Sarcolemma instability during mechanical activity in Large<sup>myd</sup> cardiac myocytes with loss of dystroglycan extracellular matrix receptor function

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The abnormal glycosylation and loss of extracellular matrix receptor function of the protein dystroglycan (DG) lead to the development of muscular dystrophy and cardiomyopathy. Dystroglycan is an important receptor for extracellular matrix proteins, such as laminin, in the basement membrane surrounding muscle. Large<sup>myd</sup> mice have a null mutation in a gene encoding the glycosyltransferase LARGE that results in abnormal glycosylation of α-DG and phenotypes similar to those in human α-DG glycosylation-deficient muscular dystrophy. Here, we show that Large<sup>myd</sup> hearts with the loss of DG extracellular matrix receptor function display a cardiomyopathy characterized by myocyte damage in patches of cells positive for membrane impermeant dyes. To examine the cellular mechanisms, we show that isolated adult cardiac myocytes from Large<sup>myd</sup> mice retain normal laminin-dependent cell adhesion, cell surface laminin deposition and basement membrane assembly. However, although isolated adult cardiac myocytes with the loss of α-DG glycosylation adhere normally to laminin substrates both passively and in the presence of mechanical activity, Large<sup>myd</sup> myocytes rapidly take up membrane impermeant dye following cyclical cell stretching. Therefore, while other cell surface laminin receptors are likely responsible for myocardial cell adhesion to the basement membrane, DG has a unique function of stabilizing the cardiac myocyte plasma membrane during repetitive mechanical activity by tightly binding the transmembrane dystrophin–glycoprotein complex to the extracellular matrix. This function of DG to stabilize the myocyte membrane during normal physiologic cell length changes is likely critical for the prevention of the myocardial damage and subsequent remodeling observed in α-DG glycosylation-deficient muscular dystrophies.

INTRODUCTION

Muscular dystrophies are a group of clinically and genetically heterogeneous diseases unified by the presence of progressive skeletal muscle weakness and wasting. Many patients with muscular dystrophies, particularly with those associated with alterations in the dystrophin–glycoprotein complex (DGC), develop cardiomyopathy (1–4). Abnormal glycosylation of the protein α-dystroglycan (α-DG) within the DGC leads to a spectrum of muscular dystrophies varying from severe congenital forms such as Fukuyama congenital muscular dystrophy (FCMD), Walker–Warburg syndrome (WWS), muscle–eye–brain disease (MEB) and congenital muscular dystrophy 1D (MDC 1D) to milder forms such as limb girdle muscular dystrophies (LGMD 2I, 2K, 2M, 2N, 2O) (5,6). The congenital forms of these muscular dystrophies, in addition to skeletal muscle and heart involvement, are characterized by varying degrees of developmental brain and eye abnormalities and peripheral neuropathy. The genetic defects underlying α-DG glycosylation-deficient muscular dystrophies reside in several genes encoding for glycosyltransferases involved in O-glycosylation of α-DG. The glycosylation steps and the precise composition of sugar residues on α-DG are not fully elucidated, but LARGE seems to be essential for sugars required for DG function (7,8). LARGE is a glycosyltransferase with two predicted catalytic domains and is highly expressed in skeletal muscle, heart and brain (9,10). Human and mouse LARGE have 98% identity in the amino...
acid sequence, and α-DG is the only known LARGE substrate that has been identified (9). Although the enzymatic activity of LARGE is still unclear, overexpression of LARGE in fibroblasts and myoblasts from human patients rescues α-DG glycosylation and laminin-binding activity (7). In humans, mutations in the LARGE gene cause congenital muscular dystrophy 1D (11). The Large<sup>myd</sup> mouse has a naturally occurring null mutation in the LARGE gene that results in skeletal muscle, cardiac and brain phenotypes similar to that observed in humans (5,9,12), and therefore can be used as a model to study pathogenesis of α-DG glycosylation-deficient muscular dystrophies resulting from the loss of DG function. While mutations in DG itself appear to be quite rare, the very first case of a genetic defect in the α-DG protein associated with human LGMD and cognitive impairment was published recently (13). Interestingly, this missense mutation also results in abnormal glycosylation of α-DG and loss of its laminin-binding ability, suggesting that the functional consequences of this particular mutation will be similar to the effects of genetic defects in the enzymes responsible for α-DG glycosylation.

