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Molecular Pathogenesis of Genetic and Inherited Diseases

Overexpression of *Galgt2* Reduces Dystrophic Pathology in the Skeletal Muscles of Alpha Sarcoglycan-Deficient Mice

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Recent studies have shown that a number of genes that are not mutated in various forms of muscular dystrophy may serve as surrogates to protect skeletal myofibers from injury. One such gene is *Galgt2*, which is also called cytotoxic T cell GalNAc transferase in mice. In this study, we show that *Galgt2* overexpression reduces the development of dystrophic pathology in the skeletal muscles of mice lacking α sarcoglycan (*Sgca*), a mouse model for limb girdle muscular dystrophy 2D. *Galgt2* transgenic *Sgca*$^{-/-}$ mice showed reduced levels of myofiber damage, as evidenced by i) normal levels of serum creatine kinase activity, ii) a lack of Evans blue dye uptake into myofibers, iii) normal levels of mouse locomotor activity, and iv) near normal percentages of myofibers with centrally located nuclei. In addition, the overexpression of *Galgt2* in the early postnatal period using an adeno-associated virus gene therapy vector protected *Sgca*$^{-/-}$ myofibers from damage, as observed using histopathology measurements. *Galgt2* transgenic *Sgca*$^{-/-}$ mice also had increased levels of glycosylation of α dystroglycan with the CT carbohydrate, but showed no up-regulation of β, γ, δ, or ε sarcoglycan. These data, coupled with results from our previous studies, show that *Galgt2* has therapeutic effects in three distinct forms of muscular dystrophy and may, therefore, have a broad spectrum of therapeutic potential for the treatment of various myopathies. *(Am J Pathol 2009, 175:235–247; DOI: 10.2353/ajpath.2009.080967)*

Recent work from a number of laboratories has identified therapeutic targets in skeletal muscle that can, when up-regulated, diminish the severity of disease despite the fact that these molecules do not replace the genetic defect. Such targets cut a wide swath across aspects of muscle biology, ranging from inhibitors of apoptosis (eg, Bcl-2[1]) to stimulators of muscle growth and regeneration (eg, myostatin inhibitors,[2–5] Igf1[6]) to inducers of, or members of, alternative transmembrane protein complexes (eg, utrophin,[7–9] agrin,[10–12] neuregulin,[13] integrin α7B,[14,15] sarcospan,[16] ADAM12,[17,18] and Galgt2[19–21]). This last category contains a glycosyltransferase, Galgt2, which can alter the expression and properties of membrane proteins in skeletal muscle.[19,22] Galgt2, also called the cytotoxic T cell (CT) GalNAc transferase by virtue of its original description in activated CD8+ T cells in mice,[23,24] is a β1,4-*N*-acetylgalactosaminyltransferase that transfers β1,4GalNAc onto a relative small number of glycoproteins and at least one glycolipid to create the CT carbohydrate antigen (Neu5Ac [or Neu5Gc]α2,3[GalNAcβ1,4]Galβ1,4GlcNAcβ-R).[23,25–27] In adult skeletal muscle, Galgt2 protein,[28] and the CT carbohydrate it creates,[29] become confined to the neuromuscular junction and are not present in extrasynaptic regions of the sarcolemmal membrane.

We have created transgenic mice that overexpress Galgt2 specifically in skeletal muscle to maintain extrasynaptic expression of the CT carbohydrate in adult animals.[22] These mice show that the predominant glycoprotein glycosylated by Galgt2 is α dystroglycan,[22] a member of the dystrophin-associated glycoprotein complex[30–32] implicated in numerous forms of muscular dystrophy.[33,34] Importantly, muscles in *Galgt2* transgenic mice show ectopic extrasynaptic expression of synaptic proteins known to bind to dystroglycan, including utrophin, laminin α4, and laminin α5.[22] In light of the changed distribution of these molecules, we have tested the therapeutic effects of *Galgt2* transgene overexpression in two

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mouse models of muscular dystrophy–mdx mice, which lack dystrophin,[35,36] and in dy^W/dy^W mice,[21] which have reduced expression of laminin $\alpha 2$.[37,38] In both instances, Galgt2 overexpression in skeletal muscle significantly reduced the development of pathology related to muscular dystrophy.[19–21] We have also used gene therapy vectors to increase Galgt2 expression in skeletal muscles postnatally. These results also demonstrate efficacy in both the mdx and dy^W/dy^W models, but with fewer molecular and developmental changes than occur in *Galgt2* transgenic animals.[20,21] Here, we have analyzed the effects of Galgt2 overexpression in a third muscular dystrophy model–-mice lacking α sarcoglycan (Sgca), a model for limb girdle muscular dystrophy 2D (LGMD2D).[39]

Sarcoglycans are transmembrane proteins that comprise a portion of the dystrophin-associated glycoprotein complex in cardiac muscle, smooth muscle, and skeletal muscle.[40–42] In skeletal muscle, the sarcoglycan complex is comprised of four proteins (α, β, γ, and δ sarcoglycan), and loss of any of proteins results in a form of Limb Girdle muscular dystrophy (LGMD2D, LGMD2E, LGMD2C, and LGMD2F, respectively). LGMD2C-2F are all autosomal recessive disorders.[43–50] Loss of any one sarcoglycan due to genetic mutation leads to the concomitant loss of the other sarcoglycan proteins in skeletal muscle membrane[50,51] (with exceptions[52]). Mouse models lacking each of these proteins has been made and these mimic important aspects of LGMD, including increased myofiber damage, necrosis, and regeneration, decreased lifespan, and variably increased cardiomyopathy.[39,53–57] Cardiomyopathy is associated with loss of β, γ, and δ sarcoglycan, but not α sarcoglycan, in patients[43] and in mice.[53] This may result from alternative sarcoglycan complexes made in heart between β, γ, and δ sarcoglycan and ε sarcoglycan, a sarcoglycan with significant homology to α sarcoglycan.[58,59] While null mouse models of sarcoglycan deficiency mimic human disease, knock-in mouse models of the most common LGMD2D missense mutation (R77C) do not, suggesting that mice have more robust quality control mechanisms for protein folding and/or protein stability than humans.[60,61] The sarcoglycan complex also tightly associates with sarcospan, another transmembrane protein whose expression is reduced in their absence.[39,62,63] Loss of sarcospan in mice does not result in muscular dystrophy,[64] though overexpression in mdx muscles can reduce disease.[16] Loss of sarcoglycans can also alter the stability of dystroglycan and dystrophin in skeletal muscle,[39,55] though these proteins are still present on the sarcolemmal membrane.[39,55,65] By contrast with sarcospan, loss of dystrophin (in the mdx mouse) results in muscular dystrophy,[35,36,66] as does loss of dystroglycan specifically in skeletal muscle.[67,68]

LGMD2D is the most common sarcoglycanopathy.[43,69] Age of disease onset can range from 3 to 40 years, usually presenting with weakness in the pelvic and girdle muscles, though truncal and distal muscles may also be affected. Disease severity can range from mild impairment with slow progression to severe disability with rapid deterioration.[43,69] While there are treatments aimed at ameliorating symptoms of LGMD2D and related sarcoglycanopathies, including medications to manage cardiac symptoms and pain,[69] there are currently no treatments that have been shown to slow disease progression in patients, though corticosteroids can have positive effects.[70] Moreover, only a scattering of approaches have shown promise in animal models for these diseases.[71–77] Here we provide a proof of principal demonstration that a surrogate gene approach can reduce muscular dystrophy in the $Sgca^{-/-}$ mouse model of LGMD2D, akin to what we have previously shown in mdx mice and dy^W/dy^W mice. This makes *Galgt2* the first surrogate gene approach shown to prevent muscle pathology in these three forms of the disease.

