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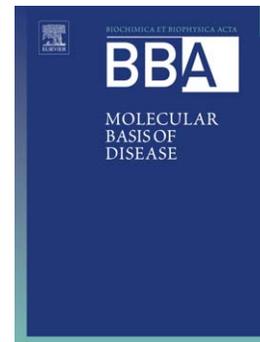
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Genetic modifiers of muscular dystrophy: implications for therapy

Ahlke Heydeman¹, Katherine R. Doherty², and Elizabeth M. McNally^{1,3}

¹Department of Medicine, Section of Cardiology

²Department of Molecular Genetics and Cell Biology

³Department of Human Genetics

The University of Chicago

Address correspondence to:

Elizabeth McNally

5841 S. Maryland, MC6088

Chicago, IL 60637

T: 773 702 2672

F: 773 702 2681

Email: emcnally@medicine.bsd.uchicago.edu

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Abbreviations: ADAM, a disintegrin and metalloproteases; BMD, Becker Muscular Dystrophy;

CK, creatine kinase; CMD, Congenital Muscular Dystrophy; DMD, Duchenne Muscular

Dystrophy; DGC, dystrophin glycoprotein complex; EBD, Evans Blue Dye; EDMD, Emery

Dreifuss Muscular Dystrophy; FKRP, fukutin related protein; IGF1, insulin like growth factor 1;

LGMD, Limb Girdle Muscular Dystrophy; MM, Miyoshi Myopathy

Abstract

The genetic understanding of the muscular dystrophies has advanced considerably in the last two decades. Over 25 different individual genes are now known to produce muscular dystrophy, and many different “private” mutations have been described for each individual muscular dystrophy gene. For the more common forms of muscular dystrophy, phenotypic variability can be explained by precise mutations. However, for many genetic mutations, the presence of the identical mutation is associated with marked phenotypic range that affects muscle function as well as cardiac function. The explanation for phenotype variability in the muscular dystrophies is only now being explored. The availability of genetically engineered animal models has allowed the generation of single mutations on the background of highly inbred strain. Phenotypic variation that is altered by genetic background argues for the presence of genetic modifier loci that can ameliorate or enhance aspects of the dystrophic phenotype. A number of individual genes have been implicated as modifiers of muscular dystrophy by studies in genetically engineered mouse models of muscular dystrophy. The value of these genes and products is that the pathways identified through these experiments may be exploited for therapy.

Introduction

The muscular dystrophies are heterogeneous, and much of the variability in disease relates to the precise gene mutation responsible for disease. Phenotypic variation most commonly affects factors such as age of onset, timecourse of disease progression, muscle group involvement, and accompanying non-muscle features such as cardiac and central nervous system impairment. Clinical markers such as serum creatine kinase (CK) may also vary considerable, although for some markers, like CK, intra-patient variability is present. For patients and clinicians, identifying the gene responsible for muscular dystrophy is helpful in predicting the onset and pace of disease. Understanding the precise allele that produces disease is also predictive of disease course. However, even with the identical gene mutation, there may be striking variability affecting age of onset and disease progression. This variation can be seen within families and suggests that other genetic or environmental factors can contribute to the manifestation of muscular dystrophy. Identifying these modifying factors is valuable for designing therapeutic strategies. Some modifiers are gene specific; namely, they improve only one genetic form of muscular dystrophy. Other modifiers may be more broadly applicable and useful for treating not only all forms of muscular dystrophy, but may also be useful for treating muscle degeneration in general. This review will discuss different genetic forms of muscular dystrophy with an emphasis on phenotypic variability and the presence of genetic modifiers where they have been identified. Most modifiers have been discussed in the context of modifying the phenotype from mutations in the genes encoding dystrophin or its associated proteins. Modifiers of these forms of muscular dystrophy may also be effective for other forms of muscular dystrophy.

Dystrophin

The dystrophin glycoprotein complex (DGC) underlies the membrane of all muscles. The DGC provides mechanical support during muscle contraction and also contributes

nonstructural support in that the DGC modifies signal transduction [1, 2]. Mutations in the dystrophin gene lead to Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD), and a reasonable genotype-phenotype correlation has been established for dystrophin gene mutations. The sarcoglycans are a subcomplex within the larger DGC. The components of the skeletal and cardiac muscle sarcoglycan subcomplex are α -, β -, γ - and δ -sarcoglycan, while the components of the smooth muscle subcomplex are ϵ -, β -, δ - and ζ -sarcoglycan [3]. Mutations in the genes encoding the sarcoglycan subunits result in a subset of the recessively inherited Limb Girdle Muscular Dystrophies (LGMDs). Mutations in the gene encoding any single sarcoglycan complex member tend to produce a secondary loss of the nonmutated sarcoglycan subunits resulting in a loss of the sarcoglycan subcomplex from the plasma membrane. The presence of residual sarcoglycans does not necessarily correlate with disease severity (see below). The dual role of the DGC, mechanical strength and signaling, are evident within the sarcoglycan subcomplex. Therefore, both of these functions must be considered when analyzing sarcoglycan-disease causing mutations.

Mutations in the dysferlin gene lead to LGMD 2B. Dysferlin mutations often, but not always, are associated with a loss of dysferlin from the plasma membrane of muscle. Dysferlin is thought to mediate plasma membrane repair in muscle since the loss of dysferlin is associated with defected repair of induced plasma membrane injury [4]. A considerably range in the degree of muscle weakness is found with dysferlin mutation indicating the presence of genetic modifiers.

Mutations that prematurely truncate dystrophin or are associated with little to no dystrophin protein product typically are associated with the more severe DMD phenotype [5, 6]. Mutations that internally truncate dystrophin but leave intact the amino and carboxy terminal portions of the protein usually produce the comparable milder BMD. An inverse correlation between the residual amount of dystrophin present and disease progression was noted within a

family. At one end was a 15 year old with severe disease and no detectable dystrophin by immunofluorescence and western blot, while an affected uncle had mild disease and positive staining for dystrophin [7]. As the first gene identified for a muscular dystrophy phenotype, it was assumed that genotype-phenotype correlation would be similarly informative for the other inherited muscular dystrophies. However, such clear structure-function relationships have not been evident when considering the range of mutations in the sarcoglycan genes or in the dysferlin gene. There are many examples of different mutations within the same protein causing drastically different diseases.