Dystroglycan (DG) is the central component of the DGC, located in the plasma membrane of the skeletal muscle fibers and cardiac muscle cells. α-DG, an extracellular subunit of DG, and β-DG, an integral membrane DG subunit, play a key role in the DGC by linking the extracellular matrix to the cell cytoskeleton (14). DG serves as a receptor for a variety of extracellular matrix proteins containing laminin-G domains, such as laminin (15), neurexin (16) and agrin (17). α-DG is heavily glycosylated, and proper glycosylation is required for DG’s function as an extracellular matrix receptor (14,16). Large<sup>myd</sup> hearts show loss of α-DG glycosylation and consequent loss of high-affinity laminin-binding activity (18).

In muscular dystrophies due to genetic defects in DGC proteins, there is a destabilization and reduction/loss of the entire complex, suggesting that the loss of proteins constituting the DGC may also impact the function of DG within the complex (12,15,19,20). Sensitivity to contraction-induced damage is thought to be one of the mechanisms causing skeletal muscle degeneration in a number of muscular dystrophies associated with alterations in DGC function. However, independent of the DGC, α-DG has been shown to play a role in cell adhesion in certain type of cells (21). For instance, aberrant glycosylation of α-DG and impaired cell adhesion are associated with a highly invasive cellular phenotype in epithelia-derived cancers (22,23). α-DG in many non-muscle tissues also anchors laminin on the cell surface to facilitate formation of laminin polymers and basement membrane assembly (24–26). A body-wide ablation of DG results in embryonic lethality due to a failure to form Reichert’s membrane, one of the earliest basement membrane thanes in rodent embryos (27).

Dilated cardiomyopathy and measureable left ventricular dysfunction are a frequent feature of α-DG-deficient muscular dystrophies (4,28–33). The majority of cardiomyopathy cases are reported in LGMD 2I and FCMD and, in most of the cases, patients develop cardiomyopathy in later stages of disease. One recent study reported that 60% of patients with LGMD 2I enrolled had reduced left ventricular ejection fraction (33). In FCMD, a significant correlation between left ventricular systolic function and age was reported, and systolic function was reduced in most patients over 15 years old (4). A severe and early onset cardiomyopathy can also be an isolated presentation of muscular dystrophy, as patients with FKRP and fukutin mutations have been reported to present with congestive heart failure in the presence of very minimal skeletal muscle weakness or asymptomatic hyperCKemia (34,35). One of those patients died from heart failure, and two underwent heart transplantation in the first and second decade of life. No correlation has been shown to exist between the severity of skeletal muscle disease and cardiomyopathy (28,30,33). To our knowledge, there have been few if any reports of cardiac involvement in WWS or MEB, but the short lifespan of these patients may preclude the development of significant heart disease.

The exact mechanism for the heart disease in α-DG glycosylation-deficient muscular dystrophy is largely unknown. Targeted deletion of DG in cardiac muscle cells is sufficient to cause a dilated cardiomyopathic phenotype characterized by focal patches of damaged cardiac myocytes that take up Evan’s blue dye prior to onset of pathological remodeling (18). Despite evidence of a potential cardiac muscle cell intrinsic phenotype of loss of DG matrix receptor function in vivo, it still is not clear how and why cardiac tissue is more sensitive to damage and why the resultant phenotype is focal in nature. The observed phenotypes in vivo could be the result of the direct effects of loss of α-DG glycosylation, or a combination of secondary factors caused by the onset of cardiac disease. Here we show that the loss of α-DG glycosylation in Large<sup>myd</sup> hearts results in patches of damaged cardiac myocytes. To determine the cellular mechanism for why abnormal α-DG glycosylation leads to membrane damage, we show that, despite little to no effects on cellular adhesion or matrix assembly, loss of α-DG extracellular matrix receptor function renders isolated Large<sup>myd</sup> cardiac myocytes sensitive to mechanical stretch-induced membrane damage. Therefore, we provide direct evidence for membrane instability caused by loss of DG function as an extracellular matrix receptor in cardiac myocytes as a primary mechanism of focal cardiac myocyte damage and fibrotic remodeling in α-DG glycosylation-deficient muscular dystrophy.

