharides created by Galgt2 overexpression were not identified.

The goal of the present study was to identify small molecules that could alter glycosylation of membrane glycoproteins on skeletal muscle myotubes. To achieve this goal, we developed a high throughput screening assay of cultured murine myotubes using WFA as an inexpensive tool to screen libraries of compounds for the ability to alter glycosylation. One critical advantage of this approach was that it focused on the phenotype (*i.e.* increased binding of WFA) rather than on selecting for increased expression of a specific glycosyltransferase or glycoprotein or a specific effect on glycoprotein processing. Screening the Prestwick library of >1200 Food and Drug Administration (FDA)-approved compounds (17), we identified a single compound that 1) increased WFA binding to cells, 2) increased abundance of several sarcolemmal glycoproteins and associated binding partners, and 3) increased laminin binding to cellular proteins. Surprisingly, we found that the increased WFA binding required *N*-glycans but not *O*-mannose glycans. This study identifies a novel role for *N*-glycans in DG stabilization and promotion of laminin binding and suggests new approaches for developing therapies to improve muscle cell survival and function in muscular dystrophy.

EXPERIMENTAL PROCEDURES

Animals and Cells—Wild type and *mdx* breeders were purchased from Jackson Laboratories (Bar Harbor, ME), and tissue sections from 8-week-old males were analyzed. α7 integrin-deficient mice were a generous gift from Dean J. Burkin (University of Nevada, Reno, NV) (18). Akt transgenic mice (19) were a generous gift from Kenneth Walsh (Boston University). Activation of the Akt transgene was accomplished by doxycycline administration as described previously (19). All procedures were carried out in accordance with guidelines set by the UCLA Institutional Animal Care and Use Committee.

Murine C2C12 cells were obtained from the American Type Culture Collection and cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM), Hepes, sodium pyruvate, Glutamax, penicillin/streptomycin (Invitrogen) with 10% fetal bovine serum (FBS, Hyclone)). Myoblast differentiation into myotubes was initiated by replacing growth medium with differentiation medium, substituting 2% equine serum (Hyclone) for FBS. For primary cultures of murine myoblasts, cells were derived from limb muscle of neonatal *mdx* and C57/BL6J mice. Cells were grown on plates coated with extracellular matrix from Engelbreth-Holm-Swarm (EHS) murine sarcoma (Sigma-Aldrich) and maintained in Ham's F-10 nutrient mixture (Mediatech) Hepes, sodium pyruvate, Glutamax, penicillin/streptomycin with 20% FBS. DMD patient dermal fibroblasts were maintained in growth medium (DMEM with 15% FBS, 1% non-essential amino acids, penicillin/streptomycin), and fibroblasts were immortalized with hTERT in a lentiviral vector and reprogrammed with a tamoxifen-inducible lentiviral MyoD

(20) (kind gift of Jeffrey S. Chamberlain, University of Washington, Seattle, WA). Reprogrammed fibroblasts were seeded onto laminin plates in growth medium, and the next day, tamoxifen (Sigma) was added in growth medium. The following day, fusion medium was added (2% equine serum, 2% insulin-transferrin-selenium (Sigma), 1:1 DMEM/Ham's F-10) with 1 μM tamoxifen.

Lectin Binding to Muscle Sections—Quadriceps muscles were mounted in 10.2% polyvinyl alcohol, 4.3% polyethylene glycol, flash frozen in liquid nitrogen-cooled isopentane, and stored at −80 °C. Transverse sections (8 μm) were mounted on positively charged glass slides (Thermo Fisher Scientific). Sections were brought to room temperature for 15 min, blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and then blocked with an avidin/biotin blocking kit (Vector Laboratories). Biotinylated WFA (B-1355, Vector), 1:1500 in PBS, was added to sections at 4 °C overnight, and sections were washed three times with PBS. Bound WFA was detected with fluorescein-avidin D (A-2001, Vector), 1:1500 in PBS, for 1 h at room temperature. Parallel sections were incubated with fluorescein-avidin alone as a control. Sections were mounted in VECTASHIELD (Vector) to prevent photobleaching. Bound WFA was visualized on a fluorescent microscope (Axioplan 2, Carl Zeiss, Inc.) equipped with a Plan Neofluar ×40, numerical aperture 1.3 oil differential interference contrast objective, and images were captured using Axiovision Rel 4.5 software (Carl Zeiss) under identical conditions.

High Throughput Screening and WFA Binding Assay—C2C12 cells were plated in growth medium at 500 cells/well in 384-well white clear bottom plates (Thermo Fisher Scientific) in 50 μl using a Multidrop 384 (Thermo Scientific). On day 3, compounds from the Prestwick library (Prestwick Chemicals) were added using a Biomek FX (Beckman Coulter) equipped with a V&P custom pin tool, resulting in a final drug concentration of 10 μM. On day 4, growth medium was removed using an ELX 405 Select (Biotek), differentiation medium was added, and compounds from the Prestwick library were added again. Vehicle control (dimethyl sulfoxide (DMSO)) was part of the plate layout for each screened plate. Liquid handling during the screening procedure was validated to a coefficient of variation of 7% over the entire plate, and duplicates were run. After 4 days (day 8 of culture), medium was removed as above, plates were washed twice with PBS with 1% BSA (Gemini BioProducts) (PBA), and cells were fixed with 15 μl of 2% paraformaldehyde in PBS at 4 °C overnight. Plates were washed three times and blocked at 4 °C overnight in 100 μl of PBA. PBA was partially removed leaving 50 μl, and 15 μl of biotin-conjugated lectin at 2 μg/ml (Zymed Laboratories Inc.) or 2 μg/ml biotin-BSA (Vector Laboratories) in PBS supplemented with 0.1% Tween 20 and 1% BSA (PBSTB) was added and incubated at 4 °C overnight. Plates were washed three times with PBSTB and incubated with streptavidin-conjugated HRP at 1 μg/ml in PBSTB for 1 h at room temperature. Plates were washed three times with PBSTB, and 40 μl of SuperSignal West Pico chemiluminescent substrate was added (Thermo Fisher Scientific). Plates were read on a Victor 3 V plate reader (PerkinElmer Life Sciences) with Wallac software. Average binding to biotin-BSA was subtracted from the value for biotin-lectin binding for each well, and results were expressed as ratios for each compound *versus* DMSO. A cut-off value of 2 was used to identify hits in the library in duplicate samples.

For confirmation of hits from the library screen, the indicated compounds were added over a range of concentrations to C2C12 cells in duplicate samples in 96-well plates on the same day that differentiation medium was added. WFA binding was measured 48 h later. Thus, in the confirmatory assay, WFA binding was measured 2 days after the addition of drug, compared with 5 days after the first addition of drug in the screen. As a positive control, C2C12 cells transfected with the pFLAGCTneo vector containing Galgt2 cDNA (a kind gift of Paul Martin (Ohio State University, Columbus, OH) and John B. Lowe (Case Western Reserve University, Cleveland, OH)) were used to detect increased WFA binding compared with cells transfected with vector alone.

Immunoblotting and Lectin Blotting—To analyze glycoprotein abundance in whole cell lysates, DMSO- and lobeline-treated muscle cells were washed with PBS and scraped into ice-cold lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) with phosphatase inhibitors (1 mM sodium orthovanadate, 100 nM okadaic acid, 5 nM microcystin-L-arginine) and protease inhibitors (0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 mM benzamidine, and 0.1 mM PMSF). Homogenates were solubilized by rotating at 4 °C for 1 h. After clarification by centrifugation at 15,000 × *g* for 15 min, cell lysates were stored at −80 °C. Protein concentrations were determined using the DC protein assay (Bio-Rad). Equal concentrations (60 μg) of protein samples were resolved by 12% isocratic or 4–20% gradient SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Primary antibodies against laminin (L9393, 1:5000; Sigma), agrin (ab12358, 1:100; Abcam), β1D integrin (MAB1900, 1:100; Chemicon), dysferlin (sc-16635, 1:200; Santa Cruz Biotechnology, Inc.), dystrophin (MANDYS1, 1:10; Developmental Studies Hybridoma Bank), utrophin (MANCHO3, 1:200; Developmental Studies Hybridoma Bank), α-DG (IIH6C-4, 1:500, Santa Cruz Biotechnology, Inc.), β-DG (MANDAG2, 1:250; Developmental Studies Hybridoma Bank), α-SG (VP-A105, 1:100; Vector Laboratories), β-SG (VP-B206, 1:100; Vector Laboratories), γ-SG (VP-G803, 1:100; Vector Laboratories), sarcospan (sc-16760, 1:200; Santa Cruz Biotechnology, Inc.), caveolin-3 (ab2912, 1:1000; Abcam), and glyceraldehyde-3-phosphate dehydrogenase (MAB374, 1:50,000; Chemicon) were incubated at 4 °C while shaking for 18 h. HRP-conjugated anti-rabbit IgG (GE Healthcare), anti-mouse IgG (GE Healthcare), anti-goat IgG (Santa Cruz Biotechnology, Inc.), and IgM (Upstate Signaling) secondary antibodies were used at 1:2000 dilution. All immunoblots were developed using enhanced chemiluminescence with SuperSignal West Pico chemiluminescent substrate.

