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Introduction of a human-specific deletion in mouse *Cmah* increases disease severity in the mdx model of Duchenne muscular dystrophy

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Abstract

The evolution of humans included introduction of an inactivating deletion in the *CMAH* gene, which eliminated biosynthesis of N-glycolylneuraminic acid from all human cells. Here we show that this human-specific sialylation change contributes to the marked discrepancy in phenotype between the mdx mouse model for Duchenne muscular dystrophy (DMD) and the human disease. Despite lacking dystrophin protein in almost all muscle cells, mdx mice show slower development, relative to overall lifespan, or reduced severity of a number of clinically relevant disease phenotypes compared to DMD patients. This is especially true for loss of ambulation, cardiac and respiratory muscle weakness, and loss of lifespan, all major phenotypes contributing to DMD morbidity and mortality. All these phenotypes occur at an earlier age or to a greater degree in mdx mice bearing a human-like mutation in the mouse *Cmah* gene. Altered phenotypes correlate with changes in two mechanisms; reduced strength and expression of the dystrophin-associated glycoprotein complex and increased activation of complement. Activation of complement may be driven by the increased expression of anti-Neu5Gc antibodies in *Cmah*^{-/-}mdx animals and ultimately by uptake of N-glycolylneuraminic acid, a foreign glycan in humans and *Cmah*-deficient mice, from dietary sources. *Cmah*-deficient mdx mice represent a new small animal model for DMD that better approximates the human glycome and its contributions to muscular dystrophy.

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AUTHOR CONTRIBUTIONS

PTM designed the study, analyzed all data, and wrote the paper, with editorial input from AV, KC, and PJ. PTM and KC analyzed all cellular and molecular data. KC did most of the experiments, with significant assistance from JHY, MC, SdV. JHY did all ECM binding experiments, while MC and SdV did most assessments of muscle histopathology. AV made the *Cmah*^{-/-} mice, provided and characterized the Neu5Gc-specific reagents, and analyzed Sia content and profiles. PJ and YX performed and analyzed all cardiac and skeletal muscle physiology studies.

COMPETING INTERESTS

AV is a co-founder of Sialix Inc. (formerly Gc-free, Inc.), a biotech company focused on solving problems arising from anti-Neu5Gc antibodies. None of the other authors have any competing interests to declare.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe and progressive disease of muscle wasting that results from X-linked recessive mutations or deletions in the dystrophin gene that cause loss of dystrophin protein expression(1,2). DMD occurs with an incidence of approximately 1 in every 3500 boys(3). Boys with DMD can show evidence of muscle damage at birth (elevated serum creatine kinase levels), but are usually diagnosed after showing delayed motor milestones at age 2-5(4). Muscle wasting, the replacement of muscle tissue with fat or extracellular matrix, progresses with age, leading to severe muscle weakness and lack of ambulation, typically by age 12(5). Children most often perish from the disease due to complications related to cardiac or respiratory failure in the second to third decade of life(4). While use of high dose corticosteroids can prolong the ability to ambulate by several years in younger DMD patients(6,7), no therapy has yet been developed that changes the ultimate course of the disease, despite the fact that the gene responsible for DMD has been known for over twenty years(1,2).

The mdx mouse, first described in 1984(8), has become the most commonly used animal model for DMD(1,2,9-11). mdx carries a point mutation in exon 23 of the mouse dystrophin gene that leads to a premature stop codon, resulting in absent dystrophin protein from almost all skeletal and cardiac muscle cells(9), just as in DMD(1). Loss of dystrophin in mdx mouse muscles and DMD muscles leads to a disruption of the dystrophin-associated glycoprotein (DAG) complex, a series of proteins that link the basal lamina of extracellular matrix that surrounds every skeletal myofiber through the membrane to the F-actin cytoskeleton(12-16). Consistent with their central role in disease, loss of function mutations in almost all DAG proteins cause forms of muscular dystrophy(4,17-20); mutations in laminin $\alpha 2$ (*LAMA2*), the principal laminin in the basal lamina surrounding every skeletal myofiber(21,22), causes congenital muscular dystrophy 1A (*MDC1A*), mutations in *POMT1*, *POMT2*, *POMGnT1*, *FKTN*, *FKRP*, or *LARGE*, genes that affect the glycosylation of α dystroglycan(23), an extracellular membrane-associated protein that binds laminins in a glycosylation-dependent manner(16,24), cause congenital or limb-girdle muscular dystrophies (Walker Warburg Syndrome, Muscle Eye Brain Disease, Fukuyama Congenital Muscular Dystrophy, *MDC1C*, *MDC1D*, *LGMD2I*, *LGMD2K*, *LGMD2L-N*), and mutations in α , β , γ and δ sarcoglycans (*SGCA*, *SGCB*, *SGCG*, and *SGCD*), all DAG transmembrane proteins, cause forms of Limb Girdle muscular dystrophy (*LGMD2D*, *2E*, *2C*, and *2F*, respectively). In DMD and mdx muscle, dystroglycan, sarcoglycans, and other DAG proteins have lowered expression(12,15), which may vary depending on detergent extraction conditions(25,26), while the utrophin, an autosomal paralogue of dystrophin(27) that can bind many of the same proteins dystrophin does(28-30), is upregulated and can ameliorate disease(31,32).

mdx mice develop a number of aspects of DMD disease biology that have allowed investigators to use them for translational studies, however, the progression and severity of muscular dystrophy in mdx mice does not completely parallel the human disease. Muscle damage and concomitant muscle regeneration are present at 3-4 weeks of age in mdx animals, and such skeletal muscle pathology progresses, along with variable muscle weakness, in extent and severity as the animals mature(33,34). Muscle damage correlates with deficits in the physiological properties of mdx skeletal myofibers, including increased damage in response to eccentric contractions(35-38), reduced muscle specific force(35,36,38-41), increased calcium leakage into myofibers(42,43), increased dye uptake into myofibers(36,38,44), and increased release of muscle enzymes into the serum(45,46). Many of these phenotypes can be increased by a regimen of forced exercise(47-49). Evidence for replacement of muscle tissue with extracellular matrix or fat (muscle wasting), the process that ultimately drives muscle weakness in DMD, becomes significant in the diaphragm at 6 months of age(40). Thus, wasting of the diaphragm muscle in mdx mice phenocopies wasting that occurs in many DMD muscles.

Most large mdx limb muscles, however, only show significant fibrosis at very old ages(50). As such, mdx mice never truly fail to ambulate. Evidence of physiological decrements in cardiac muscle are also present at 6 months of age in mdx mice(51), but as with most skeletal muscles, fibrotic changes in the heart are far more evident only in very old animals (e.g. 20 months(40,50,52)). Such older animals also show other evidence of cardiac pathology, including necrotic lesions and cardiomyopathy, and have approximately a 20% reduction in lifespan compared to normal wild type mice(10,53,54). The disease phenotypes that develop in mdx mice have allowed them to be used to great effect to demonstrate proof of principle for many therapeutic approaches, including gene replacement therapy(55-58), surrogate gene therapy(31,32,46,59-68), exon skipping(69-72), RNA splicing(73,74), stop codon read-through(75,76), myoblast transfer(77-79), stem cell therapy(80-82), and various drug or nutritional therapies(83-85). All such studies, however, must be viewed within the context that a number of the phenotypes most relevant to DMD morbidity and mortality, respiratory and cardiac muscle weakness and failure, loss of ambulation, and loss of lifespan, are either less severe or show delayed onset, relative to a normal mouse's lifespan, when compared to the human disease.

As the lack of these early onset phenotypes in mdx mice does not pertain to loss of dystrophin, which is by and large absent in mdx muscles, it is highly likely that other human-mouse genetic differences account for differences in disease presentation. While some have attributed this to issues of animal size, with mice being roughly two orders of magnitude reduced relative to humans, it is clear that mdx mice can manifest more severe dystrophic phenotypes when engineered to contain additional gene mutations (for example mutations in utrophin (mdx*Utrn* $-/-$)(86)), integrin $\alpha 7$ (mdx*Itga7* $-/-$)(87)), α dystrobrevin (mdx*Adbn* $-/-$)(88), or MyoD (mdx*Myod1* $-/-$)(89). Unfortunately, none of these engineered mice speak directly to relevant human-mouse genetic changes, as all of these genes are expressed in DMD muscles(90-92). Obviously, human-mouse differences in phenotype development arise from multiple genetic differences between the two species. Nevertheless, it is useful to examine the contributions of specific interspecies genetic changes that are appropriate from an evolutionary perspective. One aspect of molecular evolution that can be highly variable between mammalian species is the repertoire of glycans present on the cell surface.

All glycoproteins and glycolipids within the plasma membrane contribute to the expression of a glycocalyx, a highly concentrated halo of carbohydrates (glycans) that surrounds the extracellular surface of all cells. It is within this intensely glycan-rich environment that all ligand-receptor signaling is initiated, all infectious processes begin, and in which all cellular movements and adhesive changes take place. As such, fundamental changes in the glycan repertoire could alter the biology of many systems and disease processes. Importantly, because infectious agents such as viruses and bacteria often use host cell glycans as receptors, cell surface glycans are also prone to alteration during natural selection, amplifying interspecies glycan differences(93). Because the same glycan structure can be present on multiple proteins and lipids, such changes can also impact multiple cellular and molecular processes.