**RESULTS**

**Loss of DG glycosylation results in myocardial damage focal in nature**

Since hearts of patients and mice with abnormal α-DG glycosylation develop focal fibrosis (4,18), and the DGC is thought to maintain membrane stability, we hypothesized that focal cardiac necrosis as a result of ongoing cell membrane damage is a basis for fibrotic remodeling in α-DG glycosylation-deficient hearts. To test this hypothesis, we examined hearts from wild-type (WT) and Large<sup>myd</sup> mice for intracellular uptake of IgG, which is normally excluded from the cell if plasma membrane is intact. Recent work in our laboratory (18) showed that immunoglobulin uptake is identical to marking cells by Evan’s blue dye uptake without requiring dye injection (Supplementary Material, Fig. S1). Similarly, an earlier study showed an overlap of IgG and Evan’s blue dye positive areas in dystrophic skeletal muscle...
Analysis of serial sections from WT and Largemyd animals revealed a specific pattern of IgG uptake in which cells taking up IgG are clustered together forming an IgG positive patch (Fig. 1A). In each group, we quantified IgG positive areas and number of individual cells taking up IgG to find that the number of IgG-positive cell clusters was significantly greater in Largemyd hearts compared with WT hearts (Fig. 1B, left panel). We found only three one- and two-cell positive areas in 16 sections from four WT hearts, whereas in Largemyd mice there was an increase in number of observed patches as well as a manifestation of patches containing up to 30 IgG-positive cells in cross-section (Fig. 1B, right panel). Interestingly, patches often contained cells in the same orientation, i.e. same mechanical axis, suggesting that distribution of the force or stretch along this axis might play a role in how patches form. Taken together, these results show that Largemyd mice have focal areas of myocardial cell damage where clusters of neighboring cardiac myocytes appear to be preferentially affected.

Largemyd cardiac myocytes retain their adhesive properties

To investigate the potential direct cellular mechanisms underlying clustered cell damage in myocardium of Largemyd mice, we enzymatically isolated cardiac myocytes from WT and Largemyd mice. Viability of Largemyd cardiac myocytes following the enzymatic isolation was comparable with that of WT cells, and myocytes from Largemyd showed rod-shaped morphology similar to myocytes from WT mice (Fig. 2A and C). Cardiac myocytes from some Largemyd mice tend to be thinner and smaller in size than WT cells. Although this might be an intrinsic characteristic of cardiac myocytes with abnormally glycosylated α-DG, Largemyd animals are generally smaller than their WT littermates and thus have proportionally smaller hearts (Supplementary Material, Fig. S2). Biochemical analysis of isolated cardiac myocytes showed that the peripheral membrane α-DG recognized by the IIH6 antibody against fully glycosylated, laminin-binding form of α-DG detected by the IIH6 antibody due to the loss of glycosylation (top panel), but retained DG expression as detected by a β-DG antibody (middle panel). A portion of the Ponceau S-stained blot is to show equal loading (bottom panel). (C) Cardiac myocytes from Largemyd showed rod-shaped morphology similar to myocytes from WT mice. Abbreviations: glyc α-DG, glycosylated α-DG.
adhesion underlies myocardial damage observed in Large<sup>myd</sup> hearts. We developed assay conditions to quantify the laminin-dependent adhesion of cardiac myocytes to matrix-coated surfaces. Freshly isolated adult mouse cardiac myocytes show laminin dose-dependent adhesion to glass cover slips in the absence of fetal bovine serum (Fig. 3A), which is abolished by treating cells with ethylenediaminetetraacetic acid (EDTA) and trypsin but not with arginine-glycine-aspartic acid (RGD) peptide (Supplementary Material, Figs S3 and S4).