Sarcoglycan

Among the many human sarcoglycan gene mutations, a correlation between genotype and phenotype is rarely present. In large part, this relates to recessive inheritance and the observation that most mutations disrupt the entire sarcoglycan complex. The most common gene mutation in the γ -sarcoglycan in LGMD 2C is c.525delT, and this mutation has been described in muscular dystrophy patients from every continent. Because this mutation is commonly noted in linkage disequilibrium with neighboring markers, it is thought to be evolutionarily aged. The age of this mutation may have allowed for the development of modifiers since this single mutation is associated with striking phenotypic variability [8, 9]. In this report, affected members from three families have severe symptoms while those from the fourth family were largely spared. The patients were all from Brazil arguing against large environmental or diet differences. Since this early report, this phenotypic variability have been identified with this mutation [9]. The severity of phenotype did not strictly correlate with presence or absence of the remaining, unmutated sarcoglycan family members [9].

A common γ -sarcoglycan mutation, C283Y, occurs in the Roma population throughout Europe [10], and this mutation typically associates with a severe phenotype and an absence of

γ -sarcoglycan from the plasma membrane. In two sisters with LGMD 2C, the G69R γ -sarcoglycan mutation was identified on one chromosome and a large deletion in the opposite γ -sarcoglycan gene [11]. The sisters had a severe clinical presentation and required wheelchairs by age 12 years old with markedly elevated serum creatine kinase levels. Taken together we can hypothesize that the cysteine at amino acid residue 283 and the glycine at residue 69 are highly critical for proper γ -sarcoglycan expression since in common to each of these mutations is the loss of γ -sarcoglycan. C283 is highly conserved and forms a conserved motif found at the carboxyl-terminus of β - and δ -sarcoglycan and is thought to contribute to the formation of intramolecular disulfide bridges [12, 13]. The loss of a disulfide bridge likely affects the trafficking of γ -sarcoglycan to the plasma membrane.

Muscle biopsies can be helpful for determining phenotypic severity. The degree that the unmutated sarcoglycan components remain and traffic normally to the plasma membrane has an inverse correlation with phenotypic severity, although exceptions occur. For example, the severe mutation G69R demonstrated only reduced α -sarcoglycan staining, indicating that sarcoglycan subcomplex trafficking is not completely disrupted, despite the severe phenotype [11]. In addition, the C283Y mutation displays variable residual staining that does not correlate with disease severity [10].

Merosin deficient Congenital Muscular Dystrophy

Congenital Muscular Dystrophy (CMD) is present at birth, and arises from a subset of genetic defects. Mutations in the gene encoding laminin α 2, also known as merosin, lead to CMD [14]. The DGC directly interacts with laminin α 2 through an interaction of dystroglycan. However, the phenotype from laminin α 2 gene mutations is phenotypically distinct from what is seen with dystrophin or sarcoglycan gene mutations. Furthermore, there is evidence to support that laminin α 2 gene mutations produce pathology by a distinct mechanism in that murine

models support differences in membrane instability and levels of apoptosis [15-17]. The *dy/dy* model harbors a large deletion in the laminin $\alpha 2$ gene and recapitulates many aspects of CMD. A second spontaneous mutation in laminin $\alpha 2$ occurs in the *dy^{2J}/dy^{2J}* mice that have a smaller deletion and a slightly milder phenotype than what is seen in the *dy/dy* mice. At least two targeted disruptions have been generated at this locus resulting in a similar phenotype of rapidly progressive muscle weakness with paralysis affecting the hindlimbs associated with spasticity [18, 19].

Murine models and DGC modifiers

One striking discrepancy for dystrophin gene mutations is noted when comparing the muscular dystrophy phenotype between man and mice. The *mdx* mouse provides a useful model for DMD in that a premature stop codon results in the absence of full length dystrophin [20], and the histopathology in the *mdx* mouse is similar to what is seen in DMD patients. However, the loss of dystrophin does not result in a comparable degree of physical impairment in mice as it does in boys with dystrophin gene mutations. Without support, the lifespan of DMD patients is less than one third normal while the lifespan of *mdx* mice shortened only modestly. Despite the functional differences between the *mdx* mouse and boys with DMD, the histopathology in the *mdx* mouse is a good reflection of a particular dystrophin mutation. When dystrophin is engineered to lack its actin binding domain and introduced transgenically into *mdx* mice, the resultant animals have a mild Becker-like phenotype [21]. Therefore, structural-function relationships can be determined from these analyses supporting the role as the precise dystrophin mutation itself as the major determinant of phenotype. It has been suggested that the milder phenotype in the *mdx* mouse may derive, in part, from the presence of genetic modifiers that may now be present after decades of inbreeding to support the line. Nearly all *mdx* colonies are maintained as homozygous inbred lines, and therefore any genetic variation

that affects reproductive fitness will rapidly be assimilated into the colony. Therefore, genetic modifiers that affect the cardiovascular fitness are most likely to be maintained within colonies. Consistent with this, the cardiac phenotype in the *mdx* mouse is comparatively very mild compared to human DMD counterparts [22].

Mouse models of sarcoglycan gene mutations recapitulate many aspects of the LGMD muscular dystrophy phenotype including a more severe muscle and cardiac phenotype than what is seen in the *mdx* mouse. In the case of γ -sarcoglycan gene mutations, there is evidence to support the presence of genetic modifiers. Mice lacking γ -sarcoglycan were engineered by eliminating the exon that encodes the initiator methionine and the transmembrane domain. This mutation results in a complete absence of γ -sarcoglycan protein and produces partial secondary reduction of the remaining, unmutated sarcoglycan subunits, α , β and δ [23]. *Sgcg null* mice display a reduced lifespan and develop cardiomyopathy that can manifest as sudden death due to cardiac arrhythmias [23]. There is evidence for coronary artery vascular spasm that develops as a consequence of loss of sarcoglycan in cardiomyocytes. This vascular smooth muscle cell extrinsic mechanism can be rescued by a transgene expressing γ -sarcoglycan in the cardiomyocytes of *Sgcg null* mice [24, 25]. A second, independent gene targeting using the same gene targeting strategy for the γ -sarcoglycan gene was reported [26]. These mice were generated on a similar mixed 129SVE and C57Bl6 background, but these mice displayed less lethality and more muscle hypertrophy consistent with influence on the phenotype by alternative genetic loci.