Materials and Methods

Materials

Agarose-bound lectins (*Wisteria floribunda* agglutinin, WFA; and Wheat germ agglutinin, WGA) were purchased from EY Laboratories (San Mateo, CA). AAV1-CMV-Galgt2 was made and purified by Virapure (San Diego, CA). AAV8-CMV-Galgt2 was made by the Viral Vector Core at Nationwide Children's Hospital. Monoclonal antibodies to dystrophin (Dy4/6D3), utrophin (DRP3/20C5), β-dystroglycan (43DAG1/8D5), α-sarcoglycan (Ad1/20A6), β-sarcoglycan (Sarc1/5B1), δ-sarcoglycan (δSarc3/12C1), and γ-sarcoglycan (35DAG/21B5) were obtained from Nova Castra (Newcastle On Tyne, UK). A rabbit polyclonal antiserum to ε sarcoglycan (H-67) and a mouse monoclonal antibody to Plectin 1 (10F6) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody to amino acids 20 to 33 of actin (Sigma, A5060) and a polyclonal antiserum to EHS laminin (Sigma, L9393) were obtained from Sigma (St. Louis, MO). Antibody to α-dystroglycan (IIH6) was obtained from Upstate Biotechnology (Lake Placid, NY). An affinity-purified rabbit anti-peptide antibody to mouse CT GalNAc transferase (Galgt2) was produced in our laboratory (CT68[22]), as was CT2,[29] a monoclonal antibody that recognizes the CT carbohydrate.[24] Secondary antibodies conjugated to horseradish peroxidase, fluorescein isothiocyanate, Cy3, or Cy2 were purchased from Jackson Immunochemicals (Seattle, WA).

Mice

Transgenic mice bearing the CT GalNAc transferase (*Galgt2*) specifically in skeletal muscles via the human skeletal α-actin promoter (CT)[22] were described by us previously, as were *Galgt2* transgenic mdx mice.[19] $Sgca^{-/-}$ mice were originally made by Kevin Campbell and colleagues (HHMI, U. Iowa).[39] For these experiments, we generated four genotypes: $Sgca^{+/-}$, $Sgca^{+/-}$CT, $Sgca^{-/-}$, and $Sgca^{-/-}$CT. All mice were maintained on a similarly mixed background, and all control animals were age-matched littermates.

Infection of Skeletal Muscles with AAV1-CMV-Galgt2 or AAV8-CMV-Galgt2

The tibialis anterior (TA) or gastrocnemius muscle on the left side of 4- to 7-day-old *Sgca*$^{-/-}$ mice was injected with 1 to 5 $\times$ 10^{10} vector genomes (vg) of AAV1-CMV-Galgt2 or AAV8-CMV-Galgt2. AAV8 used in these experiments was isolated in Rhesus macaque strain variant 74 (rh.74). Packaged AAV vectors were produced and purified using the triple transfection method in HEK293 cells and purified by iodixinol chromatography as previously described.[78] In these vectors, the mouse CT GalNAc transferase gene (*Galgt2*) was expressed via the cytomegalovirus promoter (CMV). The gastrocnemius muscle of the left leg was injected with AAV vector in a volume of 50 μl of sterile PBS using a 0.3cc insulin syringe, whereas tibialis anterior muscle was injected with a 25-μl volume. Muscles were always injected near the midpoint of the belly of the muscle. Contralateral muscles (on the right side) were injected with the same volume of sterile PBS (mock-infected). Control infections were also done with AAV1-CMV-lacZ or AAV1-CMV-GFP to confirm that no changes came from nonspecific effects of AAV infection (not shown). After 4 to 6 weeks, mice were sacrificed and muscles dissected and snap-frozen in liquid nitrogen-cooled isopentane, as before.[20,21]

Serum Creatine Kinase Activity

Blood was collected from the tail vein and allowed to clot for 1 hour at 37°C. Clotted cells were centrifuged at 1500 $\times$ *g* for 3 minutes, and serum was collected and analyzed without freezing. Creatine kinase activity assays were done using an enzyme-coupled absorbance assay kit (CK-SL; Diagnostic Chemicals Limited; Charlottetown, PEI, Canada) following the manufacturer's instructions, as previously described.[19] Absorbance was measured at 340 nm every 30 seconds for 4 minutes at 25°C to calculate enzyme activity. All measurements were done in triplicate.

Histology

Muscles were dissected and snap-frozen in liquid nitrogen-cooled isopentane and sectioned at 8 to 10 μm on a cryostat. Sections were either stained with H&E (Sigma; St. Louis, MO), or immunostained with antibodies against the CT carbohydrate (CT2), α-, β-, δ-, ε-, and γ-sarcoglycans, α- and β-dystroglycan, dystrophin, or utrophin as previously described.[22] For use of mouse monoclonal antibodies, a mouse Ab-on-mouse blocking reagent was used (Dako). Antibody used here for δ-sarcoglycan did not stain, but worked well on Western blots, while the antibody used for ε-sarcoglycan stained but was ineffective on Western blot. All other antibodies were used with both methods. Quantification of central nuclei and myofiber diameters was done as previously described.[19–21] Briefly, cross-sections were cut from the midsection (belly) of the muscle at 8 to 10 μm and stained with H&E. For central nuclei measurements, all myofibers (ca. 500 to 1000/section) from each section were counted and the percentage of central nuclei averaged over eight sections per animal. For myofiber diameters, 50 myofibers were measured at random per section for at least five sections per animal, and the data were then averaged to generate the measures of myofiber diameter. Determinations of the presence of central nuclei versus CT carbohydrate overexpression were done at or near the midsection of infected skeletal muscles, again as previously described.[20,21] Fifty myofibers were measured in each condition (expressing or non-expressing) per section for gastroc and TA and 3 to 4 sections were averaged per animal. All myofibers were counted in each section analyzed, and all data obtained was used in determinations of significance. Averages of central nuclei represent analysis of individual myofibers where *n* is always a single muscle from a unique animal, with the exception of AAV experiments, in which data for the AAV1-CMV-Galgt2 and mock-infected muscles were obtained from the same muscles. Identical time exposures were used for all comparisons of immunostaining. Addition of secondary reagents alone did not generate significant staining.

Lectin Precipitation and Immunoblotting

Immunoblotting of whole muscle lysates and of lectin precipitations were done as previously described.[19–21] For lectin precipitations, we extracted identical amounts (50 mg) of minced gastrocnemius muscle from *Galgt2* transgenic (CT) or non-transgenic *Sgca*$^{+/-}$ or *Sgca*$^{-/-}$ mice with Nonidet P-40 buffer (1% Nonidet P-40, 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, and 1:200 protease inhibitor cocktail; Sigma, St. Louis, MO). For whole cell lysates, we extracted a similar weight of muscle with SDS/urea buffer (2% SDS, 4 mol/L urea, 50 mmol/L Tris-HCl, pH 6.8, 2 mmol/L EDTA). Extractions were performed at 4°C with light shaking overnight. Protein amounts were measured using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) versus standard curve made in the appropriate buffer. For lectin pull-downs, 150 μg of NP-40-extracted protein per sample was incubated with 50 μl WGA agarose or WFA agarose at 4°C overnight. Pellets were washed four times in excess volume of NP-40 buffer and boiled in SDS sample buffer. The entire precipitate for each genotype was then resolved by SDS-polyacrylamide gel electrophoresis (PAGE, 4% to 12% linear gradient gels, Invitrogen, CA, or standard 6% or 12% SDS-PAGE gels) and transferred to nitrocellulose membrane (Bio-Rad Laboratories, CA). Antibodies against the CT carbohydrate (CT2), Galgt2, α-, β-, δ-, and γ-sarcoglycans, α- and β-dystroglycan, dystrophin, utrophin, and actin were used in immunoblot analysis as previously described.[19–21,28] For whole cell SDS-urea extracted lysates, extracted proteins were boiled in SDS gel running buffer and 40 μg of cell lysate loaded per lane for separation on SDS-PAGE gels, as above. Antibodies against Galgt2, α-, β-, δ-, and γ-sarcoglycans, α- and β-dystroglycan, dystrophin, utrophin, plectin 1, and actin were used in immunoblot analysis as

previously described.[19,22,28] Secondary antibody alone never generated any positive bands, with the exception of mouse secondary antibodies, which recognized a 50 to 55 kDa band not shown in the results in all genotypes (presumed to be endogenous IgG Fc receptor). Protein levels were compared by scanning densitometry of the appropriate protein bands on Western blots, as previously described.[19] Data from four independent experiments were averaged to obtain measures of changed expression.