![Figure 3](https://example.com/fig3)

**Figure 3.** Loss of α-DG glycosylation is not detrimental for adhesion of cardiac myocytes to laminin substrata. (A) Freshly isolated adult mouse cardiac myocytes show laminin (LM) dose-dependent adhesion to glass cover slips in the absence of fetal bovine serum. Data are mean ± SEM; n = 3 for each group; *P < 0.05 versus all groups with 5% serum and the 50 μg/ml LM group with no serum. (B) Abnormal glycosylation of α-DG does not interfere with cardiac myocytes adhesion to laminin substratum. Data are mean ± SEM; n = 6 for each group; *P < 0.05 versus 50 μg/ml laminin groups.

![Figure 4](https://example.com/fig4)

**Figure 4.** Laminin deposition on the cell surface is independent of α-DG glycosylation. (A) Large<sup>myd</sup> cardiac myocytes show loss of staining with IIH6 antibody against glycosylated epitope of α-DG, but show the WT level of immunofluorescence when labeled with a pan-laminin antibody. Scale bar, 50 μm. (B) Western blot (WB) of freshly isolated cells showed that the amount of total laminin associated with the Large<sup>myd</sup> cardiac myocytes is similar to that of WT cells (top panel). The bottom panel is a portion of the Ponceau S-stained blot to show loading. (C) Mutant hearts showed WT levels of laminin deposition at plasma membrane as assessed with a pan-laminin and laminin α2 chain (major cardiac α chain) antibodies. Scale bar, 100 μm. (D) Membrane expression of integrin α7 and β1, major integrin subunits in the heart, was unchanged as showed by western blotting of the microsome fraction of tissue homogenates.
levels of laminin deposition around mutant and WT cardiac myocytes with both antibodies. In order to determine whether the loss of α-DG glycosylation and function caused upregulation of integrins in cardiac myocytes to compensate for loss of DG function as matrix receptor, the expression of α7 and β1 integrin in the Large<sup>myd</sup> heart was examined. Expression of integrin α7 and β1, the major integrin subunits in the heart, was unchanged (Fig. 4D).

Cardiac myocytes with abnormal α-DG glycosylation are sensitive to mechanical stretch

DG is a member of the DGC complex, which has been implicated in stabilizing muscle cells against contraction-induced damage in skeletal muscle (38). Although patients with α-DG-deficient muscular dystrophy and Large<sup>myd</sup> mice show marked muscular dystrophy phenotype and signs of cardiomyopathy, it is still not fully understood whether the observed cell degeneration is indeed due to direct membrane instability. We developed a cell stretch assay for adult cardiac myocytes that mechanically strains cells in the presence of laminin-dependent adhesion to surface substrata. Cardiac myocytes were plated on silastic membranes, and the attachment of myocytes to the silastic membrane was dependent on precoating the membranes with 50 μg/ml of laminin (Fig. 5A). Following myocyte attachment, cardiac myocytes were subjected to cyclic stretch by application of vacuum pressure to the bottom of the dish resulting in 20% stretch of the silastic membrane. The stretch was performed in the presence of propidium iodide, a membrane-impermeable fluorescent dye (39), with the rationale that this dye would permanently mark nuclei of cells that became permeant to the dye, and would not be easily washed out if cells failed to reseal during the assay because of its tight association with DNA. Myocytes were scored for their rod-shaped morphology and uptake of propidium iodide. Following this assay, we commonly observed both rod-shaped and round cells positive for propidium iodide (Fig. 5B). Presence of rod-shaped cardiac myocytes positive for propidium iodide indicates that membrane damage most likely was repaired. Observation of round cells positive for the dye is likely indicative of plasma membrane damage resulting in cell contracture due to influx of calcium from the extracellular media. Quantification of Large<sup>myd</sup> cardiac myocytes subjected to stretch showed a significantly greater number of dye-positive myocytes than of Large<sup>myd</sup> mice on silastic membranes not subjected to stretch and both stretched and non-stretched control myocytes (Fig. 6). The number of round cells combined with positive rods was also significantly greater in stretched mutant cardiac myocytes than in non-stretched Large<sup>myd</sup> myocytes, indicating some cells underwent permanent cell contracture. In contrast, stretching of parallel cultures of control myocytes subjected to stretch at the same time showed no significant increase in the number of propidium iodide-positive cardiac myocytes or an increase in the number of dye-positive round myocytes. The other interesting finding is that while Large<sup>myd</sup> cardiac myocytes showed significant sarcolemma instability in response to stretch, there was no significant effect of the mutation on the total number of cells that remain attached to the silastic membranes (Fig. 6). Thus, in agreement with our