The allele generated by Hack et al. was bred heterozygously through ten generations onto four different genetic background strains to inquire whether genetic background can influence phenotypic outcome in the muscular dystrophies [27]. For these experiments, it is critical to maintain heterozygous breeding to avoid selection through the breeding process. After breeding, heterozygous mice were interbred and the histopathologic phenotype was

compared using two different assays. The first assay measured uptake of Evans Blue Dye (EBD) into muscle. Uptake of EBD has been noted in the *mdx* mouse but not in the *dy/dy* mouse [15], and is similarly a feature of sarcoglycan gene mutations. EBD uptake into muscle reflects plasma membrane instability and is an indicator of the degree of myofiber damage within a muscle. This quantitative analysis was conducted on *Sgcg null* mice and revealed that DBA 2J results in quantitatively more EBD uptake than the *Sgcg null* allele bred into other backgrounds. Similarly, quantitative analysis of EBD uptake revealed that the *Sgcg null* allele in the 129/SVEMS+/J background was least affected. A second distinct assay was performed that analyzed the deposition of hydroxyproline into the muscle. Fibrosis and ongoing replacement of the muscle by connective tissue in muscular dystrophy can be measured by an increase in hydroxyproline content. Using this analysis, we found that, again, the *Sgcg null* allele in the DBA 2J background contained the greatest hydroxyproline content indicating a more severe phenotype in this background. Similarly, hydroxyproline content was less in *Sgcg null* mice bred into the 129/SVEMS+/J background consistent with a milder phenotype. We also compared whether the same animals that displayed increased EBD uptake were identical to those with increased collagen content and fibrosis. However, regression analysis suggests no correlation and is consistent with membrane damage being independent from collagen deposition and fibrosis in muscular dystrophy. An example of the varying histopathology associated with the *Sgcg null* allele in the different background strains is shown in Figure 2. We further determined that activity levels were not increased in DBA 2J mice excluding an increase in physical activity as responsible for the enhanced muscular dystrophy phenotype in the DBA 2J background. Through further breeding and genetic mapping, genetic modifiers will be identified.

Dihybrid genetic crosses

Many modifier loci have been identified in murine models through genetic breeding that uncovers synthetic relationships between gene products. These genetic modifiers may encode

proteins that have direct homology or other structural similarity to dystrophin or the sarcoglycan complex. Alternatively, these genetic modifiers may work in unrelated pathways that function synergistically to enhance or ameliorate the muscular dystrophy phenotype. Utrophin, a dystrophin homolog, is upregulated in DMD patients and in the muscle fibers of *mdx* animals [28]. To demonstrate the functional overlap of utrophin with dystrophin, it was shown that utrophin driven by the skeletal α -actin promoter could rescue the phenotype of the *mdx* mice [29]. Three independent transgenic lines were generated and they rescued the phenotype in a dose dependent manner. The transgenic mice had improved morphology, and mechanical properties and demonstrated decreased regeneration. Importantly, transgene expressed utrophin localized to the sarcolemma and caused α -dystrobrevin, α -sarcoglycan and syntrophin to relocalize to the sarcolemma. These experiments form the basis of determining a mechanism to increase endogenous utrophin expression as a therapy for DMD.

Further evidence to support overlapping role of utrophin and dystrophin in muscle pathology is observation that double mutant mice lacking dystrophin and utrophin displayed a much more severe phenotype [30, 31]. The *mdx/utrn*^{-/-} mice have reduced life span and increased pathology, joint contractures, weight loss and kyphosis. These data directly demonstrate that utrophin upregulation compensates in the *mdx* mouse, and increasing utrophin expression to higher levels is a valid therapeutic strategy. The singly mutated *utrn*^{-/-} mouse display only minor neuromuscular junction defects and negligible muscle pathology [32]. Importantly, utrophin over expression is not toxic to the remainder of the animal [33]. The upregulation of utrophin can be post-translational in humans and mice [34], therefore therapies that stabilize the protein may be beneficial to humans [35, 36]. One caveat to this approach is that no inverse correlation was found between amount of utrophin over expression and disease progression in DMD and BMD patients [37]. However, the limited sampling from muscle biopsies and/or the degree of utrophin upregulation may account for these negative findings.

In the 10 years since its generation the *mdx/utr^{-/-}* mouse line has been largely bred as *mdx* hemizygous or homozygous null and *utr^{+/-}*. When comparing the original phenotype with more recent publications, a trend is quickly noticed. Longevity increased from 4-14 weeks, to 4-20 weeks to most recently a 36-week maximum was observed [31, 38, 39]. Continual hemizygous and sibling intercross breeding may lead to selection of beneficial genetic modifiers and reduced phenotype and increased longevity.

Nitric oxide synthase

Neuronal nitric oxide synthase (nNOS, and also known as NOS1) is secondarily decreased from the plasma membrane in DMD and the *mdx* mouse [40, 41]. This displacement of nNOS has been implicated in the degenerative process. However, when double mutant mice (*mdx/nNOS^{-/-}*) were compared to *mdx* mice, no phenotypic modulation was detected [42, 43]. Yet when nNOS was introduced transgenically into the *mdx* background, the muscular dystrophy phenotype was greatly attenuated in both skeletal and cardiac muscle [44, 45]. The nNOS transgene restored NO levels to normal levels in *mdx* animals from a reduction of 80% in non-transgenic *mdx* mice. The transgenic mice had reduced pathology by histology, fiber size variability, infiltrating macrophages, central nuclei, EBD uptake, CK levels and MHC class II staining. The authors convincingly argue that these benefits are due to a decrease of inflammation and infiltrating macrophages. When macrophages are depleted from *mdx*, there is a 75% reduction in injured fibers. Furthermore, it was shown that *mdx* muscle macrophages are cytotoxic to myofibers *in vitro*. The *mdx/nNOS^{-/-}* mice did not demonstrate an increase of utrophin or integrin $\alpha 7$ proteins, lending further support that nitric oxide from the transgene is inhibiting immune cells [46]. Despite no reduction normal nNOS and NOS activity in *mdx* hearts, a transgene providing myocardial-specific nNOS partially rescues the cardiomyopathy associated with dystrophin loss [45]. In particular, macrophage numbers, heart rate variability, ECG abnormalities and fibrosis were returned to normal levels.

