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Disruption of *Dag1* in Differentiated Skeletal Muscle Reveals a Role for Dystroglycan in Muscle Regeneration

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Summary

Striated muscle-specific disruption of the dystroglycan *(DAG1)* gene results in loss of the dystrophin-glycoprotein complex in differentiated muscle and a remarkably mild muscular dystrophy with hypertrophy and without tissue fibrosis. We find that satellite cells, expressing dystroglycan, support continued efficient regeneration of skeletal muscle along with transient expression of dystroglycan in regenerating muscle fibers. We demonstrate a similar phenomenon of reexpression of functional dystroglycan in regenerating muscle fibers in a mild form of human muscular dystrophy caused by disruption of posttranslational dystroglycan processing. Thus, maintenance of regenerative capacity by satellite cells expressing dystroglycan is likely responsible for mild disease progression in mice and possibly humans. Therefore, inadequate repair of skeletal muscle by satellite cells represents an important mechanism affecting the pathogenesis of muscular dystrophy.

Introduction

Muscular dystrophies are a diverse group of inherited disorders characterized by progressive muscle weakness and wasting (Bushby, 2000; Cohn and Campbell, 2000). The dystrophin-glycoprotein complex (DGC) is a multisubunit complex comprised of peripheral and integral membrane proteins, which links the cytoskeleton to the extracellular matrix. The proteins that comprise the DGC are the intracellular proteins (dystrophin and the syntrophins) and the sarcolemmal proteins (dystroglycans [α and β subunits], sarcoglycans [α, β, γ, and δ subunits], and sarcospan). Disruption of this linkage, due to mutations of dystrophin or the sarcoglycans, causes sarcolemmal instability and contraction-induced tears to the sarcolemma (Alderton and Steinhardt, 2000). Subsequent to sarcolemmal damage, increased calcium influx has been observed (Alderton and Steinhardt, 2000), which in turn may render the muscle fibers susceptible to necrosis, the major pathological feature of muscular dystrophies (for review see Bushby, 2000; Cohn and Campbell, 2000).

Dystroglycan was first identified in skeletal muscle as a central component of the DGC (Ervasti et al., 1990). It is composed of α and β subunits, which are posttranslationally derived from a single mRNA species encoded by a single gene (Ibraghimov-Beskrovnaya et al., 1992). In skeletal muscle, α-dystroglycan is an extracellular membrane glycoprotein that binds laminin-2 in the extracellular matrix (Sunada et al., 1994), whereas β-dystroglycan is an integral membrane glycoprotein that anchors α-dystroglycan to the membrane. When the gene encoding dystroglycan was cloned, expression pattern analysis suggested that dystroglycan has important roles outside of muscle, which cover a broad range of biological inquiry (for review see Henry and Campbell, 1999). Generation of dystroglycan null mice indicated that dystroglycan is required for the formation of Reichert's membrane during early embryonic development, disruption of which causes embryonic lethality (Williamson et al., 1997). Subsequent studies have demonstrated that dystroglycan is required for the formation of the subendodermal basement membrane in embryoid bodies (Henry and Campbell, 1998). Mice chimeric for dystroglycan expression in all tissues have been reported to develop severe muscular dystrophy (Cote et al., 1999). Recently it has been demonstrated that tissue-specific disruption of dystroglycan in the brain is sufficient to cause neuronal migration errors that closely resemble the brain abnormalities seen in congenital muscular dystrophy (Moore et al., 2002). Moreover, we have shown that mutations in glycosyltransferases (POMGnT1, fukutin, and LARGE) result in posttranslational disruption of dystroglycan-ligand interactions in the skeletal muscle of patients with a severe phenotype of congenital muscular dystrophy (Michele et al., 2002).

In order to better understand the function of dystroglycan in mature, differentiated skeletal muscle, we have used the Cre-loxP system to specifically inactivate the dystroglycan gene in skeletal muscle using the muscle creatine kinase (MCK) promoter in transgenic mice (Bruning et al., 1998). Mice with skeletal muscle-specific disruption of dystroglycan (*MCK-DG null*) develop myonecrosis around 4–6 weeks of age, indicating that loss of dystroglycan and accompanying disruption of the DGC at the sarcolemma alone is sufficient to cause muscular dystrophy. Given the previously demonstrated findings in functionally dystroglycan null patients and mice (Michele et al., 2002) we were surprised to find that despite ongoing cycles of muscle degeneration, *MCK-DG null* mice with advanced age do not exhibit

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signs of fibrosis and fat replacement generally seen in other mouse models of muscular dystrophy. Instead, they present with a remarkably mild phenotype with signs of efficient muscle regeneration and marked skeletal muscle hypertrophy similar to findings observed in early stages of human and mouse models with muscular dystrophy.

Here we find that dystroglycan is expressed in satellite cells. During cycles of degeneration, satellite cells are repetitively activated in *MCK-DG null* mice. Because satellite cells have not been targeted by the MCK Cre promoter, this activation leads to expression of dystroglycan and other components of the DGC in muscle fibers undergoing regeneration. Our results reveal the first in vivo evidence that self-renewing potential does not necessarily decrease in aging mice undergoing repetitive cycles of de- and regeneration during the course of muscular dystrophy. Our findings provide new insights into the pathogenesis of muscular dystrophy by demonstrating that perturbation of satellite cell function leading to insufficient repair of skeletal muscle cells represents a major pathogenetic mechanism in the course of muscular dystrophy.

Results

Generation of Dystroglycan (lox) Mice and Breeding Strategy

Dystroglycan (DG), the central component of the dystrophin-glycoprotein complex (DGC), is expressed in virtually every tissue in humans and other mammals. To investigate selectively the role of dystroglycan expression and function in striated muscle of the mouse, we have employed conditional gene inactivation using Cre-loxP-mediated recombination (Gu et al., 1994). To this end, we generated a mouse bearing a "floxed" allele of dystroglycan in which loxP sites were introduced flanking exon 2 of the mouse dystroglycan gene. Breeding of DG (lox/+) and MCK-Cre (+/−) mice resulted in double heterozygous animals that were then bred with DG (lox/+) mice to obtain DG (lox/lox):MCK-Cre offspring, that is, mice with a disruption of dystroglycan specifically in striated muscle (*MCK-DG null*). There was no embryonic lethality, and these mice were obtained with the expected frequency of 12.5% for a trait requiring two independent loci. Newborn *MCK-DG null* mice were indistinguishable from their DG (lox/lox) and wild-type littermates and grew normally. In a different breeding strategy, we generated mice heterozygous for the floxed gene and for the original dystroglycan knockout allele (L/−). These mice were then bred to MCK-Cre mice in order to obtain MCK-Cre/L/− mice. However, no difference in protein expression pattern as well as in phenotype was observed between MCK-Cre/L/− and MCK-Cre/L/L mice. RT-PCR showed loss of mRNA for dystroglycan in *MCK-DG null* mice. Western blot analysis and immunohistochemistry revealed loss of dystroglycan in skeletal muscle of *MCK-DG null* mice (Cre/L/L and Cre/L/−) and normal expression in wild-type, heterozygous dystroglycan null mice (L/−), and homozygous floxed mice (L/L) (Figure 1). Interestingly, dystroglycan staining was solely lost from the sarcolemma in skeletal muscle and still preserved in myotendinous junctions and neuromuscular junctions, suggesting that MCK-Cre either might not be active in myonuclei associated with these sites or that dystroglycan half-life and turnover might be different (not shown).