Laminin deposition on the cell surface is independent of α-DG glycosylation

DG has been shown to be involved in the assembly of basement membranes by nucleating and depositing laminin on cell surface (24–27,37). To test the hypothesis that DG function as a laminin receptor is essential for laminin deposition and basement membrane assembly, we looked at cell surface and total laminin expression on adult mouse cardiac myocytes. During isolation of single cardiac myocytes, a considerable amount of basement membrane remains tightly associated with myocytes on the cell surface as shown by immunostaining for surface laminin (Fig. 4A). In addition, IIH6-reactive α-DG is present on the cell surface of WT myocytes. While in myocytes from Large<sup>myd</sup> hearts, IIH6-reactive laminin-binding glycoform of α-DG was undetectable, no difference in laminin staining on the surface of freshly isolated Large<sup>myd</sup> and WT cardiac myocytes was observed, suggesting the basement membrane remains tightly associated with the cardiac myocyte surface membrane even under fairly harsh conditions of cell isolation (Fig. 4A). Western blotting of cell pellets prepared from freshly isolated cardiac myocytes showed that the amounts of laminin associated with the Large<sup>myd</sup> cells are similar to WT cells (Fig. 4B). Immunostaining of heart sections from Large<sup>myd</sup> and WT mice with a pan-laminin as well as laminin α2 chain antibody (Fig. 4C) showed similar

Figure 5. Stretch assay to reveal membrane instability. (A) Cardiac myocytes attach to silastic membranes in a laminin-dependent fashion. No attachment is observed on non-coated membranes. (B) Representative images of propidium iodide exclusion tested in Large<sup>myd</sup> cardiac myocytes subjected to stretch. When cell membrane integrity gets compromised propidium iodide, gains access to the nucleus, where it complexes with DNA and renders the nucleus highly immunofluorescent. In our experiments, propidium iodide was added to wells prior to stretch and washed out after stretch. Number of rod-shaped and round cells with bright red fluorescence was quantified. Images have same exposure time. Scale bar, 100 μm.
In the present study, we show that the loss of α-DG function on cell adhesion is not likely explained by compensatory upregulation of β1 containing integrins. There was no observable effect of RGD peptide on cardiac myocyte adhesion to laminin, at concentrations reported to inhibit cell adhesion of COS-7 cells to fibronectin (44). Furthermore, the expression of heart-specific integrin isoforms was unchanged in Large<sup>mvd</sup> mice. Therefore, normal levels of laminin-binding integrins or alternative laminin receptors are likely sufficient for cell adhesion and basement membrane assembly in cardiac myocytes, and loss of α-DG glycosylation is dispensable for these functions.

Cardiac muscle cells are tightly surrounded by basement membrane, a specialized form of extracellular matrix that has a very complex organization allowing it to be flexible and strong (41,45). Previous studies suggested that DG is required for basement membrane formation (26,27,37). A body-wide deletion of DG in a mouse leads to a failure to form Reichert’s basement membrane (27). α-DG regulates laminin expression and nucleates laminin on the cell surface to facilitate basement membrane assembly, at least in ES cells (26,37). Skeletal muscle of patients with α-DG glycosylation-deficient muscular dystrophy shows slightly reduced amounts of laminin α2 chain, the major laminin α chain in striated muscle (28,46). In the present study, we did not observe any effects of loss of α-DG glycosylation on laminin deposition on the surface of cardiac myocytes from Large<sup>mvd</sup> hearts, suggesting that basement membrane formation in the heart is not affected by the loss of α-DG laminin-binding activity and that cell damage in vivo is not likely due to abnormal basement membrane assembly.