Further evidence for immunopathology contributing to the muscular dystrophy phenotype comes from T cell ablation experiments. Antibody depletion of CD8+ or CD4+ T cells early in disease reduces pathology of *mdx* mice [47]. CD4+ T cell depletion reduced skeletal muscle pathology by 61%, and CD8+ T cell depletion reduced skeletal muscle pathology by 75%. In addition, breeding *mdx* mice with T cell deficient nude mice reduced fibrosis [48]. However, not all immune system inhibition is beneficial to the *mdx* mouse. When tumor necrosis factor null mice were bred to *mdx* mice the pathology became enhanced and benefit was only observed in 8-week quadriceps muscle [49].

Integrin

Increased expression in the major laminin-binding integrin of mature muscle, integrin α , has been noted in both DMD patients and *mdx* mice [50]. Based on this observation, it was examined whether manipulation of integrin expression could alter the muscular dystrophy phenotype in mouse models [39]. The rat α 7 integrin chain was introduced into *mdx/utr^{-/-}* mice transgenically under the control of the muscle creatine kinase promoter. The resultant transgenic animals demonstrated reduced disease with enhanced longevity, mobility, and weight maintenance and reduced kyphosis, joint contractures, mononuclear cell infiltrate, and expression of fetal myosin heavy chain. Therefore, overexpression of a specific component in the actin-DGC-extracellular matrix complex compensated for absence of another component. In support of this data are observations from mice lacking both sarcoglycan and integrin α 7 [51]. As in the *mdx* mouse, there is an upregulation of integrin α 7 in sarcoglycan mutant muscle. Mice lacking both γ -sarcoglycan and integrin α 7 display a severe phenotype with early lethality no animal survived beyond one month and, in addition, there was significant prenatal lethality. The double mutant mice had enhanced muscle degeneration determined by EBD uptake while regeneration appeared intact. These results indicate that the two major laminin receptors of

muscle, sarcoglycan-dystroglycan and integrin α 7, are compensatory transmembrane linkers. Additionally, these data demonstrate that upregulation of integrin α 7 is compensatory, and, like utrophin upregulation, is a viable therapeutic target for treating muscular dystrophy.

Myostatin

Myostatin is a TGF β family member that is an inhibitor of skeletal muscle growth [52]. Because the myostatin null mouse (*Mstn*^{-/-}) has an increased muscle mass due to hyperplasia and hypertrophy, it was bred to the *mdx* mouse model to determine its effect on muscular dystrophy [53]. The resultant mice had a reduced severity of disease as measured by muscle weight, grip strength, muscle fiber diameter, histology and hydroxyproline content. These findings have translated closer to therapy in that myostatin inhibitors also attenuate the *mdx* phenotype [54, 55]. Although these results have been encouraging and lead to human clinical trials, a second dihybrid cross was undertaken which cautions against the positive results of the *mdx/Mstn*^{-/-} data. The same myostatin null mutation was bred onto the laminin-deficient mice, *dy*^W/*dy*^W, a model for merosin deficient CMD. In this cross, increased muscle regeneration and muscle mass, due primarily to hyperplasia, were present. However, no improvement of muscle pathology and an increase in pre-weaning mortality was described [56]. The difference between the two models may relate to intrinsic pathologic defects between the two forms of muscular dystrophy. The more severe phenotype of the *dy*^W/*dy*^W mice and/or the lack of tolerance for decreased brown fat in the *dy*^W/*dy*^W mice may also contribute. An alternative strategy that would directly test the contribution of brown fat would utilize the cre-lox technology to eliminate myostatin shortly after birth, beyond the window where brown fat may be required [57]. Over-expression of a dominant negative myostatin exclusively in differentiated muscles may also reflect what can be achieved through pharmacologic inhibition [58].

Insulin-like growth factor

Insulin like growth factor 1 (IGF1) regulates skeletal muscle growth and regeneration [59, 60]. Indeed, IGF-I overexpression in *mdx* mice results in increased muscle fiber size and number [61, 62]. These transgenic mice also had normalized fibrosis as measured by hydroxyproline content and increased total force, but not specific force [61, 63]. Functionally, mice receiving 1 mg/kg IGF-I for 8 weeks demonstrated resistance to fatigue in *ex vivo* experiments [63]. This coincided with a phenotypic shift to more fatigue-resistant and oxidative slow fibers.

ADAM12

ADAM (a disintegrin and metalloprotease)-12 is expressed in several tissues, and most predominantly in muscle during embryogenesis [64]. Postnatally, ADAM12 is not normally expressed in muscle. However, it is transiently re-expressed during regeneration and it is believed to have a role in membrane fusion [65]. ADAM12 null animals have intact muscle regeneration, and constitutive ADAM12 overexpression in adult muscle transgenically under the control of the muscle creatine kinase promoter had beneficial effects upon *mdx* mice [66]. The animals had less necrosis, inflammation, creatine kinase and EBD uptake. Therefore, it is believed that ADAM12 aids muscle cell regeneration in the *mdx* mouse. Importantly, beneficial effects were also seen in cardiac tissue, consistent with a role for ADAM12 in myocyte survival [66]. When the identical transgene expressing ADAM12 was bred into the more severely affected *dy^W/dy^W* laminin α 2 null animals, enhanced regeneration was seen supported by increased numbers of central nuclei and increased fetal myosin expression. However, the disease progression was the same in transgenic and littermate control animals [67]. Both groups displayed the same muscle pathology, degeneration, inflammation, fibrosis and had reduced weight. Interestingly, the overexpression of ADAM12 in the *mdx* background caused an increase of both utrophin and integrin α 7 [68], while only utrophin was upregulated in the

dy^W/dy^W transgenic animals [67]. The muscle creatine kinase promoter that drives expression in these experiments may also influence the difference in outcome. This promoter is highly expressed only in fully differentiated fibers, and these fibers may be more at risk in the dy^W/dy^W mice. Supporting this mechanism is that expression of ADAM12 from the same integration event was lower in dy^W/dy^W mice than in littermate controls [67].