Measurement of Gene Expression by TaqMan

Total RNA was isolated using Trizol reagent (Invitrogen; Carlsbad, CA) from gastrocnemius, quadriceps, tibialis anterior, diaphragm, or triceps muscle samples stabilized in RNALater (Ambion; Austin, TX). RNA was further purified on a silica-gel-based membrane (RNeasy-Mini; Qiagen, Valencia, CA) and the integrity of RNA was determined by capillary electrophoresis using 6000 Nano LabChip kit on a Bioanalyzer 2100 (Agilent; Foster City, CA). RNA content was measured using an ND-1000 spectrophotometer (Nanodrop; Wilmington, DE). Only samples with no evidence of RNA degradation were used for TaqMan gene expression studies. A high capacity cDNA archive kit (Applied Biosystems; Foster City, CA) was used to reverse transcribe 3 μg of total RNA as per the manufacturer's guidelines. Samples were subjected to real-time PCR in triplicate using TaqMan ABI 7500 sequence detection system (Applied Biosystems; Foster City, CA) with 18S ribosomal RNA (product no. 4308329, Applied Biosystems) as internal control. Primers and probe against CT GalNAc transferase (*Galgt2*) were custom-made and provided as a 20× reaction mix containing 18μmol/L each of primers (forward primer sequence: 5′-GATGTCCTGGAGAAAACCGAACT-3′; reverse primer sequence: 5′-GCAGCCTGAACTGGTAAGTATTCC-3′) and 5μmol/L of probe (probe sequence: 5′-CCGCCCACCACATCC-3′) (Applied Biosystems). Each 25 μl PCR reaction mix consisted of 1× primer-probe mix, 1× TaqMan Universal PCR master mix (product no. 4304437; Applied Biosystems). After an initial hold of 2 minutes at 50°C to allow activation of AmpErase and 10 minutes at 95°C to activate the AmpliTaq polymerase, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 1 minute. Gene expression was determined as relative changes by the $2^{-}\Delta\Delta$Ct method.[79] Data are presented as fold difference normalized to 18S ribosomal RNA. All measures were done in triplicate for each data point.

Treadmill and Evans Blue Dye uptake

To measure exercise physiology, a treadmill experiment was performed on mice that were 3 months old. All strains of mice (*Sgca*$^{+/-}$, *Sgca*$^{+/-}$CT, *Sgca*$^{-/-}$, and *Sgca*$^{-/-}$CT) underwent a 10-minute run on a horizontal treadmill (Treadmill Simplex II, Columbus Instruments) at a speed of 10 m/min twice a week for 2 to 3 weeks. Time remaining on the treadmill, up to a total time of 10 minutes, was recorded. For Evans blue dye (EBD) uptake analysis, EBD was injected intraperitoneally (50 μg/g body weight in 100 μl of sterile PBS). Five hours later, mouse activity was normalized by subjecting them to 15 minutes of exercise on a treadmill at a constant speed of 15m/min, as before.[39] Thirty-six hours after the injection, mice were sacrificed, and skeletal and heart muscles were snap-frozen in liquid nitrogen-cooled isopentane. Quantification of EBD-positive stained areas in sections of skeletal muscle was done using Olympus imaging program (Slidebook) and Olympus BX61 epifluorescence microscope to quantify the area of the muscle with spontaneous fluorescence (in the rhodamine channel) of EBD. Percentage area with dye uptake in skeletal muscle was calculated by dividing the area of positive EBD staining by the total area of the muscle section analyzed for at least eight sections per muscle.

Statistics

Determinations of significance for comparisons of transgenic animals were done using analysis of variance with pre- and posthoc Bonferroni analysis. Comparisons for AAV-infected muscles versus non-infected ones were done using a paired Student's *t*-test with equal weighting between samples.

Results

Characterization of Galgt2 *Transgenic (CT) α Sarcoglycan-Deficient (*Sgca$^{-/-}$*) Mice*

We first measured the level of Galgt2 transgene (CT) overexpression in *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT skeletal muscle compared with non-transgenic *Sgca*$^{+/-}$ and *Sgca*$^{-/-}$ controls (Figure 1). *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT muscles had equivalent levels of overexpression of CT carbohydrate, the product of Galgt2 activity,[25,27] as determined by immunostaining (Figure 1A). They also had equivalent levels of overexpressed Galgt2 protein, as determined by immunoblotting of whole muscle SDS cell lysates (Figure 1B). For the gastrocnemius muscle (shown in Figure 1), Galgt2 protein was increased by 9.7 ± 1.7-fold in *Sgca*$^{+/-}$CT versus *Sgca*$^{+/-}$ and by 9.3 ± 1.8-fold in *Sgca*$^{-/-}$CT versus *Sgca*$^{-/-}$ ($P < 0.001$ for both comparisons versus non-transgenic littermates, $n = 4$). There was no change in *Sgca*$^{-/-}$ vs. *Sgca*$^{+/-}$ (1.1 ± 0.7). The fold-overexpression of Galgt2 mRNA was also consistent with our previous studies in wild-type, mdx, and dy^{W}/dy^{W} transgenic mice,[19–22] though the amplitude of increased expression was higher (Figure 1C). Galgt2 mRNA levels were increased between 2000- and 10,000-fold in both *Sgca*$^{+/-}$CT versus *Sgca*$^{+/-}$ and *Sgca*$^{-/-}$CT versus *Sgca*$^{-/-}$ comparisons. *Sgca*$^{-/-}$ muscles, however, showed reduced Galgt2 mRNA expression compared with *Sgca*$^{+/-}$, with decreases ranging from two- to sevenfold depending on the muscle (Figure 1D). This stands in contrast to previous mdx versus wild-type and dy^{W}/dy^{W} vs. dy^{W}/+ comparisons, where Galgt2 mRNA expression was increased.[21] The lower denominator in *Sgca*$^{-/-}$ mus-