In striated muscle, DG and the DGC are hypothesized to anchor the sarcolemma to the basement membrane at costamers (47,48). Costameres and the DGC are also believed to be sites of lateral transmission of force from sarcomeres to the matrix (48,49). An earlier study reported that Large<sup>mvd</sup> mice show slightly reduced expression of dystrophin, β-DG and γ-sarcoglycan in the heart but not in the skeletal muscle (12). Although the reason for such tissue-dependent differences is unknown, the reduction in the expression of these proteins might alter stoichiometry of the DGC and along with the loss of α-DG glycosylation contribute to the observed phenotypes. However, skeletal muscle of Large<sup>mvd</sup> mice with...
preserved expression of the DGC proteins shows more severe phenotype than cardiac muscle. Sensitivity to contraction-induced plasma membrane damage is thought to be one of the mechanisms causing skeletal muscle degeneration in a number of muscular dystrophies associated with alterations in DGC function. The evidence supporting the membrane instability hypothesis comes from previous studies showing uptake of Evan’s blue dye and leakage of muscle enzymes by dystrophic skeletal muscle (38,50). Leaky membranes may lead to influx of calcium ions and activation of calcium-activated proteolysis of sarcomeric and cytoskeletal proteins that perhaps contribute additionally to muscle instability and muscle dysfunction (51,52). Relative to skeletal muscle, considerably less data are available on the dystrophic heart disease. Heart sections of dystrophic animals show areas of focal myocardial necrosis and dye uptake (12,53). Cardiac myocytes from dystrophin-deficient mdx mice show abnormal calcium influx in response to stretch (54). Mice with cardiac-specific deletion of DG develop focal areas of myocardial damage positive for Evan’s blue dye (18). In the present study, we observed similar patches of myocardial damage in Large myd mice deficient in α-DG glycosylation and extracellular ligand receptor function. Importantly, we now show that adult cardiac myocytes isolated from these mice show signs of membrane fragility in response to mechanical stretching, demonstrating direct evidence for the membrane instability hypothesis at the cellular level as a mechanism for patch formation. What is remarkable about this effect on membrane stability is that it occurs due to the loss of function of a single laminin receptor on the cell surface and in the presence of normal laminin-mediated cell adhesion. Therefore, α-DG appears to have a direct function in stabilizing the sarcolemma over the normal physiological range of cardiac myocyte mechanical activity by tightly binding the transmembrane DGC and likely the intracellular costamere to the extracellular matrix. The anchorage of the costamere to the matrix may confer membrane stability by distributing the membrane deformation uniformly over the cell surface proportional to the underlying sarcomere length changes in response to stretch. A remaining question is how this membrane instability at the single cell level leads to a cascade of events that cause the focal patches of myocardial damage. One possibility is that the spontaneous damage of a single myocyte results in greater load or mechanical activity on neighboring myocytes. Because cardiac myocytes with the loss of DG function are also more sensitive to mechanical stretching, those neighboring cells experiencing greater mechanical load are more likely to experience subsequent membrane damage. In vivo, the secondary consequences following cardiac muscle damage are certainly complex but also likely significant to the progressive worsening of cardiac disease with age in dystrophic patients and in mouse models. The initial injuries to myocardium may trigger local inflammation at the site of the injury and surrounding tissue and could contribute to the expansion of patches of cell damage. Infiltration by macrophages and cytotoxic T-lymphocytes has been proposed to contribute to myofiber injury in dystrophin-deficient skeletal muscle (51,55). The pathological hallmark of dystrophic cardiac muscle of older patients and mouse models appears to be focal myocardial fibrosis. It seems likely that local inflammation and the release of cytokines such as transforming growth factor beta (TGF-beta) may contribute to the focal activation of fibroblasts and development of tissue fibrosis in dystrophic cardiac muscle. Indeed, treatment of dystrophin-deficient mice with losartan, which indirectly antagonizes TGF-beta function, appears to decrease fibrosis and improve cardiac function (56), although the mechanism of benefit of losartan and mechanisms of fibrosis in dystrophic myocardium remain to be investigated.