For enrichment in GalNAc modified proteins, C2C12 cells were lysed in ice-cold Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and primary mouse muscle cells were lysed as above. Lysates (5 mg of total protein) were added to 1 ml of agarose-WFA (Vector Laboratories) overnight at 4 °C. Beads were washed with cold lysis

buffer, and bound proteins were eluted with 0.3 M GalNAc (Sigma-Aldrich) and concentrated by filter centrifugation (Centricon Ultracel, Millipore) at 4000 × *g* for 20 min. C2C12 eluates were separated on 4–12% NuPAGE gels (Invitrogen) and transferred to PVDF membranes (Bio-Rad). Wild type and *mdx* muscle cell eluates were separated on 4–20% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore). Membranes were probed with biotinylated WFA (1:5000; Vector Laboratories) overnight at 4 °C or the indicated antibodies, as above. Blots were washed with TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20) and incubated with HRP-streptavidin (1:10,000; Bio-Rad), HRP-avidin D (1:2000; Vector Laboratories), or HRP-anti-IgM, and bound reagent was detected by enhanced chemiluminescence.

Laminin Overlay Assay—C2C12, wild type, or *mdx* myoblasts were differentiated in differentiation medium with 100 μM lobeline or DMSO control, with or without DMNJ, for 72 h. Cells were collected by scraping and lysed in Nonidet P-40 lysis buffer, and lysates were separated on a 4–12% BisTris gel and immunoblotted, as above. Membranes were blocked in 5% nonfat dry milk in laminin binding buffer (10 mM triethanolamine, 140 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) and incubated sequentially with 5 μg/ml of EHS laminin, rabbit anti-laminin (1:1000), and goat anti-rabbit HRP (1:3000). Bound antibody was detected by enhanced chemiluminescence.

Microarray Analysis of Lobeline-treated Versus DMSO-treated Cells—Whole transcriptome analysis was performed in the UCLA DNA Microarray Facility using Mouse 430_2.0 arrays and 3′ IVT Express Kit using the manufacturer's recommended protocols for RNA amplification and labeling. Gene expression measurements were calculated using RMA and a standard reference RNA panel, as described previously (21). Pairwise comparisons of gene expression were performed in custom R scripts to measure -fold differences.

siRNA Reduction of Glycosyltransferases—Large, protein *O*-mannosyltransferases 1 and 2 (POMT1/POMT2), and control scrambled siRNAs were obtained from Santa Cruz Biotechnology, Inc. C2C12 cells were transfected using the Amaxa Nucleofector II and Nucleofector V kit (Amaxa), following the manufacturer's recommendations. C2C12 myoblasts were transfected 5 h prior to the addition of differentiation medium with 100 μM lobeline or vehicle control. After 48 h in differentiation medium, cells were fixed for WFA binding or were collected for lectin blotting or immunoblotting, as above.

Inhibitor Treatment and Lectin Binding by Flow Cytometry—Deoxymannojirimycin (2 mM) (DMNJ) was added to C2C12 myoblasts 24 h prior to the addition of differentiation medium. Differentiation medium with lobeline or buffer control was added for 48 h, and cells were analyzed by lectin binding in plates or by flow cytometry. For flow cytometry, adherent cells were lifted by gentle agitation in PBS/EDTA, pH 8.0. Cells were dispersed by pipetting. 4×10^4 cells/sample were incubated with biotinylated BSA, as control, or biotinylated PHA (8 μg/ml), and bound lectin was detected with streptavidin-conjugated FITC (30 μg/ml). Cells were analyzed on a FACScan flow cytometer (BD Biosciences).

Glycan Release and Permethylation—Isolation and permethylation of *N*-glycans was performed as described previously (22). Analysis of *N*-glycans was performed in duplicate, mixing control- and lobeline-treated cells at a 1:1 ratio based upon protein concentration. *N*-Glycan analysis was performed in duplicate with cell lysates prepared from control C2C12 cells grown in regular L-glutamine ^{14}N or amide-^{15}N medium mixed 1:1 with lobeline-treated C2C12 cells grown in regular L-glutamine ^{14}N or amide-^{15}N medium. Samples were subjected to delipidation using a chloroform/methanol/water extraction. The resulting protein pellets were digested with trypsin, and *N*-glycans were released with protein:*N*-glycosidase F overnight at 37 °C. Released *N*-glycans were separated from the peptide mixture based upon the inability to bind to a C18 reverse phase column (Mallinckrodt Baker). The *N*-glycans collected from each sample mixture were permethylated to facilitate analysis by mass spectrometry. Following permethylation, the *N*-glycans were dried to dryness in a SpeedVac prior to analysis.

Glycan Analysis—*N*-Glycans were analyzed as described previously (22, 23). Briefly, permethylated *N*-glycans were resuspended in 50 μl of 100% methanol. For analysis, 30% of the resuspended *N*-glycans were set aside, and 35 μl of 1 mM NaOH in 50% methanol was added. The total sample volume of 50 μl was directly infused into an Orbitrap mass spectrometer (ThermoFisher) at a syringe flow rate of 0.4 μl/min. A full MS profile of all released and permethylated *N*-glycans was acquired over the 200–2000 *m*/*z* range. MS/MS fragmentation spectra were acquired using a total ion mapping method, where parent mass was increased stepwise by 2.0 *m*/*z* within overlapping collection windows with an isolation width of 2.8 *m*/*z*. The resulting MS/MS spectra were manually interpreted, and glycan structures were assigned based upon fragmentation rules and Glycoworkbench (24). For quantification of glycans in the 1:1 mixture of the heavy and light *N*-glycans as measured by protein concentration, in-house software was used. The representation and nomenclature used to represent the observed *N*-glycan structures shown in the figures and Table 1 is in accordance with guidelines outlined by the Consortium of Functional Glycomics.

RESULTS

As mentioned above, DG on skeletal muscle cells can be differentially glycosylated. In normal muscle, DG localized at the sarcolemma preferentially binds WGA, whereas DG localized at neuromuscular junctions preferentially binds WFA (6). Substitution of utrophin for dystrophin to ameliorate pathophysiology in specific models of muscular dystrophy coincides with an increase in the WFA-binding fraction of DG and increased binding of WFA to the sarcolemma (6). In dystrophin-deficient *mdx* muscle, WFA binding to skeletal muscle DG is substantially increased, consistent with the increased substitution of utrophin for dystrophin around the extrasynaptic sarcolemma. This is shown in Fig. 1, *A* and *B*, demonstrating WFA binding to quadriceps muscle sections from wild type and *mdx* mice; note the robust WFA binding completely surrounding the myofibers in *mdx* mice, compared with the sparse, punctuate WFA binding to neuromuscular junctions in wild type muscle. A similar increase in WFA binding is seen in muscle sections from mice overexpressing constitutively active Akt (Fig. 1*D*), which promotes utrophin usage in skeletal muscles of wild type mice (19).