Sialic acids (Sias) are an important class of monosaccharides expressed on the terminal ends of glycan structures on many glycoproteins and glycolipids(94). While there are dozens of possible Sia forms, the two most common in most mammals are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Neu5Gc differs from Neu5Ac only by having an additional oxygen atom at the 5-*N*-acyl position (Fig. 1A). Neu5Gc expression requires the *CMAH* gene, which encodes the CMP-Neu5Ac hydroxylase, an enzyme that hydroxylates the 5-*N*-acetyl group of Neu5Ac on CMP-Neu5Ac to make CMP-Neu5Gc(95, 96). Both CMP-Neu5Ac and CMP-Neu5Gc are sugar nucleotide donors used by the some 20 mammalian sialyltransferases to incorporate these Sias into glycans on proteins and lipids. Because most mammals, including mice, express a functional *CMAH* gene (Fig. 1A)(95,96),

they typically express a 50:50 mixture of Neu5Gc and Neu5Ac on glycoproteins and glycolipids in skeletal and cardiac muscles, the two cell types most effected in Duchenne muscular dystrophy. By contrast, dogs, for which there are also more human-like DMD models (10,97,98), contain almost undetectable Neu5Gc levels in skeletal muscle (2% of Sias on muscle gangliosides(99)) and humans express no Neu5Gc, and instead have an excess of Neu5Ac(100). This altered Sia expression is due to the presence of an inactivating deletion in human *CMAH* that occurred approximately 2-3 million years ago, after the divergence of modern humans from the great apes(101). All humans are null for Neu5Gc biosynthesis and human cells should be devoid of Neu5Gc expression(93), however, pathological human cells, for example cancer cells, can incorporate Neu5Gc from dietary sources via a salvage pathway which leads to Neu5Gc expression on their cell surface(100,102). Because all humans also express antibodies that recognize Neu5Gc(103,104) as a foreign antigen, such incorporation has the effect of creating Neu5Gc-glycan “xeno-auto-antigens” on such cells, which can alter cancer progression(105) and also allow toxins that recognize Neu5Gc to affect humans(106). Because glycosylation can be a strong modifier of disease in mdx mice(46) and in other mouse models of muscular dystrophy(107,108), we have tested whether *Cmah* gene affects disease biology by introducing a human-like inactivating *Cmah* gene deletion into mdx mice. In doing so, we have developed a more genetically appropriate small animal disease model for DMD that also has significant advantages for translational research.

RESULTS

Expression of Neu5Gc in *Cmah*-deficient mdx mice and in humans with Duchenne muscular dystrophy

To assess the role of *CMAH* in muscle disease, we incorporated a human-like inactivating deletion into the mouse *Cmah* gene(109) and bred it into the mdx mouse model for Duchenne muscular dystrophy for over 15 generations to create *Cmah*^{-/-}mdx mice on the same genetic background as mdx. To assess Neu5Gc expression, we used a monospecific affinity-purified Neu5Gc-specific IgY polyclonal antibody from chickens(110), a species also with no detectable Neu5Gc(110), to immunostain normal and diseased mouse and human muscle (Figs 1B-D). *Cmah*^{-/-} mice expressed no Neu5Gc in skeletal or cardiac muscle tissue (Figs. 1B, 1C), though occasional expression could be seen in blood vessels, as described previously (109). This expression results from Neu5Gc incorporation at low levels into endothelial cells and can be avoided by feeding the mice a diet devoid of Neu5Gc(109). As expected, both wild type (WT) and mdx mice expressed easily detectable Neu5Gc on the surface of both skeletal (Fig. 1B) and cardiac (Fig. 1C) muscle, as mice normally express *Cmah* in these tissues. In all cases, staining with non-immune chicken IgY gave no detectable staining of myofibers, but did show occasional staining of mononucleated cells (S1A). In addition, small regenerating muscles in mdx tissue showed higher levels of Neu5Gc expression than did mature myofibers, even in *Cmah*^{+/-}mdx muscles (S1B). Like *Cmah*^{-/-} mice, most skeletal myofibers in mice deficient for both *Cmah* and dystrophin (*Cmah*^{-/-}mdx) showed no Neu5Gc expression (S1B), however, a small number (less than 1%) of myofibers in *Cmah*^{-/-}mdx muscles were strongly Neu5Gc-positive (Fig. 1B), as were some *Cmah*^{-/-} mdx cardiomyocytes (Fig. 1C). Some Neu5Gc-positive fibers in *Cmah*^{-/-}mdx skeletal muscles appeared to be undergoing necrosis, as they lacked complete cytoplasm, but others appeared as normal regenerating myofibers or myoblasts (Fig. 1B and D). Likewise, normal human skeletal muscle expressed no detectable Neu5Gc, but small regenerating and necrotic muscles in DMD samples showed easily detectable expression of Neu5Gc on the sarcolemmal membrane (Fig. 1B). Regenerating myofibers (and/or myoblasts) were identified in DMD and *Cmah*^{-/-}mdx skeletal muscle by co-staining with embryonic myosin and were Neu5Gc-positive at a very early stage of development (Fig. 1D), often prior to expression of laminin α 2 (S1C). Biopsies from patients with inflammatory myopathy (inclusion body myositis), by contrast to DMD, showed no

Neu5Gc staining of myofibers (Fig. 1D). All muscles studied showed no gross deficit in overall sialic acid expression, as evidenced by staining with lectins, such as *Maackia amurensis* agglutinin (MAA), which bind both Neu5Ac and Neu5Gc (S1D-S1E). Total sialic acid content of the muscles also showed no obvious differences, as determined by DMB-derivatization and HPLC analysis (not shown). This assay could not detect the trace amounts of Neu5Gc in *Cmah*^{-/-}mdx muscles seen by the antibodies (Fig. 1B-D), but this is not surprising, given the focal Neu5Gc staining pattern and the likely low level of Neu5Gc incorporation into muscle cells.

Accelerated disease onset and increased disease severity in *Cmah*^{-/-}mdx mice

The muscle histopathology of the mdx mouse is consistent with what is observed in DMD, yet many major phenotypic milestones of the clinical disease, including early loss of muscle tissue (wasting) and respiratory and cardiac failure, do not often appear until the animals are near the end of their normal lifespan(10,11). As a likely consequence, lifespan is reduced in mdx mice by less than 20% when compared to wild type controls(54). *Cmah*^{-/-}mdx mice, by contrast to mdx animals, showed a clinical progression and physiologic decrements more representative of what is seen in DMD patients, strongly arguing that *Cmah* is a genetic modifier of disease in mdx mice (Fig. 2). *Cmah*^{-/-}mdx mice showed highly significant decreases in lifespan compared to mdx, with almost half of all animals dying by 11 months of age (Fig. 2A). At 8 months of age, most aged *Cmah*^{-/-}mdx mice showed impaired ambulation, having a 70% decrease on constant-speed (5 rpm) rotarod test when compared to mdx littermates (Fig. 2B). Thus, while mdx mice are also impaired in ambulation at this age, *Cmah*^{-/-}mdx mice are impaired to a much greater extent. In DMD, failure of diaphragm and cardiac muscles are the most predominant contributors to mortality. Aged (8-month-old) *Cmah*^{-/-}mdx mice showed highly significant deficits in diaphragm (Fig. 2C) and cardiac (Fig. 2D) muscle strength (specific force; force normalized to muscle weight) compared to age-matched mdx controls. *Cmah*^{-/-}mdx diaphragm showed a reduction in peak force of 88% (at 125Hz), and cardiac trabeculae of 60% (at 6Hz), compared to WT (Fig. 2C and D). mdx diaphragm also showed reduced specific force compared to WT, consistent with the fact that the diaphragm is the one muscle in mdx animals that shows significant fibrosis beyond 6 months of age(40). By contrast, peak isometric active developed tension of mdx cardiac trabeculae were only changed at the highest frequencies used (10-12Hz) compared to WT, while *Cmah*^{-/-}mdx trabeculae were reduced at all frequencies between 4 and 12Hz ($P < 0.01$ for all vs. WT). The frequencies used here for heart muscle measures are relevant in the mouse; the resting mouse heart beats at roughly 600 beats per minute (or 10 Hz) and can increase up to 800 beats per minute upon stimulation (or ca. 13Hz)(111). We also measured Evan's blue dye uptake, a measure of myofiber membrane damage, into skeletal myofibers after exercise and found that all *Cmah*^{-/-}mdx skeletal muscles had significantly more damage than mdx muscles (Fig. 2E). Similarly, *Cmah*^{-/-}mdx muscles showed increased loss of force during repeated eccentric contractions (in the extensor digitorum longus muscle) compared to mdx, with a small (but insignificant) decrease in maximal specific force as well (Fig. 2F). In none of these instances did *Cmah*-deficient mice expressing dystrophin (*Cmah*^{-/-}) show a significant change compared to wild type animals (Figs. 2A-2F). Thus, loss of *Cmah* in mdx mice led to significant weakness of both cardiac and skeletal muscles relative to mdx mice that was specific to dystrophin-deficiency, and this was particularly significant in diaphragm and heart, the two muscles whose failure typically causes death of DMD patients.

Although mdx mice display histopathology that is reflective of DMD(10,11), loss of *Cmah* increased many relevant histopathology measures. Of particular note, *Cmah*^{-/-}mdx mice had increased necrotic foci (regions of damage with immune cell infiltrates) by 3 months of age in the heart (Fig. 3A) and increased fibrosis (replacement of skeletal muscle with extracellular matrix (ECM)) by 6 weeks of age in the quadriceps (Fig. 3B). Normally these findings are not present in the larger limb muscles of mdx mice until the mice are very old(10,11). In addition,

the extent of fibrosis in *Cmah*^{-/-}mdx diaphragm, the only mdx muscle where fibrosis is routinely found(40), at 6 months of age was increased relative to mdx(Fig. 3C). Evan's blue dye uptake was also increased, indicating increased myofiber damage in *Cmah*^{-/-}mdx (Fig. 3D and 2E). Overall, measures of muscle damage (regions with necrotic myofibers or where muscle tissue was replaced by ECM or inflammatory infiltrates) were significantly increased in the gastrocnemius, quadriceps, tibialis anterior, diaphragm, and heart (Fig. 3E). Thus, while the extent of muscle damage varied from muscle to muscle, as it does in DMD(5), there was generally increased muscle damage in *Cmah*^{-/-}mdx muscles compared to mdx. Other measures of muscle histopathology or damage, including skeletal myofibers with centrally located nuclei (S2A), coefficient of variance in skeletal myofiber diameter (S2B), average myofiber diameter (S2C), and serum creatine kinase activity (S2D) were increased in *Cmah*^{-/-}mdx muscles relative to mdx, but these changes were not statistically significant. Mouse (2SE) and muscle (2SF) weights at 5 months of age were decreased in *Cmah*^{-/-}mdx relative to mdx. These decreases were independent of changes in average myofiber diameter (S2C), and were therefore also suggestive of increased muscle damage.

