Role of dystroglycan in limiting contraction-induced injury to the sarcomeric cytoskeleton of mature skeletal muscle

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Dystroglycan (DG) is a highly expressed extracellular matrix receptor that is linked to the cytoskeleton in skeletal muscle. DG is critical for the function of skeletal muscle, and muscle with primary defects in the expression and/or function of DG throughout development has many pathological features and a severe muscular dystrophy phenotype. In addition, reduction in DG at the sarcolemma is a common feature in muscle biopsies from patients with various types of muscular dystrophy. However, the consequence of disrupting DG in mature muscle is not known. Here, we investigated muscles of transgenic mice several months after genetic knockdown of DG at maturity. In our study, an increase in susceptibility to contraction-induced injury was the first pathological feature observed after the levels of DG at the sarcolemma were reduced. The contraction-induced injury was not accompanied by increased necrosis, excitation–contraction uncoupling, or fragility of the sarcolemma. Rather, disruption of the sarcomeric cytoskeleton was evident as reduced passive tension and decreased titin immunostaining. These results reveal a role for DG in maintaining the stability of the sarcomeric cytoskeleton during contraction and provide mechanistic insight into the cause of the reduction in strength that occurs in muscular dystrophy after lengthening contractions.

muscular dystrophy | eccentric contraction | titin | skeletal muscle | dystroglycan

Muscular dystrophies are a heterogenous group of genetic disorders characterized by progressive muscle weakness and wasting (1, 2). Mutations in genes encoding proteins of the dystrophin–glycoprotein complex (DGC) are associated with various muscular dystrophies (3–10). The DGC is a multimeric complex comprising both transmembrane proteins [β-dystroglycan (DG), sarcoglycans (α, β, γ, and δ), and sarcospan] and membrane-associated proteins (α-DG, dystrophin, syntrophin, dystrobrevin, and neuronal nitric oxide synthase). α- and β-DG are produced by posttranslational cleavage of DG, which is encoded by *DAG1* (11). These subunits are central to linking the extracellular matrix to the intracellular cytoskeleton (6, 11). α-DG undergoes posttranslational glycosylation, which is required for its binding to extracellular-matrix proteins, such as laminin, agrin, neurexin, pickachurin, and perlecan, which bear laminin globular domains (11, 12). One such glycosylation modification is mediated by the glycosyltransferase LARGE and requires the presence of the N terminus of α-DG (12, 13), which is truncated during subsequent processing of the protein (12). The C terminus of α-DG is noncovalently associated with β-DG, a protein that is key to DGC function because it associates with α-DG extracellularly and with dystrophin intracellularly (11). Dystrophin, in turn, binds to the cytoskeleton, through an interaction between its N terminus and F-actin (12, 14). The mechanical link provided by DG and the DGC enables the cytoskeleton to impart tension to the extracellular matrix (15).

Many muscular dystrophies are associated with reduced, but detectable, sarcolemmal immunofluorescence staining of glycosylated α-DG or core α- and β-DG. The extracellular matrix receptor function of α-DG is impaired when either *DAG1* is itself mutated (limb-girdle muscular dystrophy type 2P) (10, 16, 17) or genes that encode putative or known glycosyltransferases that act on α-DG are mutated (Walker–Warburg syndrome, muscle–eye–brain disease, Fukuyama congenital muscular dystrophy, congenital muscular dystrophy types 1C and 1D, or limb-girdle muscular dystrophy) (12, 18, 19). Sarcolemmal expression of α-DG, sarcospan, and the sarcoglycan complex is reduced in patients with distinct sarcoglycan mutations (limb-girdle muscular dystrophy type 2C-F) (3–5, 20). Levels of α-DG, β-DG, the sarcoglycan complex, and sarcospan are reduced at the sarcolemma of patients with dystrophin mutations (Duchenne and Becker muscular dystrophy) (6–9).

Defects in the DGC are associated with high susceptibility to severe muscle injury caused by lengthening contractions (21–24). Muscles of mice deficient for dystrophin (*mdx* mice) display increased sarcolemmal damage, destabilization of sarcomeres, hypercontraction, and contraction-induced force deficits (22, 25–27). Muscles of mice that either lack DG or have mutations that render α-DG hypoglycosylated are significantly more susceptible to severe contraction-induced injury than those of WT counterparts (21).