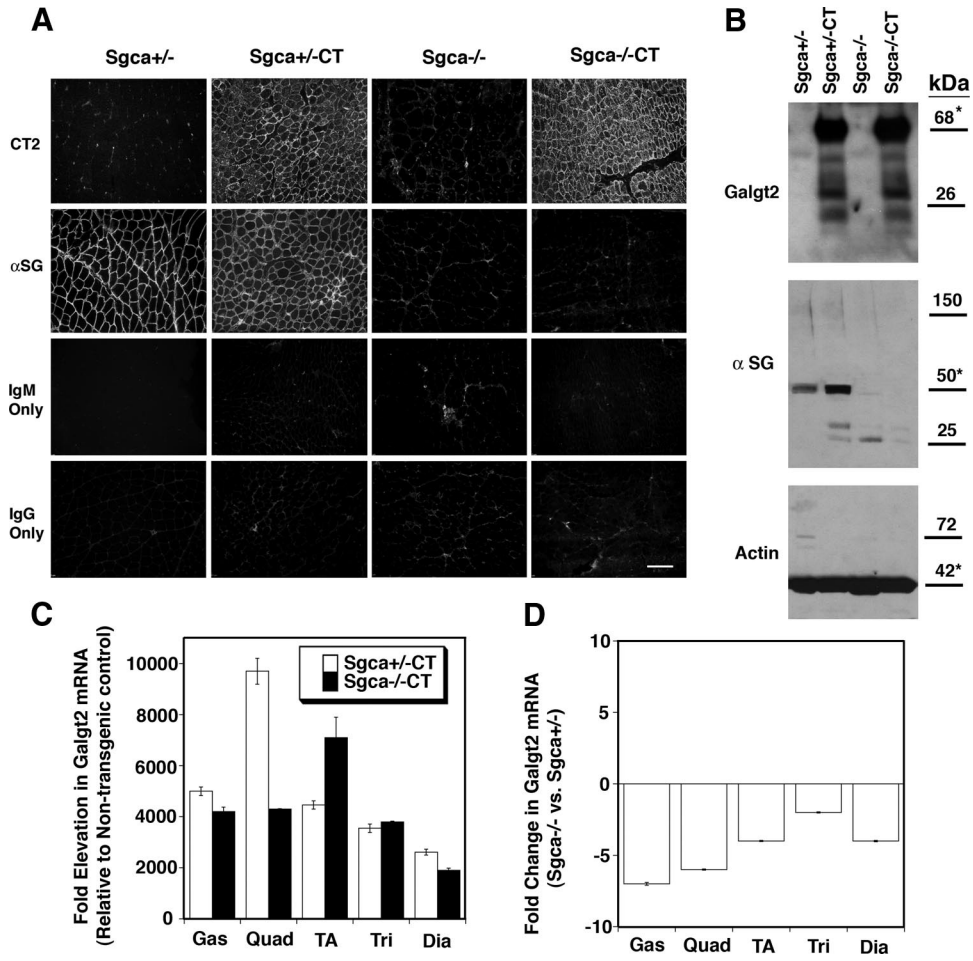

Figure 1. Creation of *Galgt2* transgenic α sarcoglycan-deficient (*Sgca*$^{-/-}$) mice. **A:** *Galgt2* transgenic (CT) mice and non-transgenic littermates that were either heterozygous (*Sgca*$^{+/-}$) or homozygous (*Sgca*$^{-/-}$) for a deletion of α sarcoglycan were stained with a monoclonal antibody to α sarcoglycan or with CT2, an antibody that recognizes the CT carbohydrate. Control secondary antibody for CT2 (IgM only) and α sarcoglycan (IgG only) are also shown. Scale bar = 100 μm. **B:** 40 μg of whole muscle SDS protein lysate was blotted for Galgt2 protein and for α sarcoglycan protein. Actin was used as a control for protein loading and transfer. **Asterisk** indicates native molecular weight for the protein of interest. **C:** *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT muscles show similar increases in Galgt2 mRNA, relative to *Sgca*$^{+/-}$ and *Sgca*$^{-/-}$, respectively, as measured by quantitative RT-PCR. **D:** Galgt2 mRNA was reduced in *Sgca*$^{-/-}$ muscles relative to *Sgca*$^{+/-}$. Gas, gastrocnemius; Quad, quadriceps; TA, tibialis anterior; Tri, triceps; Dia, diaphragm. Errors are SD for 3 to 4 animals per condition (in **C** and **D**).

cle may explain the 0.5–1-log increase in Galgt2 mRNA expression in *Sgca*$^{-/-}$CT compared with mdxCT and dy^{W}/dy^{W}CT muscles.[21] As expected,[39] immunostaining for α sarcoglycan was lost in both *Sgca*$^{-/-}$ and *Sgca*$^{-/-}$CT muscles (Figure 1A), and α sarcoglycan protein was absent in *Sgca*$^{-/-}$ and *Sgca*$^{-/-}$CT muscles by Western blot (Figure 1B). As we have previously reported, *Galgt2* transgenic wild-type muscles have increased levels of sarcoglycan proteins.[19] Consistent with this, *Sgca*$^{+/-}$CT muscles had increased levels of α sarcoglycan relative to Sgca$^{+/-}$ (a 1.6- ± 0.9-fold increase, $n = 4$, Figure 1B). Control secondary antibodies for CT2 (mouse IgM only) or α sarcoglycan (mouse IgG only) yielded only minimal background (Figure 1A), and blots for actin showed equivalent levels of protein loading and transfer (Figure 1B).

Analysis of Muscular Dystrophy in Galgt2 *Transgenic (CT)* Sgca$^{-/-}$ *Animals*

Having made *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT animals, we proceeded to determine whether *Galgt2* transgenic *Sgca*$^{-/-}$ mice would reduced levels of muscle pathology (as we had shown previously in mdxCT[19] and dy^{W}/dy^{W}CT[21] mice). We began by analyzing the histopathology of individual skeletal muscles from *Sgca*$^{+/-}$, *Sgca*$^{+/-}$CT, *Sgca*$^{-/-}$, and *Sgca*$^{-/-}$CT mice (Figure 2, A–C). *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT muscles were reduced in size relative to their *Sgca*$^{+/-}$ and *Sgca*$^{-/-}$ littermates (Figure 2, A and B), a finding we had observed previously in all other *Galgt2* transgenic strains we have studied. Reductions in myofiber diameter were highly significant in the diaphragm, gastrocnemius, quadriceps, tibialis anterior, and triceps for both *Sgca*$^{+/-}$CT muscles (compared with *Sgca*$^{+/-}$) and *Sgca*$^{-/-}$CT muscles (compared with *Sgca*$^{-/-}$) ($P < 0.01$ for all such comparisons, Figure 2B). The percentage reduction ranged from 20% to 50%, with the diaphragm being least affected. Diaphragm, gastrocnemius, and triceps muscles were all reduced as well for *Sgca*$^{-/-}$ muscles compared with *Sgca*$^{+/-}$ ($P < 0.05$ for all). As expected,[39] *Sgca*$^{-/-}$ muscles also showed increased variability in myofiber diameter relative to *Sgca*$^{+/-}$, which is likely due to the presence of smaller regenerating myofibers in these dystrophic muscles (Figure 2B). *Sgca*$^{-/-}$ muscles also had high percentages of myofibers with centrally located nuclei (ca. 80% for all muscles studied), again indicating the presence of muscular dystrophy (Figure 2C). This elevation was highly significant ($P < 0.001$) for all *Sgca*$^{-/-}$ vs. *Sgca*$^{+/-}$ comparisons. By contrast, *Sgca*$^{-/-}$CT muscles, like mdxCT[19] and dy^{W}/dy^{W}CT muscles,[21] showed very reduced levels of myofibers with central nuclei ($P < 0.001$ for all *Sgca*$^{-/-}$CT versus *Sgca*$^{-/-}$ compar-

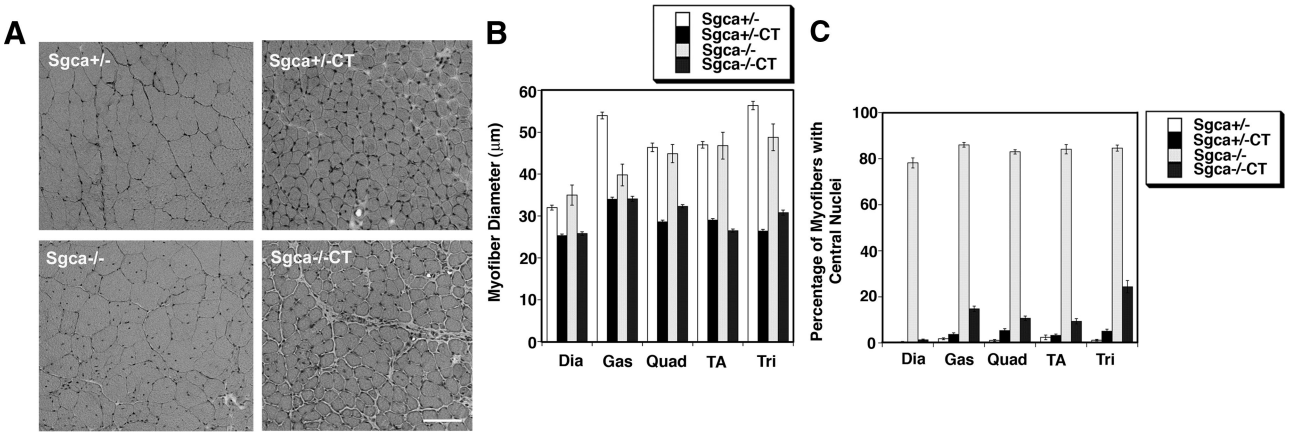

Figure 2. Characterization of muscle pathology in *Galgt2* transgenic $Sgca^{+/-}$ and $Sgca^{-/-}$ mice. **A:** H&E staining of the gastrocnemius muscle of *Galgt2* transgenic (CT) $Sgca^{+/-}$ and $Sgca^{-/-}$ mice and non-transgenic littermates. Scale bar =100 μm. **B:** Myofiber diameters were measured in the diaphragm (Dia), gastrocnemius (Gas), quadriceps (Quad), tibialis anterior (TA) and triceps (Tri) muscle of *Galgt2* transgenic (CT) $Sgca^{+/-}$ and $Sgca^{-/-}$ mice and their non-transgenic, age-matched, littermates. CT muscles were reduced in size, regardless of *Sgca* genotype ($P < 0.01$ for all). **C:** Percentage of myofibers with central nuclei was greatly increased in $Sgca^{-/-}$ muscles ($P < 0.001$ for all versus $Sgca^{+/-}$). *Galgt2* transgene expression significantly lowered central nuclei in $Sgca^{-/-}$ muscles relative to $Sgca^{-/-}$ ($P < 0.001$ for all). Errors are SD from $n = 3$ to 4 animals per genotype (in **B** and **C**).

isons). As with dy^{W}/dy^{W}CT muscles,[21] this level was not quite reduced to wild-type (non-dystrophic) levels, but was nevertheless a highly significant in all muscles studied (Figure 2C).