Apoptosis

Reduction of apoptosis by Bax inactivation or Bcl-2 overexpression increases the life span of laminin- α 2 (merosin) mutant mice [69, 70]. In addition, Bax inactivation increases the weight of the animals through an increase in muscle mass and reduces myofiber pathology. Apoptotic myonuclei have been detected in DMD and *mdx* [71, 72]. Despite this, muscle-specific expression of Bcl-2 does not decrease pathology in *mdx* mice [70].

Fukutin-related protein (FKRP) and dystroglycan

Enzymatic proteins that are thought to post-translationally modify components of the DGC have been implicated in the Congenital Muscular Dystrophies (CMDs). Fukuyama muscular dystrophy is caused by mutations in the gene encoding fukutin [73], and congenital muscular dystrophy type 1C (MDC1C) and LGMD-2I are both associated with distinct mutations in the fukutin related protein (FKRP) [74, 75]. FKRP is thought to O-glycosylate proteins, and dystroglycan, a key component of the DGC, is thought to be the primary defective target in these disorders [76]. MDC1C is a severe muscular dystrophy in which patients typically cannot stand. Alternatively, the allelic LGMD-2I is less severe, although there is phenotypic variability that can also affect the heart.

Dystroglycan is expressed on nearly all cells, but is essential for normal muscle function [77]. Dystroglycan is produced from a single gene, and after translation, it is cleaved into α - and

β -dystroglycan subunits. β -dystroglycan is only modified at N-linked sugars, but α -dystroglycan is modified by both N- and O-linked glycosylation. The subunits of dystroglycan assemble, and at the membrane link dystrophin and cytoskeletal actin to the extracellular matrix, thereby preserving sarcolemmal integrity. Mice with a targeted deletion of dystroglycan are embryonic lethal due to basement membrane defects in many tissues [78]. Tissue specific gene deletions of dystroglycan correlate with human diseases, demonstrating muscle pathology and CNS involvement in the appropriate cre-expressing mouse lines [77, 79].

With the identification of muscular dystrophy causing mutations in these post-translation modifying enzymes, a large new group of proteins must be considered as potential modifiers. Many proteins are involved in post-translational modifications and mutational impairment of these may result in frank disease as with FKRP, or may modify phenotype severity of other mutations. In addition, FKRP itself is modified by other factors. A specific mutation L276I is associated with a mild but variable phenotype: age of onset can range from 5 to 25 years old, and variable muscle groups are affected to differing levels [80].

Dysferlin

Mutations in the dysferlin gene produce Miyoshi myopathy (MM), LGMD 2B and/or distal anterior compartment myopathy [81, 82]. MM is characterized as an early-adult onset, autosomal recessive, distal muscular dystrophy with calf involvement and elevated CK levels (Miyoshi, 1986). LGMD 2B usually begins with proximal muscle weakness and progresses more rapidly than MM. It has been difficult to correlate genotype with phenotype in dysferlin mutations [83], and the identical mutation can yield both MM and LGMD-2B even within the same family. The presence of the identical mutation associating with both mild and severe forms of the disease argues for the presence of genetic modifier loci (Liu, 1998, Weiler, 1999). In particular, the P791R mutation was found in a large family that contained both MM and LGMD-2B patients (Weiler, 1999). The LGMD-2B patients within this family presented with

rapidly progressing muscular dystrophy and required wheelchairs by their third decade. The MM patients were more mildly affected. Interestingly, the amount of dysferlin protein retained by these patients was independent of disease type and progression (Weiler, 1999).

Evidence is accumulating that the dysferlin family of proteins function in membrane fusion events (Bansal, 2003). The dysferlin gene contains six C2 domains and a transmembrane domain at its carboxy-terminus. A point mutation in the first C2A domain, V67D, was reported in both MM and LGMD 2B [84]. When engineered into dysferlin, this mutation was found to disrupt calcium sensitive phospholipid binding [85]. The highly related family member myoferlin is important in myoblast fusion to myotubes during development and muscle regeneration [86]. The roles of myoferlin, in myoblast fusion after cardiotoxin injury, implicate it as a potentially interesting modifier and therapeutic target [86, 87]. Supporting this, myoferlin is increased in response to a number of muscular dystrophies [88].

There is evidence from animal models to support the existence of genetic modifiers of muscular dystrophy produced by dysferlin gene mutations. Four separate dysferlin null animals have been described. The first mutation in the murine dysferlin gene was described in the SJL strain [89]. The SJL strain is highly inbred and is a model for experimental autoimmune encephalitis. Analysis of the locus indicated a splice site mutation of the dysferlin locus and the loss of a 171 base pair exon. This exon is in frame but is reported to decrease the level of dysferlin expression [90]. Two additional dysferlin mutant models were described and compared [91]. In this work, the authors describe a second spontaneous mutation in the A/J line and compared its phenotype to a targeted deletion of the same exon implicated in the SJL mice. The mutation in the A/J mice was from a retrotransposon insertion that affects mRNA and protein expression. This insertion was thought to have occurred in the strain more than three decades ago. The maintenance of this as an inbred strain has resulted in a milder phenotype than the engineered mutation. An example of the milder histopathology is seen in Figure 3 and compared to the phenotype in mice lacking δ -sarcoglycan. The muscular pathology in these

three lines is similar with the same muscles being progressively affected. However, the A/J strain has a delayed onset of disease and decreased EBD uptake. Because both these lines are null for dysferlin, by immunoblot and mRNA, differences of phenotype do not result from different mutations or residual protein production. The SJL mice clearly contain a divergent genotype as they are aggressive, susceptible to autoimmune disease, B-cell lymphomas, and blind by weaning [92-95]. Lastly, Bansal et al. reported a targeted disruption of dysferlin and identified a defect in membrane resealing [4].