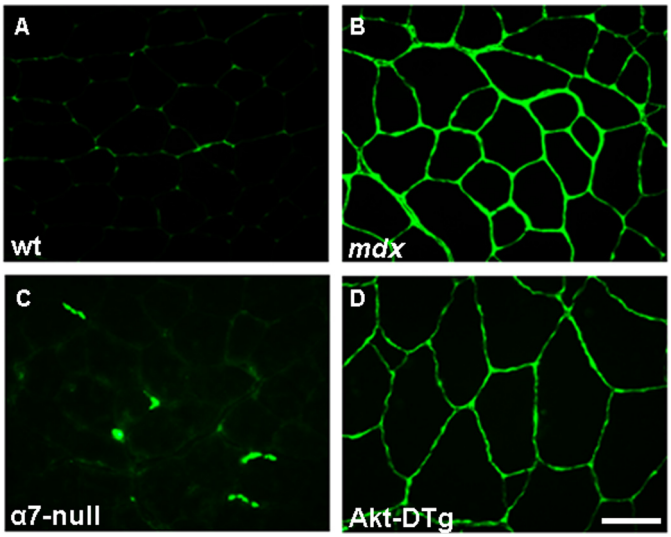

FIGURE 1. **WFA binding to sections of murine muscle**. Transverse sections of quadriceps from wild type (*wt*) (*A*), *mdx* (*B*), α7 integrin null (*C*), or Akt-overexpressing (*Akt-DTg*) (*D*), were stained with biotinylated WFA, and bound lectin was detected with fluorescein-streptavidin using indirect immunofluorescence microscopy. Note punctuate staining in wild type and α7-null, compared with robust staining surrounding muscle cells in *mdx* and Akt-DTg mice. *Bar*, 50 μM.

Thus, increasing utrophin usage in skeletal muscle correlates with increased WFA binding to the sarcolemma. Importantly, WFA binding to muscle from mice lacking the α7 integrin chain is comparable with that observed for binding to wild type muscle (Fig. 1, *A* and *C*), indicating that α7 integrin is not essential for WFA binding to skeletal muscle.

We thus designed a high throughput screen to identify compounds that would increase WFA binding to cultured mouse myotubes derived from C2C12 cells. We reasoned that any pharmacologic effect that increased WFA binding could result in a concomitant increase in utrophin usage in muscle cells, a long term goal in developing novel therapies for DMD. The screen was mechanism-blind, in that we only measured the end point of increased WFA binding; for example, increased WFA binding could be due to altered expression of glycosyltransferases or of specific glycoprotein acceptors or to increased transport or decreased turnover of WFA binding glycoproteins at the cell surface. For the screen, C2C12 myoblasts were cultured in 384-well plates in growth medium for 2 days, and then a single concentration of test compounds (10 μM) or vehicle was added to duplicate wells for an additional day. On day 3, differentiation medium containing the test compound was added to the cells, and WFA binding to fused myotubes was measured by chemiluminescence after 4 days. We screened the Prestwick library of ~1200 FDA-approved drugs, and an increase in WFA binding of 2-fold or greater was considered a positive response. As a control, we used C2C12 cells stably overexpressing Galgt2 to demonstrate that we could detect increased WFA binding to myotubes derived from these cells compared with C2C12 transfected with vector alone (data not shown).

Table 1 lists the six compounds that gave a ≥2-fold increase in WFA binding to C2C12 myotubes in two consecutive screens. Importantly, none of the six compounds have similar

TABLE 1
Compounds identified in high throughput screen of Prestwick library
Values in screens 1 and 2 are ratios of WFA binding to cells treated with drug *versus* vehicle control.

Name	Uses	Screen 1	Screen 2
Norgestrel	Female contraceptive	2.9	7.3
Felbinac	Anti-inflammatory	2.7	4.3
N-acetylleucine	Vertigo	2.8	3.0
Liothyronine	Hypothyrodism	2.0	2.9
Calciferol	Vitamin D	2.1	2.8
Lobeline	Smoking cessation	2.6	3.0

known mechanisms of action; nor do they group into common families based on structure. We reassayed the six compounds in confirmatory assays to determine dose-response characteristics over a range of concentrations from 10 to 100 μM. Only one of the six compounds, lobeline, demonstrated a consistent dose-dependent effect on WFA binding to C2C12 myotubes in the confirmatory assay format. As shown in Fig. 2*A*, lobeline treatment increased WFA binding to C2C12 myotubes 2–4-fold compared with vehicle control. The maximum increase in WFA binding was observed at a concentration of 100 μM; although we observed no effect of lobeline on myotube viability at or below this concentration, we did detect loss of C2C12 myotube viability at higher concentrations of lobeline (data not shown), so a maximal concentration of 100 μM was used for experiments with C2C12 cells. Although we observed increased WFA binding to lobeline-treated myotubes, we did not detect increased binding of two other lectins, peanut agglutinin and phytohemagglutinin (PHA) in the screen (data not shown).

We confirmed the effect of lobeline on primary myoblasts cultured from limb muscles of neonatal wild type and *mdx* mice. As described for C2C12 cells, lobeline was added to myoblast cultures at the indicated concentrations at the time that differentiation was induced, as in the confirmatory assays. Two days after incubation in the presence or absence of lobeline, WFA binding was measured. As shown in Fig. 2, *B* and *C*, lobeline increased the binding of WFA to both wild type and *mdx* myotubes, with the maximal effect observed at 200 μM; this concentration had no effect on the viability of primary muscle cells (data not shown). Thus, lobeline increased binding of WFA to *mdx* myotubes lacking dystrophin, indicating that the effect of lobeline was not dystrophin-dependent, as well as to cultured C2C12 cells and wild type myotubes.

In designing the screen, we reasoned that, if changes in cell surface glycosylation were required for increased WFA binding, it would be essential to add drugs at the initiation of myotube differentiation, to ensure that new glycoproteins entering the Golgi encountered appropriate glycosyltransferase enzymes; this was the rationale for adding the drugs with the differentiation medium in the original high throughput screen. However, we found that, when lobeline was added to confluent, differentiated C2C12 myotubes, we still observed a 2–4-fold increase in WFA binding (Fig. 2*D*), demonstrating that lobeline could increase WFA binding to differentiated cells *in vitro* and suggesting that any activity of lobeline might be effective in mature skeletal muscle. In contrast, we saw no increase in WFA binding to C2C12 myoblasts maintained in growth medium in the presence of lobeline (supplemental Fig. 1), indicating that either the glycoprotein backbones or the glycan structures rec-

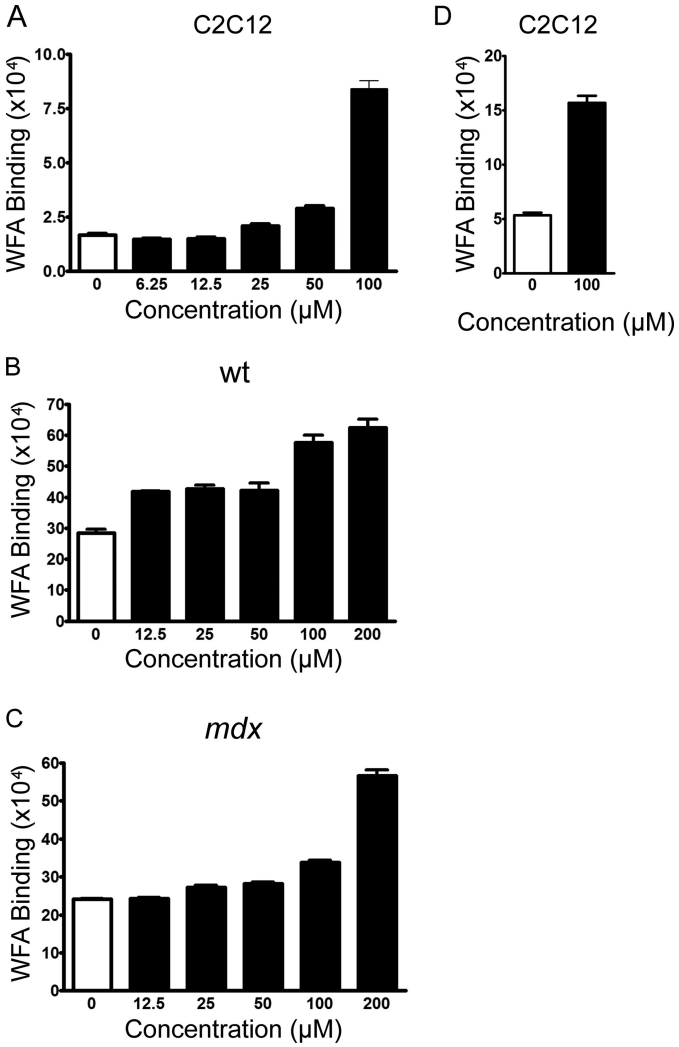

FIGURE 2. **Lobeline increases WFA binding to myotubes *in vitro*.** *A*, dose-response curve of WFA binding to lobeline-treated (*filled bars*) *versus* control (DMSO, *0*; *open bars*) C2C12 myotubes. Lobeline or vehicle was added to myoblasts at the time of differentiation for 48 h. *B*, dose-response curve of WFA binding to lobeline-treated *versus* control myotubes derived from wild type primary murine myoblasts, as in *A*. *C*, dose-response curve of WFA binding to lobeline-treated *versus* control myotubes derived from *mdx* myoblasts, as in *A*. *D*, WFA binding to differentiated C2C12 myotubes treated with 100 μM lobeline or control. Data are mean ± S.D. (*error bars*) for triplicate samples from one of three or more independent experiments for each *panel*.

ognized by WFA are preferentially expressed on differentiated myotubes rather than on myoblasts.