Our findings indicate that cardiac myocytes void of the glycosylated α-DG epitope are more sensitive to stretch-induced membrane damage at levels of stretch that reflect the length changes that occur during normal myocardial function. Normal deposition of laminin on the surface of myocytes and retained attachment of α-DG glycosylation-deficient myocytes to laminin during stretching indicates that the stretch-induced damage in the plasma membrane is not due to the weaker attachment of the cells to underlying matrix, but rather due to primary instability of the plasma membrane. Therefore, anchorage of the DGC to the basement membrane through α-DG is not essential for integrity of the basement membrane but is essential for stabilizing the plasma membrane against normal mechanical activity of myocardial cells.

MATERIALS AND METHODS

Mice

Homozygous mutant Large myd mice as well as heterozygous and homozygous WT littermates were generated from mating heterozygous mice in a colony of B6C3Fe-a/a-LARGEmyd originally purchased from Jackson Laboratories. For our experiments, we used male and female age-matched littermate animals. Genotyping was performed using previously published primers (9). The procedures used in this study were approved by the University of Michigan Committee on the Use and Care of Animals. Animal care was provided by the University of Michigan Unit for Laboratory Animal Medicine in accordance with National Research Council’s Guide for the Care and Use of Laboratory Animals (57).

Antibodies

We used following primary antibodies: rabbit polyclonal L9393 pan-laminin (Sigma, St Louis, MO, USA), rat monoclonal anti-laminin α-2 chain (Alexis, San-Diego, CA, USA), monoclonal anti-β-DG (Vector Labs, Burlingame, CA, USA), monoclonal anti-glycosylated epitope of α-DG IIH6 (kind gift of K. P. Campbell, University of Iowa), monoclonal anti-integrin α7 (Sigma) and rabbit polyclonal anti-integrin β1 antibody (Chemicon Billerica, MA, USA). Secondary antibodies used for western blotting were conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA). Secondary antibodies for immunofluorescent imaging were conjugated to Alexa-488 (Molecular Probes, Invitrogen, Grand Island, NY, USA), DyLight™488 (Jackson ImmunoResearch) and Cy3 (Jackson ImmunoResearch).
Assessment of myocardial membrane damage
Mice were anesthetized i.p. with a mixture of 100 μg/g ketamine and 8 μg/g xylazine. Hearts were quickly excised, weighed and snap frozen in liquid nitrogen cooled isopentane. Frozen hearts were sectioned in 8 μm thick slices in a cryostat and co-stained with antibodies against mouse IgG and pan-laminin. A goat anti-mouse IgG antibody conjugated to DyLight™488 was from Jackson ImmunoResearch. Pan-laminin antibody (see above) was followed by secondary antibody conjugated to Cy3. We analyzed four serial sections at a 300 μm interval for each animal.

Cardiac myocyte primary culture
Adult cardiac myocytes were enzymatically isolated from 9 to 15 weeks old mice following a protocol we previously published (58). Plating and culture media contained 25 μM S-(−)-blebbastatin, a contraction inhibitor (Toronto Research Chemicals, North York, ON, Canada), unless otherwise stated (58). Cells were isolated from WT and Large myd mice with 30–60 min interval and were treated similarly.

Quantification of cardiac myocytes adhesion
Cardiac myocytes were plated on glass cover slips placed in a 24-well plate in media supplemented with 30 mM of contraction inhibitor 2,3-butanedione monoximine (Sigma). Plating density was ≏350 cells/cm² with a total of 400 cells/cover slip. The cover slips were coated with 50 and 1 μg/ml mouse laminin-111 (Invitrogen) and Dulbecco’s phosphate buffered saline (PBS) as a control. Cells were left to attach for 30 min at 37°C in media containing no serum. The membrane impermeant dye, propidium iodide (Sigma), was added to final concentration of 50 ng/μl without serum and blebbistatin. The membrane impermeant dye, propidium iodide-positive nuclei above background, and cell morphology were visualized using the red channel of an Olympus BX51 microscope (Olympus). Myocytes from WT and mutant mice were always isolated in pairs and tested in parallel wells under identical conditions with identical dye solutions. Digital images of five to six random fields of cells per sample were taken. The images were thresholded in parallel using Adobe Photoshop to eliminate background autofluorescence, and cells were then scored blindly for propidium iodide-positive nuclei above background, and cell morphology.