We next assessed resistance to membrane damage *in vivo* by measuring Evans blue dye uptake into myofibers. Only $Sgca^{-/-}$ muscles had increased dye uptake (Figure 3, A and B). Area of uptake ranged from 5% in the diaphragm to 20% in the gastrocnemius, quadriceps, and tibialis anterior ($P < 0.05$ for all $Sgca^{-/-}$ muscles versus $Sgca^{+/-}$, Figure 3B). By contrast, no $Sgca^{+/-}$, $Sgca^{+/-}$CT, or $Sgca^{-/-}$CT muscle exceeded 1% of total area with dye uptake (Figure 3B), and no significant uptake was observed in the heart (not shown). Thus, overexpression of Galgt2 prevented dye uptake in all $Sgca^{-/-}$ skeletal muscles studied, indicating prevention of muscle damage ($P < 0.05$ for all comparisons of $Sgca^{-/-}$CT to $Sgca^{-/-}$).

Overexpression of the Galgt2 was similarly effective in preventing other whole animal measures of muscular dystrophy in $Sgca^{-/-}$ animals (Figure 4). $Sgca^{-/-}$ mice had, on average, over a tenfold elevation in their serum creatine kinase activity, a measure of acute damage in skeletal muscles throughout the animal (Figure 4A). As with mdx mice,[19,80] this measure is highly variable from mouse to mouse, nevertheless, serum CK activity levels in $Sgca^{-/-}$ mice were significantly elevated compared with $Sgca^{+/-}$ or $Sgca^{+/-}$CT ($P < 0.01$ for both comparisons). $Sgca^{-/-}$CT animals showed a significant decrease in serum CK activity relative to $Sgca^{-/-}$ ($P < 0.05$), reaching levels indistinguishable from $Sgca^{+/-}$ controls. $Sgca^{-/-}$ animals perform poorly on a constant treadmill measure of locomotor activity when compared with $Sgca^{+/-}$ littermates (Figure 4B). We therefore also assessed the ability of the four strains of mice to maintain walking on a treadmill of constant speed for a period of 10 minutes. All $Sgca^{+/-}$ and $Sgca^{+/-}$CT mice studied were able to stay on the apparatus for the duration of the experiment. By contrast, $Sgca^{-/-}$ mice lasted, on average, only 1 minute, while $Sgca^{-/-}$CT mice lasted 9 minutes. Thus, locomotor activity of $Sgca^{-/-}$CT mice was significantly different from $Sgca^{-/-}$ ($P < 0.001$) and approached levels found in non-dystrophic $Sgca^{+/-}$ mice (Figure 4B). In summary, using histopathology measures of muscle regeneration (Figure 2), muscle membrane damage (Figure 3), and measures of muscular dystrophy

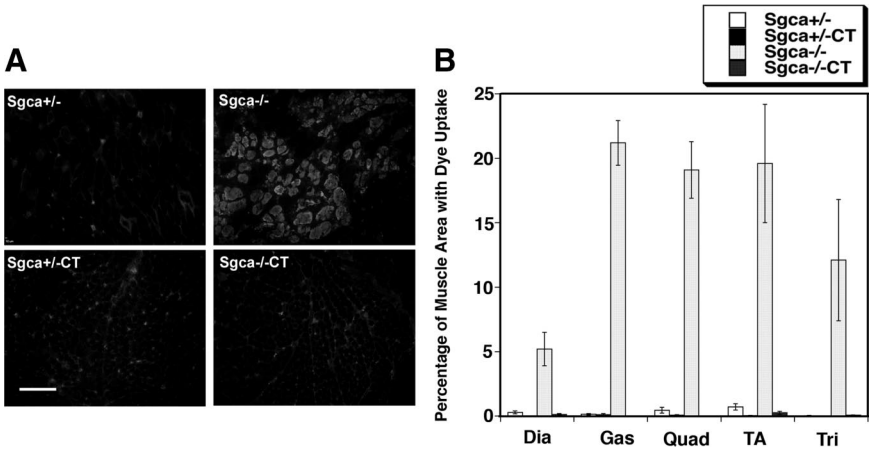

Figure 3. Evans blue dye uptake is reduced in exercised *Galgt2* transgenic $Sgca^{-/-}$muscles. *Galgt2* transgenic (CT) $Sgca^{+/-}$ and $Sgca^{-/-}$ mice were compared with their non-transgenic littermates for uptake of Evans blue dye. **A:** Dye uptake is increased in the quadriceps muscles in $Sgca^{-/-}$ animals and is not elevated in $Sgca^{+/-}$, $Sgca^{+/-}$CT, and $Sgca^{-/-}$CT animals. Scale bar = 100 μm. **B:** Quantification of the percentage of total area with dye uptake in the diaphragm (Dia), gastrocnemius (Gas), quadriceps (Quad), tibialis anterior (TA) and the triceps (Tri) muscle. Dye uptake was reduced in all $Sgca^{-/-}$CT muscles compared with $Sgca^{-/-}$ ($P < 0.05$ for all). Errors are SD for $n = 3$ to 4 animals per condition.

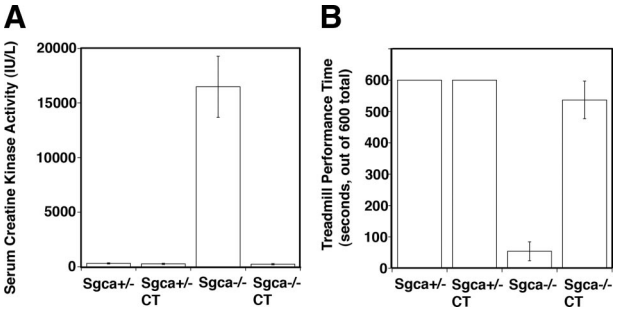

Figure 4. Whole animal measures of muscular dystrophy are reduced in *Galgt2* transgenic *Sgca*$^{-/-}$ mice. *Galgt2* transgenic (CT) and non-transgenic *Sgca*$^{+/-}$ and *Sgca*$^{-/-}$ mice were compared for levels of (**A**) serum creatine kinase activity and (**B**) locomotor activity. **A:** Serum creatine kinase activity was increased by an order of magnitude in *Sgca*$^{-/-}$ animals and this was significantly reduced, to wild-type levels, in *Sgca*$^{-/-}$CT mice ($P < 0.01$ for *Sgca*$^{-/-}$ vs. *Sgca*$^{+/-}$ and $P < 0.05$ for *Sgca*$^{-/-}$CT versus *Sgca*$^{-/-}$). **B:** *Sgca*$^{-/-}$ mice had significantly reduced locomotor activity, and this too was significantly reversed in *Sgca*$^{-/-}$CT mice ($P < 0.001$ for *Sgca*$^{-/-}$ vs. *Sgca*$^{+/-}$ and for *Sgca*$^{-/-}$CT versus *Sgca*$^{-/-}$). Errors are SEM for $n = 11$(*Sgca*$^{+/-}$), 7 (*Sgca*$^{+/-}$CT), 6 (*Sgca*$^{-/-}$), or 17 (*Sgca*$^{-/-}$CT) in (**A**) and $n = 8$ (*Sgca*$^{+/-}$ and *Sgca*$^{-/-}$) or 7 (*Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT) animals in (**B**).

in the whole animal (Figure 4), the overexpression of the *Galgt2* transgene inhibited the development of muscular dystrophy in Sgca$^{-/-}$ mice.