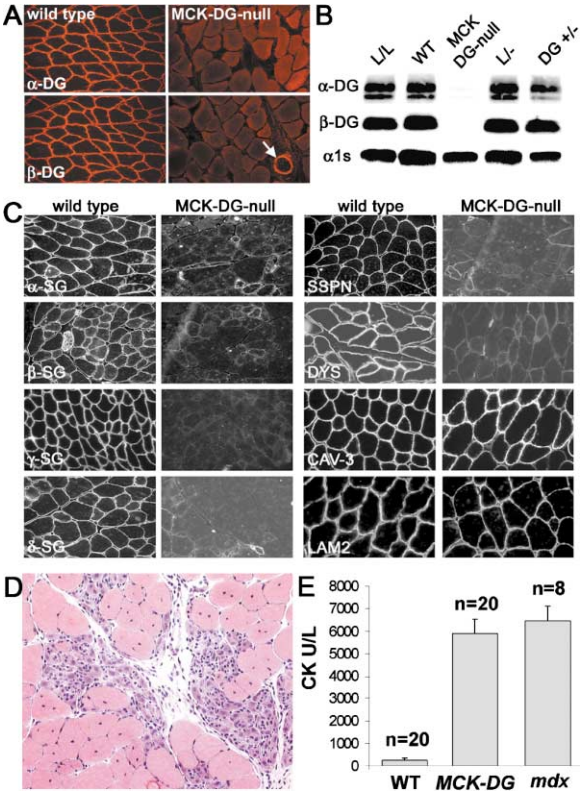

Figure 1. Skeletal Muscle-Specific Disruption of Dystroglycan

(A) Immunohistochemical analysis reveals loss of α- and β-dystroglycan (αDG, βDG) at the sarcolemma of *MCK-DG null* mice. Arrow marks dystroglycan in vascular smooth muscle.

(B) Western blot analysis of skeletal muscle KCL-washed microsomes shows almost complete loss of dystroglycan in *MCK-DG null* mice. In contrast, normal levels of dystroglycan expression can be observed in wild-type (wt), heterozygous dystroglycan null (DG+/−), heterozygous floxed/dystroglycan null (L/−), and homozygous floxed (L/L) mice. Note that α1S calcium channel is normally expressed in all membrane preparations studied.

(C) Absence of dystroglycan leads to perturbation of the DGC (DYS, dystrophin; α-SG, α-sarcoglycan; β-SG, β-sarcoglycan; γ-SG, γ-sarcoglycan; δ-SG, δ-sarcoglycan; SSPN, sarcospan). Note that caveolin-3 (cav-3) and laminin α2 (LAM2) expression is not affected by loss of dystroglycan.

(D) Hematoxylin- and eosin-stained sections of tibialis anterior muscle demonstrate hallmarks of muscular dystrophy in *MCK-DG null* mice at 6 weeks of age.

(E) *MCK-DG null* mice exhibit significant elevation of serum creatine kinase (error bars represent SD).

MCK-DG Null Mice Show Disruption of the DGC and Develop Muscular Dystrophy

Immunohistochemical analysis from 6-week-old *MCK-DG null* mice revealed that the absence of dystroglycan expression in skeletal muscle was accompanied by loss of dystrophin, dystrobrevin, and the sarcoglycan-sarcospan complex (Figure 1). Interestingly, in *MCK-DG null* mice, dystrophin sarcolemma localization varied from normal staining (e.g., tibialis) to reduced staining (e.g.,

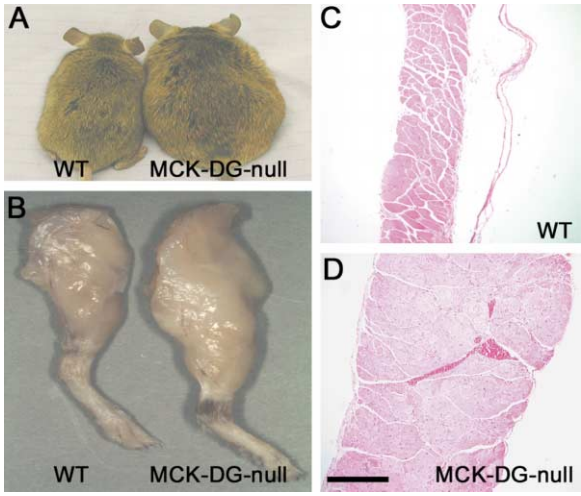

Figure 2. Gross and Histological Analysis of Skeletal Muscle Hypertrophy

MCK-DG null mice are larger (A) and the musculature of the hindlimb is increased in size (B). Note the significant increase in thickness and increased muscle fiber diameter in diaphragm of *MCK-DG null* mice (C and D). Bar represents 350 μm.

quadriceps) depending on muscle group. The immunofluorescent data were confirmed by Western blot studies of KCL-washed microsomal fractions (data not shown). In addition, neuronal nitric oxide synthase and α1-syntrophin were also absent from the sarcolemma in *MCK-DG null* mice (data not shown). Expression of laminin α2 was normal in *MCK-DG null* mice, which can most likely be explained by normal sarcolemmal expression levels of α7 integrin (data not shown), which serves as an additional receptor for laminin α2 in skeletal muscle.

Histological studies of various skeletal muscles, including quadriceps, gastrocnemius, tibialis anterior, biceps, gluteus maximus, and diaphragm, displayed histological hallmarks of muscular dystrophy such as myonecrosis, central nucleation, and variation of fiber size in *MCK-DG null* mice at around 6 weeks of age. Furthermore, significant elevation of serum creatine kinase was observed in these mice (Figure 1).

MCK-DG Null Mice Develop Significant Skeletal Muscle Hypertrophy with Age

As *MCK-DG null* mice aged, we observed an interesting phenomenon. In marked contrast to other models of muscular dystrophy such as the dystrophin-deficient *mdx* mice or the sarcoglycan null mice, which show significant muscle atrophy at 12–18 months of life, *MCK-DG null* mice developed substantial hypertrophy of the skeletal muscle. As shown in Figure 2 and Table 1, 15-month-old *MCK-DG null* mice were larger than littermate controls only carrying the Cre transgene. Similar results were observed when *MCK-DG null* mice were compared to age-matched homozygous dystroglycan-floxed mice (data not shown). Determination of wet muscle weights showed widespread increase of muscle mass, particularly in the quadriceps and gastrocnemius muscles, where the weight doubled (Table 1). Histological evaluation of muscle fibers as well as the full diaphragm thickness confirmed the observed increase in wet muscle weight (Figure 2). Fiber diameter of quadriceps and soleus muscle in 18-month-old MCK transgenic mice revealed a mean value of 35 ± 8 μm and 23 ± 5 μm, respectively, as compared to 79 ± 15 μm and 56 ± 18 μm in quadriceps and soleus muscle of 18-month-old *MCK-DG*-null mice ($p < 0.0001$). Moreover, up to 95% of the muscle fibers exhibited centrally located nuclei, indicating that regeneration had occurred in these fibers.

Table 1. Increased Muscle Weight in *MCK-DG Null* Mice

	MCK-Cre	*MCK-DG Null*
Quadriceps[a]	300.5 ± 10.3	620.5 ± 11.7[b]
Soleus	31.4 ± 1.2	36.4 ± 2.1
EDL	22.2 ± 1.1	45.3 ± 1.5[b]
Gastroc.	460.5 ± 11.7	922.8 ± 9.8[b]
Triceps	280 ± 5	420 ± 20[b]
Heart	190.4 ± 12.1	290.4 ± 26.6[b]
Body weight[c]	40.32 ± 1.90	47.24 ± 1.30[b]

Male littermate mice carrying only the MCK-Cre transgene and *MCK-DG null* wet muscle weights. Mean volumes were compiled from five mice of each genotype at 15 months of age. In contrast to MCK-Cre, *MCK-DG null* mice show widespread increase in muscle weight.

[a] Muscle weight in milligrams; mean ± SD ($n = 5$)

[b] Statistically significant $p < 0.001$.