We next asked if specific glycoproteins on muscle cells demonstrated increased WFA binding after lobeline treatment. As shown in Fig. 3*A* (*left*), treatment of C2C12 cells with lobeline increased the abundance of several glycoproteins that were precipitated with WFA from cell lysates. After these samples were probed with WFA (Fig. 3*A*, *left*), the blot was reprobed with mAb IIH6, which recognizes *O*-mannose glycans that are critical for laminin binding (*right*). WFA-binding was detected in control treated cells, but the abundance of WFA binding increased dramatically in lobeline-treated cells. Similarly, lobeline treatment increased the abundance of WFA-binding glycoproteins in primary myotubes derived from wild type (Fig. 3*B*) and *mdx* (Fig. 3*C*) mouse muscle. In primary mouse myotubes, we again observed a dramatic increase in WFA-precipitation of IIH6-reactive glycoproteins, as well as β-DG, after treatment with lobeline. These data demonstrate that lobeline treatment affects multiple glycoproteins that bear GalNAc moieties recognized by WFA, including DG; an increase in WFA-reactive α-DG was also observed in mice overexpressing Galgt2 (6).

In patients with DMD, loss of dystrophin results in loss of the entire DGC and thus reduced muscle cell adhesion to extracellular matrix. Specifically, reduction in sarcolemmal α-DG results in decreased adhesion to laminin in the extracellular matrix. We asked if lobeline treatment affected laminin binding to proteins from control or treated wild type or *mdx* myotubes. To detect laminin binding to cell surface proteins, equal amounts of protein from each sample were separated by SDS-PAGE, laminin was added to blots, and bound laminin was detected with anti-laminin (Fig. 4). Samples were also probed with IIH6 mAb. We observed a dramatic increase in the abundance of IIH6 reactivity and in laminin binding to glycoproteins from lobeline-treated cells.

To further examine the effect of lobeline on the abundance of DGC/UGC components, as well as other sarcolemmal proteins involved in muscle cell adhesion and function, we compared the abundance of several proteins from control- or lobeline-treated myotubes derived from wild type and *mdx* primary muscle cells. Lysates from equal numbers of control- or lobeline-treated cells were separated by SDS-PAGE, and sarcolemmal proteins were examined by immunoblotting. As shown in Fig. 5*A*, lobeline treatment of wild type myoblasts increased the abundance of several proteins, including agrin, dystrophin, utrophin, α- and β-DG, sarcospan, sarcoglycans, and β1D integrin, whereas the abundance of caveolin-3 was not affected. A similar effect was found when lobeline-treated *mdx* myotubes were examined (Fig. 5*B*). In these cells, the increased abundance of agrin, utrophin, and α- and β-DG was particularly striking. Overall, in control-treated *mdx* cells, there was increased abundance on a per cell basis of many proteins, including agrin, utrophin, and β1D integrin, compared with control-treated wild type cells; this most likely reflects the compensatory mechanisms that occur in *mdx* mouse muscle. However, even with higher base-line abundance of many proteins in *mdx* cells, lobeline further increased the abundance of sarcolemmal proteins. Intriguingly, the increased abundance of agrin, α- and β-DG, and utrophin in cells treated with lobeline was consistent with the increased abundance of these proteins with Galgt2 overexpression in various dystrophic mouse models (6, 8, 9), suggesting that increasing WFA binding to muscle cells by either pharmacologic or genetic approaches results in increasing abundance of sarcolemmal proteins.

We next asked what types of glycans are required for WFA binding to myotubes. Because lobeline increased laminin binding to myotube glycoproteins (Fig. 4), an effect involving *O*-mannose glycans on muscle cell glycoproteins, and because *O*-mannose glycans in general are known to be critical for muscle cell viability and function, we asked if the lobeline-mediated increase in WFA binding required *O*-mannose glycans. Although the precise structure of the complete laminin-binding glycans elaborated on *O*-mannose structures is not known, it is intriguing that one branch of the

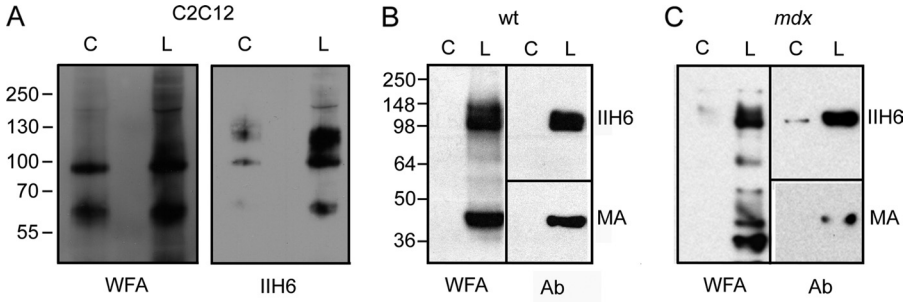

FIGURE 3. **Identification of WFA binding glycoproteins in control-treated *versus* lobeline-treated cells.** *A*, equal amounts of cell protein from lysates of C2C12 myotubes treated with 100 μM lobeline (*L*) or control (*C*) for 48 h were precipitated with WFA beads, bound proteins were eluted with GalNAc, eluted proteins were separated by SDS-PAGE, and blots were probed with WFA or mAb IIH6. Equal amounts of cell protein from lysates of wild type (*wt*) (*B*) or *mdx* myotubes (*C*) treated with lobeline or control were precipitated with WFA beads, bound proteins were eluted with GalNAc, eluted proteins were separated by SDS-PAGE, and blots were probed with WFA or mAbs IIH6 or MANDAG2 (*MA*), which recognizes β-DG. Results are representative of four independent experiments.

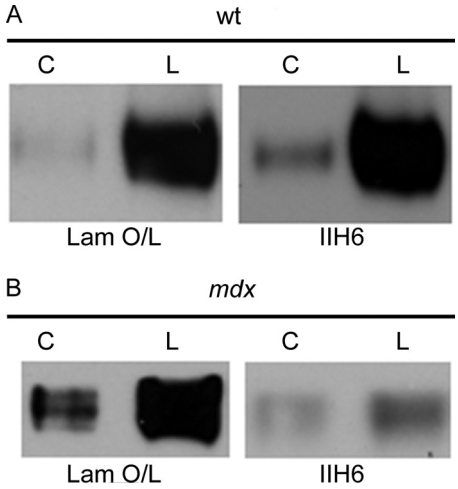

FIGURE 4. **Lobeline treatment increases laminin binding.** Lysates of surface biotinylated wild type (*wt*) (*A*) or *mdx* myotubes (*B*) treated with lobeline (*L*) or control (*C*) were separated by SDS-PAGE and blotted, laminin was incubated with the blots, and bound laminin was detected with anti-laminin. Blots were stripped and reprobed with mAb IIH6.

O-mannose glycan bears the SAα2,3Galβ1,4GlcNAc trisaccharide that could be modified to create the Sda tetrasaccharide, SAα2,3Gal (β1,3GalNAc)β1,4GlcNAc (12). We reduced expression of POMT1/POMT2, the enzyme complex that adds the initiating mannose residue, in C2C12 myoblasts by siRNA; transfections were performed just prior to the addition of differentiation medium with lobeline or vehicle control, and WFA binding was measured after 48 h. We confirmed that reduction in POMT1/POMT2 mRNA resulted in reduced expression of *O*-mannose glycans detected by mAb IIH6, demonstrating loss of *O*-mannose glycans (Fig. 6*A*). However, reduction in POMT1/POMT2 expression resulted in no loss of WFA binding to lobeline-treated cells; rather, there was a modest but reproducible increase in WFA binding to lobeline-treated myotubes in the absence of *O*-mannose glycans (Fig. 6*B*). We also reduced expression of Large, which modifies *O*-mannose structures to create laminin binding glycans, and also observed reduced IIH6 binding but increased WFA binding to lobeline-treated cells (not shown).