Significance

Dystroglycan (DG) is an extracellular matrix receptor that is linked to the cytoskeleton and critical for the development of skeletal muscle. DG deficiency throughout development is associated with multiple pathological features and muscular dystrophy. However, the direct consequence of DG disruption in mature muscle is not known. Here, we investigate muscles of transgenic mice after genetic knockdown of DG at maturity. The results demonstrate early susceptibility to contraction-induced injury accompanied by reduced passive tension and decreased titin immunostaining, rather than increased necrosis, excitation–contraction uncoupling, or sarcolemmal fragility. These results suggest a need for critical rethinking of both current theories regarding contraction-induced injury in muscular dystrophy and therapeutic strategies.

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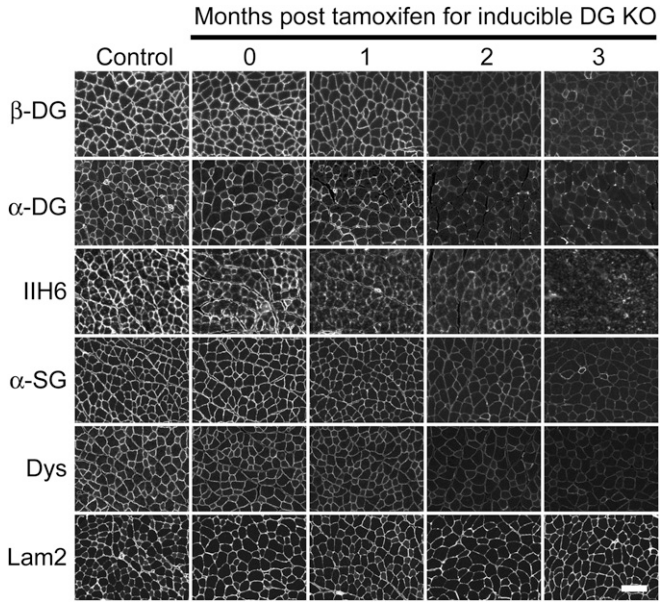

Fig. 1. Upon tamoxifen exposure in skeletal muscle of inducible DG KO mice, DG expression is progressively reduced. Time-dependent reductions in sarcolemma immunostaining were observed for core α-DG, glyco-α-DG (IIH6), and the C terminus of β-DG. A reduction in staining was also observed for α-sarcoglycan (α-SG) and dystrophin (Dys), which are also DGC components. This reduction was not the case for laminin α2 (Lam2). Values are means ± SE. (Scale bar: 100 μm.)

These findings are consistent with the proposal that severe contraction-induced injury exacerbates the dystrophic condition.

Previous research regarding the association of DG function with contraction-induced injury was based on studies in which DG was defective throughout development (21). These analyses failed to distinguish whether increased susceptibility to contraction-induced injury is a direct consequence of DG disruption or an indirect consequence of abnormal development and growth. Here, we find that, in an inducible DG knockout (inducible DG KO) mouse model, susceptibility to injury from lengthening contractions is increased at maturity and in the absence of overt necrosis, which implies that this form of injury is a direct consequence of disruption of the DGC. Interestingly, lengthening contractions reduce immunostaining levels of titin whereas membrane permeability and excitation–contraction uncoupling seem to be unaffected. Overall, these findings contribute to the current theory regarding the mechanism of contraction-induced injury in the dystrophic condition by characterizing the sensitivity of the sarcomeric cytoskeleton to reductions in DG, and the DGC more generally, at maturity.