[c] Body weight in grams; mean ± SD ($n = 5$)

Synchronized Reexpression of Dystroglycan during Regenerative Cycles

The unexpected finding of skeletal muscle hypertrophy and morphological signs of muscle regeneration even in 15- to 18-month-old *MCK-DG null* mice prompted us to more closely investigate this phenomenon. One interesting observation we made during our analysis of *MCK-DG null* mice was that clusters of dystroglycan-positive muscle fibers were consistently present in older mice. Therefore, we studied expression levels of dystroglycan in *MCK-DG null* mice at various ages using an immunohistochemical approach. Remarkably, dystroglycan was expressed at normal levels in newborn *MCK-DG null* mice (Figure 3A). By around 4 weeks of age, dystroglycan expression was nearly absent from the sarcolemma (fewer than 5% fibers were positive). In contrast, analysis of dystroglycan expression in *MCK-DG null* mice at numerous intervals between 10 weeks and 18 months of age revealed muscle fiber groups expressing dystroglycan with a frequency of dystroglycan-positive fibers of around 40% and 54%, respectively (Figure 3A). Dystroglycan-positive clusters were present only after the onset of the dystrophic process, which began at about 6 weeks of age. These results led us to hypothesize that dystroglycan might be reexpressed during regenerative cycles caused by the ongoing dystrophic process and that activated satellite cells or other myogenic precursor cells, which are not targeted by the MCK-Cre promoter, might initiate the expression of dystroglycan during the course of regeneration.

Consequently, we analyzed frozen crosssections of skeletal muscle from wild-type and *MCK-DG null* mice for satellite cell expression of dystroglycan and M-cadherin, a cell adhesion molecule that has been reported to

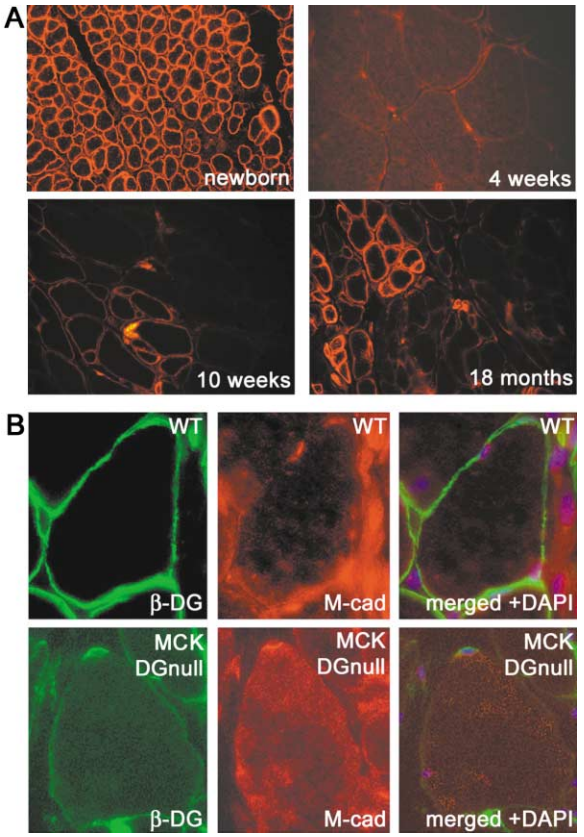

Figure 3. Dystroglycan Is Expressed in Satellite Cells

(A) Analysis of dystroglycan expression at various ages in *MCK-DG null* mice. Interestingly, dystroglycan is normally expressed in newborn skeletal muscle. In contrast, at 4 weeks of age, dystroglycan is nearly absent from the sarcolemma. Subsequently, *MCK-DG null* mice develop muscular dystrophy with ongoing cycles of necrosis and regeneration. After onset of the dystrophic process, clusters of dystroglycan-positive muscle fibers are observed in *MCK-DG null* mice between 10 weeks and 18 months of age (antibody AP 83 against β-dystroglycan).

(B) Expression of dystroglycan in satellite cells of wild-type and *MCK-DG null* mice. Double labeling with M-cadherin reveals enhanced membrane staining of the satellite cell toward the cytoplasm of the myofiber, whereas dystroglycan expression seems to be enhanced toward the basal lamina. The merged image including DAPI demonstrates the single nucleus of the satellite cell.

be expressed in quiescent, activated, and proliferating satellite cells (Irintchev et al., 1994). Figure 3B reveals dystroglycan expression in satellite cells of *MCK-DG null* mice, with an enhanced membrane labeling toward the basal lamina of the parent muscle fiber. Double labeling with M-cadherin, which has enhanced staining at sites of contact between satellite cells and the parent muscle fiber, and nuclear staining using DAPI identified the dystroglycan-positive cell as a satellite cell (Figure 3B). Moreover, in vitro isolation of satellite cells showed positive staining for dystroglycan in myoblasts of homozygous floxed (L/L) and *MCK-DG null* mice and loss of dystroglycan in differentiated myotubes of *MCK-DG null* mice (not shown).

Given these data, we sought to induce more extensive and synchronized regeneration in wild-type and *MCK-DG null* mice by injecting cardiotoxin into the calf muscle. Remarkably, dystroglycan was strongly expressed in regenerating fibers 4 days after cardiotoxin injection (Figure 4A), and strong sarcolemmal expression of dystroglycan was still observed 14 days after injection of cardiotoxin. In contrast, 28 days after administration of cardiotoxin, expression of dystroglycan was markedly reduced in *MCK-DG null* muscle, indicating that MCK-Cre-mediated recombination had again occurred in these muscle fibers (Figure 4A). These data supported our hypothesis that activated satellite cells during muscle regeneration led to reexpression of dystroglycan in dystrophic muscle of *MCK-DG null* mice.

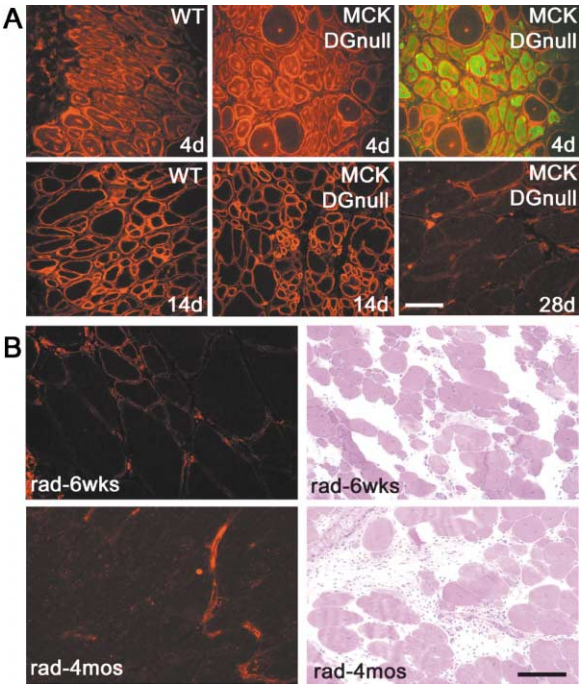

Figure 4. Reexpression of Dystroglycan in Regenerating Fibers of *MCK-DG Null* Mice

(A) Challenging muscle fibers with cardiotoxin leads to synchronized reexpression of dystroglycan in *MCK-DG null* mice at 4 and 14 days of toxin injection. Actively regenerating fibers are positive for neonatal myosin (top right, green color). In contrast, 28 days after administration of cardiotoxin, expression of dystroglycan was markedly reduced, indicating that MCK-Cre-mediated recombination has again occurred in these muscle fibers (bottom right). Bar represents 50 μm.

(B) Exposure of single legs from *MCK-DG null* mice to 25 Gy irradiation (rad) leads to almost complete loss of dystroglycan expression 6 weeks and 4 months after irradiation. Morphological analysis of skeletal muscle demonstrates the development of a more severe dystrophic phenotype with endomysial fibrosis and adipose tissue replacement. Bar represents 50 μm.