As mentioned above, muscle cell glycoproteins, such as α-DG, also bear mucin type *O*-glycans and *N*-glycans; for example, α-DG has three *N*-glycosylation sites, and β-DG has one *N*-glycosylation site (15, 16). Thus, we asked if *N*-glycans were important for WFA binding to control- and lobeline-treated cells. To globally reduce complex *N*-glycans on C2C12 cells, we treated myoblasts with DMNJ, which inhibits mannosidase I and prevents creation of complex *N*-glycan structures from high mannose precursors (16). Complex *N*-glycans could bear GalNAc residues, recognized by WFA, either on Sda tetrasaccharides or as terminal structures on *N*-glycan branches. As shown in Fig. 7*A*, DMNJ had the predicted effect on C2C12 cells because DMNJ treatment abrogated binding of the lectin PHA that recognizes branched complex *N*-glycans. Surprisingly, DMNJ also dramatically reduced WFA binding to control- or lobeline-treated cells. As shown in Fig. 7*B*, the loss of complex *N*-glycans reduced WFA binding by >90% to both control- and lobeline-treated C2C12 myotubes. Thus, complex *N*-glycans bear the glycan structures recognized by WFA on muscle cells, and increased WFA binding after lobeline treatment requires expression of complex *N*-glycans.

To analyze the effect of lobeline on the abundance of *N*-glycans involved in increased WFA binding, *N*-glycans of C2C12 cells grown either in the presence or absence of lobeline were analyzed. In order to quantify the abundance of observed N-glycan structures from the treated and control populations, an IDAWG tandem mass spectrometry-based strategy was applied (22). After characterizing the released *N*-glycans and quantifying the abundance of the observed glycan structures, we determined the relative abundance for *N*-glycans released from lobeline-treated samples over the control (supplemental Tables 1 and 2). Specifically, the majority of the *N*-glycans released from lobeline-treated C2C12 cells were observed to be elevated over control. A total of 32 glycan masses were observed, of which 30 were able to be quantified, and 84% of the quantified glycans showed an increase of at least 1.5-fold. Of particular interest, two proposed GalNAc-containing structures were elevated 2.8–5.5-fold (Fig. 8); however, glycans bearing the Sda epitope were not substantially elevated.

N-Glycosylation of numerous glycoproteins on C2C12 cells has been described (25); however, in contrast to the extensively studied *O*-mannose glycans on α-DG, which are known to participate in laminin binding, no clear functions for *N*-glycans on many muscle cell glycoproteins have been proposed. Because increased WFA binding correlated with increased laminin

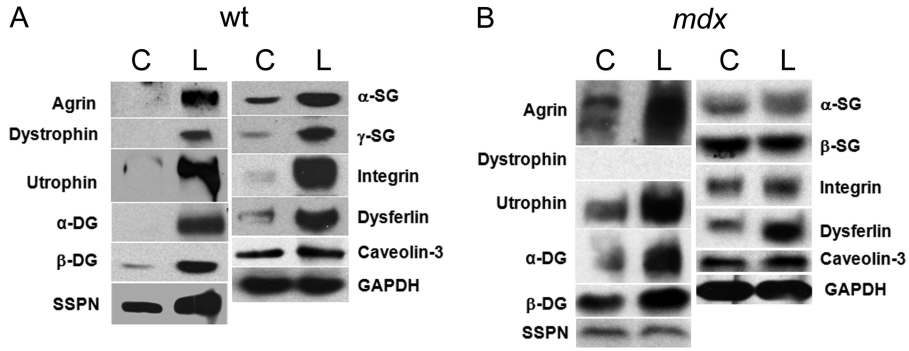

FIGURE 5. **Lobeline increases abundance of multiple muscle cell proteins.** *A*, wild type (*wt*) primary myoblasts differentiated in the presence of 100 μM lobeline (*L*) or control (*C*) were solubilized in radioimmune precipitation assay buffer, and 60 μg of protein was separated by SDS-PAGE and blotted to nitrocellulose. Membranes were probed with antibodies against agrin, dystrophin, utrophin, dystroglycans (α- and β-DG), sarcospan (*SSPN*), sarcoglycans (α-, β-, and γ-SG), β1D integrin, dysferlin, and caveolin-3. GAPDH was a loading control. Lobeline treatment increased agrin, integrin, dysferlin, DGC, and UGC protein levels. *B*, *mdx* primary myoblasts were treated as described in *A*. Membranes were probed similarly with the addition of antibody to β-SG. Dystrophin protein was not detected due to mutation in the dystrophin gene in *mdx* cells. Increases were observed for agrin, dysferlin, utrophin, and α- and β-dystroglycans.

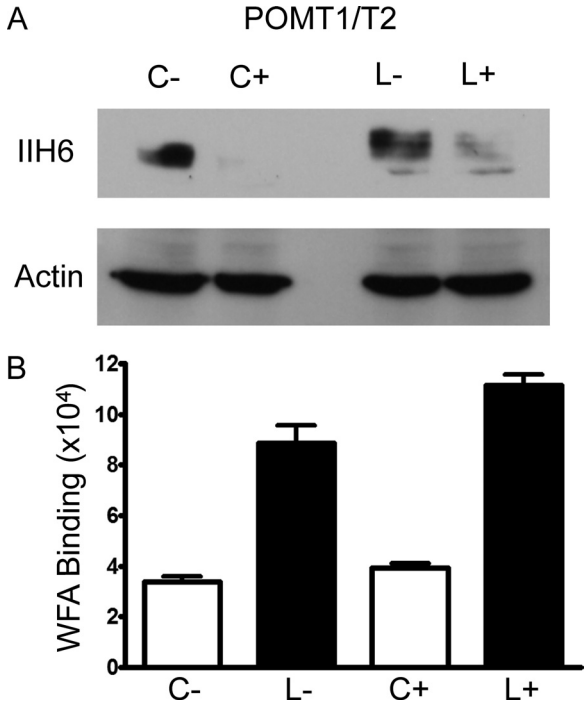

FIGURE 6. ***O*-Mannose glycans are not required for increased WFA binding after lobeline.** *A*, reduction in POMT1/POMT2 by siRNA (+) *versus* scrambled control (−) in C2C12 cells treated with lobeline (*L*) or control (*C*) resulted in loss of mAb IIH6 binding, indicating loss of POMT1/POMT2 activity. *B*, reduction in POMT1/POMT2 activity did not reduce WFA binding. Increased WFA binding to cells treated with lobeline *versus* vehicle control was observed in cells transfected with scrambled siRNA (*C*− and *L*−) or POMT1/POMT2 siRNA (*C*+ and *L*+). Data are mean ± S.D. (*error bars*) of six replicate samples from one of three independent experiments.

binding to cell surface glycoproteins from lobeline-treated cells (Fig. 4), we asked if loss of WFA-binding glycans would affect laminin binding by muscle cell glycoproteins. Cells were treated with or without DMNJ, and extracts were analyzed by immunoblotting. Loss of complex *N*-glycans did not reduce reactivity with IIH6; in fact, reactivity of DMNJ-treated cells with an antibody recognizing an α-DG core epitope actually increased (Fig. 9*A*), suggesting that loss of complex *N*-glycans may have increased availability of this specific epitope. Also, there was no significant difference in overall IIH6 reactivity in DMNJ-treated *versus* control-treated cells. However, there was a significant decrease in laminin binding to glycoproteins from DMNJ-treated cells compared with controls (Fig. 9*B*). These data imply that complex *N*-glycans may indirectly participate in promoting laminin binding or, alternatively, that loss of complex *N*-glycans in DMNJ-treated cells may reduce laminin binding by altering glycoprotein structure or the accessibility of laminin binding sites.

DISCUSSION

The present study demonstrates that high throughput screening of muscle cells using a plant lectin, WFA, can identify compounds that alter cellular glycosylation and laminin binding. Moreover, the compound identified in this screen, lobeline, appears to stabilize one or more membrane glycoproteins in treated cells, rather than acting by altering expression of genes encoding glycan-modifying enzymes or the glycoprotein backbones. The increased WFA binding observed in lobeline-treated cells requires hybrid or complex *N*-glycans, and prevention of *N*-glycan processing on α-DG alters laminin binding, revealing a novel role for *N*-glycans in the function of this critical muscle cell glycoprotein.