Calpain

Calpain 3 is a calcium-activated protease, and mutations in the gene encoding calpain 3 produce recessively inherited muscular dystrophy. Calpain 3 has a nuclear localization signal and has been detected in myonuclei [96, 97]. The molecular mechanism whereby calpain 3-mutation causes LGMD-2A is unknown, but several hypotheses explain the pathologic mechanism. Calpain 3 cleaves filamin 3 (a cytoskeletal protein that binds directly to the cytoplasmic tails of γ -sarcoglycan and δ -sarcoglycan), ezrin, talin, fodrin and titin [98, 99]. It has also been shown that calpain 3 deficiency is associated with a marked increase in apoptosis, where a 100 fold increase of apoptotic nuclei are found in muscle biopsies from LGMD-2A patients compared to other genetic forms of muscular dystrophy [97]. As an alternative but not mutually exclusive hypothesis, calpain 3 also has a role in muscle maturation and sarcomere remodeling [100].

Patients, with calpain 3 mutations, present with variable clinical phenotypes. There is a large range in age of onset, serum creatine kinase levels are usually elevated, but not always [101]. de Paula *et al.* performed a large study of calpain 3 mutations to analyze genotype phenotype correlations [102]. As expected, missense mutations usually cause milder disease than non-sense or frame-shift mutations. Age of onset for missense mutations was 21 years while the average for nonsense and frame-shift mutations was 11.5 years. Similarly, wheelchair

requirement was greater for frame-shift mutations [103]. The severity of disease loosely correlated with calpain deficiency by immunoblot [102].

Variable phenotypes for identical mutations have also reported for LGMD-2A [102, 104]. In an analysis of 20 patients with the same mutation, age of onset and clinical disease progression were highly variable [102]. The earliest diagnosis was at 2 years of age, while the oldest was 15 years old. Furthermore, in a single family three siblings were wheelchair bound at 13, while other siblings and cousins were only mildly affected in their twenties. Of particular note within LGMD-2A, female patients have a better prognosis than their male counterparts [102, 105]. The reason for this trend is as yet unknown, but the factor(s) mediating the effect would be modifiers worth future investigation for possible therapeutic intervention sites.

Calpain 3 null mice were generated and develop a progressive and mild muscular dystrophy with increasing CK values, histopathology, myonuclei apoptosis and Evan's blue dye uptake [106]. The histopathology revealed that muscle groups were differentially affected. The authors also compared pure 129Sv mice to C57BL/6/129Sv hybrid mice. The hybrid animals displayed a slower progressing disease when analyzed with histopathology.

Lamin A/C

Mutations in the gene encoding lamins A and C (LMNA) produce an array of phenotypes including Emery-Dreifuss MD (EDMD), LGMD-1B, Hutchinson-Gilford progeria syndrome, familial partial lipodystrophy, dilated cardiomyopathy, mandibulosacral dysplasia and others [107]. EDMD, both dominant and recessive, are characterized by early contractures of the elbows, Achilles tendons and neck and a slow progression of muscle weakness. EDMD also involves dilated cardiomyopathy and conduction abnormalities. LGMD-1B patients present with proximal muscular dystrophy and weakening, and dilated cardiomyopathy with conduction abnormalities. Hutchinson-Gilford progeria is evident within two years of birth and is characterized by short stature, alopecia with small facial features. As the disease progresses,

cardiovascular issues arise. Hutchinson-Guilford progeria is accelerated aging at a rate six to eight times faster than normal. The partial lipodystrophies are usually diagnosed at puberty as a decrease of subcutaneous fat from the upper and lower extremities; type II diabetes usually follows by age 20.

Two nonexclusive mechanisms have been developed to explain the divergent phenotypes seen with LMNA gene mutations [108]. The first hypothesis emphasizes lamin A/C's role in nuclear membrane structural integrity. The second pathogenic mechanism implicates lamin A/C's ability to modulate nuclear functions such as DNA replication, gene expression and nuclear export. The structural integrity of nuclei containing mutated lamin has been demonstrated *in vitro* and *ex vivo*. Cells expressing mutant lamin have severe nuclear abnormalities; these abnormalities include mislocalization of other inner-nuclear membrane proteins such as emerin [109, 110]. Likewise, fibroblasts taken from patients with lipodystrophy also have severe nuclear abnormalities and demonstrated increased susceptibility to stress [111]. Mice deficient for lamin A/C confirm the disorganized nucleus found in cell culture and in primary patient fibroblasts [112]. The gene expression hypothesis has been investigated in lamin A/C deleted mice that display impressive chromatin changes and possible defects in gene expression [113].

Although attempts have been made at identifying functional domains within the Lamin A/C protein, no clear correlation exists between mutated residues or mutated protein domains and disease phenotype [114]. A difficult step in attempting this genotype/phenotype correlation with lamin A/C is that mutation of this ubiquitously expressed protein causes tissue-specific diseases [115]. A further confounding consideration is the difference between human and mouse mutations with respect to lamin A/C diseases. In an effort to generate mice with autosomal dominant EDMD, a heterozygous LMNA dominant mutation was engineered [116]. Surprisingly no heterozygote phenotype existed. Homozygous animals displayed Hutchinson-Guilford progeria instead of a muscular dystrophy.

Identical mutations within lamin cause vastly different phenotypes. Variations are found in age of onset, cardiac involvement, muscle group involvement, CK elevation and other parameters [75]. In addition, penetrance is different even within a single family: ranging from silent to completely penetrant [117].

Conclusions and perspectives

The variable phenotype in the muscular dystrophies presents complications for physicians and genetic counselors seeking to advise patients and families with genetic mutations. Some phenotypic variability can reliably be predicted from genotype-phenotype correlations. In general dystrophin gene mutations are predictive of outcome, although exceptions are present. Additional examples are seen in some LGMD patients, although in general structure function relationships have been less evidence in LGMD than in DMD. Despite having some clear genotype-phenotype relationships, there is an increasing body of evidence indicating that modifier genes greatly affect disease phenotype. The evidence stems from both human and murine analyses.