Altered protein binding and expression in *Cmah*^{-/-}mdx skeletal muscle

Because sialic acids are abundantly present in the membranes of all mammalian cells, altering the sialic acid repertoire by eliminating Neu5Gc could alter multiple molecular processes (112). There are, however, two particular mechanisms known either to cause or worsen muscular dystrophy that merited a detailed investigation, the relative strength (and composition) of the dystrophin-associated glycoprotein (DAG) complex and the relative immune response to dystrophic muscle tissue. We first investigated the role of the sialyl-containing glycans and their modulation of ECM binding on α dystroglycan (α DG). Dystroglycan is an essential membrane component of the DAG complex and binding of muscle ECM proteins, including laminins and agrin, to α DG requires the O-mannosyl-linked sialylated tetrasaccharides (Neu5Ac(or Neu5Gc) α 2-3Gal β 1-4GlcNAc β 1-2Man- α -O-Ser/Thr)(24) present in the mucin domain of the α DG polypeptide(113). To assess the role of sialyl-containing glycans in ECM binding to α DG, we purified α DG from skeletal muscle of *Cmah*^{-/-} mice, where none of the Sia on α DG is Neu5Gc (but instead is all Neu5Ac(109,110)), and from wild type skeletal muscle, where Neu5Gc is present (S3A, B). We also purified recombinant forms of muscle laminins (α 2, α 4, and α 5) and agrin (α 0, muscle, and α 8, neural) (S3A). The amount of α DG immobilized was verified to be the same using α DG antibodies that recognize both glycoforms equally well and the presence or absence of Neu5Gc was confirmed using a Neu5Gc-specific antibody (S3B). Both recombinant laminin α 2 (G1-G5 domains) (Fig. 4A) and agrin (S4A) showed decreased binding to *Cmah*^{-/-} α DG relative to WT α DG (36 $\pm$ 1% decrease for laminin α 2 and 39 $\pm$ 3% for agrin at the highest concentration used, P<0.001 for both).

To more directly demonstrate the relevance of Neu5Gc to ECM binding, we also measured binding of sialyl-containing glycans (Neu5Gc(or Neu5Ac) α 2-3Gal β 1-4GlcNAc-biotinylated-polyacrylamide (PAA)) or Neu5Gc(or Neu5Ac) α 2-6Gal β 1-4GlcNAc-biotinylated-PAA) to the same proteins (S4B-C). Again, we found significant decreases in maximal binding of Neu5Ac-containing glycans to ECM proteins, relative to glycans containing Neu5Gc (P<0.05 for all Neu5Gc-vs. Neu5Ac-PAA glycans at 500nM). Similarly, measures of apparent solid-phase binding affinity of α 2-3- and α 2-6-linked Neu5Gc glycans to ECM proteins were increased relative to their Neu5Ac counterparts (S4D). These data show that laminins and agrins have properties consistent with sialic acid binding lectins, showing significant binding affinity for sialic acid-containing glycans, particularly α 2-6-linked Neu5Gc glycans, that is independent of the α DG polypeptide. While the affinity of these glycans for ECM proteins does not match that of the native α dystroglycan glycoprotein, the affinity of some glycans was within one log of this value (S4D). These data indicate that the presence of Neu5Gc on the

sarcolemmal membrane strengthens ECM binding, particularly when Neu5Gc glycans are presented in a multivalent form, as done here using PAA conjugation. Such changes are similar to changes in laminin binding to α dystroglycan when terminal β 1-4-linked GalNAc is present (92). Overexpression of Galgt2, the enzyme that produces this carbohydrate, can inhibit mdx muscle damage(38) and pathology(46,114). Such changes could therefore help explain the increased damage observed in *Cmah*^{-/-}mdx muscles.

Other proteins, especially utrophin, α dystrobrevin, and α - δ sarcoglycans, are also important components of the DAG complex in skeletal and cardiac muscle(115). Utrophin, a dystrophin orthologue that is naturally upregulated in mdx skeletal muscles(116), can ameliorate mdx muscle disease when overexpressed(32) and can increase mdx disease severity when deleted (86,117), even when only one of the two utrophin alleles is absent(118). Similarly, deletion of α dystrobrevin, which binds dystrophin and utrophin, in mdx mice amplifies the severity of muscular dystrophy(88). Accordingly, we measured utrophin, α dystrobrevin, and other DAG member protein (Fig. 4E, 4H-J) and mRNA expression (Fig. 4F-G) in skeletal muscle and heart. While DAG protein expression in KCl-washed purified sarcolemmal membranes was originally found to be severely reduced in mdx skeletal muscle(15), such findings were due in part to detergent extraction conditions(26,119). We(46) and others(119) have found that DAG protein expression is more modestly changed in crude mdx muscle extracts compared to wild type, and we have used such lysates here(46). mdx skeletal muscles have increased utrophin protein expression relative to wild type, but this compensatory increase was reduced by 30 $\pm$ 2% for utrophin, by 36 $\pm$ 9% for α dystrobrevin 2 (Fig 4H). By contrast, we observed no change in *Cmah*^{-/-}mdx muscle (vs. mdx) for α - δ sarcoglycans, α - β dystroglycan, or α dystrobrevins 3-4, though all were reduced in both mdx and *Cmah*^{-/-}mdx muscles compared to WT and *Cmah*^{-/-} (Figs. 4E, 4H), as previously reported(15,46). While utrophin and α dystrobrevin protein were significantly reduced in *Cmah*^{-/-}mdx relative to mdx, mouse IgM and Collagen I and Collagen III were significantly increased (Fig. 4E, H). By contrast to skeletal muscle, no decrease in utrophin or α dystrobrevin protein was observed in *Cmah*^{-/-}mdx heart relative to mdx (Fig. 4E, 4I). Here, however, *Cmah*^{-/-} cardiac muscle, in addition to mdx, and *Cmah*^{-/-}mdx muscle, showed increased expression of Collagen I and Collagen III (Fig. 4I, J). This finding suggests the presence of fibrosis in *Cmah*^{-/-}, mdx and *Cmah*^{-/-}mdx heart muscle. In fact, the only protein significantly reduced in *Cmah*^{-/-}mdx heart relative to mdx was Serca2 (Fig. 4I, J). The reduced expression of Serca2 and the elevated expression of Col1 and Col3 all are potential indicators of increased cardiac hypertrophy(120). Shorter versions of dystrobrevin (Dbn3-4) were significantly increased in both mdx and *Cmah*^{-/-}mdx heart, while dystrobrevin 2, α dystroglycan, and α , β , and δ sarcoglycan were significantly decreased in mdx and *Cmah*^{-/-}mdx animals (compared to either WT or *Cmah*^{-/-}, Fig. 4E, I, P<0.05 for all).

Consistent with changed protein expression, compensatory increased mRNA expression was observed for utrophin, α dystrobrevin, and other DAG and extracellular matrix genes in mdx skeletal muscle by qRT-PCR (Fig. 4F). The extent of increased expression in mdx muscle (vs. WT) was reduced in *Cmah*^{-/-}mdx skeletal muscle (vs. WT) for a number of muscle ECM and DAG genes (Fig. 4F, 65 $\pm$ 3% reduction for utrophin, 56 $\pm$ 2% for α dystrobrevin, 84 $\pm$ 2% for laminin α 2, 56 $\pm$ 2% for laminin α 4, 100 $\pm$ 2% for collagen IV(α 1), 100 $\pm$ 2% for collagen IV (α 2), 56 $\pm$ 2% for agrin, 48 $\pm$ 1% for dystroglycan, and 64 $\pm$ 2 for β sarcoglycan, P<0.01 for all). Thus, *Cmah* deletion may globally affect DAG and ECM gene expression in mdx skeletal muscle. Conversely, such changes may reflect the increased dystrophy present in *Cmah*^{-/-}mdx skeletal. mRNA expression levels for many DAG genes were unchanged in *Cmah*^{-/-}mdx heart (Fig. 4G). The two exceptions here were laminin α 5 and integrin α 7, which were elevated in mdx heart (vs. WT) and significantly reduced in *Cmah*^{-/-}mdx heart (Fig. 4G).

To explore molecular changes more broadly, we analyzed transcriptional changes using Affymetrix microarrays to compare WT, *Cmah*^{-/-}, mdx, and *Cmah*^{-/-}mdx skeletal muscle and heart (Fig. 4K). In analyzing the DAG gene expression specifically in such arrays, we identified reduced expression of utrophin and α dystrobrevin in *Cmah*^{-/-}mdx skeletal muscle relative to mdx (not shown). More broadly, in skeletal muscle we identified 618 unique gene expression changes (out of 39,000 mouse transcripts analyzed, $P < 0.05$) in *Cmah*^{-/-}mdx skeletal muscle relative to mdx, *Cmah*^{-/-}, and WT, and 404 such unique changes in heart. The majority of genes with changed expression in skeletal muscle (79%) and heart (78%) did not overlap with gene changes found in mdx vs. WT or *Cmah*^{-/-} vs. WT. Thus, most gene changes in *Cmah*^{-/-}mdx are unique to the combination of dystrophin-deficiency and *Cmah* deletion and are not simply an amplification of dystrophic changes found in mdx muscle.

Altered immune responses in *Cmah*^{-/-}mdx mice

Last, we assessed immune responses in mouse muscles (Figs. 5, 6), as T cell-, macrophage/monocyte-, and antibody-mediated inflammatory responses are all known to play a role in DMD pathogenesis (121). We first looked at expression of markers of muscle inflammation, focusing on factors known to play a role in recruitment of immune cells to skeletal muscles. Gene expression markers of muscle inflammation (CD68, IL-1 β , MCP-1, MIP-1a, RANTES, TNF α) in skeletal muscle were generally higher in both mdx and *Cmah*^{-/-}mdx muscles, compared to wild type (WT) and *Cmah*^{-/-}, as expected for dystrophic muscles (Fig. 5A). At 2 months, when dystrophy is first present in mdx muscles, we found reduced levels of several markers in *Cmah*^{-/-}mdx mice relative to mdx (IL-1 β and MIP-1a, Fig. 5A), while IL-1 β and MCP-1 were increased (by $100 \pm 7\%$ and $35 \pm 3\%$, respectively) in *Cmah*^{-/-}mdx relative to mdx at 5 months of age, when dystrophy is more severe (Fig. 5A). Expression of CD68, a macrophage/monocyte marker, was high in both genotypes of mice at both ages. In heart, markers of inflammation were not evident until 5 months of age (Fig. 5B). Here, as with elevated collagen protein levels (Fig. 4I, J) and gene expression levels (Fig. 5C), we found elevated expression of CD68 and MCP-1 in *Cmah*^{-/-} muscle in addition to mdx and *Cmah*^{-/-}mdx, while we observed no signal for MIP-1a or RANTES (Fig. 5B). Natriuretic peptide precursor type A (ANP) levels, which are elevated with cardiac hypertrophy, were also elevated in all three of these genotypes (Fig. 5C). These data suggest that heart muscle may be subject to Neu5Gc-dependent phenotypes that are independent of loss of dystrophin. We also quantified (using immunostaining) the numbers of intramuscular resident macrophage/monocytes (CD68), T helper cells (CD4), and cytotoxic T cells (CD8) (Fig. 5D). All three of these cell populations were elevated in most mdx and *Cmah*^{-/-}mdx muscles compared to WT and *Cmah*^{-/-}. As expected, the majority of immune cells in mdx and *Cmah*^{-/-}mdx muscles were CD68+ macrophages/monocytes, the cells primarily responsible for clearing damaged myofibers. While there was some muscle-to-muscle variability both in resident numbers of intramuscular immune cells and in molecular markers of muscle inflammation, *Cmah*^{-/-}mdx muscles showed no generalized increase in inflammation compared to mdx that would explain their increased severity of muscle disease. Several muscles (gastroc, TA, and diaphragm, but not quad, triceps or heart), however, did show increased numbers of CD68+ mononuclear cells at 5 months of age (Fig. 5E, $P < 0.05$ for gastroc and TA). This increase in intramuscular macrophage/monocytes could be a reflection of increased dystrophy at this age in *Cmah*^{-/-}mdx muscles. Alternatively, it could reflect increased inflammation in *Cmah*^{-/-}mdx muscles that is in turn driving increased dystrophy.