Postnatal Overexpression of Galgt2 Reduces Muscular Dystrophy in Sgca$^{-/-}$ *Muscle without Affecting Myofiber Size*

We infected *Sgca*$^{-/-}$ mice with AAV1-CMV-Galgt2 at 1 week of age in the tibialis anterior and gastrocnemius muscle and then compared infected and contralateral mock-infected muscles for Galgt2 overexpression at 5 weeks of age (Figure 5A). We performed four experiments where we dosed AAV-CMV-Galgt2 injections over a range from 1×10^{10} to 5×10^{10} vector genomes (vg). QRT-PCR showed a range of increased Galgt2 mRNA expression (Figure 5A). At the high end, this equaled levels measured in Galgt2 transgenic *Sgca*$^{-/-}$ mice (Figure 1C). Here, as before,[20] the range of expression in the gene therapy experiments was wider than was seen in transgenic mice (10-fold to 3800-fold). To some extent this reflected altered percentages of myofibers that were infected (which ranged from 25% for lowest expressing muscles to 95% for the highest expressing ones). As before, increased expression was elevated by 0.5 to 1 log compared with similar experiments in mdx muscles.[20] This may be due to a changed denominator in such measures between strains; Galgt2 levels in mdx are increased compared with wild-type,[21] while levels in *Sgca*$^{-/-}$ were reduced (Figure 1D). Despite this larger range, myofibers within muscles with only 40-fold increased Galgt2 expression overexpressed the CT carbohydrate at robust levels and showed reduced to absent dystrophic pathology (Figure 5B). This was assessed by immunostaining for the CT carbohydrate and counterstaining with an antibody to β dystroglycan (Figure 5B), which reveals central nuclei due to non-specific binding of the goat anti-mouse secondary antibody.[20] Similar results were obtained if we analyzed serial sections where

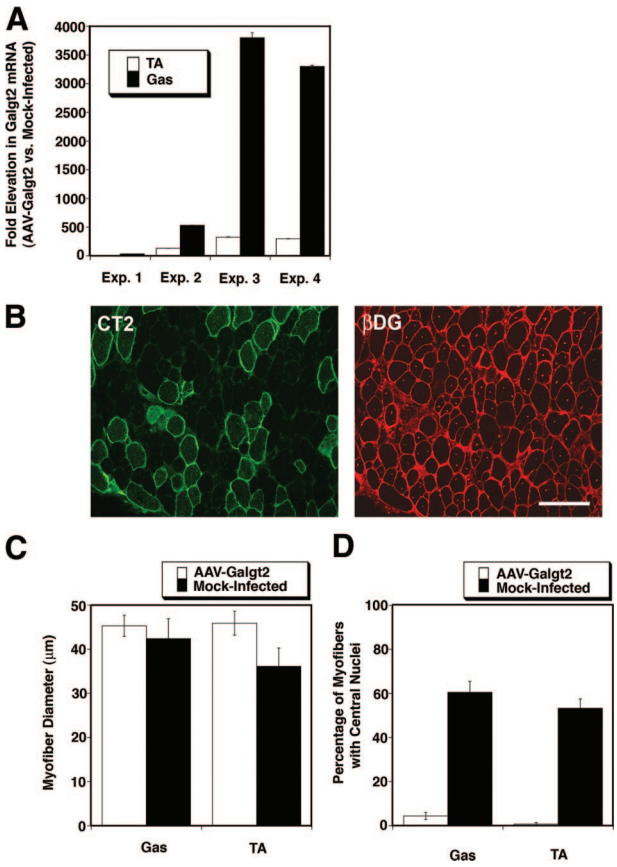

Figure 5. Postnatal overexpression of Galgt2 inhibits the development of muscle pathology in *Sgca*$^{-/-}$ mice. **A:** *Sgca*$^{-/-}$ muscles infected with AAV1-CMV-Galgt2 show a range of increased Galgt2 mRNA expression, as measured by QRT-PCR, in four separate experiments. TA, tibialis anterior; Gas, gastrocnemius. **B:** Infected muscles overexpressing Galgt2 were visualized by immunostaining with CT2, an antibody to the CT carbohydrate (green). Central nuclei were evident on counterstaining with an antibody to β dystroglycan (red). Scale bar =100 μm. **C:** Myofiber diameters were measured in Galgt2-overexpressing myofibers and compared with muscles not overexpressing transgene. **D:** Muscles overexpressing Galgt2 had significantly fewer myofibers with central nuclei than muscles not overexpressing transgene ($P < 0.001$ for comparison in Gastroc and TA). Errors are SD for $n = 3$ to 4 animals per condition in **C** and **D**. Errors in (**A**) are SEM for $n = 9$ measurements per condition.

staining for CT2 (to score overexpression) and hematoxylin and eosin (to score central nuclei) were done on subsequent sections (not shown).

Almost all AAV1-CMV-Galgt2-infected myofibers in all experiments did not display muscle pathology or centrally located nuclei. *Sgca*$^{-/-}$ myofibers overexpressing Galgt2 and *Sgca*$^{-/-}$ myofibers not overexpressing Galgt2 were compared for myofiber diameter (Figure 5C) and myofibers with centrally located nuclei (Figure 5D). As before,[20,21] Galgt2 overexpression at this time point did not inhibit muscle growth; Galgt2-overexpressing myofibers were, on average, significantly larger than non-expressing myofibers in the gastrocnemius (and insignificantly larger the tibialis anterior). This is not a function of Galgt2 expression inducing muscle growth, but instead results from the presence of small regenerating myofibers in non-expressing muscle, which result from dystrophic processes in unprotected muscles. As in previous mdx and dy^{W}/dy^{W} experiments,[20,21] postnatal overex-

pression of Galgt2 prevented histopathology associated with muscular dystrophy; Galgt2 overexpressing myofibers had normal (wild-type) levels of centrally located nuclei, while non-expressing myofibers had significantly elevated levels ($P < 0.001$ for gastroc and TA, Figure 5D). Allowing transgene overexpression to continue for longer time periods of time (up to 3 months) led to the maintenance of these normal CT-positive *Sgca*$^{-/-}$ myofibers (not shown), and this suggests that postnatal Galgt2 overexpression in fact protects skeletal muscles against the development of the dystrophic phenotype.

Glycosylation of α Dystroglycan in Skeletal Galgt2 *Transgenic* Sgca$^{+/-}$ *and* Sgca$^{-/-}$ Muscle

We had previously shown that α dystroglycan was the predominant glycoprotein glycosylated with the CT carbohydrate in *Galgt2* transgenic skeletal muscle, including in transgenic mdx[19] and dy^{W}/dy^{W} mice.[21] This can be shown by differential lectin precipitation of α dystroglycan from non-denaturing muscle cell extracts; Wheat germ agglutinin (WGA) normally binds α dystroglycan by virtue of its dense concentration of sialic acid (and also GlcNAc), but WGA binds very poorly to the CT-glycosylated form of the protein.[19,22] By contrast, *Wisteria floribunda* agglutinin (WFA) only binds CT-glycosylated α dystroglycan,[19,22] which it does because WFA is a βGalNAc-binding lectin and β1,4GalNAc is an essential glycan defining the CT carbohydrate.[81] α dystroglycan was precipitated equally well by WFA in *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT muscle, while it precipitated no α dystroglycan from *Sgca*$^{+/-}$ or *Sgca*$^{-/-}$ muscle. WGA, however, precipitated α dystroglycan from both *Sgca*$^{+/-}$ and *Sgca*$^{-/-}$ muscle (and also *Sgca*$^{-/-}$CT), demonstrating that α dystroglycan was present in all four lysates in roughly equal amounts. Immunoblotting with antibody to the CT carbohydrate (CT2) identified multiple glycoforms of α dystroglycan containing this glycan in WFA precipitates, and these forms were equivalent in *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT muscle. These blots were analyzed using a low (6%) percentage SDS-PAGE gel to clarify the glycoforms present. Thus, loss of α sarcoglycan did not inhibit Galgt2 glycosylation of α dystroglycan with the CT carbohydrate. In general, similar amounts of β dystroglycan co-precipitated with α dystroglycan, to which it binds tightly.[30,31] The one exception was *Sgca*$^{-/-}$CT precipitates, where far more α dystroglycan was present than β dystroglycan (Figure 6). α, β, and γ sarcoglycan, utrophin, and dystrophin were all similarly co-precipitated by WFA in *Sgca*$^{+/-}$CT muscle but were reduced or absent in *Sgca*$^{-/-}$CT muscle (despite abundant α dystroglycan being present). This reduction in sarcoglycans may reflect their reduced expression in *Sgca*$^{-/-}$ muscle. Both utrophin and dystrophin were enriched in WGA and WFA precipitates of *Sgca*$^{+/-}$CT muscle compared with *Sgca*$^{+/-}$ (and to a lesser degree in *Sgca*$^{-/-}$CT compared with *Sgca*$^{-/-}$). While dystrophin was precipitated as a full-length native protein of 427 kDa, utrophin was only precipitated as a proteolytic fragment (51 kDa) and not as a native (ca. 400 kDa) protein.