In order to more firmly establish that satellite cells are responsible for the dystroglycan staining observed in *MCK-DG null* mice, we exposed single legs of 4-month-old mice to 18 Gy and 25 Gy of irradiation to inactivate the satellite cell population. Analysis of dystroglycan expression 3 weeks, 6 weeks, and 4 months after irradiation showed complete absence of dystroglycan at the sarcolemma of *MCK-DG null* mice. Moreover, morphological analysis exhibited a considerably more severe dystrophic phenotype with endomysial fibrosis and adipose tissue replacement (Figure 4B).

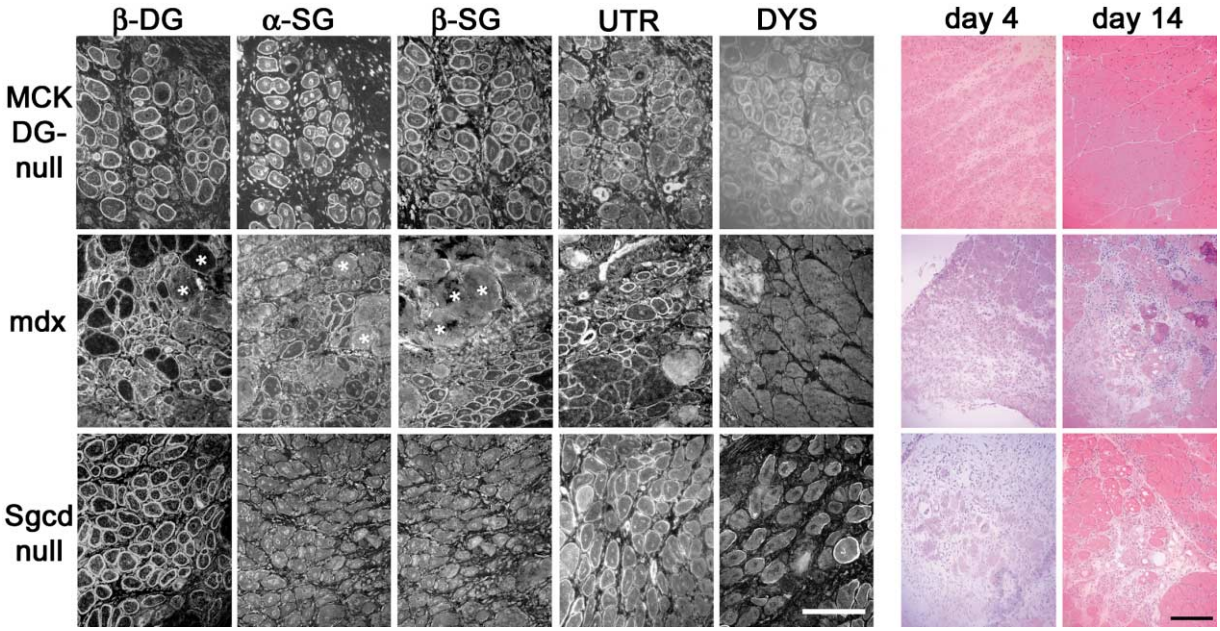

Figure 5. Characterization of the DGC in Regenerating Fibers of Various Muscular Dystrophy Mouse Models

Analysis of DGC expression 4 days after cardiotoxin injection reveals normal expression levels for β-dystroglycan (β-DG), α-sarcoglycan (α-SG), β-sarcoglycan (β-SG), utrophin (UTR), and dystrophin (DYS) in *MCK-DG null* mice. In contrast, upregulation of utrophin compensates in part for the loss of dystrophin during the regeneration process in *mdx* mice (middle), as shown by almost normal expression levels for dystroglycan and sarcoglycan. However, some *mdx* fibers (asterisks) express reduced amounts of dystroglycan and sarcoglycan. *Sgcd null* mice lack upregulation of a protein to compensate for δ-sarcoglycan and display no expression for α- and β-sarcoglycan in regenerating fibers. Interestingly, dystrophin expression at the sarcolemma is reduced in actively regenerating muscle fibers. Bar represents 50 μm. Induction of regeneration in 15-month-old *MCK-DG null*, *mdx*, and δ-sarcoglycan null mice (*Sgcd null*) by administration of cardiotoxin (right). Note the impaired regeneration capacity of *mdx* and δ-sarcoglycan null mice 4 days and 14 days after cardiotoxin challenge as compared to the effective regeneration in *MCK-DG null* mice. Bar represents 100 μm.

MCK-DG Null Mice Maintain Their Regenerating Capacity in Contrast to *mdx* and Sarcoglycan Null Mice

In order to evaluate whether the dystrophin-glycoprotein complex might have an impact on muscle regeneration, we studied by immunohistochemistry the expression of DGC components in 4-month-old wild-type, *MCK-DG null*, *mdx*, and δ-sarcoglycan null mice 4 days after cardiotoxin challenge. *MCK-DG null* mice showed expression levels that were similar to wild-type muscle of β-dystroglycan, α-sarcoglycan, β-sarcoglycan, utrophin, and dystrophin during regeneration (Figure 5). In contrast, expression levels of DGC components were markedly abnormal in *mdx* and δ-sarcoglycan null mice.

Although upregulation of utrophin in actively regenerating muscle fibers does seem to partially compensate for the absence of dystrophin in *mdx* mice, expression levels for β-dystroglycan and the sarcoglycans decrease shortly (about 7–10 days) after the acute phase of regeneration (Figure 5). Mice deficient for δ-sarcoglycan apparently lack upregulation of a compensatory protein for the missing sarcoglycan; thus, no sarcoglycan expression was observed during the acute phase of muscle regeneration. Interestingly, analysis of dystrophin expression revealed that sarcolemmal localization of dystrophin is reduced in muscle fibers of δ-sarcoglycan null mice 4 days after cardiotoxin injection. These data indicate that the regeneration process in *mdx* and sarcoglycan null mice led to repopulation by myofibers with perturbed expression of the DGC, while regenerating myofibers in *MCK-DG null* mice expressed relatively normal DGC.

In order to compare the regenerative capacity of *MCK-DG null* mice to dystrophin-deficient *mdx* mice and sarcoglycan null mice, we exposed 4-month-old and 15-month-old mice to calf injections of cardiotoxin and analyzed the phenotype 4 days and 14 days after injection of the toxin. At 4 months of age, wild-type and *MCK-DG null* mice as well as *mdx* and δ-sarcoglycan null mice showed signs of effective regeneration, as assessed by the presence of small rounded muscle fibers with centrally located nuclei, a basophilic cytoplasm, and neonatal myosin immunoreactivity 4 days after injection (data not shown). Fourteen days after cardiotoxin injection, muscle architecture was largely restored, as demonstrated by the presence of normal diameter and centrally nucleated myofibers in *MCK-DG null*, *mdx*, and δ-sarcoglycan null mice (data not shown). In marked contrast, cardiotoxin challenge in 15-month-old mice exhibited a difference between *MCK-DG null* mice on the one hand and *mdx* and δ-sarcoglycan null mice on the other. *MCK-DG null* mice showed signs of effective regeneration 4 days after cardiotoxin injection, whereas *mdx* mice and δ-sarcoglycan null mice responded only poorly to the cardiotoxin-induced regeneration challenge (Figure 5). Interestingly, the regenerative response was less efficient in sarcoglycan null mice compared to *mdx* mice. The histological observations were confirmed