While overall intramuscular T cell burden was only minimally changed in *Cmah*^{-/-}mdx animals compared to mdx, it was possible that the T cells present in *Cmah*^{-/-}mdx animals might still be more active than those present in mdx. We tested this in several ways. First, we performed ELISPOT assays of T cell activation using both Interleukin 4 (IL4) and Interferon gamma (IFN γ) as reporters of Th2 and Th1 responses, respectively, as we have done previously

(122) (Fig. 5E). Non-stimulated mdx and *Cmah*^{-/-}mdx splenocytes showed a slight elevation in IL4⁺ ELISPOTs compared to WT and *Cmah*^{-/-}, and both mdx and *Cmah*^{-/-}mdx splenocytes showed higher ConA induction of ELISPOTs for IL4 and IFN γ compared to WT and *Cmah*^{-/-} (Fig. 5E). There were no significant differences, however, between *Cmah*^{-/-}mdx and mdx using either measure. We also performed T cell proliferation assays using uptake of ³H-thymidine, as previously(122), to assess whether *Cmah*^{-/-}mdx splenocytes would show a difference in activation of T cell division (Fig. 5F). ConA increased the T cell stimulation index for all four genotypes of mice, but again there was no difference between mdx and *Cmah*^{-/-}mdx splenocytes. Thus, we found no evidence of a difference in the activation potential of T cells between *Cmah*^{-/-}mdx and mdx mice. We did not assess activation by Neu5Gc directly in these assays, as there are no known T cell receptors that can recognize terminal sialic acids on glycans.

Because we had observed incorporation of dietary Neu5Gc specifically in *Cmah*^{-/-}mdx cardiac and skeletal muscle (Fig. 1B-D), we wanted to determine if this expression would increase the production of anti-Neu5Gc antibodies, as Neu5Gc is a foreign glycan in *Cmah*^{-/-}mdx mice, as it also is in humans(103). We used α 2-3- or α 2-6-specific sialic acid lectins to purify skeletal muscle proteins from WT muscle, which contains Neu5Gc (S3C), and from *Cmah*^{-/-} muscle, which does not (S3C), to compare serum antibody binding at varying dilutions. While serum from WT, *Cmah*^{-/-}, and mdx mice showed no Neu5Gc-specific titer to either α 2-3- or α 2-6-linked glycoproteins, serum from *Cmah*^{-/-}mdx mice showed anti-Neu5Gc-specific titers as high as 13 μ g/ml (and on average 2 $\pm$ 1 μ g/mL) to α 2-6-linked structures (Fig. 6A). This is almost one log below the average level identified in studies of human serum, where anti-Neu5Gc-specific antibody titers are also higher to α 2-6-linked Sia-containing glycans than to α 2-3-linked ones(103).

We next wanted to determine if the increased Neu5Gc-dependent antibodies in *Cmah*^{-/-}mdx mouse serum (Fig. 6A) might stimulate complement-mediated killing of Neu5Gc-expressing cells. To do this, we compared the serum of *Cmah*^{-/-}mdx and mdx mice in complement-dependent killing assays (Fig. 6B). Using varying dilutions, we found that serum from *Cmah*^{-/-}mdx mice uniformly increased complement-mediated killing of Neu5Gc-rich cells (wild type, WT) relative to Neu5Gc-deficient cells (*Cmah*^{-/-}, Fig. 6B). This was true both for cultures of primary myoblasts and myotubes (Fig. 6B). Serum from mdx mice, by contrast, showed no such difference at any dilution tested (Fig. 6B). This suggests that anti-Neu5Gc antibodies *Cmah*^{-/-}mdx serum can drive activation of complement and the killing of Neu5Gc-expressing myoblasts and myotubes. Consistent with this notion, we found that *Cmah*^{-/-}mdx skeletal and cardiac muscles had increased deposition of mouse antibody (Fig. 4E and S6) and activated (C5b-9) complement (Figs. 6C, D and S5-6). By contrast, there was no deposition of activated (C5b-9) complement or mouse antibody in WT or *Cmah*^{-/-} muscle (S6). Deposition of activated complement was evident on small regenerating myofibers (or differentiating myoblasts), which were marked by co-staining for embryonic myosin, and some such muscles were also positive for Neu5Gc expression (S6). Many myofibers with deposited C5b-9 complement also had deposited endogenous mouse antibody, suggestive of activation of the classical (antibody-mediated) complement pathway (S6). This was not, however, the case in all myofibers (S6). The increased deposition of activated C5b-9 complement on *Cmah*^{-/-}mdx muscles *in vivo* would be expected to destroy the myofibers to which it is bound, as C5b-9 is a cytotoxic complement protein complex.

DISCUSSION

The evolution of modern humans from the great apes coincided with a series of genetic changes that altered both the composition of sialic acids on the surface of human cells and the properties of sialic acid-binding proteins(112). While there may have been advantages to such changes,

for example blunting infections by lethal pathogens like malaria(123), our experiments suggest that deletion of *CMAH* came at the cost of worsening the severity of Duchenne muscular dystrophy. While there are certainly additional genetic changes in humans that reflect altered DMD disease presentation relative to mdx animals and also additional evolutionary changes in humans resulting from loss of *CMAH* that have impacted, and perhaps muted, loss of Neu5Gc function, the fact that the single genetic change of deleting *Cmah* in mice results in accelerated presentation of dystrophic phenotypes in mdx animals is striking and suggests a direct role for Sia modifications in muscle disease. Importantly, regardless of the exact mechanism of action, *Cmah*^{-/-}mdx mice represent both a biologically and genetically improved small animal model for DMD that should facilitate the testing of therapeutics in a context of morbidity and mortality measures more akin to the human disease. As the currently most accepted severe model for DMD is the dystrophin-deficient golden retriever (GRMD)(98), having a mouse model that can better mimic these more severe aspects of DMD, including increased mortality, increased cardiac and respiratory muscle weakness, and increased deficits in ambulation, should significantly facilitate translational work on these important aspects of DMD by allowing drug and gene therapy tests on a model that does not require such significant scaling of reagent. As dogs are breeds and not pure genetic strains, the genetic variability between animals would also be reduced in *Cmah*^{-/-}mdx mice compared to GRMD dogs. It is interesting to note that dog skeletal muscles also tend to be poor in Neu5Gc content(99), perhaps accounting for the increased severity, as in *Cmah* null mice.

We have further described two, not mutually exclusive, mechanisms that may contribute to the increased disease severity found in *Cmah*^{-/-}mdx animals. The first involves both weakened expression and function of the DAG complex, including reduced ECM binding to α dystroglycan and reduced expression of utrophin, a dystrophin surrogate that when overexpressed can ameliorate disease(61), as well as reduced expression of other dystrophin-associated glycoproteins (DAGs). While the reduction in DAG protein levels shown here may seem modest (30-35% for utrophin and α 2 dystrobrevin relative to mdx), loss of only one allele of utrophin has recently been shown to increase the severity of muscle damage in mdx animals (118). Loss of DAG expression or strength would contribute to the weakening of sarcolemmal membrane and the integrity in muscle fibers. The second potential mechanism involves the metabolic accumulation of dietary Neu5Gc(102), generation of Neu5Gc-specific antibodies (103), and the deposition of activated (C5b-9) complement on muscle fibers. Both activated C5b-9 complement(124) and CD8⁺ cytotoxic T cells(125) are known to be present in DMD muscle, and we have shown here high C5b-9 deposition in young DMD muscle. Because *Cmah*-deficient mdx myofibers, like DMD myofibers, appear to preferentially take up Neu5Gc from diet, they may better mimic the role of dietary Neu5Gc in priming immune responses to regenerating muscle, a process that would seed the destruction of the very cells needed to overcome dystrophic muscle damage. This could speed the wasting of skeletal muscle in DMD, the mechanism that ultimately causes muscle failure and mortality in patients. Because all humans express antibodies that recognize Neu5Gc(103,104), a foreign antigen in all humans, and because most humans eat large amounts of Neu5Gc in their diet from Neu5Gc-rich food sources, particularly red meat, that can be incorporated into human cells under certain conditions(100,105), this mechanism may be particularly significant. Importantly, the levels of Neu5Gc antibodies we have measured in *Cmah*^{-/-}mdx mice are lower than the levels found in normal human subjects(103). Therefore, this mechanism may be even more significant were *Cmah*^{-/-}mdx anti-Neu5Gc antibody levels normalized to levels found in humans. Because sialic acids are present on many proteins and lipids, and because they are present in all tissues, removal of Neu5Gc in mice likely has affects on many signaling pathways beyond those described here. The broad spectrum of functions influenced by individual cell surface carbohydrates makes the creation of animal disease models that mimic the human glycome all the more important.