Results

Induction of *Dag1* Disruption by Tamoxifen Gradually Decreases DG Levels. The effectiveness of tamoxifen in rapidly inducing recombination and decreasing levels of the *Dag1* mRNA in inducible DG KO mice was confirmed by RT-PCR (Fig. S1). A reduction in sarcolemmal staining for core α-DG, glycosylated α-DG, and β-DG was evident by 2 mo after tamoxifen exposure (Fig. 1). A comparable pattern of reduction was observed for the DGC proteins α-sarcoglycan and dystrophin whereas expression of the extracellular laminin α2 protein remained unchanged (Fig. 1). By 2–3 mo after tamoxifen exposure, levels of the DG protein decreased by ~80%, as assessed by immunoblotting with antibodies for core α-DG, glycosylated α-DG, and β-DG (Fig. S2). Thus, the combination of immunofluorescence staining and Western blotting demonstrates that this inducible mouse model leads to a dramatic knockdown of DG expression at both the mRNA and protein levels after 3 mo, but clearly the mice are not null for DG protein.

Susceptibility to Contraction-Induced Force Deficits Precedes Overt Necrosis. Despite the fact that DG levels were reduced by 3 mo after tamoxifen exposure, no overt necrosis was observed at that time (Fig. S3). Moreover, the percentage of centrally nucleated fibers (0.5 ± 0.3%, n = 5) and variability in muscle fiber area (coefficient of variance = 440 ± 47, n = 5) for inducible DG KO mice did not differ significantly from values for control mice (0.6 ± 0.3% and 478 ± 20 coefficient of variance, n = 5). However, necrosis became apparent later, at 6 mo post-tamoxifen, at which point both the number of centrally nucleated fibers (20.3 ± 4.5%, n = 7) and the coefficient of variance for muscle fiber area (604 ± 42, n = 7) were elevated relative to control values (1.1 ± 0.7% and 486 ± 20, respectively, n = 7, $P < 0.05$). To evaluate muscle function before the onset of overt necrosis, specific force and contraction-induced force deficits were evaluated for muscle ex vivo up to 3 mo after tamoxifen exposure (Fig. 2 and Table S1). At 1 and 2 mo post-tamoxifen exposure, the specific forces in muscles of inducible DG KO mice (237 ± 8 kN/m^2 at 1 mo and 239 ± 5 kN/m^2 at 2 mo, n = 5–8 per group) did not differ significantly from those in controls (243 ± 4 kN/m^2 and 246 ± 7 kN/m^2, respectively, n = 6 per group). At 3 mo post-tamoxifen exposure, however, the forces generated by muscles of inducible DG KO mice (206 ± 8 kN/m^2, n = 5) were subtly but significantly ($P < 0.05$) lower than both those at the earlier time points and those for muscles of control mice at 3 mo (237 ± 10 kN/m^2, n = 5). Increased susceptibility to contraction-induced injury was observed at 2 mo and remained at 3 mo (Fig. 2 and Table S1). These results indicated that the increase in sensitivity to contraction-induced injury precedes necrosis, suggesting that this injury is a direct result of DGC disruption rather than a secondary consequence of necrosis or developmental defects.

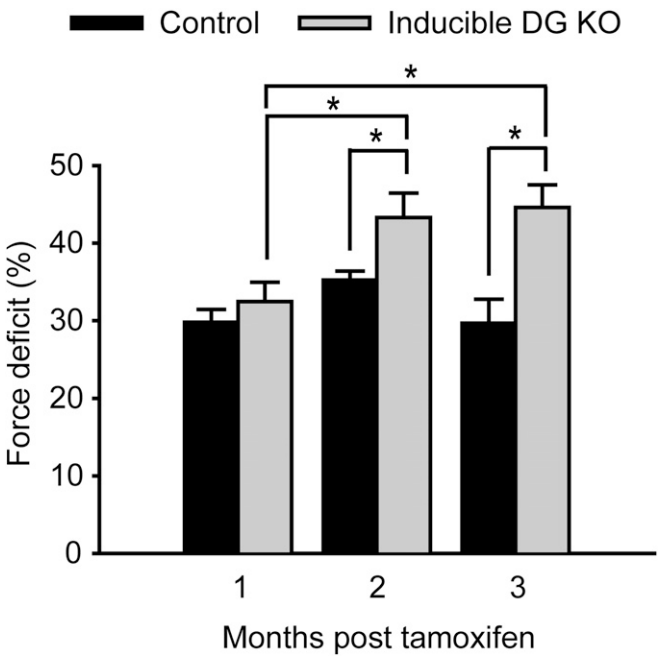

Fig. 2. Increased susceptibility to contraction-induced injury accompanies the reduction in DG levels. Shown are contraction-induced force deficits for EDL muscles (n = 5–9 per group) at various time points after exposure to tamoxifen. Values are means ± SE; *$P < 0.05$.