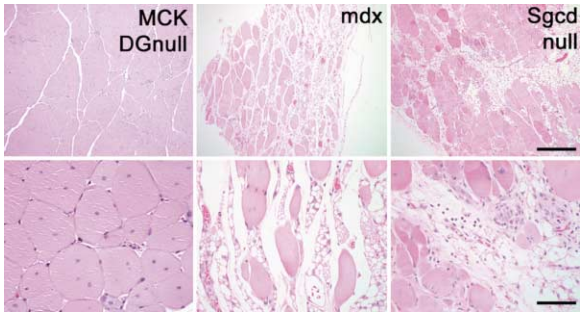

Figure 6. Comparison of Muscular Dystrophy in 18-Month-Old Mice

The capability of *MCK-DG null* mice to efficiently maintain skeletal muscle regeneration not only leads to significant muscle hypertrophy, but also prevents the development of severe dystrophic alterations as observed in mdx and *Sgcd null* mice. Bars represent 50 μm and 120 μm, in the upper and lower right panels, respectively.

by counting of neonatal myosin-positive fibers as a marker for acute regeneration in 300 fibers randomly chosen from 5–10 regions per toxin-challenged muscle in wild-type, *MCK-DG null*, *mdx*, and sarcoglycan null mice ($n = 4$, each group). The quantification demonstrated similar numbers of neonatal myosin-positive fibers in wild-type and *MCK-DG null* mice (230 ± 25 and 220 ± 32, respectively) and dramatically fewer neonatal myosin-positive fibers in *mdx* and δ-sarcoglycan null mice (109 ± 14 and 62 ± 9, respectively, $p < 0.0002$).

Fourteen days after cardiotoxin injection, *MCK-DG null* mice exhibited a nearly full restoration of the challenged muscle fiber groups, whereas *mdx* mice and sarcoglycan null mice still had regions of hypercellular myonecrotic response, indicating impaired regeneration capacity (Figure 5). Moreover, impaired muscle regeneration in *mdx* and δ-sarcoglycan null mice was eventually accompanied by extensive replacement of muscle by adipose tissue 4 weeks after cardiotoxin injection (data not shown).

In order to finally demonstrate that dystroglycan has an essential role in the biological function of satellite cells in vivo, we studied the effect of cardiotoxin on skeletal muscle regeneration in mice lacking dystroglycan at the sarcolemma of skeletal muscle as well as in satellite cells using *MORE-DG null* mice. *MORE-DG null* mice lack dystroglycan in *all* tissues in the embryo. However, dystroglycan is still expressed in extraembryonic membranes to circumvent embryonic lethality. Interestingly, cardiotoxin challenge in *MORE-DG null* mice (2 weeks of age) revealed not only lack of reexpression of dystroglycan but also severely impaired regenerative response 4 and 7 days after toxin injection (see Supplemental Figure S2 at http://www.cell.com/cgi/content/full/110/5/639/DC1), demonstrating that dystroglycan expression in satellite cells is essential for the regenerating capacity in skeletal muscle.

Morphological comparison of *MCK-DG null*, *mdx*, and δ-sarcoglycan mice at 18 months of age ($n = 6$ for each group), which have not been challenged by any toxin injections, demonstrated the development of significant endomysial fibrosis and extensive replacement of muscle by adipose tissue in *mdx* and δ-sarcoglycan null mice (Figure 6). In marked contrast, *MCK-DG null* mice exhibited extensive skeletal muscle hypertrophy but did not develop the devastating histopathology observed in *mdx* and/or δ-sarcoglycan null mice. Despite the relatively mild changes in 18-month-old *MCK-DG null* mice, elevation of serum creatine kinase levels and histological analysis of serial sections of various muscle types ($n = 6$ mice) confirmed ongoing cell necrosis, indicating the continuation of the dystrophic process.

Transient Reexpression of Dystroglycan in Regenerating Fibers of Human Limb-Girdle Muscular Dystrophy

Recent evidence shows that altered α-dystroglycan expression in recently described forms of limb-girdle muscular dystrophy does not necessarily lead to a severe phenotype, and a subset of these patients presents with marked muscle hypertrophy (Brockington et al., 2001). Thus, we started to analyze dystroglycan expression in patients with milder forms of muscular dystrophy.

Interestingly, we find that glycosylated dystroglycan is reexpressed in regenerating fibers in three patients with a mild form of limb-girdle muscular dystrophy caused by disruption of posttranslational dystroglycan processing (Figure 7A). In marked contrast, no residual glycosylated dystroglycan is expressed in regenerating fibers of patients with Fukuyama muscular dystrophy and muscle-eye-brain disease, which present with a severe clinical phenotype (D.E.M. and K.P.C., unpublished data). Biochemical characterization of dystroglycan expression in one of these patients with mild limb-girdle muscular dystrophy revealed that glycosylated α-dystroglycan is not significantly detected while the core protein shows a shift in apparent molecular weight of about 55 kDa (Figure 7B). No laminin binding was detected in the hypoglycosylated dystroglycan from the patient. Moreover, comparison of laminin binding activity of the patient to *MCK-DG null* mice shows similar reductions of total high-affinity laminin binding activity in WGA-enriched muscle homogenates from both sources. This indicates considerable loss of dystroglycan ligand binding activity in both muscle samples (Figure 7C).

Discussion

Several forms of muscular dystrophy caused by mutations of components of the DGC have been identified during the past two decades. However, we lack a clear understanding of the pathogenetic mechanism(s) responsible for the severe progressive and devastating course of these disorders. Current hypotheses developed through many years of research suggest two main biological cascades involved in the dystrophic disease process. First, the DGC plays an essential role in protecting the sarcolemma against muscle contraction-induced injury, and perturbation of the DGC causes sarcolemmal instability and structural damage subsequently leading to increased calcium influx followed by muscle cell death. Second, while skeletal muscle seems capable of efficiently repairing itself during the early phase of the disease, it is believed that the ongoing stimulus and activation of the repair mechanism eventually exhaust the satellite cell pool. Consequently, failure of the myogenic

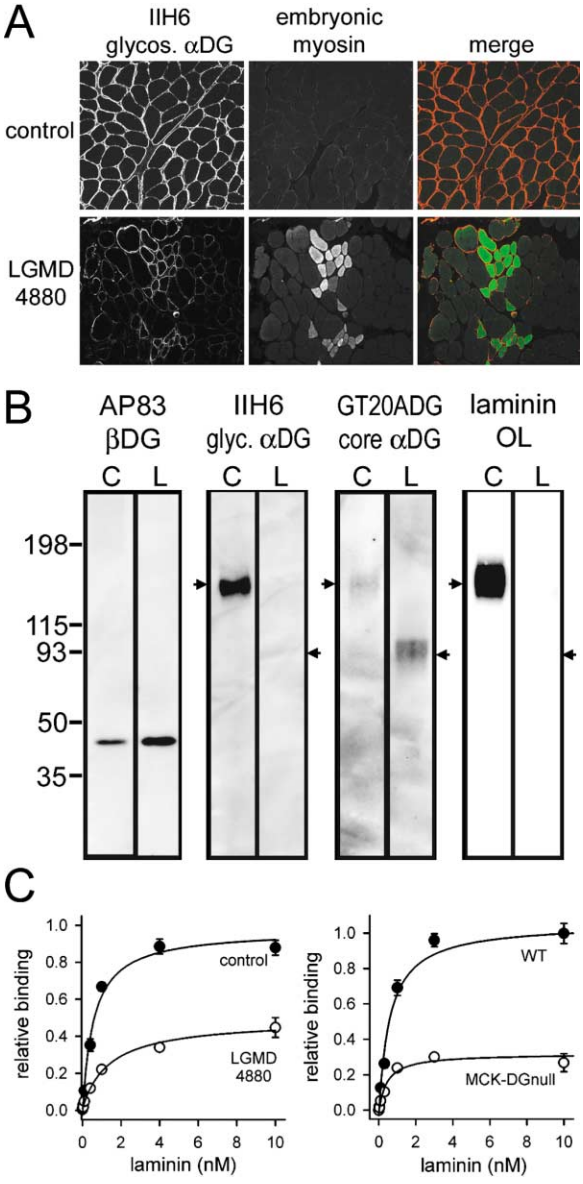

Figure 7. Loss of Fully Glycosylated α-Dystroglycan (IIH6) in a Mild Form of Limb-Girdle Muscular Dystrophy

(A) Residual glycosylated α-dystroglycan can be detected in regenerating fibers labeled with embryonic myosin. Similar results were obtained in the additional patients.