MATERIALS AND METHODS

Materials

Antibodies to dystrophin (NCL-DYS1), utrophin (NCL-DRP2), α -sarcoglycan (NCL-a-SARC), β -sarcoglycan (NCL-b-SARC), δ -sarcoglycan (NCL-d-SARC), γ -sarcoglycan (NCL-g-SARC), Myosin heavy chain (developmental) (NCL-MHC-d), and β -dystroglycan (NCL-b-DG) were purchased from Novocastra (Newcastle Upon Tyne, UK). Antibody to α -dystroglycan (IIH6C4) was purchased from Upstate Biotechnology (Lake Placid, NY). Antibody to α -dystrobrevin (610766) was purchased from BD Transduction Laboratories (San Jose, CA). Antibodies to actin (A5060) and FLAG (M2, horseradish peroxidase (HRP)-conjugated and agarose-conjugated) were purchased from Sigma Aldrich (St. Louis, MO). Antibody to mouse CD68 (MCA1957Ga) was purchased from Serotec (Oxford, UK). Antibodies to mouse CD4 (550278) and CD8 (550281) were purchased from BD Pharmingen (San Diego, CA). Anti-sera specific for the activated state of complement C5b-9 complex (55811), rabbit polyclonal antibody to collagen I (ab34710) and rabbit polyclonal antibody to collagen III (ab7778) were purchased from Abcam (Cambridge, MA). Mouse monoclonal antibody (MA3-919) to SERCA2 was purchased from Thermo Scientific (Rockford, IL). An additional rabbit polyclonal antiserum specific to SERCA2a was a gift from Muthu Periasamy (Ohio State). *Maackia amurensis* agglutinin (MAA, FITC-or -agarose conjugated), *Sambucus nigra* agglutinin (SNA, FITC- or agarose-conjugated), and Wheat germ agglutinin (WGA, agarose-conjugated) were purchased from EY laboratories (San Mateo, CA). Species- and/or antibody-specific secondary antibodies conjugated with FITC, rhodamine, Cy2, Cy3, or HRP were purchased from Jackson Immunochemicals (West Grove, PA). Methods for production of anti-Neu5Gc antibody and its specificity for Neu5Gc have been previously described(109, 110).

Glycans conjugated to polyacrylamide (and containing biotin), including Neu5Gc α 2-3Gal β 1-4GlcNAc-PAA, Neu5Ac α 2-3Gal β 1-4GlcNAc-PAA, Neu5Gc α 2-6Gal β 1-4GlcNAc-PAA, Neu5Ac α 2-6Gal β 1-4GlcNAc-PAA, and Gal α 1-3Gal β 1-4GlcNAc-PAA, were obtained from the Consortium for Functional Glycomics (Core D) or were purchased from Glycotech (Gaithersburg, MD). All plasmids used in the production of recombinant proteins have been previously described(126,127).

Animals

All mice were housed on a 12:12-h light-dark cycle and had access to a pellet diet (Cat # 2919, Teklad Global Rodent diet, Harlan, USA) and water ad libitum. The content of Neu5Gc in the diet was measured as 6 μ g Neu5Gc per g mouse chow. This gives a daily consumption dosage, assuming 4g of mouse chow consumed per day and an average mouse weight of 25g, of 0.96mg/kg/day for Neu5Gc. This dosage is perhaps 3-fold higher than that of a human 30kg child, whose dose would be 0.33mg/kg/day based on an estimated 10mg Neu5Gc consumed in the diet daily(100). However, absorption of Neu5Gc from the gut into the blood-stream could be considerably different between species and was not studied here. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The Research Institute at Nationwide Children's Hospital and/or The Ohio State University. Wild type (WT) C57Bl/6J and mdx (C57Bl/10ScSn-Dmd^{mdx/Y} and C57Bl/10ScSn-Dmd^{mdx/mdx}) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine), bred, and maintained in the transgenic barrier facility at Nationwide Children's Hospital or at The Ohio State University. Breeding pairs of *Cmah*-null mice (*Cmah*^{-/-}) were made and described previously(109). These mice carried a human-like deletion of exon 6 of the *Cmah* gene and were generated by embryonic stem cell targeting by the introduction of loxP sites flanking exon 6 and Cre-mediated recombination in ES cells(109). *Cmah*^{-/-}mdx mice were obtained through creating first and second filial generations by the following strategy. Female mdx/mdx

mice were bred with male *Cmah*^{-/-} mice. F1 males (*Cmah*^{+/-mdx/Y}) were then mated with mdx/mdx females to create F2 generation that were all dystrophin-deficient (mdx) and were either heterozygous for deletion of the *Cmah* locus or contained two wild type *Cmah* alleles. *Cmah*^{+/-mdx/Y} and *Cmah*^{+/-mdx/mdx} mice were then interbred to obtain *Cmah*^{-/-mdx} mice. *Cmah*^{+/-mdx} and *Cmah*^{+/+} mdx mice generated from the same crosses were used as controls for all experiments, and did not differ from one another for any of the measures used (not shown). DNA extracted from toe clips were used, as described previously by Hedlund et al (109), to genotype mice for the *Cmah* deletion by polymerase chain reaction.

Histology and Morphometric Analysis

Animals were anesthetized and skeletal muscles were rapidly excised and snap-frozen in liquid nitrogen-cooled isopentane. Hearts were rinsed free of blood using ice-cold saline and embedded in optimal cutting temperature (OCT) embedding medium in a dry ice isopentane slurry. 8 µm sections were cut in a cryostat and used for histological and immunohistochemical studies. Histomorphological changes in the muscle were analyzed by routine hematoxylin (72404, Richard Allan Scientific) and eosin (318906, Sigma) staining, as previously described (107) or using Masson's trichrome stain (HT15-1KT, Sigma). Myofiber diameter and area, central nuclei, necrotic and fibrotic area were determined by using the measurement parameters in the imaging software, AxioVision LE 4.1 (Zeiss; Jena, Germany), as previously described (107).

For immuno- or lectin-staining, muscles were frozen and sectioned as above. For staining with MAA, SNA or antibody to activated C5b-9 complement, sections were blocked in 5% bovine serum albumin (BSA) in phosphobuffered saline (PBS), pH 7.2. Sections were incubated with FITC-conjugated lectin (10 µg/mL) or with antibody, washed, probed with fluorophore-conjugated secondary antibody if necessary, and then mounted. For Neu5Gc staining (and non-immune chicken IgY), sections were blocked in PBS with 10% human serum that was confirmed to be Neu5Gc free. Sections were incubated with anti-Neu5Gc or control antibody (1:500) in blocking solution overnight at 4°C, washed in PBS, and incubated with goat anti-chicken IgY secondary antibody conjugated to fluorophore (1:250; 703-095-155, Jackson ImmunoResearch). For all other antibodies used, which were antibody raised either in rats, rabbits, mice or chickens, sections were blocked in PBS containing 10% goat serum, incubated in primary antibody overnight, washed in PBS, and incubated with the appropriate secondary reagent. Quantitation of immune cells per unit area or antibody staining per unit area was done on a Zeiss Axiophot epifluorescence microscope using AxioVision LE 4.1 imaging software (Zeiss; Jena, Germany). At least 10 sections per muscle were analyzed for each animal to obtain measures, with the entire cross-sectional area considered in each case for regions comprising the muscle in question.

Survivability

Growth and death of C57BL/6J (Wild type, WT), *Cmah*^{-/-}, *Cmah*^{+/-mdx}, *Cmah*^{+/+mdx}, and *Cmah*^{-/-mdx} mice was recorded daily and death events were used to plot the survivability curve using GraphPad Prism 4 (Version 4.03; GraphPad Software; La Jolla, CA).

Assessment of Motor Function by Rotarod

Rotarod sessions were performed in a room adjacent to the housing room in the vivarium. The mice were first allowed to acclimatize to the new surroundings and train on the rotarod treadmill (Economex Rotarod, Columbus Instruments, USA). To ascertain motor function, mice were individually placed in a neutral position on the immobile rotarod bars. Then the rotarod was activated at a constant speed of 5 rpm and the time for each mouse fall off the rod was noted. Motor function was assessed on consecutive days and the average latency to fall was determined.

Measurements of Cardiac and Skeletal Muscle Physiology

Assessment of contractile properties of isolated cardiac trabeculae and papillary muscles was done as previously described(52). Briefly, small, thin preparations were dissected from the right ventricle and placed in between a force transducer and stimulator hook in an experimental set-up, superfused with Krebs-Henseleit solution at 37°C. Twitch contractions were initiated by delivering 3 ms-wide pulses and the force-response was recorded. This force response was assessed at different lengths, different frequencies, and during infusion of the β -adrenergic agonist isoproterenol. After completion of measurements, muscles were weighed and measures normalized to muscle weight as before(52).

Assessment of diaphragm muscle strip contractility was done as previously described(68). Briefly, small linear strips, were suspended between a force transducer and stimulator hook in an experimental set-up, superfused with Krebs-Henseleit solution at 37° C. Tetanic force was assessed by tetani of 600 ms duration, with frequencies ranging from 20 to 250 Hz, pulse width 1 ms. After measurements, muscles were weighed and cross-sectional area calculated as previously described(38).

Assessment of EDL contractile parameters was done as previously described(38). Briefly, isolated EDL muscles were tied to a force transducer and linear servomotor. Twitch contractions at 30°C were elicited and the muscle was stretched to optimal length. Next, 1-3 tetani, 500 ms in duration using 1 ms pulses at 150 Hz were imposed on the muscle. This was followed by 10 repeats of eccentric contractions. Per repeat, the muscle was tetanized for 700 ms, and stretched by 5% of its initial length during the last 200 ms of the tetanus. After stimulation was halted at t=700 ms, the muscle was taken back to its original length in 200 ms. In between repeats, the muscle remained unstimulated for 2 minutes. After measurements, muscles were weighed and cross-sectional area calculated as previously described(38).

Sarcolemmal Membrane Integrity by Evans Blue Dye Uptake

Eight hours prior to treadmill exercise, mice were injected intraperitoneally with Evan's Blue Dye (500 μ g/10g body weight; EBD; E2129, Sigma) dissolved in sterile-filtered phosphate-buffered saline. Mice were exercised for a total of 45 minutes at a speed of 12 m/min for the first 15 minutes and at 24 m/min for the rest of the period. All animals were sacrificed at 36 hours later, and serial sections of skeletal and cardiac muscles were analyzed for EBD uptake by fluorescence microscopy using rhodamine-specific optical filters. EBD uptake in the skeletal muscle was quantified using measurement parameters in the imaging software, AxioVision LE 4.1 (Zeiss; Jena, Germany), as previously described(128).