Evidence of Titin Disruption Rather than Increased Sarcolemma Damage or Excitation–Contraction Uncoupling Followed the Lengthening Contractions. To evaluate whether sarcolemmal damage contributed to increased susceptibility to contraction-induced injury, several experiments were performed in inducible DG KO mice. In dystrophic muscles, damage mediated by extracellular calcium or reactive oxygen species is a consequence of increased membrane permeability (28–30). Exposure of muscles of *mdx* mice to either calcium-free buffer or buffer supplemented with the antioxidant *N*-acetylcysteine (NAC) decreased contraction-induced force deficits to 50% of those in control buffer (Fig. 3*A* and Table S2), confirming earlier reports regarding *mdx* mice (28–31). In contrast, muscles of inducible DG KO mice were unresponsive to the conditions of calcium-free buffer and antioxidant supplementation (Fig. 3*A* and Table S2). To test membrane permeability more directly, Evans Blue Dye (EBD) uptake was quantified for muscle, both at rest and after completing a lengthening contraction protocol (LCP) (Fig. 3*B*). Although a contraction-induced increase in EBD uptake was observed in muscles of *mdx* mice, no such increase was detected in muscles of inducible DG KO mice. These results indicated that the sarcolemma of the inducible DG KO mouse maintained its integrity during the LCP.

A demanding protocol of lengthening contractions induces excitation–contraction uncoupling in muscles of WT mice (32, 33). To test whether heightened excitation–contraction uncoupling accounted for increased contraction-induced force deficits in the muscles of inducible DG KO mice, post-LCP forces were measured in the presence and absence of 10 mM caffeine (Fig. 3*C* and Table S3); caffeine at this concentration increases sarcoplasmic release of Ca^{2+} during muscle activation (32, 33). The presence of caffeine decreased the force deficits in both the control and inducible DG KO mice, which is consistent with a deficit in calcium release from the sarcoplasmic reticulum. However, the two-factor ANOVA interaction term (i.e., genotype × caffeine status) was not significant ($P = 0.48$), and no significant differences were observed between control and inducible DG KO mice with respect to the extent to which caffeine diminished the force deficits (by 40 ± 6% for control mice, and by 35 ± 8% for inducible DG KO mice). These results suggested that excitation–contraction uncoupling does not account for the increased susceptibility to contraction-induced injury in the muscles of the inducible DG KO mice.

Passive elements of the sarcomeric cytoskeleton, such as titin, are susceptible to damage from lengthening contractions (31, 34).