Importantly, the target of this high throughput screen, increased WFA binding, made no assumptions about how altered glycosylation might occur. We chose WFA as a target because increased expression of Galgt2 in several mouse models of dystrophy resulted in increased WFA binding to muscle cells, along with increased abundance of utrophin in *mdx* muscle, and amelioration of dystrophy. However, using increased WFA binding as a target could identify drugs that act by multiple mechanisms to promote expression or stabilization of glycoproteins, such as α-DG. Although we considered other reagents for the screen, such as the mAb IIH6 or α-DG-binding proteins, such as laminin or agrin, these reagents would have been prohibitively expensive, especially in future studies, where much larger libraries will be screened. Plant lectins have the additional advantage of typically recognizing only a glycan epitope, rather than a glycan displayed on a particular polypeptide as detected by many mAbs; thus, using lectins for screens does not limit detection of changes to a single protein.

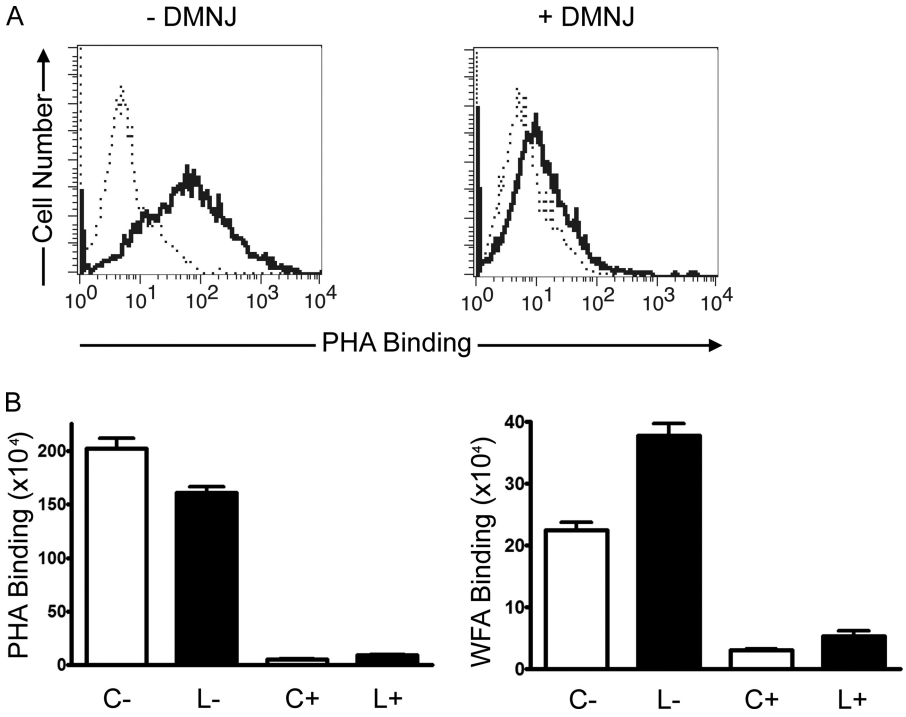

FIGURE 7. **Complex *N*-glycans are required for WFA binding.** *A*, C2C12 myoblasts were treated with DMNJ or vehicle control, and binding of biotinylated PHA (*solid line*) was determined by flow cytometry, using biotinylated BSA as a control (*dotted line*). PHA binding was virtually eliminated by treatment with DMNJ, which inhibits mannosidase I and prevents conversion of high mannose *N*-glycans to complex *N*-glycans. *B*, loss of complex *N*-glycans abrogated WFA binding in cells treated with or without lobeline. *Left*, PHA binding determined in plate binding assay; PHA binding is not affected by lobeline (*L*−) but is abolished by DMNJ in both control (*C*+) and lobeline-treated (*L*+) cells. *Right*, DMNJ treatment markedly abrogates WFA binding to cells treated with control (*C*+) and lobeline (*L*+), whereas cells bearing complex *N*-glycans demonstrate increased WFA binding after lobeline treatment (*L*−). Data are mean ± S.D. (*error bars*) of six replicate samples from one of three independent experiments.

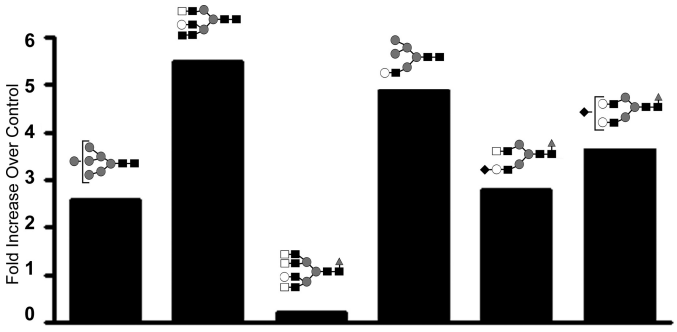

FIGURE 8. **Abundance of *N*-glycans from lobeline-treated cells relative to control.** *N*-Glycans were released from lysates of C2C12 cells grown in the presence or absence of lobeline, in both regular ^{14}N medium and ^{15}N medium. Analysis was performed in duplicate with lobeline/^{15}N control and ^{15}N lobeline/control. The majority of characterized *N*-glycans from cells treated with lobeline were elevated relative to the control. A sample of glycans is shown, including terminal GalNAc-containing structures (see supplemental Tables 3 and 4 for all structures identified and quantified).

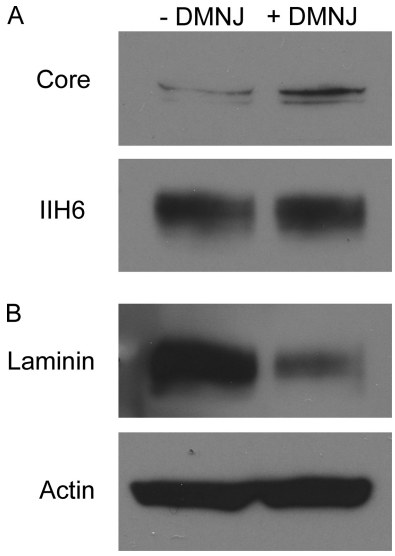

FIGURE 9. **Loss of complex *N*-glycans reduces laminin binding.** Cells treated with or without DMNJ were analyzed by immunoblotting to assess effects on laminin binding. *A*, loss of complex *N*-glycans did not affect production or processing of α-DG; α-DG from cells treated with or without DMNJ was detected with an antibody that recognizes a core epitope on α-DG polypeptide. Loss of complex *N*-glycans did not affect *O*-mannosylation, as shown by reactivity with mAb IIH6. *B*, loss of complex *N*-glycans in cells treated with DMNJ reduced laminin binding to the IIH6-reactive band, detected by a laminin overlay assay. Actin control demonstrates no significant difference in total protein loaded for the two samples.

Lobeline-treated cells had increased abundance of both membrane and intracellular proteins (Fig. 5) as well as increased abundance of virtually all *N*-glycans (Fig. 8). Moreover, microarray analysis comparing control- and lobeline-treated cells demonstrated no increase in mRNA transcripts encoding proteins in the DGC/UGC, including those examined in Fig. 5 (data not shown); thus, the increased abundance of proteins may result from stabilization of the proteins at the sarcolemmal membrane. This could be a general effect on glycoprotein stabilization or could result from stabilization of one or a few scaffolding proteins that associate with other proteins.

DG has been described as a scaffolding protein in a number of studies, because of the ability of DG to promote association of sarcolemmal glycoproteins that contribute to membrane sta-

bility and extracellular matrix binding, such as the sarcoglycans (1, 2). Thus, stabilization of one or more scaffolding proteins, such as DG, could result in increased abundance of associated proteins.

Why would enriching for WFA binding glycans on α-DG and other glycoproteins result in the effects that we observed for lobeline treatment and that Martin and co-workers (6) observed for Galgt2 overexpression? Altered glycosylation could affect the turnover rate of membrane glycoproteins, promote interactions of those glycoproteins with other binding partners, or stabilize such interactions to retain glycoprotein complexes at the plasma membrane. It is important to consider that the amelioration of dystrophic symptoms in *mdx* mice by Galgt2 was seen in mice expressing a high level of Galgt2 (26). Although overexpression of glycosyltransferases may be an effective experimental approach to ameliorating dystrophic pathology (27–29), pharmacologic approaches may have several advantages. Also, it is not known if the Sda modification is restricted to *N*-glycans in mice overexpressing Galgt2; modifying other glycans with GalNAc residues may have different effects than stabilizing or enriching for existing glycoproteins, as we appear to have done with lobeline, and the determination that we did not specifically increase Sda-containing *N*-glycans (Fig. 8 and supplemental Tables 1 and 2) implies that the Sda epitope *per se* may not be required for the lobeline effect.