Serum Creatine Kinase Assays

Blood cells were allowed to clot and serum was separated by centrifugation of clotted cells at 1500 $\times$ g for 10 minutes. The serum creatine kinase activity was determined in triplicate by an enzyme-coupled absorbance-based kit (CK-SL; Diagnostic Chemicals Limited; Charlottetown, PEI, Canada) following manufacturer's protocol before storage by freezing at -20°C. The enzyme activity was monitored and calculated by measuring the absorbance at 340 nm every 30 seconds for 4 minutes at 25°C, as done previously(107).

Extraction Sia-linkage-Specific Glycoproteins From Cardiac or Skeletal Muscle and ELISAs

Pooled skeletal muscles and heart tissues from wild type (WT) or *Cmah*^{-/-} mice were solubilized in Tris-buffered saline (50 mM Tris, pH 7.4, 250 mM NaCl) containing 1% NP-40 and protease inhibitors (78425, Pierce). Total protein was measured by BCA protein assay (23227, Pierce) and used for Western blot analysis or further glycoprotein enrichment. Three different glycan-specific lectins were used for isolating glycoproteins needed for different

experimental purposes: Wheat Germ Agglutinin (WGA), *Maackia amurensis* agglutinin (MAA), and *Sambucus nigra* agglutinin (SNA-I). WGA selectively binds to N-acetylglucosamine and N-acetylneuraminic acid residues, while MAA and SNA-I bind to α 2-3-linked and α -2-6-linked sialic acids, respectively. For WGA purification, 1.5 mg total protein extracted from wild type and *Cmah*^{-/-} skeletal muscle was enriched for WGA glycoproteins using a glycoprotein isolation kit (89805, Pierce) following the instructions provided. WGA glycoprotein-enriched eluates, rich in dystroglycan, were used for solid-phase binding of laminin and agrin to α dystroglycan, with WT muscle protein showing high Neu5Gc expression and *Cmah*^{-/-} muscle showing none, despite having equivalent amounts of α dystroglycan (S3B). For MAA or SNA-I purification, 3 mg of muscle proteins from wild type or *Cmah*^{-/-} skeletal muscle or heart were enriched for sialic acid-rich glycoproteins using immobilized MAA and SNA-I (AK-7801 and A-6802; EY Labs Inc., San Mateo, CA). Proteins were incubated overnight with 200 μ L of MAA- and SNA-linked agarose beads at 4°C in a total volume of 2 mL. The beads were washed extensively in TBS with 1% NP-40 at 4°C and eluted in 50mM Tris (pH 6.8) with 1% Sodium dodecyl sulfate (SDS) with 0.1% glycerol in a volume of 600 μ L. These samples were used to detect Neu5Gc specific serum antibodies.

To immobilize MAA- or SNA-purified proteins on ELISA plates, proteins were diluted 1:50 into 50mM sodium carbonate:bicarbonate (pH 9.6) buffer (to yield 0.02% total SDS) at 4°C overnight onto 96-well ELISA plates in a volume of 100 μ L. Subsequently, plates were washed with TBS and blocked with TBST (TBS with 0.1% Tween 20) for 2 hours at room temperature. Amounts of loaded protein were first measured using micro BCA kit (Pierce, Cat # 23235) and 5-10 μ g of protein (an excess of the well's binding capacity) loaded per well. Presence of Neu5Gc on glycoproteins in WT muscles, and absence in *Cmah*^{-/-} muscles, was confirmed with the Neu5Gc-specific IgY antibody (S3C). Wells used to determine Neu5Gc-specific titers were blocked with 10% human serum for 1 hour, and were then incubated with dilutions (from 1:50 to 1:1600) of mouse serum for 3 hours. After extensive washing in TBST, plates were incubated in TBST containing goat anti-mouse secondary antibody conjugated to HRP for 1 hr and then washed again in TBST. Signal was developed using SIGMA Fast OPD (P9187, Sigma) and absorbance read at 30 min at 450nm on a Plate Reader (Spectramax M2, Molecular Devices, USA). Binding of Neu5Gc-specific mouse antibodies from the serum WT, *Cmah*^{-/-}, mdx, and *Cmah*^{-/-}mdx mice was determined by measuring the positive Neu5Gc-specific signal (WT-(*Cmah*^{-/-}), both corrected for background) and comparing this to goat anti-mouse IgG and IgM secondary antibody immobilized at different concentrations on the same plate and developed using secondary reagent. Binding of mouse serum antibodies to wells not coated with protein was uniformly minimal (less than 10% of signal) as was binding of secondary reagents to wells containing immobilized laminin or agrin proteins. Only signals comparing quantities in the linear range (OD 0.1-1) were used for measurements of antibody levels.

Growth and transfection of cultured cell lines

HEK293T cells were grown in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal calf serum, 50 μ g/ml streptomycin, and 50U/ml penicillin. Cells were transfected using Effectene Reagent (Qiagen; Valencia, CA) according to the manufacturer's instructions to produce recombinant laminin or agrin proteins.

Expression and purification of recombinant laminins and agrins

Partial cDNAs encoding recombinant, secreted, and FLAG-tagged muscle agrin (G2-G3, z0), neural agrin (G2-G3, z8), and the G1-G5 domains of laminin α 2, laminin α 4, or laminin α 5, were done as previously described(126). Briefly, plasmids were transfected into HEK293T cells using Effectene (301425, Qiagen) and culture supernatants or cell lysates were collected after 48 hours, as previously described(126,127). After the addition of protease inhibitors, the culture supernatant or lysate was subjected to affinity purification on M2 agarose and FLAG-

labeled proteins were eluted with 3X FLAG peptide (F4799, Sigma). 3X FLAG peptide was eliminated by extensive washing using a 10,000Da molecular weight filter. The expression of recombinant, epitope-tagged proteins was verified by Western analysis using monoclonal anti-FLAG M2-HRP-conjugated antibody (A8592, Sigma) and purity assessed by silver stain (24597, Pierce) of eluates after separation by SDS-PAGE, all as before(126).

Western Blot Analysis

40µg of total muscle cell protein was separated on 10%, 12% or 4-15% gradient gels by SDS-PAGE and transferred to nitrocellulose membrane. For the detection of α -dystroglycan, the membranes were blocked and washed in low (100mM) salt Tris-buffered saline, pH 7.4 (TBS). Anti-Neu5Gc blots were blocked in TBS containing 0.1% Tween 20 and 20% human serum and all washes were performed in TBS containing 0.1% Tween 20. For all the other immunoblots, the membranes were blocked with TBS containing 5% non-fat dry milk, 1% bovine serum albumin, and 0.05% Tween 20. Western blot protein band intensities were quantified as previously described(46,129). Densitometric scanning of Western blots to quantify relative changes in protein expression between genotypes, relative to WT, was done as previously described(129,130).

Solid-Phase Binding Assay of ECM proteins to α dystroglycan

Solid-phase assays were carried out following a method previously published(24). WGA glycoproteins were diluted 1:25 in carbonate-bicarbonate buffer, pH 9.6 and coated overnight on polystyrene ELISA microplates (9018, Costar) at 4°C. Plates were washed six times in binding buffer (BB; 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂) and blocked for 2 h in BB containing 3% bovine serum albumin (BSA). Purified recombinant laminin α 2 (G1-G5 domains) and muscle agrin (G2-G3z0) were diluted in BB containing 3% BSA and incubated for 2½ h. Wells were washed six times with 3% BSA in BB, exposed to monoclonal anti-FLAG M2-peroxidase antibody (1:500; A8592, Sigma) for 1 h, washed and developed with SIGMAFAST™ OPD (P9187, Sigma). The absorbance was detected at 450 nm (Spectramax M2, Molecular Devices, USA).

Solid-Phase Binding of Synthetic Carbohydrates to ECM proteins

Polyacrylamide (PAA)-linked glycans also containing biotin were obtained from the Consortium for Functional Glycomics (Core D:Scripps Research Institute; La Jolla, CA) or were purchased from Glycotech (Gaithersburg, MD). All glycans were purified to ca. 95% purity as analyzed by thin layer chromatography and ¹H NMR and/or mass spectrometry (<http://www.functionalglycomics.org/static/consortium/resources/resourcecored1.shtml>). PAA-glycans used were 30kDa and contained 20% glycan and 5% biotin. As such, the valency of glycan to PAA was ca. 6:1 for each glycan used. All glycans were first verified to have equivalent levels of biotin by immobilizing them on ELISA plates, as before(131), and probing with streptavidin-HRP.

To study PAA-glycan binding to recombinant laminins or agrins, a monoclonal anti-FLAG antibody (M2) was immobilized on ELISA plates at a concentration of 500ng/well in carbonate/bicarbonate buffer (50mM, pH 9.5) overnight at 4°C. Wells were blocked with ELISA-buffer for 1hr. Purified agrins (C45(z0) or C45(z8)) or laminins (G1-G5; α 2, α 4 or α 5) were added to each well at 200ng/well and incubated overnight at 4°C. PAA-glycan binding to all five proteins was done in each experiment. After washing, representative wells were incubated in SDS denaturing buffer, separated by SDS-PAGE, and immunoblotted with M2 antibody to verify equivalent amounts of protein were present and analyzed by silver staining (24597, Pierce) to determine their relative purity. PAA-glycans were added in 100µL binding buffer (BB) at concentrations ranging from 0.25µg/well to 3µg/well for 2hrs (yielding an effective concentration range of 42-500nM), followed after washing and incubation with peroxidase-

conjugated streptavidin (1:1000, for 1hr). After subsequent washes, plates were developed in SIGMA Fast™ OPD.

All binding was followed at 5-minute intervals for 30-60 minutes and only data representative of the linear range of response was used for analysis. OD signals varied between 0.1-1.0. All data points used represent triplicate measures of each condition. Binding of PAA-glycans to wells coated with M2 antibody but not with ECM protein was negligible (less than 10% of signal), as was binding of secondary reagents to wells containing immobilized laminin or agrin proteins. Aliquots of PAA-glycans added for the 3µg/well concentration were immobilized on ELISA plates and probed with streptavidin-HRP to verify equivalent levels of biotin were present (S3D). Estimates of solid-phase binding affinity (S4D) were done by plotting binding curve using a standard receptor binding model (fractional ligand binding= $[L]/(K_d+[L])$) with XLfit software (ID Business Solutions, Guildford UK).