Immunofluorescent staining
Cells were plated on 22 mm square glass cover slips (Corning, NY, USA) coated with mouse collagen IV at a concentration of 50 ng/μl (Sigma). Cells were collected immediately after 2 h plating time or after 24–48 h in culture. Cells were fixed in 3% paraformaldehyde before subjecting to immunofluorescent staining. For heart tissue staining, hearts were collected as described above, and 8 μm cryosections were used.

Western blotting
Freshly isolated and cultured cells were washed with PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, 1% Triton X-100, 1 mM EDTA and protease inhibitors (0.6 mM benzamidine, 0.4 mM PMSF, 0.5 μg/ml pepstatin, 2 kallikrein inhibitor units/ml aprotinin, 1 μg/ml leupeptin). Samples were sonicated for 10 s and centrifuged at 14 000 rpm for 5 min to pellet undissolved debris. Total protein content was determined using DC Protein Assay (Biorad, Hercules, CA, USA). Proteins were separated by a 3–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a polyvinylidene fluoride membrane, which was stained with Ponceau S to verify equal loading. The membranes were blocked with 5% nonfat milk in TBS (100 mM NaCl, 50 mM Tris pH 7.5) and followed by incubation with primary and secondary antibodies (see above). The membranes were developed using an enhanced chemiluminescence assay (Pierce, Rockford, IL, USA).

Mechanical stretch in cardiac myocytes
Freshly isolated adult cardiac myocytes were plated on six-well plates containing silastic membranes (Flexcell, Hillsborough, NC, USA) coated with 50 μg/ml mouse laminin-111 for 30–40 min (Invitrogen). The plating density was ～60 cells/mm² with the total of 20 000 cells/well. Following 2 h of attachment, media were switched to media without serum and blebbistatin. The membrane impermeant dye, propidium iodide (Sigma), was added to final concentration of 50 μg/ml for 15 min before stretch. Cells were stretched for 1 h at 1 Hz using a custom-made cylinder-based pump and platform which replicated the platforms commercially available from Flexcell International, to apply vacuum pressure to the bottom of a six-well dish with silastic membrane bottoms. The device was calibrated using a depth micrometer to measure the displacement of the membrane at the center, and an ellipse equation was used to calculate a stretch of the silastic membrane of 20%. After completion of stretch, cells were let rest for 30 min and then washed with media to remove extracellular propidium iodide. Cardiac myocytes were fixed in 3% paraformaldehyde for 15 min and washed three times with PBS. Silastic membranes were cut off the plates and mounted on glass slides with PermaFluor (Thermo Electron Corporation, Pittsburg, PA, USA). Cells were visualized using the red channel of an Olympus BX51 microscope (Olympus). Myocytes from WT and mutant mice were always isolated in pairs and tested in parallel wells under identical conditions with identical dye solutions. Digital images of five to six random fields of cells per sample were taken. The images were thresholded in parallel using Adobe Photoshop to eliminate background autofluorescence, and cells were then scored blindly for propidium iodide-positive nuclei above background, and cell morphology.

Microsome preparation
Snap frozen heart samples were homogenized in pyrophosphate buffer, pH 7.1, containing 1 mM MgCl, 0.303 M sucrose, 1 mM EDTA and protease inhibitors in concentrations indicated above. Homogenates were centrifuged at 14 000g at 4°C for 30 min, and supernatants were then centrifuged at 30 000g at 4°C for 30 min. Microsome pellets were washed twice with homogenization buffer and resuspended in 20 mM Tris, pH 7.0 containing 0.303 M sucrose. After quantification, microsomes were separated on SDS–PAGE and western blotted as described above.
**Statistics**

Student’s t-test or one-way analysis of variance with Newman–Keuls post-test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). A P-value <0.05 was considered significant. Values are expressed as mean ± SEM unless otherwise stated.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement** None declared.

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