(B) Western blots and laminin overlay (OL) of WGA-enriched homogenates of the patient's muscle biopsy. Glycosylated α-dystroglycan is not significantly detected, and the core protein shows a shift in apparent molecular weight of about 55 kDa. No laminin binding was detected in the hypoglycosylated dystroglycan from the patient.

(C) Similar reduction of total high-affinity laminin binding activity in WGA-enriched homogenates of the limb-girdle muscular dystrophy patient muscle biopsy and WGA-enriched homogenates of *MCK-DG null* mice.

satellite cells to maintain muscle regeneration ultimately leads to severe fibrosis and adipose tissue replacement (Cossu and Mavilio, 2000; Heslop et al., 2000), a process that has not been attributed to any physiological function of the DGC. The current manuscript reveals that dystroglycan and the DGC are indeed essential for the protection and stability of the sarcolemma in skeletal muscle. However, our data elaborate on the second paradigm. We find that dystroglycan plays an essential role in satellite cell function by demonstrating that muscle-regenerating capacity can be sustained until senescence despite repetitive activation of degeneration/regeneration cycles, thereby preventing the development of severe tissue fibrosis and fat replacement. Thus, dysfunction of the satellite cell population leading to impairment of the repair mechanism in skeletal muscle represents a key mechanism in the pathogenesis of muscular dystrophy.

Absence of Dystroglycan Leads to Disruption of the DGC and Development of Muscular Dystrophy

Dystroglycan is a central component of the DGC in skeletal muscle. As our current data demonstrate, dystroglycan plays a key role in the expression of the DGC and in maintaining normal muscle function. Mice with tissue-specific ablation of dystroglycan show disruption of the DGC and develop muscular dystrophy. In vivo restoration of dystroglycan via adenovirus injection into skeletal muscle leads to expression of dystroglycan at the sarcolemma of *MCK-DG null* mice and consequently prevents the development of muscular dystrophy (data not shown). *MCK-DG null* mice show normal expression of laminin α2 and no perturbation of the basement membrane as revealed by electron microscopy studies (data not shown). Importantly, *MCK-DG null* mice show a specific absence of dystroglycan at the sarcolemma, while expression is still preserved in other muscle structures such as neuromuscular junctions and myotendinous junctions as well as vascular smooth muscle and peripheral nerve. Numerous studies have demonstrated alterations of neuromuscular junctions and myotendinous junctions in dystrophin-deficient *mdx* mice (for review see Winder, 1997). Moreover, recent studies have shown that alterations of vascular smooth muscle can have an impact on the severity of the dystrophic process in mouse models deficient for β- or δ-sarcoglycan (Cohn and Campbell, 2000). In contrast, the development of muscular dystrophy in *MCK-DG null* mice can be attributed solely to the loss of dystroglycan at the sarcolemma, emphasizing the essential role of dystroglycan for muscle function and stability.

Dystroglycan Is Expressed in Satellite Cells and Regenerating Muscle Fibers of *MCK-DG Null* Mice

Studies in dystroglycan chimeric mice (Cote et al., 1999) showed that these mice develop severe muscular dystrophy with increasing age with remarkable tissue fibrosis and fat replacement. In addition, patients with abnormal dystroglycan formation due to posttranslational disruption of dystroglycan-ligand interaction present with severe muscular dystrophy (Michele et al., 2002). To our surprise, *MCK-DG null* mice developed a mild phenotype with significant skeletal muscle hypertrophy with age instead of the expected severe dystrophic features such as tissue fibrosis and replacement of muscle by adipose tissue. Interestingly, we observed synchronized reexpression of dystroglycan in *MCK-DG null* mice when they were challenged to regenerate muscle fibers by intramuscular injection of cardiotoxin. Thus, we hy-

pothesized that dystroglycan might be expressed in satellite cells, which are not targeted by the MCK-Cre-driven promoter and that the satellite cell response to muscle injury was responsible for repopulation by transiently dystroglycan-positive myofibers.

Here we were able to demonstrate in vivo and in vitro that dystroglycan is indeed expressed in wild-type satellite cells. Our current animal model exhibits a specific disruption of skeletal muscle dystroglycan at the sarcolemma. Hence, it provides a unique tool to study dystroglycan expression in other cells present in skeletal muscle that are not targeted by the MCK promoter. Interestingly, we show that dystroglycan expression in satellite cells is enriched at the membrane site toward the basal lamina of the surrounding muscle fiber. This finding suggests a role for dystroglycan in the attachment and/or stability of satellite cells between the basal lamina and the sarcolemma of their associated myofiber. This may have an important impact on satellite cell function during regeneration.

In order to further support our hypothesis that the satellite response to muscle injury was responsible for repopulation of dystroglycan-positive cells, we used γ irradiation to render satellite cells incapable of proliferation. Indeed, irradiation of satellite cells in 4-month-old *MCK-DG null* mice resulted eventually in complete loss of dystroglycan at the sarcolemma of skeletal muscle as well as an increased amount of fat and fibrotic tissue replacement.

MCK-DG Null Mice Maintain Regeneration Capacity

MCK-DG null mice represent an in vivo animal model that maintains its regenerating capacity until senescence despite ongoing degeneration/regeneration cycles in the course of muscular dystrophy. Even induction of regeneration by administration of cardiotoxin into 15-month-old *MCK-DG null* mice demonstrated efficient regeneration of the challenged muscle fibers. The essential biological role for dystroglycan in satellite cell function in muscle regeneration is further substantiated by the severely impaired regeneration response to cardiotoxin challenge in *MORE-DG* null mice (R.B. and K.P.C., unpublished data), which lack dystroglycan in satellite cells *and* the sarcolemma of skeletal muscle. Moreover, this idea would also explain why chimeric mice for dystroglycan exhibit a more severe dystrophic phenotype in skeletal muscle as described by Cote et al. (1999).

In marked contrast to *MCK-DG null* mice, which develop significant skeletal muscle hypertrophy, old dystrophin-deficient *mdx* mice and mice deficient for δ-sarcoglycan exhibit severe endomysial fibrosis and adipose tissue replacement in skeletal muscle. Analysis of DGC components in *MCK-DG null*, *mdx*, and δ-sarcoglycan mice in regenerating muscle fibers demonstrated that satellite cells of *mdx* and δ-sarcoglycan mice respond to the injury by repopulating the injured skeletal muscle with myofibers defective in the expression of DGC components. *MCK-DG null* mice, on the other hand, regenerate muscle fibers with an intact DGC. In this regard it is interesting to note that *mdx* mice show upregulation of utrophin in actively regenerating muscle fibers. This partially compensates for the loss of dystrophin and leads to increased amounts of dystroglycan and sarcoglycan protein expression in these fibers as compared to muscle fibers, which do not exhibit signs of muscle regeneration and have perturbed expression of the DGC (Ervasti et al., 1990; current manuscript). In contrast, mice deficient for δ-sarcoglycan lack upregulation of a sarcoglycan-compensatory protein during their regeneration process. The observed differences might explain the milder morphological phenotype observed in *mdx* mice during their first year of life as compared to δ-sarcoglycan null mice, which develop severe fibrosis and adipose tissue replacement during the first 9 months of life. However, it is interesting to note that initial utrophin upregulation during regeneration in *mdx* mice is not sufficient to prevent the development of fibrosis later in life. Thus, our data together with previous reports of dystrophin expression in satellite cells (Mussini et al., 1995; Kong and Anderson, 2001) suggest a direct role of the DGC in satellite cell function and muscle repair. Experiments in our laboratory are currently under way to specifically characterize the expression pattern of DGC components in satellite cells in order to further delineate the role of the DGC in muscle regeneration.