However, although lobeline had a consistent effect on increasing WFA binding to myotubes from C2C12 cells or primary myoblasts, the mechanism of lobeline action is still unclear. Lobeline is described as a nicotinic acetylcholine receptor agonist/antagonist, and lobeline was evaluated in animal models as an anti-smoking agent and has also been administered to patients as a safe, short-acting respiratory stimulant (30, 31), although due to side effects, such as nausea and convulsions, and the availability of more effective agents, lobeline is not currently used for these indications. Although classified as a nicotinic receptor agonist, lobeline has no structural similarity to nicotine (31), and the Prestwick library contains several other acetylcholine receptor agonists/antagonists, none of which increased WFA binding on the initial high throughput screen (supplemental Table 3). In addition, we specifically examined the effects of two related acetylcholine receptor agonists/antagonists, gallamine and condelphine, and observed no effect on WFA binding (supplemental Fig. 2*A*). We also added bungarotoxin, to irreversibly inhibit the acetylcholine receptor, and detected no difference in the lobeline-induced increase in WFA binding (supplemental Fig. 2*B*). Finally, we reduced expression of the α-chain of the acetylcholine receptor by siRNA. Immunoblotting revealed a significant reduction in α-chain expression in cells treated with the specific siRNA; however, despite loss of α-chain expression, which is essential for trafficking of the intact receptor to the plasma membrane, we observed no difference in WFA binding to lobeline-treated cells compared with lobeline-treated cells transfected with scrambled siRNA (supplemental Fig. 2*C*). Thus, the acetylcholine receptor does not appear to be required for the effect of lobeline on muscle cell expression of WFA-binding glycans. Lobeline is also known to act through alternative receptors, including the dopamine transporter and the vesicular monoamine transporter (30–32). A role for lobeline as a protein folding chaperone has been reported, suggesting that lobeline may increase protein abundance by reducing protein misfolding and degradation (33). Lobeline has also been found to be a nonselective Ca^{2+} channel antagonist (30). Thus, the mechanisms responsible for the lobeline effects that we have observed remain to be elucidated.

Laminin binding decreased significantly when cells were treated with DMNJ (Fig. 9*A*). Why might *N*-glycan processing be important for laminin binding? Campbell and co-workers (34) found that the N terminus of α-DG was essential for proper Large modification of and laminin binding by *O*-mannose glycans in the mucin domain of α-DG, indicating that the N terminus of α-DG is important for Large recognition as an acceptor substrate, for binding, or for activity. However, we did observe IIH6 binding to glycoproteins from DMNJ-treated C2C12 cells (Fig. 9), indicating that at least some *O*-mannose glycans were modified by Large in these cells. Thus, preventing *N*-glycan processing may affect presentation or processing of a subset of specific *O*-mannose or other glycans that are required for laminin binding or may sterically prevent access of laminin to these glycans; several reports have shown that, in addition to Large-mediated laminin binding by α-DG, other glycoproteins and glycans may also be modified by Large and participate in laminin binding (28, 29, 35–37). A recent report identified two *O*-mannose glycans on the mucin domain immediately adjacent to the N-terminal domain of α-DG as essential for laminin binding, so that altering *N*-glycans on the α-DG N-terminal domain could affect accessibility of such adjacent glycans (14). Although the functions of most *N*-glycans on muscle cell glycoproteins are unknown, it is intriguing that congenital disorder of glycosylation type Ie, a disorder that disrupts dolichol-phosphate-mannose synthesis and affects synthesis of *N*-glycans, *O*-mannose glycans, and glycophospholipid anchors, has been described in a patient presenting with muscular dystrophy (38). This suggests that proper processing of specific *N*-glycans may also contribute to muscle cell development, differentiation, or function.

In addition to α-DG, integrins are important mediators of muscle cell binding to extracellular matrix, and integrins on C2C12 cells are known to be heavily *N*-glycosylated (25). However, although DG was a significant component of the glycoproteins precipitated by WFA from cells treated with or without lobeline (Fig. 3), we did not detect α7β1D integrin in these WFA precipitates despite repeated attempts (data not shown). Moreover, the presence of WFA binding to skeletal muscle from α7-null mice (Fig. 1) further indicates that this integrin is not required for presentation of WFA-binding glycans on the sarcolemmal membrane *in vivo*.

A critical unanswered question is why glycan modification of DG, which promotes WFA binding, correlates with preferential association of utrophin, rather than dystrophin, with the cytoplasmic tail of β-DG. It is remarkable that this phenomenon has been used for several years to enrich for dystrophin-containing *versus* utrophin-containing complexes, whereas the precise types of glycans recognized, the mechanism of subcellular site-specific glycosylation of DG, and the rationale for dystrophin *versus* utrophin association are completely unknown. More-

over, although glycan modifications that promote WFA binding are found during differentiation of murine myoblasts into myotubes (Fig. 2), it is not clear that the identical glycan changes occur during differentiation of human muscle cells. Little is known about cellular glycosylation in normal and dystrophic human muscle (39). We have examined myotubes derived from dermal fibroblasts from DMD patients and parental controls and have not observed an increase in WFA binding to these differentiated myotubes.[4] Thus, although the high throughput screening approach using plant lectins described here should be feasible with human cells, it will be essential to first define appropriate glycosylation targets that correlate with a therapeutic goal (*e.g.* increased utrophin usage) in Duchenne muscular dystrophy. Ultimately, this approach to the identification of compounds that stabilize DG and associated proteins at the sarcolemma may lead to development of novel therapeutics for multiple types of muscular dystrophy.