Extraction and Purification of Total Skeletal or Cardiac Muscle mRNA

Gastrocnemius and cardiac muscles dissected out under RNase-free conditions were stored overnight at 4°C in RNALater (Ambion; Austin, TX). After decanting the RNALater, tissues were kept frozen at -80°C until RNA extraction. Total RNA was isolated using Trizol reagent (Invitrogen) and further purified on a silica-gel-based membrane (RNeasy-Mini; Qiagen, Valencia, CA). Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the purity/integrity of RNA samples using Agilent Bioanalyzer 2100. RNA content and quality was assessed using a ND-1000 spectrophotometer (Nanodrop; Wilmington, DE). Only samples with an 260/280 absorbance ratio of 2.0-2.1 were used for subsequent analysis.

cDNA Synthesis and TaqMan Gene Expression Assays

High Capacity cDNA Archive Kit (Applied Biosystems; Product # 4322171) was used to reverse transcribe 3 µg of total RNA following the instructions provided. Samples were subjected to real-time PCR in triplicate, on a TaqMan ABI 7500 Sequence Detection System (Applied Biosystems; Foster City, CA) with 18S ribosomal RNA (4308329, Applied Biosystems) as internal control. Primers and probes were purchased as pre-developed 20X TaqMan assay reagents from Applied Biosystems and the details are provided in a Supplementary Table (S7). 18S ribosomal RNA probe contained VIC™ dye as the reporter whereas the other probes had FAM™ reporter dye at the 5' end. Each 25 µL PCR reaction mix consisted of 1X primer-probe mix, 1X TaqMan Universal PCR master mix with AmpliTaq Gold DNA polymerase, uracil-N-glycosylase (AmpErase), dNTPs with dUTP, and a passive reference to minimize background fluorescence fluctuations (4304437, Applied Biosystems). After an initial hold of 2 min at 50°C to allow activation of AmpErase and 10 minutes at 95°C to activate the AmpliTaq polymerase, the samples were cycled 40 times at 95°C for 15 s and 60°C for 1 min. Gene expression was determined as relative changes by the $2^{-\Delta\Delta C_t}$ method compared to 18S RNA(132), and the data presented as fold difference relative to wild type (WT).

Affymetrix GeneChip® Expression Profiling and Analysis

The Ovation™ Biotin RNA Amplification and Labeling System (NuGen Technologies, Inc., San Carlos, CA) was used to prepare amplified biotin-labeled cDNA from total RNA following manufacturer's instructions. Briefly, first strand cDNA was synthesized from 25 ng of total RNA using a unique first strand DNA/RNA chimeric primer and reverse transcriptase. Following double strand cDNA generation, amplification of cDNA was achieved by utilizing an isothermal DNA amplification process that involves repeated SPIA™ DNA/RNA primer binding, DNA duplication, strand displacement and RNA cleavage. The amplified SPIA™ cDNA was purified and subjected to a two-step fragmentation and labeling process. The fragmented/biotinylated cDNA content was measured in a ND-1000 spectrophotometer and

the quality was analyzed on an RNA 6000 Nano LabChip (Agilent) using Agilent Bioanalyzer 2100.

For each microarray, cDNA was hybridized onto 430 2.0 GeneChips® (Affymetrix), containing probe sets that measure 39,000 transcripts from mouse RNA. The probes were designed based on the gene sequences available in GenBank®, dbEST, and RefSeq. The sequence clusters were created from the UniGene database and then refined by analysis and comparison with the publicly available draft assembly of the mouse genome from the Whitehead Institute for Genome Research. Hybridization was allowed to continue for 16 hours at 45°C followed by washing and staining of microarrays in a Fluidics Station 450 (Affymetrix Inc., USA). GeneChips were scanned in a GeneChip Scanner 3000 (Affymetrix) and CEL files were generated from DAT files using GeneChip® Operating Software (GCOS) software (Affymetrix Inc., USA). The probe set signals were generated using the RMA algorithm in ArrayAssist 3.4 (Stratagene) and were used to determine differential gene expression by pairwise comparisons. The genes that were altered by two-fold either ways were sorted and used for further interpretation of the microarray data. Array data is available from the GEO repository (<http://www.ncbi.nlm.nih.gov/geo>) under the accession numbers GSM413160-GSM413182.

Hierarchical Clustering and Venn Diagram

The clustering analysis was performed and Venn diagrams were created by GeneMaths Software 2.01; Applied Maths, Austin, TX). Array-based dendrograms were generated by subjecting the differentially expressed genes to an unsupervised 2-dimensional hierarchical clustering algorithm with Pearson correlation as similarity coefficient and UPGMA (unweighted pair-group method using arithmetic averages) as clustering method. Independent Welch t tests followed by Bonferroni procedure were carried out to estimate the number of gene expression changes in *Cmah*^{-/-}, mdx, and *Cmah*^{-/-}mdx relative to wild type and Venn diagrams were generated to determine the number of unique and common gene expression changes within each group.

ELISPOT Assays

Spleens were dissected from WT, *Cmah*^{-/-}, mdx, and *Cmah*^{-/-}mdx mice and splenocytes made by titration in RPMI 1640 media and filtration through a cell strainer. Cells were then collected by gentle centrifugation (1000g for 3 minutes). After further purification, splenocytes were cultured at 4×10⁵ cells/well and stimulated with 10µg/mL Concanavalin A (ConA, a positive control) or buffer alone (a negative control) in the presence of tissue culture media (RPMI 1640 with 10% FCS and P/S) for 48 hours. Plates were washed in PBS 6 times and subjected to ELISPOT assays for Interleukin 4 (IL4), to detect Th2-type responses (U-CyTech CT319-PB5) or Interferon gamma (IFNγ), to detect Th1-type responses (U-CyTech, CT317-PB5), according to the manufacturer's instructions. Spots per well were counted manually after imaging of plates on a Zeiss bright field microscope using Zeiss imaging software.

T cell proliferation Assays

Analysis of T cell proliferation was performed using splenocyte cultures from individual animals. Splenocytes were isolated from wild type (WT), *Cmah*^{-/-}, mdx, and *Cmah*^{-/-}mdx animals. At the time of sacrifice, the spleen was dissected and splenocytes isolated as above (ELISPOT assays). For proliferation assays, 6×10⁵ isolated splenocytes were cultured per well (in 96-well ELISA plates) and incubated with control buffer or with 10µg/mL Concanavalin A (ConA) for 48 hours in RPMI media. After 48 hours stimulation, 2µCi of ³H thymidine (TRK 424, GE Healthcare) was added for an additional 24 hours. Cells were subsequently washed and lysed using a Wallac harvester and ³H measured using a scintillation counter. Stimulation index was determined as previously described(133).

Isolation and Growth of Primary Myoblasts and Myotubes

Primary myoblasts were isolated following previously published protocol(134). Briefly, leg skeletal muscles were dissected after euthanasia under sterile conditions and minced into tiny bits in phosphate-buffered saline (PBS) containing 1.5 mg/ml trypsin (Mediatech Inc., Manassas, VI), 1.0 mg/ml collagenase D (Roche, Mannheim, Germany), and 2 mg/ml DNase I (Boehringer Mannheim; Indianapolis, IN) for 20 min. Tissue digest was triturated and trypsin was inactivated with DMEM containing 20% fetal calf serum, 4% chick embryo extract (US Biologicals, Massachusetts), and antibiotics. Cells were centrifuged, re-triturated in growth medium, pre-plated on tissue culture plastic for 20 min at 37 °C to remove fibroblasts, and the supernatant plated on cell culture dishes coated with calf skin collagen (Sigma Aldrich, Saint Louis, MO). Nearly confluent myoblasts were differentiated into myotubes by replacing the medium with DMEM containing 2% horse serum, and antibiotics, with repeated feeding, for 6 days.

Complement-Mediated Cell Killing Assays

Myoblasts were seeded in 96-well tissue culture plates at a density of 5×10^4 cells per well. After 12-18 h, the growth medium was replaced with 50 μ L of DMEM without phenol red (Mediatech Inc., Manassas, VI). Test serum was diluted 1:100 in DMEM without phenol red and added to the cells in a volume of 50 μ L. Following a 15 min incubation at 37°C, the cells were washed in DMEM without phenol red and exposed to 5% Low-Tox®-H rabbit complement (Cedarlane Labs, Ontario, Canada) in DMEM without phenol red. The cells were incubated at 37°C for 1 hour and centrifuged. 50 μ L of the supernatant was mixed with 50 μ L of lactic acid dehydrogenase (LDH) substrate and LDH activity was measured following manufacturer's instruction (Roche Applied Science, Mannheim, Germany). The percentage of lysis was calculated using the formula $100 \times [(A-C)/(B-C)]$, where A represents an absorbance obtained with test serum (experimental release), B represents an absorbance obtained by lysing all of the target cells with 1% Triton X-100 (maximum release), and C represents an absorbance obtained with target cells incubated in serum-free medium containing rabbit complement at 5% (minimum release). Experimental release typically ranged between 10 and 30% of maximal release after correction for background. To measure complement-mediated killing in myotubes, the myoblasts were differentiated into myotubes before conducting the complement-dependent cytotoxicity assay.

Statistics—Either ANOVA with repeated measures, where applicable (3 or more groups), followed by a post-hoc t-test, or paired or unpaired 2-tailed Student's t-test, where applicable (only 2 groups), was used to test for statistical differences. Values of $P < 0.05$ (two-tailed) were considered significant.

One sentence summary

This study describes the development of a new mouse model for Duchenne muscular dystrophy where a human-specific change in glycosylation has been introduced into the mdx mouse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression of Neu5Gc in normal and dystrophic mouse and human muscles. (A) N-glycolylneuraminic acid (Neu5Gc) differs from N-acetylneuraminic acid (Neu5Ac) by an additional oxygen at the 5 position of this sialic acid (modified from(112)). Because mice express a functional *Cmah* gene, they incorporate sialic acids (Sias) at the outer ends of glycolipids and glycoproteins that is usually either Neu5Gc or Neu5Ac. Humans and *Cmah*^{-/-} mice, by contrast, contain an inactivating deletion in *Cmah*, and therefore do not express Neu5Gc on the surface of glycoconjugates but instead have increased Neu5Ac. Skeletal muscles (B) and cardiac muscles (C) were immunostained with a Neu5Gc-specific antibody. (D) DMD and *Cmah*^{-/-}mdx skeletal muscles were co-stained for Neu5Gc (green) and

embryonic myosin (eMyosin, red), which marks regenerating myofibers/myoblasts. Bar is 100µm in B, 200µm in C, and 50µm in D. Abbreviations: WT, wild type; DMD, Duchenne muscular dystrophy; IM, inflammatory myopathy (inclusion body myositis); N-glycolylneuraminic acid, Neu5Gc; embryonic myosin, eMyosin.