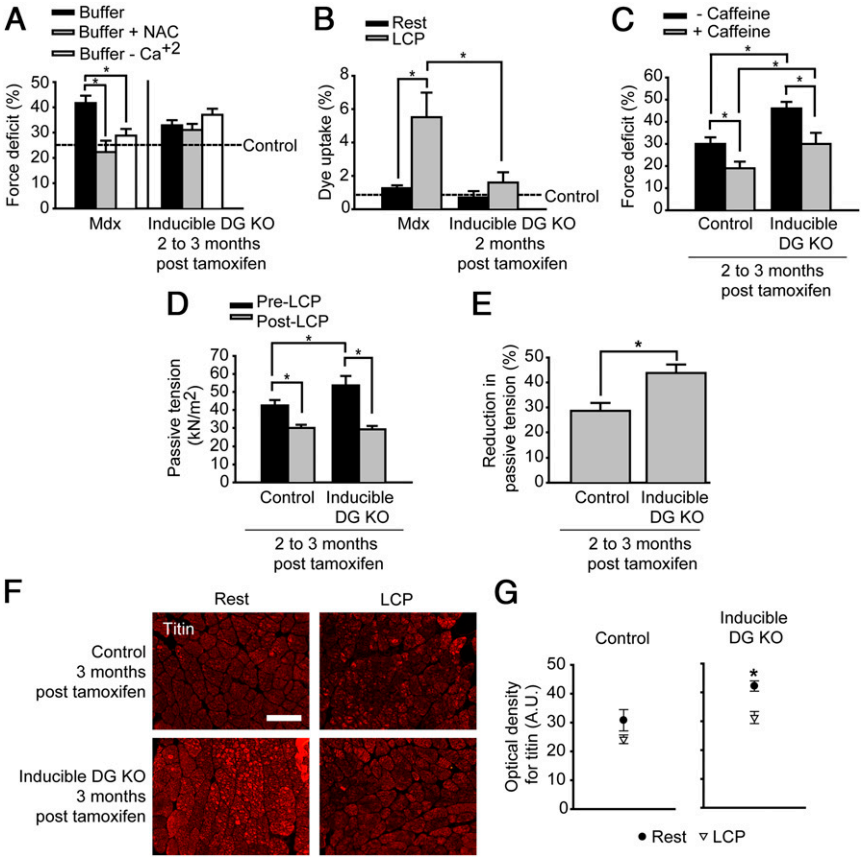

Fig. 3. Evidence for passive element disruption rather than excitation–contraction uncoupling or sarcolemma permeability as underlying the increased susceptibility to contraction-induced injury with DG knockdown. The EDL muscles of inducible DG KO mice were evaluated 2–3 mo after exposure to tamoxifen. (*A*) The antioxidant, *N*-acetylcysteine (NAC) or calcium-free buffer decreased the contraction-induced force deficits of *mdx* mice ($n = 5$–9 per group), but not those of the inducible DG KO mice ($n = 5$–6 per group). The mean control level (dashed line) was measured for control mice ($n = 5$) under normal buffer conditions. (*B*) Contraction-induced Evans Blue Dye uptake was increased in *mdx* mice ($n = 5$), but not in inducible DG KO mice ($n = 6$). The mean dye uptake for control muscle was quantified under rest conditions (dashed line). (*C*) The administration of caffeine led to comparable decreases in force deficits in muscles of control and inducible DG KO mice ($n = 5$ per group). (*D*) Passive tension occurred at 115% of optimal fiber length (L_o) before and after the lengthening contraction protocol (LCP) ($n = 8$ per group). (*E*) Passive tension was reduced at 115% of L_o, which was defined as the difference in passive tension pre- and post-LCP, and was expressed as the percentage of pre-LCP passive tension ($n = 8$ per group), $P = 0.003$. (*F*) Titin immunofluorescence in cryosections of muscles at rest and muscles after the LCP. (Scale bar: 100 μm.) (*G*) For muscles 2–3 mo post-tamoxifen exposure, optical density measures of titin immunofluorescence in cryosections were quantified for control and inducible DG KO mice ($n = 3$ per group). Comparison of muscles exposed to the LCP with those at rest revealed a significant difference exclusively for inducible DG KO mice, $P = 0.0009$. Values are means ± SE; $^*P < 0.05$.

To investigate this phenomenon in our model, passive tension at 115% of optimal muscle fiber length was determined pre- and post-LCP (Fig. 3*D* and Table S4). Before the LCP, passive tensions for inducible DG KO muscles were 1.3-fold greater than for control muscles ($P = 0.029$). The LCP induced a reduction in passive tension, which was more severe for the muscles of inducible DG KO mice than for those of control mice, as confirmed by a significant ($P = 0.02$) two-factor ANOVA interaction term between genotype and LCP exposure (Fig. 3*E*). Titin expression was investigated by observing immunofluorescence in transverse muscle sections (Fig. 3*F*). In the case of muscles at rest, titin immunofluorescence in the inducible DG KO muscles was high, which was consistent with high pre-LCP passive tension observed in inducible DG KO muscles. For muscles subjected to the LCP, the optical density of titin (by 26%) decreased relative to that in muscles at rest exclusively in the muscles of inducible DG KO mice, $P = 0.0009$ (Fig. 3*G*). These findings indicate that a decrease in DG levels is accompanied by a compensatory increase in titin levels at rest, but that, in the context of lengthening contractions, this increase is insufficient to prevent a loss of titin and the development of severe force deficits. We also investigated the immunofluorescence levels of desmin, nebulin, and α-actinin in muscle sections. In contrast with the data regarding titin, optical density values for desmin, nebulin, and α-actinin were unaltered by the LCP, thereby implying that the extent of disruption among cytoskeletal/sarcomeric components is variable (Table S5).