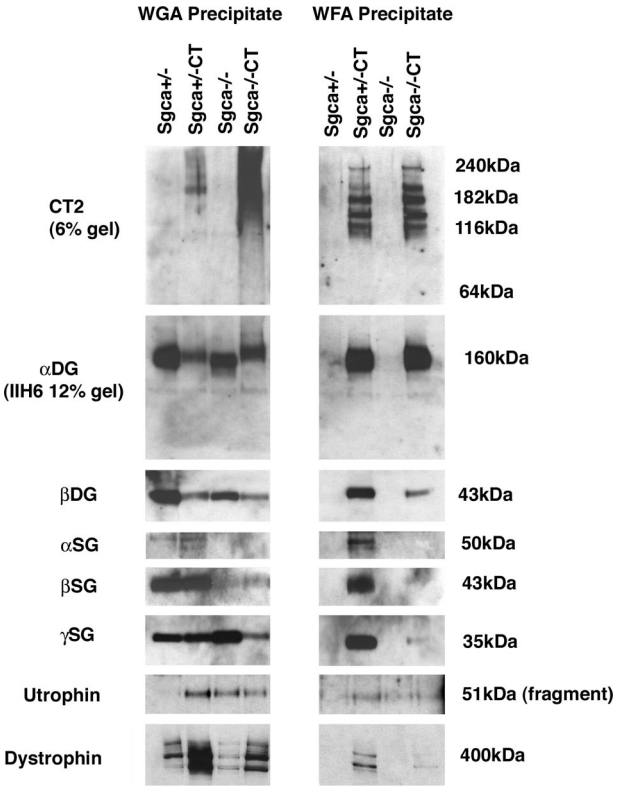

Figure 6. Loss of α sarcoglycan does not affect glycosylation of α dystroglycan with the CT carbohydrate in *Galgt2* transgenic mice. Gastrocnemius muscle was solubilized in non-ionic detergent and precipitated with Wheat germ agglutinin (WGA), a control lectin known to bind α dystroglycan, and *Wisteria floribunda* agglutinin, a βGalNAc-binding lectin that binds the CT-glycosylated form of α dystroglycan. α dystoglycan was glycosylated by Galgt2 such that it could be precipitated by WFA equally well in *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT muscle. In non-CT muscle, WGA bound as much α dystroglycan as WFA did in CT muscle. Precipitates resolved on a low percentage gel (6%) and blotted with CT2 show equivalent glycoforms of α dystroglycan precipitated by WFA in *Sgca*$^{+/-}$CT and *Sgca*$^{-/-}$CT muscle. β dystroglycan was co-precipitated with α dystroglycan in all muscles, but was relatively reduced in WFA precipitates from *Sgca*$^{-/-}$CT, as were β and γ sarcoglycan, due to their reduced overall expression in these lysates (see Figure 7). Full-length dystrophin (427 kDa) and a utrophin protein fragment (51 kDa) were also enriched in CT muscles. Data are representative of three experiments with similar results.

Expression of Sarcoglycans, Dystroglycan, Dystrophin, and Utrophin in Galgt2 *Transgenic* Sgca$^{+/-}$ *and* Sgca$^{-/-}$ *Muscle*

Last, we analyzed the expression of members of the dystrophin-associated glycoprotein complex, including dystrophin, utrophin, α dystroglycan, β dystroglycan, β sarcoglycan, γ sarcoglycan, δ sarcoglycan, and ε sarcoglycan by immunostaining (Figure 7, A and B), immunoblotting (Figure 7C), or both in *Sgca*$^{+/-}$, *Sgca*$^{+/-}$CT, *Sgca*$^{-/-}$, and *Sgca*$^{-/-}$CT muscle. Importantly, β, γ, and ε sarcoglycan were not increased in expression along myofibers in *Sgca*$^{-/-}$CT muscle (Figure 7A), suggesting that the inhibition of muscular dystrophy seen in these muscles (Figure 2–4) did not come from compensation by an alternative sarcoglycan protein complex. Immuno-

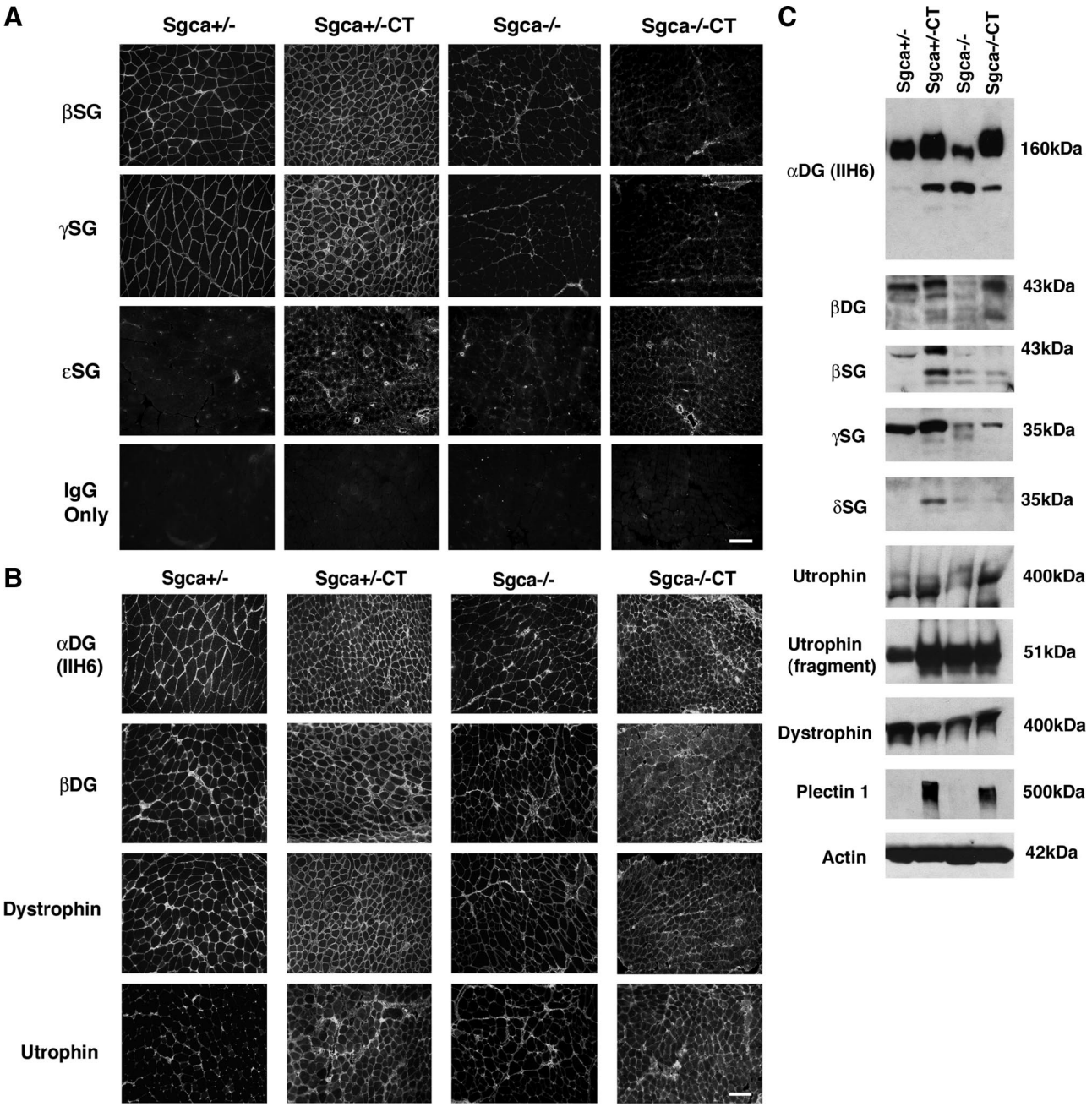

Figure 7. β, γ, δ, and ε sarcoglycan are not overexpressed in *Galgt2* transgenic $Sgca^{-/-}$ muscle. **A:** Immunostaining of gastrocnemius muscle in *Galgt2* transgenic (CT) and non-transgenic $Sgca^{+/-}$ and $Sgca^{-/-}$ mice. β, γ, and ε sarcoglycan were not increased in expression along myofibers in $Sgca^{-/-}$CT muscle, while (**B**) immunostaining of α dystroglycan, β dystroglycan, dystrophin, and utrophin was high in $Sgca^{+/-}$CT and $Sgca^{-/-}$CT muscle. Scale bar =100 μm (**A** and **B**). **C:** 40 μg of SDS whole muscle lysate from gastrocnemius is loaded per lane. β, γ and δ sarcoglycan protein levels were not increased in $Sgca^{-/-}$CT muscle as they were in $Sgca^{+/-}$CT muscle. Utrophin, α dystroglycan, and plectin 1, by contrast, were similarly increased in both $Sgca^{-/-}$CT and $Sgca^{+/-}$CT muscle. Actin is shown as a control for protein loading and transfer.