Figure 2.

Increased mortality and functional deficits in *Cmah*^{-/-}*mdx* skeletal and cardiac muscle. (A) *Cmah*^{-/-}*mdx* mice showed a highly significant decrease in lifespan relative to *mdx*, *Cmah*^{-/-}, and wild type (WT) mice ($P < 0.001$ for *Cmah*^{-/-}*mdx* vs. all others). (B) By 8 months of age, *Cmah*^{-/-}*mdx* showed a 70% reduction in time able to walk on a constant speed rotarod for 5 minutes relative to *mdx* littermates ($P < 0.001$), while *Cmah*^{-/-} mice did not show a significant decrease relative to WT ($P > 0.05$). (C) Developed tension (specific force in mN/mm²) of isolated cardiac trabeculae muscles was reduced in 8 month-old *Cmah*^{-/-}*mdx* relative to *mdx*. (D) Specific force of diaphragm muscle was reduced in 8 month-old *Cmah*^{-/-}*mdx* animals relative to *mdx*. (E) Evan's blue dye uptake into myofibers was increased after exercise in the gastrocnemius (Gastroc), quadriceps (Quad), tibialis anterior (TA), triceps, and diaphragm muscles of 2 month-old *Cmah*^{-/-}*mdx* mice relative to *mdx*. *Cmah*^{-/-} and WT muscles showed no dye uptake in any muscle. (F) Normalized specific force during repeated eccentric contractions (left) in the extensor digitorum longus muscle was reduced in *Cmah*^{-/-}*mdx* compared to *mdx*, as was maximal specific force. Errors are SEM for $n = 75$ -100 animals per condition in A, 12-25 in B, 5-9 in C, 3-6 in D, 5-6 in E, and 11-12 in F. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 3.

Increased histopathology in *Cmah*^{-/-}mdx skeletal and cardiac muscle. (A) 3 month-old (mo) heart sections were stained with hematoxylin and eosin. *Cmah*^{-/-}mdx heart muscle had large mononuclear infiltrates normally not seen in age-matched mdx heart muscle. (B) 2 mo mdx and *Cmah*^{-/-}mdx skeletal muscle (quadriceps) was stained with modified Mason's Trichrome. Fibrosis, replacement of muscle tissue with collagen (blue), was evident in some regions of the *Cmah*^{-/-}mdx quadriceps muscle, while it was never observed in mdx muscle at this age. (C) 6 mo diaphragm muscle was stained with hematoxylin and eosin. *Cmah*^{-/-}mdx diaphragm showed increased fibrosis relative to mdx. (D) Evan's blue dye uptake in 2 mo quadriceps muscle was increased, after exercise, in *Cmah*^{-/-}mdx muscle relative to mdx. (E) Areas of muscle damage were quantified for skeletal muscles and heart muscles. No significant damage was measured in any WT or *Cmah*^{-/-} muscle. Bar is 200μm in A, B and C and 100μm in C. Errors are SEM for n=6-12 per condition in E.

Figure 4.

Molecular changes resulting from loss of Neu5Gc expression. (A) Loss of Neu5Gc (*Cmah*^{-/-}) on α dystroglycan (α DG) led to decreased laminin α 2 (LN α 2) binding ($P < 0.001$ for WT vs. *Cmah*^{-/-} at 20nM). Affinity purified recombinant G1-G5 domain of laminin α 2 was used as the ligand. (B-D) Neu5Gc(α 2-3 or α 2-6)Gal β 1-4GlcNAc (3' or 6'SLN)-biotin-polyacrylamide (PAA-glycan) had significantly higher binding to laminin α 2 (LN α 2, B), laminin α 4 (LN α 4, C) and laminin α 5 (LN α 5, D) than did Neu5Ac (α 2-3 or α 2-6)Gal β 1-4GlcNAc-biotin-PAA ($P < 0.001$ for Neu5Gc vs. Neu5Ac for all α 2,3- or α 2,6 Sia-linked comparisons at 500nM). Gal α 1-3Gal β 1-4GlcNAc (GalLN)-PAA, by contrast, showed only very weak binding to laminins. (E) Immunoblot analysis of DAG protein changes in skeletal muscle and heart. Expected molecular weights for proteins are: Dystrophin (Dys)-427kDa, Utrophin (Utrn)-400kDa, α dystrobrevin (α Dbn) 2-65kDa, α dystrobrevin 3-4(α Dbn 3-4)-44kDa, α dystroglycan (α DG)-160kDa, β dystroglycan (β DG)-43kDa, α sarcoglycan (α SG)-50kDa, β sarcoglycan (β SG)-43kDa, γ sarcoglycan (γ SG)-35kDa, δ sarcoglycan (δ SG)-35kDa, mouse IgG (55kDa), mouse IgM* (70kDa), Actin-42kDa. (F-G) Gene expression changes in skeletal muscle and heart at 5 months of age. Abbreviations: Collagen IV, α 1 chain (Col4(α 1)), Collagen IV, α 2 chain (Col4(α 2)), laminin (LN) α 2, α 4, and α 5, dystroglycan (Dag1), Integrin (Int) α 7 or β 1. (H-I) Protein expression was quantified by densitometric scanning of Western blots in skeletal muscle or heart. Abbreviations: Collagen I (Col1), Collagen III (Col3), ATPase, Ca²⁺ transporting, cardiac muscle, slow twitch 2 (Serca2). (J) Western blots of heart muscle protein show elevated collagen 1 and 3 expression in *Cmah*^{-/-}, *mdx*, and *Cmah*^{-/-}*mdx* cardiac muscle and reduced Serca2 in *Cmah*^{-/-}*mdx* muscle. Expected molecular weights are: Col1(precursor) 140-210kDa, Col3 110-140kDa, Serca2 100kDa (K) 2 month-old heart or

skeletal muscle (gastrocnemius) mRNA was compared using microarrays (n=3 per condition). Genes that were significantly up or down-regulated by 2-fold or more ($P < 0.05$ between *Cmah*^{-/-}, mdx, or *Cmah*^{-/-}mdx and wild type) were identified and subdivided based on whether they overlapped between groups or not. Errors are SEM for n=6-8 per condition in A-D and n=9-10 in F-I.

Figure 5.

Measures of immune response in *Cmah*^{-/-}mdx muscle. (A) mRNA expression for markers of muscle inflammation (IL-1 β , MCP-1, and MIP-1a) and macrophage/monocyte infiltration (CD68) were elevated in 2 month-old (mo) and 5mo mdx and *Cmah*^{-/-}mdx skeletal muscles, relative to wild type. 5mo MIP-1a levels for WT and *Cmah*^{-/-} were undetectable. (B) mRNA expression of MCP-1 and CD68 were elevated in 5 mo *Cmah*^{-/-}, mdx, and *Cmah*^{-/-}mdx heart. (C) 5 mo *Cmah*^{-/-}, mdx and *Cmah*^{-/-}mdx hearts all showed elevations in some markers of cardiac hypertrophy ($P < 0.05$ for all vs. WT for CD68 and MCP-1). (D) Number of cells per unit muscle area expressing CD4, CD8, or CD68 was quantified in 2 mo skeletal muscles and heart. (E) ELISPOT assays for stimulation by Concanavalin A (ConA) of T cell interleukin 4 or interferon gamma were compared. No stimulant is buffer alone. (F) Stimulation Index was calculated to assess T cell proliferation in response to ConA as compared to no stimulant (buffer alone). Abbreviations: ANP, atrial natriuretic peptide; AT1a, angiotensin II receptor, type 1a; BNP, B-type natriuretic peptide; Col1(α 1), collagen type I, alpha 1; Col3(α 1) collagen type III, alpha 1; ET1, endothelin 1; Serca2, sarco/endoplasmic reticulum calcium transporting ATPase 2; TNF α , tumor necrosis factor alpha; IL1 β , interleukin 1 beta; MCP-1, monocyte chemoattractant protein 1 (also chemokine (C-C motif) ligand 2 (Ccl2)); MIP-1a, macrophage inflammatory protein 1a (also chemokine (C-C motif) ligand 3 (Ccl3)); RANTES, regulation upon activation, normally T-expressed, and presumably secreted (also chemokine (C-C motif) ligand 5 (Ccl5)). Errors are SEM for $n = 9-12$ in A-C, $n = 6$ in D-F. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 6.

Anti-Neu5Gc titers and complement deposition and killing in *Cmah*^{-/-}mdx mice. (A) Serum antibody titers to Neu5Gc-containing glycoproteins were compared to titers against Neu5Ac-containing glycoproteins to determine Neu5Gc-specific antibody titers. Skeletal muscles proteins for wild type or *Cmah*^{-/-} skeletal muscles were purified using *Maackia amurensus* agglutinin (MAA), which purifies α 2-3-linked sialylglycoproteins, or *Sambucus nigra* agglutinin (SNA), which purifies α 2-6-linked sialylglycoproteins (see S3C). Only *Cmah*^{-/-}mdx mouse serum showed measurable Neu5Gc-specific titers, and only to α 2-6-linked glycoprotein. (B) Serum from *Cmah*^{-/-}mdx and mdx mice was compared for complement-mediated cell killing of Neu5Gc-expressing myoblasts and myotubes, from wild type (WT) mice, and Neu5Gc-deficient myoblasts and myotubes, from *Cmah*^{-/-} mice. (C) Quantification of the area of muscle deposited with activated C5b-9 complement. Levels were elevated in *Cmah*^{-/-}mdx gastrocnemius (Gastroc), quadriceps (Quad), tibialis anterior (TA), diaphragm (Diaph) and heart relative to mdx. (D) Increased staining of activated C5b-9 complement in *Cmah*^{-/-}mdx and DMD quadriceps relative to mdx or normal human muscle. Errors are SEM for n=6-12 in A-C. *P<0.05, **P<0.01, ***P<0.001