Transient Reexpression of Dystroglycan in Regenerating Fibers of Human Limb-Girdle Muscular Dystrophy

Recent studies have shown that abnormal expression of dystroglycan in skeletal muscle due to posttranslational defects leads to several forms of human muscular dystrophy (Brockington et al., 2001; Michele et al., 2002). Interestingly although most of the patients exhibit severe congenital/limb-girdle forms of muscular dystrophy, a subset of these patients present with a milder phenotype mostly characterized by skeletal muscle hypertrophy (Brockington et al., 2001). Here we report data from three patients with mild human muscular dystrophy and posttranslational functional disruption of dystroglycan, displaying transient reexpression of normally processed dystroglycan in regenerating muscle fibers. We are currently in the process of screening muscle biopsies from patients with mild muscular dystrophy, muscle hypertrophy, and abnormal dystroglycan expression in order to more firmly establish the correlation between transient reexpression of dystroglycan in regenerating fibers and mild clinical phenotype. The findings of these patients described in the current manuscript represent a phenomenon of transient reexpression of functional dystroglycan from activated satellite cells similar to that seen in *MCK-DG null* mice. These findings are in marked contrast to Fukuyama congenital muscular dystrophy and muscle-eye-brain disease patients, who exhibit complete loss of dystroglycan posttranslational modification but do not show expression of fully processed dystroglycan in regenerating fibers and have a more severe dystrophic phenotype and more severe disease progression (D.E.M. and K.P.C., unpublished data). In addition to the known proteins that effect dystroglycan glycosylation, fukutin, POMGnT-1, and LARGE, the human data presented here (Figure 7) suggest that additional proteins may participate in functional modification of dystroglycan. The data further suggest that some of

these proteins may be differentially expressed during myofiber regeneration. In future experiments, it will be important to define both the temporal and spatial expression of the components of this enzymatic pathway in order to better delineate the pathogenesis of a variety of human muscular dystrophies by understanding the effect of the mutant protein on regenerative capacity of muscle. Other target tissues with potential clinical involvement may also be identified.

Taken together, our data suggest that perturbation of satellite cell function and insufficient ability to repair skeletal muscle cells represents a major mechanistic modifier in the pathogenesis of muscular dystrophy. Moreover, maintenance of regenerative capacity by satellite cells expressing dystroglycan may be responsible for mild disease progression in mice and humans, which opens up a new avenue of research involving the role of dystroglycan and associated proteins in muscle regeneration capacity and its relationship to human muscle disease. As various studies promote myogenic stem cells as a therapeutic option for primary myopathies such as muscular dystrophy (Ferrari et al., 1998; Gussoni et al., 1999; Cossu and Mavilio, 2000), our current data represent unequivocal evidence that maintenance of self-renewing potential of skeletal muscle can largely prevent the development of severe dystrophic alterations such as tissue fibrosis and fat replacement. Thus, future efforts should be directed toward identifying ways to maintain muscle regeneration capacity.

Experimental Procedures

Generation of Floxed Dystroglycan Mice

A floxed dystoglycan targeting construct was generated by first inserting a BamHI-SalI fragment from the intron of the mouse DG gene 5′ of a floxed PGK-neo expression cassette (Potocnik et al., 2000). Subsequently, a HSV-tk cassette was added to this construct. Next, a SalI to EcoRV fragment containing the entirety of exon 2 was inserted 3′ of the PGK-neo cassette. Finally, an EcoRV to NotI fragment bearing a loxP site (in the same orientation as those on the floxed neo cassette) at its 5′ end was blunt-end ligated to the EcoRV site of the genomic fragment containing exon 2 and, using a NotI/KpnI adaptor, was placed into the final targeting plasmid named DG-floxtk. Taken together, this construct results in the insertion of a floxed neo cassette at the SalI site in the intron 5′ of exon 2 and a loxP site at an EcoRV site 3′ of exon 2, at a distance from the polyadenylation signal sequence. DG-floxtk was electroporated into R1 ES cells and clones were screened by Southern blot with external probe A (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/110/5/639/DC1). Positive clones were rescreened with Probe B (see Supplemental Figure S1) to ensure that the 3′ loxP site was intact. We were unsuccessful at deleting the neo marker in vitro by transfection with a Cre expression plasmid; all recombinants lost exon 2 as well as neo. However, we do not believe that the intronic PGK-neo expression cassette interferes with expression of DG because there are no overt phenotypic abnormalities in homozygous DG-flox mice, and the levels of DG protein in skeletal muscle of those mice are similar to wild-type controls (data not shown). Targeted R1 ES cell clones were injected into C57Bl6 host blastocysts to generate chimeric animals. Germline transmission of the floxed DG allele was established by Southern blot and PCR screening of agouti offspring. Mice bearing the floxed DG allele were mated to the MCK-Cre mice (Bruning et al., 2000) as described in the Results section.

Generation of MORE-DG Mice

Heterozygous floxed dystroglycan/null (L/−) mice were bred to mice hemizygous for Mox2 Cre transgene (MORE mice, Tallquist and Soriano, 2000). The heterozygous mice Dag1+/− carrying Mox2 Cre transgene (Cre/Dag1+/−) were than bred with Dag1lox/lox mice, and heterozygous mice for Dag1lox/− carrying the Mox2 Cre (Cre/Dag1lox/−) transgene were obtained. In an alternative breeding strategy, we bred heterozygous mice Dag1lox/+ carrying the Mox2 Cre transgene (Cre/Dag1lox/+) with Dag1lox/− mice to obtain Cre/Dag1lox/− and homozygous mice for Dag1lox carrying the Mox2 Cre transgene(Cre/Dag1lox/lox). Cre/Dag1lox/lox and Cre/Dag1lox/− mice (MORE-DG mice) were viable, and the analysis of skeletal muscle revealed complete loss of dystroglycan (R.B. and K.P.C., unpublished data). MORE mice were generously supplied from Drs. Soriano and Tallquist.

Histology and Immunohistochemistry

Histopathological studies were performed as described before (Coral-Vazquez et al., 1999; Durbeej et al., 2000). Polyclonal antibodies against β-dystroglycan (rabbit 83), α-sarcoglycan (rabbit 98), β-sarcoglycan (goat 26), δ-sarcoglycan (rabbit 214), sarcospan (rabbit 256), dystrophin (rabbit 31), and utrophin (rabbit 56) were described previously (Durbeej et al., 2000). Polyclonal antibodies against α-1 syntrophin (kind gift of Dr. Stanley Froehner), dystrobrevin (kind gift of Dr. Joshua Sanes), and laminin α2 (kind gift of Dr. Peter Yurchenko) were used for immunohistochemistry and Western blot experiments. Monoclonal antibody against α-dystroglycan (IIH6) was described previously (Ervasti et al., 1990). Monoclonal antibody against neonatal myosin (Novocastra) and goat-polyclonal antibody against M-cadherin (Santa Cruz) were used for immunohistochemistry. Immunohistochemical staining of satellite cells was performed on frozen sections fixed in 4% paraformaldehyde, then washed in PBS containing 0.3% Triton X for 15 min and subsequently blocked with 5% bovine serum albumin. Fiber diameter was determined by measuring the shortest axis for all fibers within a crosssection to the nearest 10 μm counting a total of 200 fibers of 5–10 randomly chosen regions within the quadriceps and soleus muscle of 18-month-old *MCK* and *MCK-DG*-null mice ($n = 4$). These data were analyzed statistically using Student's t test.