Many different single genes have been shown to alter the pathogenesis of muscular dystrophy in the *mdx* mouse. The functions of these proteins fall into one or more categories: structural, signaling, reduction of necrosis, reduction of immune infiltration, regeneration promotion, and survival factors (Table 2). Some of the proteins products of the manipulated genes may compensate structurally such as utrophin and integrin- α 7. Others appear to re-establish important signaling cascades including nNOS and ADAM12. Others, such as calpastatin, reduce necrosis. Manipulation of the immune system is also an effective modifier, although one that is genetically complex. Other factors serve to promote regeneration and increase muscle mass, and these genes include myostatin inhibition and IGF1. The illumination of these many pathways will aid in understanding disease progression and prognosis and present novel targets for therapy.

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Figure Legends

Figure 1. Schematic of the plasma membrane of muscle and nucleus indicating proteins associated with muscular dystrophy. The dystrophin glycoprotein complex consists of dystrophin, dystroglycan, the dystrobrevins, syntrophins, sarcospan, and the sarcoglycan complex. Dysferlin is not associated with the dystrophin glycoprotein complex. Modifiers that affect the dystrophin glycoprotein complex include utrophin and integrin $\alpha 7$.

Figure 2. Comparison of diaphragm muscles from *Sgcg*^{-/-} mice on different genetic backgrounds. Phenotypic variability is demonstrated through differences in number of centrally placed nuclei, fiber size, fibrotic infiltrate, hypertrophy and hyperplasia. The mutant mice on the DBA background have enhanced phenotype by all of these parameters; note, the marked pseudohypertrophy related to fibrofatty infiltration. Eight-week old diaphragms are shown, scale bar represents 100 μ M.

Figure 3. Comparison of fibrosis, fatty infiltrate and regeneration in *A/J* mice, a model for dysferlin mutations and LGMD 2B, and *Sgcd*^{-/-}, a model for LGMD 2F. The dysferlin null animals have increased fibro-fatty infiltrate and centrally placed nuclei compared to control mice. However, *A/J* mice have much milder histopathology compared to *Sgcd*^{-/-} mice animals. The *A/J* mice have been maintained as an inbred strain and may have acquired genetic modifiers that attenuate the phenotype. Bars are 100 μ m.

Table 1. Phenotypic variability within the LGMD 2C mutation c.525delT

Disease Severity	Creatine kinase	Age of Onset (yrs)	a-sg	b-sg	d-sg	dys	References
M	ND	9	-	-	-	+	[118]
S	25000	4	+	+	+	+++	[13]
S	8 X nl	10	+	-	-	+++	[8]
S	15 X nl	5	+	-	-	+++	[8]
S	11 X nl	5	+	-	-	+++	[8]
M	40 X nl	asx	+	-	-	+++	[8]
S	Elevated	6	++	+++	+++	+++	[119]
S	8000	9	+++	+++	+++	+++	[120]

α -sg, β -sg, δ -sg and dys refer to the degree of residual staining of these α -sarcoglycan, β -sarcoglycan, δ -sarcoglycan and dystrophin, respectively. S, severe; I, intermediate; M, mild; ND, not determined. Asx refers to asymptomatic relatives with elevated CK but minimal to no weakness at ages 22 and 23.

Table 2. Modifier effects by functional interaction in *mdx* mice, unless otherwise noted.

Function	Implicated genes	Mouse data	Reference
Structural	Utrophin	increased in regenerating fibers <i>mdx/utr</i> ^{-/-} worsens <i>mdx</i> phenotype transgene aids <i>mdx</i> phenotype	[121] [30] [29]
	Integrin $\alpha 7$	increased in regenerating fibers, <i>mdx</i> and <i>sgcg</i> transgene aids <i>mdx/utr</i> ^{-/-} phenotype <i>Itga7</i> ^{+/-} / <i>sgcg</i> worse phenotype	[50] [39] [51]
Signaling	IGF-I	signaling is increased in transgenic IGF-I, <i>mdx</i> mouse	[61]
Necrosis	IGF-I	necrosis is decreased in transgenic IGF-I, <i>mdx</i> mouse	[61]
Immunity	nNOS	decreased in <i>mdx</i> transgene lessens <i>mdx</i> phenotype	[40] [44]
	CD4+ T cells	antibody depletion lessens phenotype	[47]
	CD8+ T cells	antibody depletion lessens phenotype	[47]
	T cells	<i>mdx</i> on nude mouse background lessens phenotype	[48]
	TNF	Double knockouts <i>tnf/mdx</i> enhanced pathology	[49]
Regeneration, and muscle formation	Myostatin	<i>mstn</i> ^{-/-} / <i>mdx</i> mice have reduced pathology	[53]
	IGF-I	transgene lessens <i>mdx</i> pathology by increasing regeneration	[61]
		1 mg/kg increased fatigue resistance in <i>mdx</i>	[63]
	ADAM12	re-expressed in regenerating fibers	[65]
		transgene lessens <i>mdx</i> phenotype	[66]
	Dysferlin	important during membrane fusion	[4]
Calpain	role in muscle maturation	[100]	
Survival	Bax	inactivation increased lifespan in <i>Lama2</i> ^{-/-} mice	[69]
	Bcl-2	transgene increased lifespan in <i>Lama2</i> ^{-/-} mice	[69]
	ADAM12	transgene lessens <i>mdx</i> cardiomyopathic phenotype	[69]
	Calpain	100 fold increase in apoptotic nuclei in LGMD 2A	[97]
Fibrosis	IGF-I	fibrosis is decreased in transgenic IGF-I mouse	[61]