Increased Susceptibility to Contraction-Induced Injury Is Prevented by Maintaining WT Levels of β-DG and Core α-DG Expression. To determine which domain of DG accounts for increased susceptibility to injury, we investigated an additional mouse model—the inducible α-DG$^{\Delta N\text{-term}}$ mouse. This model enabled us to examine the effects of selectively maintaining levels of β-DG and core α-DG while reducing those of the glycosylated form of α-DG that is required for binding to the extracellular matrix (i.e., functionally glycosylated α-DG). In these animals, tamoxifen exposure leads to the replacement of a subpopulation of fully glycosylated α-DG with α-DG lacking the N-terminal domain, which is essential for glycosylation by the LARGE enzyme. No muscle pathology was observed in these mice after tamoxifen exposure. Levels of glycosylated α-DG, as assessed using the IIH6 glyco-specific antibody, were reduced to levels comparable with those in muscles of inducible α-DG$^{\Delta N\text{-term}}$ mice and inducible DG KO mice (Fig. S4*A*). By 2 mo post-tamoxifen exposure, levels of β-DG, core α-DG, and dystrophin differed between the two models—the levels were reduced in muscles of tamoxifen-exposed inducible DG KO mice, but not in muscles of inducible α-DG$^{\Delta N\text{-term}}$ mice (Fig. 1 and Fig. S4*A*). At 2–3 mo post-tamoxifen, no differences in specific force were observed in the muscles of inducible α-DG$^{\Delta N\text{-term}}$ mice (247 ± 15 kN/m^2, $n = 5$) vs. control mice (268 ± 15 kN/m^2, $n = 5$). In contrast with the inducible DG KO mice, which exhibited a high level of contraction-induced force deficits, the α-DG$^{\Delta N\text{-term}}$ mice generated forces comparable with those observed in controls (Fig. S4*B* and Table S6). These results indicate that preservation of the expression of core α-DG, β-DG, and dystrophin is sufficient to prevent severe contraction-induced force deficits.

Discussion

The association of heightened contraction-induced injury with DGC-related muscular dystrophies was established more than 20 y ago (22, 25, 26). Based on these early reports, the DGC was proposed to directly limit susceptibility to contraction-induced injury, and this injury was proposed as a primary trigger of disease onset. However, more recent reports have raised the possibility that the extent of contraction-induced injury may be affected indirectly by the absence of the DGC. Specifically, they demonstrated that, during either development or cycles of degeneration/regeneration, lack of the DGC leads to deleterious outcomes, such as inflammatory signaling (35, 36) or abnormal localization/function of caveolae (37), membrane-bound enzyme complexes (38, 39), and ion channels (40–42). These features have the potential to alter calcium influx/handling, the levels of reactive oxygen species, and cellular signaling, thereby exacerbating contraction-induced injury by increasing permeability of the sarcolemma, intensifying reactive oxygen species/calcium-mediated damage, and reducing release of calcium from the sarcoplasmic reticulum calcium (43). In the present study, we have demonstrated that, when a reduction in DG levels is induced at maturity, increased susceptibility to contraction-induced injury occurs in the absence of overt necrosis, which is consistent with a direct role for the DGC in limiting contraction-induced injury independent of development or necrosis.

We have also demonstrated that the stability of titin, rather than sarcolemmal integrity or excitation contraction coupling, is especially sensitive to reductions in levels of DG and the DGC at maturity. Lastly, this work demonstrates that preservation of the levels of β-DG in the context of reduced levels of the fully glycosylated form of α-DG prevents both disruption of the DGC and increased contraction-induced injury, which implies that α- and β-DG have distinct roles and are required at different levels—with α-DG preserving sarcolemmal integrity and being required in only minimal amounts, and β-DG stabilizing dystrophin and the sarcomeric cytoskeleton and being required in greater amounts. These findings suggest that the cytoskeleton of mature muscle is especially sensitive to reductions in DG levels.