staining of α dystroglycan, β dystroglycan, dystrophin, and utrophin was high in both $Sgca^{+/-}$CT and $Sgca^{-/-}$CT muscle. Staining showed modestly increased intracellular expression in $Sgca^{-/-}$CT muscle compared to $Sgca^{+/-}$CT. (Figure 7B). Analysis of these same proteins using immunoblotting of whole muscle SDS lysates showed that expression of β, γ, and δ sarcoglycan protein were not increased in $Sgca^{-/-}$CT muscle compared with $Sgca^{-/-}$ (1.03 ± 0.19-fold, 0.98 ± 0.14 and 1.52 ± 0.47-fold, respectively, $n = 4$ for all), while the increase in utrophin and α dystroglycan found in $Sgca^{+/-}$CT muscle (5.4 ± 1.4-fold and 1.22 ± 0.02-fold versus $Sgca^{+/-}$) was still evident in $Sgca^{-/-}$CT muscle (2.03 ± 0.27 and 1.3 ± 0.19-fold versus $Sgca^{-/-}$) (Figure 7C). As previously found,[39] $Sgca^{-/-}$ muscle had reduced levels of α-δ sarcoglycans and also α and β dystroglycan (compared with $Sgca^{+/-}$) (Figures 1B and 7C). Last, we found a dramatic increase in Plectin 1, a protein that can bind both to cytoskeletal elements such as vimetin, desmin, and F-actin[82–84] and to dystroglycan, dystrophin, and

utrophin,[85] in both *Sgca*$^{+/-}$CT (20 ± 10-fold versus *Sgca*$^{+/-}$) and *Sgca*$^{-/-}$CT muscle (14.0 ± 3-fold versus *Sgca*$^{-/-}$). Actin was used as a control for protein loading and transfer and showed equivalent signals (no more than 9% difference between strains) in all blotting experiments.

Discussion

We have provided proof of principal evidence that overexpression of the *Galgt2* gene in skeletal muscles of α sarcoglycan-deficient (*Sgca*$^{-/-}$) mice lessens the development of dystrophic skeletal muscle pathology. Overexpression of Galgt2 inhibits the increased incidence of myofibers with central nuclei, the increased uptake of Evans blue dye into myofibers, the increase in serum creatine kinase activity, and the decrease in locomotor activity that occur in *Sgca*$^{-/-}$ animals. Thus, by a variety of measures that pertain to muscle degeneration and regeneration, membrane permeability, and muscle activity, Galgt2 overexpression reduces dystrophic changes to near wild-type levels. Use of gene therapy techniques to overexpress Galgt2 in skeletal muscles of young adult *Sgca*$^{-/-}$ mice led to equivalently dramatic improvements in histopathology measures, but without impacting important developmental aspects such as muscle growth. These experiments suggest that Galgt2 should be considered as a therapeutic target for limb girdle muscular dystrophy 2D (LGMD2D).

The results we have shown here are very similar to our previous results showing reduced muscular dystrophy in *Galgt2* transgenic mdx mice[19,20] and dy^{W}/dy^{W} mice.[21] As such, the experiments presented suggest that Galgt2 may have a broader impact on muscular dystrophies than other surrogate gene therapies previously tested. Many therapies that have been shown to be effective in one of these mouse muscular dystrophy models have not been as effective when tested in others. For example, Bcl2 overexpression ameliorates disease in dy/dy mice but not in mdx mice,[1] ADAM12 overexpression is effective in mdx mice but not in dy/dy mice,[18,86] myostatin inhibition is effective in boosting muscle mass in mdx mice but not in dy/dy or *Sgca*$^{-/-}$ mice,[2–4,87,88] stimulation of calcineurin signaling is effective in mdx mice but not in dy/dy mice,[89–91] and integrin α7B overexpression ameliorates disease in mdxutrn$^{-/-}$ mice but not in *Scgd*$^{-/-}$ mice.[14,92] By contrast, proteosome inhibitors appear to promote membrane localization of sarcoglycan complexes in Sgca-deficient cells[93] and have also been shown to be therapeutic in mdx mice.[94,95] Perhaps the most promising surrogate gene therapy thus far for LGMD2D has been transgenic overexpression of ε sarcoglycan, which appears to inhibit muscular dystrophy for many months and increase expression of β-δ sarcoglycan on the muscle membrane.[96] However, endogenous ε sarcoglycan, which other groups have reported is present in skeletal muscle,[39,59] does not seem to inhibit the disease process or maintain elevated β-δ sarcoglycan levels in α sarcoglycan-deficient mice,[39,59] even though it clearly can associate with these subunits in *Sgca*$^{-/-}$ muscle.[97] In our study, we found no increase in ε sarcoglycan or in β, γ, or δ sarcoglycan to suggest that alternative sarcoglycan complexes were responsible for the therapeutic effects of the *Galgt2* transgene.

While all of these models show defects in a member of the dystrophin-associated glycoprotein complex or laminin α2, which one presumes are all linked together in a functional complex, many of these differences in therapeutic efficacy could be nevertheless be attributed to fundamental differences in the disease models. For example, dy^{W}/dy^{W} mice have very impaired muscle regeneration, while mdx mice do not.[18,98,99] Similarly, mdx muscles show decreased resistance to injury in eccentric contraction paradigms, while *Sgcg*$^{-/-}$ muscles, which also lack Sgca, do not.[100] Thus, there are distinct differences in these disease models, despite the fact that they all possess deletions in proteins that work, at least to some extent, in concert. As such, differences in therapeutic efficacy of different surrogate genes may also reflect underlying mechanistic differences that occur in different forms of muscular dystrophy. Galgt2 appears to overcome such differences in a manner that many other therapies do not.

The fundamental questions for Galgt2, going forward, are: 1. How does altering membrane glycosylation reduce muscular dystrophy in these different disease forms and 2. How can these effects be translated into a therapy? As to question 1, it is certainly beneficial from a mechanistic standpoint that glycosylation, by its very nature, affects multiple protein targets and multiple protein functions. While Galgt2 overexpression in skeletal muscle predominantly leads to increased CT-glycosylation of only several molecules, in particular α dystroglycan[22,28,101] and an as yet defined glycolipid,[21] these glycosylation changes could impact multiple biological systems that would be beneficial in these diseases. It is clear from the seminal work of Ervasti, Campbell and colleagues that the glycans on α dystroglycan are required for the binding of extracellular matrix proteins, including laminins, agrin, perlecan, and also neurexins.[30,31,102] Preliminary studies from our lab suggest that CT-glycosylation of α dystroglycan increases extracellular matrix binding. As such, CT-glycosylation of α dystroglycan may tighten extracellular matrix-membrane adhesion via the dystrophin-associated glycoprotein complex. The lectin precipitation studies shown here suggest a tight association of dystrophin and perhaps also utrophin with dystroglycan in CT transgenic muscles, which could be consistent with such a notion. Similarly, the increased expression of Plectin1, which could serve to link cytoskeletal-associated proteins including dystrophin and utrophin with membrane proteins such as dystroglycan,[85] may facilitate these interactions. Regardless of the complexities of the mechanism involved, this study, coupled with studies in mdx[19,20] and in dy^{W}/dy^{W} mice,[21] suggest that elevation of Galgt2 expression in skeletal muscles would be therapeutic in these diseases.

How to answer question 2 is of primary importance going forwards. We have undertaken two strategies in this regard. First, we have developed a gene therapy approach to overexpress the Galgt2 cDNA using adeno-

associated virus. This approach works to prevent dystrophic changes in infected muscles in *Sgca*$^{-/-}$, mdx,[20] and dy^{W}/dy^{W} mice.[21] Future work will entail developing methods to allow systemic delivery of such gene therapy vectors using the human Galgt2 cDNA driven by muscle- or muscle/heart-specific promoters. A second approach is to use pharmacology. *Galgt2* gene expression is very low in skeletal muscle,[21] consistent with the synaptic distribution of the protein[22,28] and its carbohydrate product.[29] Identification of drugs that would increase extrasynaptic expression in muscle, therefore, is another approach to stimulate the therapeutic effects of Galgt2 overexpression.

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