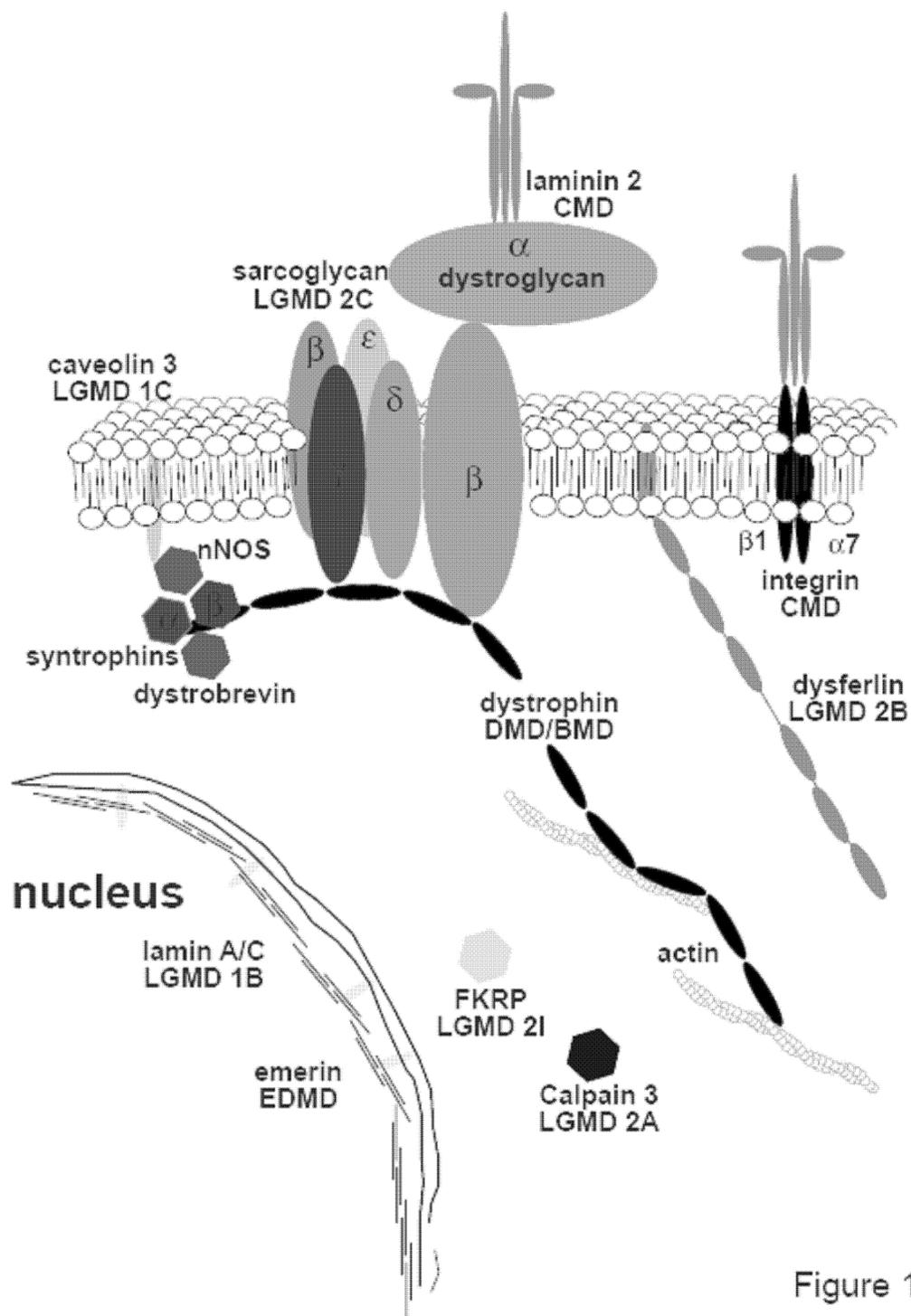


Figure 1

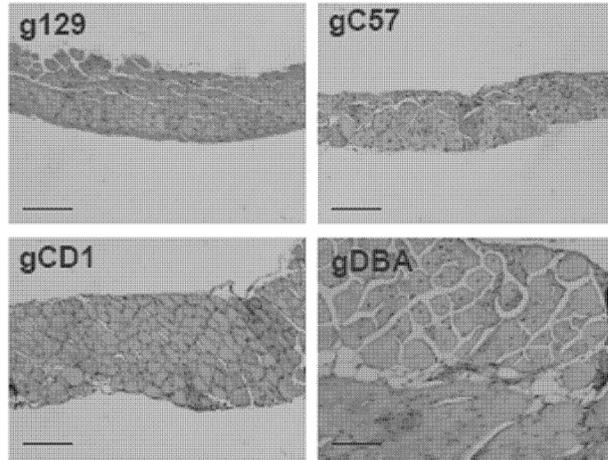


Figure 2

AC

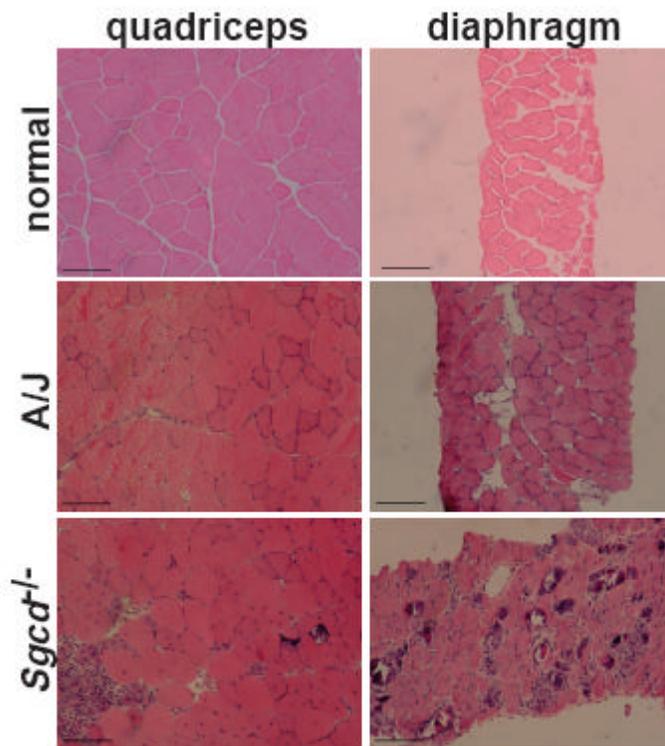


Figure 3

A