The roles of DG and the DGC in contraction-induced injury were previously investigated in mouse models in which a genetic defect was present throughout development. During this process and the accompanying growth, skeletal muscle fibers increase significantly in breadth (the mean diameter of hind limb muscle fibers increases fivefold) and even more in length (the average muscle fiber length for an adult is 20–30 mm, which is 1,000-fold longer than a mononucleated cell) (44). DG and the DGC are critical during this dynamic phase of sarcolemmal expansion (45–47). Contraction-induced injury before the onset of necrosis has been investigated in muscles of mouse pups deficient for both dystrophin and utrophin (48). β-DG is able to bind each of these proteins, and a deficiency for both completely eliminates the link between β-DG and the cytoskeleton. Muscles from mouse pups with such a deficiency sustained twofold greater force deficits than muscles from control mice in the absence of necrosis (48). These results complement the present research. In the present investigation, the separation of susceptibility to contraction-induced injury and necrosis was established at reduced DGC levels rather than abolished levels. Because comparable results were observed in the previous research regarding mouse pups completely deficient in intact DGC levels, the indication is that the onset of increased injury precedes muscle degeneration when intact DGC levels are compromised in general (i.e., significantly reduced or completely absent) (48). The present study furthers the previous findings by investigating the susceptibility to contraction-induced injury at maturity. Because the dystrophin-deficient pups developed in the absence of an intact DGC, it could not be determined in that study whether the increased contraction-induced injury was a consequence of abnormal development rather than a more direct result of DGC disruption. In the present study, induction of the knockdown in mature mice demonstrates that the DGC can restrict injury independently of the effects of the DGC on development.

Various inducible systems have been used to study the histopathology that results from disrupting the DGC. These systems include tamoxifen-induced disruption of fukutin (46), RNA-mediated knockdown of fukutin-related protein (49), and dystrophin knockdown using a tetracycline-responsive transactivator or RNAi system (50, 51). Interestingly, knockdown of dystrophin at maturity did not result in a discernible phenotype (48). However, these investigations were limited, in that only histology, and not muscle function, was tested. In the present study, we found that a reduction in

sarcolemmal expression of dystrophin accompanied DG knockdown. Such a reduction is expected to have had a major impact on the results for contraction-induced injury in the present study because dystrophin links DG to the cytoskeleton, and dystrophin deficiency in other mouse models exacerbates contraction-induced force deficits (22, 25–27). Therefore, the data of the present study suggest that increased contraction-induced injury susceptibility was present in the past studies regarding dystrophin knockdown at maturity but were simply not detected because analysis was restricted to histological assessment alone. This possibility demonstrates the importance of complementing histological analysis with physiology measures when performing research on skeletal muscle.

Results of the present study support the concept that, in the context of an intact DGC, only minimal levels of glycosylated α-DG are required for limiting contraction-induced injury. When we reduced levels of the fully glycosylated form of α-DG using the inducible α-DG$^{\Delta N\text{-term}}$ mouse model, susceptibility to contraction-induced injury remained at WT levels. This finding is consistent with a previous report showing that low amounts of intact α-DG are necessary for preserving the function of receptors on the basal lamina—a feature that is essential for maintaining the integrity of the sarcolemma (52). For example, muscles of knock-in mice carrying a retrotransposon insertion of the gene encoding fukutin maintained only a small level of α-DG hypoglycosylation yet retained 50% of their laminin-binding capacity, whereas mice null for the glycosyltransferase LARGE (*Large^{myd}* mice), in which glycosylated α-DG was absent, retained less than 5% of laminin-binding capacity (52). Also, the extracellular matrix receptor α7β1 integrin is thought to help preserve the integrity of the basal lamina and limit contraction-induced injury, especially when α-DG function is disrupted (24).