RT-PCR

Total RNA was isolated from skeletal muscle tissue of wild-type and *MCK-DG* null mice using RNAzolB (Tel-Test) according to the manufacturer's specification. Gene-specific sense primer (5′-GCT CATTTCGAGTGAGCATTCC-3′) and antisense primer (5′-CTAGTT TCCAGGACAGGAGA-3′) were designed to anneal to the sequences in exon 1 and exon 2, respectively, which allow differentiation between amplification of cDNA and potential contaminating genomic DNA. Reverse transcription and PCR were performed with SuperScript One Step RT-PCR with PLATINUM Taq (GIBCO-BRL) according to the manufacturer's protocol, and the amplified DNA was separated by 2% agarose gel electrophoresis.

Immunoblot Analysis

Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 3%–15% linear gradient gels and transferred to nitrocellulose membranes. Immunoblot analysis was performed as previously described (Durbeej et al., 2000) with the addition of 5 nM EDTA to the homogenizing buffer. Laminin overlay and binding activity experiments have been described previously (Michele et al., 2002).

Cardiotoxin Experiments

All studies were performed in accordance with the guidelines of the animal care facility at the University of Iowa. One hundred microliters of 10 μM cardiotoxin (purified from the venom of Naja nigricollis snake; Sigma) was injected intramuscularly (Garry et al., 2000). Injected gastrocnemius was examined at 4, 7, 14, 21, and 28 days after injection. For each experiment, 3 to 6 wild-type, *MCK-DG null*, *mdx*, δ-sarcoglycan null mice were used. In addition, cardiotoxin was injected into two MORE DG null mice, which lack dystroglycan in all tissues including satellite cells and the δ-sarcolemma of skeletal muscle. Quantification of neonatal myosin-positive fibers in wild-type, *MCK-DG null, mdx*, and sarcoglycan null mice after cardiotoxin challenge was determined by counting 300 fibers randomly chosen from 5–10 regions per toxin-challenged muscle in wild-type, *MCK-*

DG null, *mdx*, and δ-sarcoglycan null mice (n = 4). These data were analyzed statistically using Student's t test.

Irradiation of the Mouse Hind Limb

Mice were anesthetized with Ketamine and the hind limb irradiated with a single dose of 18.0 or 25.0 Gy 137Cs γ rays (J.L. Shepherd and Assoc., Glendale, CA, model 81-16 beam irradiator). Anesthetized air-breathing mice were placed inside a purpose-built lead-lined irradiation jig with an opening that exposed the hind limb but shielded the remainder of the body. In those studies where both hind limbs were irradiated with a dose of 18 Gy, each hind limb was sequentially irradiated. The dose rate at a source-to-skin distance of 15 cm was 2.66 Gy/min, measured using radiochromic film (International Specialty Products, Wayne, NJ, type HS prototype film).

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Supplemental Data for:
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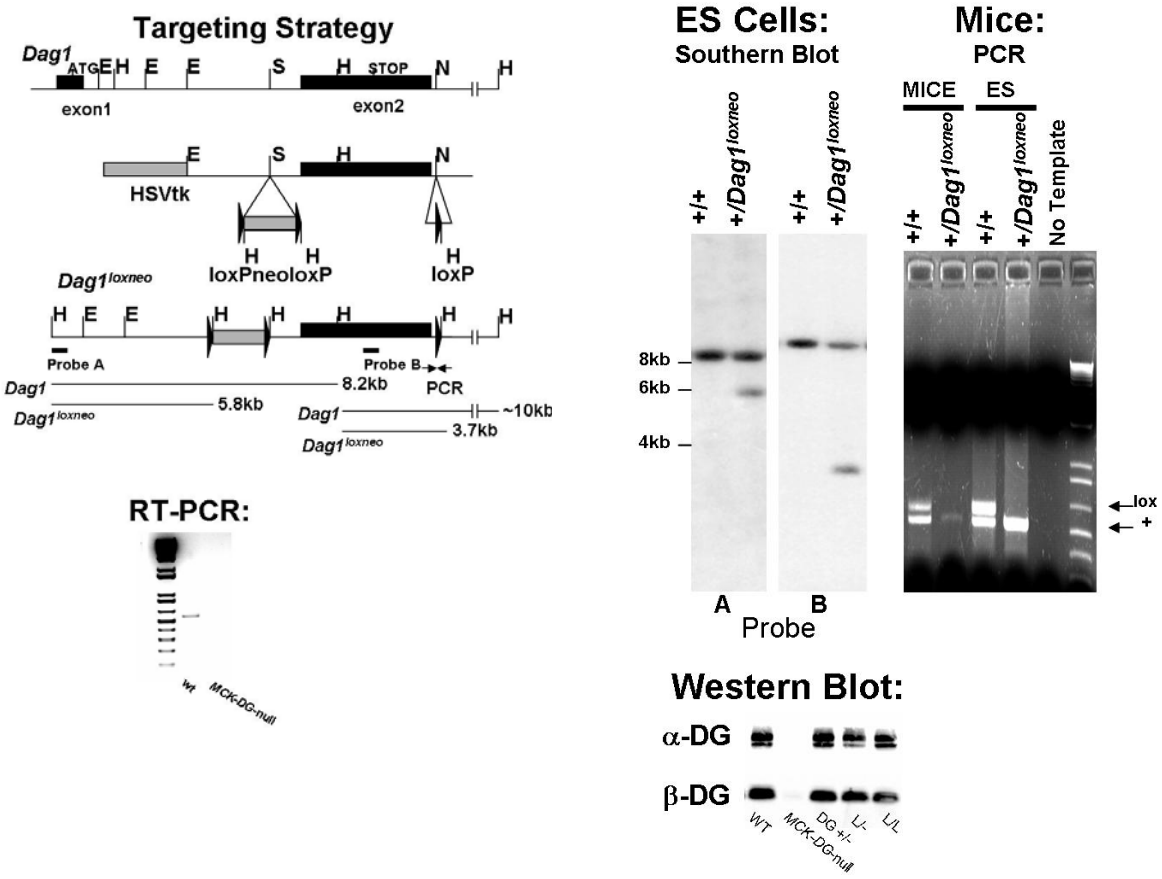
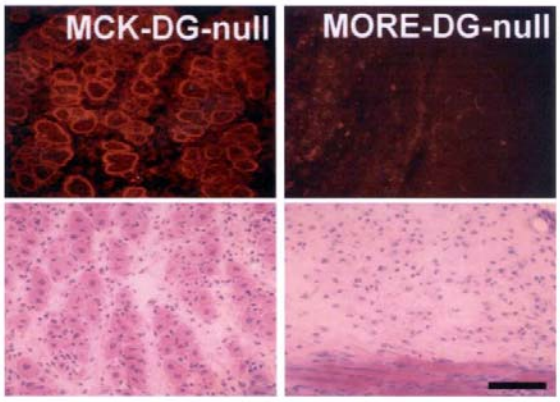
Supplemental Experimental Procedures



Supplemental Figure S1. Targeting Strategy for Tissue-Specific Disruption of Dystroglycan

A floxed dystoglycan targeting construct was generated by first inserting a BamHI-SalI fragment from the intron of the mouse DG gene 5′ of a floxed PGK-neo expression cassette. DG-floxtk was electroporated into R1 ES cells and clones were screened by Southern blot with external probe A. Positive clones were rescreened with Probe B to ensure that the 3′ loxP site was intact.



Supplemental Figure S2 *MORE-DG Null* Mice Do Not Reexpress Dystroglycan after Cardiotoxin Challenge and Exhibit Severely Impaired Regeneration Capacity

MORE-DG null mice lack dystroglycan in all tissues in the embryo. However, dystroglycan is still expressed in extraembryonic membranes to circumvent embryonic lethality. Bar represents 50 mm.