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REFERENCES

1. Michele, D. E., and Campbell, K. P. (2003) Dystrophin-glycoprotein complex. Post-translational processing and dystroglycan function. *J. Biol. Chem.* **278,** 15457–15460
2. Ervasti, J. M. (2007) Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. *Biochim. Biophys. Acta* **1772,** 108–117
3. Blake, D. J., Tinsley, J. M., and Davies, K. E. (1994) The emerging family of dystrophin-related proteins. *Trends Cell Biol.* **4,** 19–23
4. Ohlendieck, K., Ervasti, J. M., Matsumura, K., Kahl, S. D., Leveille, C. J., and Campbell, K. P. (1991) Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* **7,** 499–508
5. Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J. M., and Davies, K. (1998) Expression of full-length utrophin prevents muscular dystrophy in *mdx* mice. *Nat. Med.* **4,** 1441–1444
6. Nguyen, H. H., Jayasinha, V., Xia, B., Hoyte, K., and Martin, P. T. (2002) Overexpression of the cytotoxic T cell GalNAc transferase in skeletal muscle inhibits muscular dystrophy in *mdx* mice. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 5616–5621
7. Jayasinha, V., Hoyte, K., Xia, B., and Martin, P. T. (2003) Overexpression of the CT GalNAc transferase inhibits muscular dystrophy in a cleavage-resistant dystroglycan mutant mouse. *Biochem. Biophys. Res. Commun.* **302,** 831–836
8. Xu, R., Chandrasekharan, K., Yoon, J. H., Camboni, M., and Martin, P. T. (2007) Overexpression of the cytotoxic T cell (CT) carbohydrate inhibits muscular dystrophy in the dyW mouse model of congenital muscular dystrophy 1A. *Am. J. Pathol.* **171,** 181–199
9. Xu, R., DeVries, S., Camboni, M., and Martin, P. T. (2009) Overexpression of Galgt2 reduces dystrophic pathology in the skeletal muscles of α-sarcoglycan-deficient mice. *Am. J. Pathol.* **175,** 235–247
10. Yoon, J. H., Chandrasekharan, K., Xu, R., Glass, M., Singhal, N., and Martin, P. T. (2009) The synaptic CT carbohydrate modulates binding and expression of extracellular matrix proteins in skeletal muscle. Partial dependence on utrophin. *Mol. Cell Neurosci.* **41,** 448–463
11. Bozzi, M., Morlacchi, S., Bigotti, M. G., Sciandra, F., and Brancaccio, A. (2009) Functional diversity of dystroglycan. *Matrix Biol.* **28,** 179–187
12. Yoshida-Moriguchi, T., Yu, L., Stalnaker, S. H., Davis, S., Kunz, S., Madson, M., Oldstone, M. B., Schachter, H., Wells, L., and Campbell, K. P. (2010) *O*-Mannosyl phosphorylation of α-dystroglycan is required for laminin binding. *Science* **327,** 88–92
13. Stalnaker, S. H., Hashmi, S., Lim, J. M., Aoki, K., Porterfield, M., Gutierrez-Sanchez, G., Wheeler, J., Ervasti, J. M., Bergmann, C., Tiemeyer, M., and Wells, L. (2010) Site mapping and characterization of *O*-glycan structures on α-dystroglycan isolated from rabbit skeletal muscle. *J. Biol. Chem.* **285,** 24882–24891
14. Hara, Y., Kanagawa, M., Kunz, S., Yoshida-Moriguchi, T., Satz, J. S., Kobayashi, Y. M., Zhu, Z., Burden, S. J., Oldstone, M. B., and Campbell, K. P. (2011) Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 17426–17431
15. Sgambato, A., and Brancaccio, A. (2005) The dystroglycan complex. From biology to cancer. *J. Cell Physiol.* **205,** 163–169
16. Esapa, C. T., Bentham, G. R., Schröder, J. E., Kröger, S., and Blake, D. J. (2003) The effects of post-translational processing on dystroglycan synthesis and trafficking. *FEBS Lett.* **555,** 209–216
17. Kawahara, G., Karpf, J. A., Myers, J. A., Alexander, M. S., Guyon, J. R., and Kunkel, L. M. (2011) Drug screening in a zebrafish model of Duchenne muscular dystrophy. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 5331–5336
18. Rooney, J. E., Welser, J. V., Dechert, M. A., Flintoff-Dye, N. L., Kaufman, S. J., and Burkin, D. J. (2006) Severe muscular dystrophy in mice that lack dystrophin and α7 integrin. *J. Cell Sci.* **119,** 2185–2195
19. Peter, A. K., Ko, C. Y., Kim, M. H., Hsu, N., Ouchi, N., Rhie, S., Izumiya, Y., Zeng, L., Walsh, K., and Crosbie, R. H. (2009) Myogenic Akt signaling up-regulates the utrophin-glycoprotein complex and promotes sarcolemma stability in muscular dystrophy. *Hum. Mol. Genet* **18,** 318–327
20. Kimura, E., Han, J. J., Li, S., Fall, B., Ra, J., Haraguchi, M., Tapscott, S. J., and Chamberlain, J. S. (2008) Cell-lineage regulated myogenesis for dystrophin replacement. A novel therapeutic approach for treatment of muscular dystrophy. *Hum. Mol. Genet* **17,** 2507–2517
21. Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J., and Speed, T. P. (2002) Normalization for cDNA microarray data. A robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res.* **30,** e15
22. Aoki, K., Perlman, M., Lim, J. M., Cantu, R., Wells, L., and Tiemeyer, M. (2007) Dynamic developmental elaboration of *N*-linked glycan complexity in the *Drosophila melanogaster* embryo. *J. Biol. Chem.* **282,** 9127–9142
23. Orlando, R., Lim, J. M., Atwood, J. A., 3rd, Angel, P. M., Fang, M., Aoki, K., Alvarez-Manilla, G., Moremen, K. W., York, W. S., Tiemeyer, M., Pierce, M., Dalton, S., and Wells, L. (2009) IDAWG. Metabolic incorporation of stable isotope labels for quantitative glycomics of cultured cells. *J. Proteome Res.* **8,** 3816–3823
24. Ceroni, A., Maass, K., Geyer, H., Geyer, R., Dell, A., and Haslam, S. M. (2008) GlycoWorkbench. A tool for the computer-assisted annotation of mass spectra of glycans. *J. Proteome Res.* **7,** 1650–1659
25. Gundry, R. L., Raginski, K., Tarasova, Y., Tchernyshyov, I., Bausch-Fluck, D., Elliott, S. T., Boheler, K. R., Van Eyk, J. E., and Wollscheid, B. (2009) The mouse C2C12 myoblast cell surface *N*-linked glycoproteome. Identification, glycosite occupancy, and membrane orientation. *Mol. Cell Proteomics* **8,** 2555–2569
26. Xia, B., Hoyte, K., Kammesheidt, A., Deerinck, T., Ellisman, M., and Martin, P. T. (2002) Overexpression of the CT GalNAc transferase in skeletal muscle alters myofiber growth, neuromuscular structure, and laminin expression. *Dev. Biol.* **242,** 58–73
27. Barresi, R., Michele, D. E., Kanagawa, M., Harper, H. A., Dovico, S. A., Satz, J. S., Moore, S. A., Zhang, W., Schachter, H., Dumanski, J. P., Cohn, R. D., Nishino, I., and Campbell, K. P. (2004) LARGE can functionally bypass α-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. *Nat. Med.* **10,** 696–703
28. Patnaik, S. K., and Stanley, P. (2005) Mouse large can modify complex *N*- and mucin *O*-glycans on α-dystroglycan to induce laminin binding. *J. Biol. Chem.* **280,** 20851–20859
29. Zhang, Z., Zhang, P., and Hu, H. (2011) LARGE expression augments the

[4] M. Pang, M. C. Miceli, and L. G. Baum, unpublished observations.

glycosylation of glycoproteins in addition to α-dystroglycan conferring laminin binding. *PLoS One* **6,** e19080
30. Sántha, E., Sperlágh, B., Zelles, T., Zsilla, G., Tóth, P. T., Lendvai, B., Baranyi, M., and Vizi, E. S. (2000) Multiple cellular mechanisms mediate the effect of lobeline on the release of norepinephrine. *J. Pharmacol. Exp. Ther.* **294,** 302–307
31. Dwoskin, L. P., and Crooks, P. A. (2002) A novel mechanism of action and potential use for lobeline as a treatment for psychostimulant abuse. *Biochem. Pharmacol.* **63,** 89–98
32. Wilhelm, C. J., Johnson, R. A., Eshleman, A. J., and Janowsky, A. (2008) Lobeline effects on tonic and methamphetamine-induced dopamine release. *Biochem. Pharmacol.* **75,** 1411–1415
33. Lee, W. C., Kang, D., Causevic, E., Herdt, A. R., Eckman, E. A., and Eckman, C. B. (2010) Molecular characterization of mutations that cause globoid cell leukodystrophy and pharmacological rescue using small molecule chemical chaperones. *J. Neurosci.* **30,** 5489–5497
34. Kanagawa, M., Saito, F., Kunz, S., Yoshida-Moriguchi, T., Barresi, R., Kobayashi, Y. M., Muschler, J., Dumanski, J. P., Michele, D. E., Oldstone, M. B., and Campbell, K. P. (2004) Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell* **117,** 953–964
35. Zhang, P., and Hu, H. (2012) Differential glycosylation of α-dystroglycan and proteins other than α-dystroglycan by like-glycosyltransferase. *Glycobiology* **22,** 235–247
36. Hu, Y., Li, Z. F., Wu, X., and Lu, Q. (2011) Large induces functional glycans in an *O*-mannosylation dependent manner and targets GlcNAc terminals on α-dystroglycan. *PLoS One* **6,** e16866
37. Aguilan, J. T., Sundaram, S., Nieves, E., and Stanley, P. (2009) Mutational and functional analysis of Large in a novel CHO glycosylation mutant. *Glycobiology* **19,** 971–986
38. Lefeber, D. J., Schönberger, J., Morava, E., Guillard, M., Huyben, K. M., Verrijp, K., Grafakou, O., Evangeliou, A., Preijers, F. W., Manta, P., Yildiz, J., Grünewald, S., Spilioti, M., van den Elzen, C., Klein, D., Hess, D., Ashida, H., Hofsteenge, J., Maeda, Y., van den Heuvel, L., Lammens, M., Lehle, L., and Wevers, R. A. (2009) Deficiency of Dol-P-Man synthase subunit DPM3 bridges the congenital disorders of glycosylation with the dystroglycanopathies. *Am. J. Hum. Genet.* **85,** 76–86
39. Capaldi, M. J., Dunn, M. J., Sewry, C. A., and Dubowitz, V. (1985) Lectin binding in human skeletal muscle. A comparison of 15 different lectins. *Histochem. J.* **17,** 81–92