Notwithstanding the lack of evidence for sarcolemma fragility in the muscles of inducible DG KO mice, instability of myofibrillar cytoskeleton was evident from the contraction-induced decrease in titin immunostaining and the reduction in passive tension. In a relevant report, muscles of *mdx* and WT mice were exposed to lengthening contractions in vivo, and then individual, permeabilized fibers from these muscles were tested (27). These muscle fibers were activated by direct addition of calcium to the buffer, thereby bypassing the sarcolemma and excitation–contraction coupling. The finding that, for *mdx* mice, permeabilized fibers from injured muscles generated 28% less force than fibers from uninjured muscles indicated that myofibrillar disruption contributed to the contraction-induced injury. Myofibrillar disruption would help explain why contraction-induced force deficits typically exceed the extent predicted based on the uptake of procion orange dye or EBD, indicators of breaches of the sarcolemma, in *mdx* muscle (22, 28, 40, 53). The extent of disruption of distinct components of the cytoskeleton/sarcomere may be unequal, as suggested by our finding that the expression of titin was disrupted by lengthening contractions whereas that of desmin, nebulin, and α-actinin was unperturbed. Our data are consistent with two other reports describing abnormal titin staining and reduced passive tension in WT muscle after lengthening contractions (31, 34), as well as with the fact that titin fragmentation is detected in serum and urine, in dystrophin-deficient mice as well as Duchenne muscular dystrophy patients (54–56). Disruption of the DGC is accompanied by compromised lateral force transmission (31, 34). Thus, the excessive reduction in titin staining in muscles of the inducible DG KO mice may have been due to excessive strain on titin during contractions, when such transmission was disrupted. Therefore, our results are consistent with a mechanism of contraction-induced injury whereby the ability to laterally shunt tension to β-DG is decreased, resulting in the development of excessive tension in specific components of the sarcomeric cytoskeleton.

The finding that the DGC limits contraction-induced injury in mature muscle has implications for approaches to the treatment of muscular dystrophy. One is that it would likely be beneficial to extend therapeutic treatment into maturity. Previous reports describing a delay in the onset of histopathology (up to 1 y) after disruption of the DGC in mature mice led to the proposal that infrequent treatment may be sufficient at maturity (15). In the present work, the finding that increased susceptibility to contraction-induced injury occurs in the absence of necrosis when the DGC is compromised at maturity stresses the necessity to treat the disease frequently. In addition, the finding that the sarcomeric cytoskeleton protein titin is especially sensitive to contraction-induced damage when DGC levels are reduced provides a better understanding of the pathophysiology, which will facilitate the development of more effective therapeutic strategies.

Materials and Methods

Refer to *SI Materials and Methods* for details regarding mice, antibodies, reagents, and analysis.

Inducible Mouse Models. All mice were maintained at The University of Iowa Animal Care Unit, and the animal studies were authorized by the Animal Care Use and Review Committee of The University of Iowa. Mice homozygous for a floxed allele of DG, in which loxP sites flank exon 4 of *Dag1* (49–51), were crossed with transgenic mice with a tamoxifen-inducible cre-mediated recombination system (004682; The Jackson Laboratory). This recombination system was driven by the chicken β-actin promoter/enhancer coupled with the cytomegalovirus (CMV) immediate-early enhancer. Male offspring heterozygous for the floxed allele and Cre-positive were crossed with female mice heterozygous for the floxed allele. Inducible DG KO mice were obtained from this cross. Control mice were Cre-negative and homozygous for floxed *Dag1*. Inducible α-DG$^{\Delta N\text{-term}}$ mice were obtained by crossing cre-positive male mice heterozygous for floxed *Dag1* with mice heterogeneous for deletion of the N-terminal domain of α-DG. At 10–12 wk of age, mice were gavaged with tamoxifen (200 mg/kg) on two occasions within a 1-wk period.

Measurement of Contractile Properties. At various time points after *Dag1* disruption, contractile properties of extensor digitorum longus (EDL) muscles were assessed ex vivo (45). Mice were anesthetized before surgical removal of the EDL. Muscle mass, fiber length, and maximum isometric tetanic force were measured. These measurements were used to determine total cross-sectional area and specific force (kN/m^2) (53, 57). The susceptibility to contraction-induced injury was determined after lengthening contractions. Differences between control and inducible DG KO mice were assessed by analysis of variance testing. Muscles were prepared for immunofluorescence or Western blot analysis as described in *SI Materials and Methods*.

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