Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex in skeletal muscle

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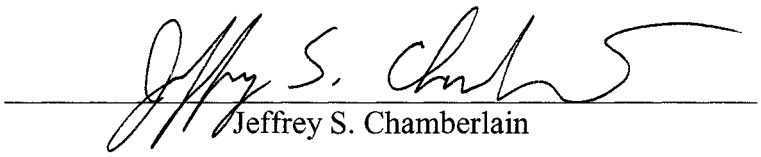

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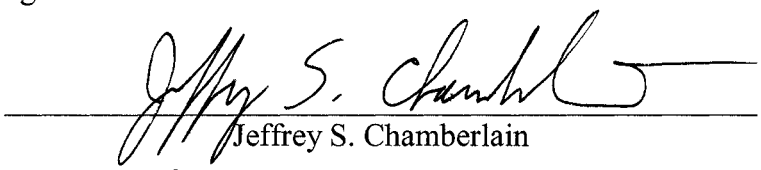

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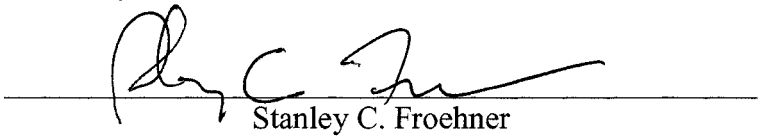

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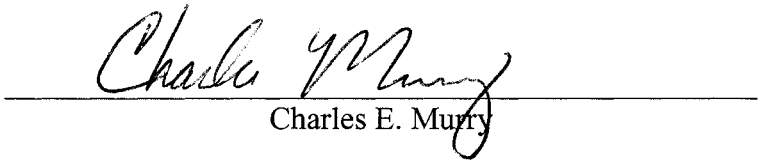

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Abstract

Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex in skeletal muscle

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Duchenne Muscular Dystrophy (DMD) is a fatal genetic disorder characterized by severe and progressive muscle degeneration, which is caused by mutations in the gene encoding the dystrophin protein. Dystrophin is required for assembly of the dystrophin-glycoprotein complex (DGC) and provides a mechanically strong link between the cytoskeleton and the extracellular matrix. Several DGC proteins also participate in signaling cascades, but the relationship between these signaling and mechanical functions in the development of muscular dystrophy is unclear. To explore the mechanisms of myofiber necrosis in dystrophin-deficient muscle, we tested the hypothesis that restoration of the DGC without a link to the cytoskeleton could ameliorate dystrophic pathology. Transgenic mice were generated that express Dp116, a non-muscle isoform of dystrophin that assembles the DGC but lacks actin-binding domains, specifically in skeletal muscle. Expression of Dp116 in skeletal muscles of dystrophin-deficient

mice exacerbated muscular dystrophy. Conversely, expression of Dp116 in severely affected mice deficient in both dystrophin and utrophin (a closely related homologue) prevented muscle wasting and increased survival. Dp116 did not restore the signaling molecule neuronal nitric oxide synthase (nNOS) to the DGC, and specific parts of the dystrophin rod domain not found in utrophin were shown to participate in localization of nNOS. However, association of nNOS with the DGC was not required for amelioration of dystrophy. The results suggest that mechanical destabilization, rather than signaling dysfunction, is the primary cause of myofiber necrosis in dystrophin-deficient muscle, but that the severe consequences of the additional loss of utrophin may be due to the loss of homeostatic signaling pathways.

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Finally I wish to thank my parents John and Mary Ann, my wife Telli, and my son Cyrus for all their support.

DEDICATION

In memory of Buzzy, Kate, and K.C.

CHAPTER 1:

Background and Introduction

Structure and function of the dystrophin gene and protein

Clinical consequences and treatment of DMD

Duchenne muscular dystrophy (DMD) is a debilitating disease characterized by progressive destruction of the striated musculature. The eventual result is severe weakness of the skeletal muscles and often cardiomyopathy. Inevitably, DMD patients lose the ability to walk and die at an early age, most commonly from complications of respiratory and/or cardiac insufficiency. Microscopically, muscle tissue in early stages of DMD shows infiltration with inflammatory cells and a mixture of necrotic and regenerated muscle fibers. In later stages the regenerative capacity of myogenic cells is exhausted and muscle tissue is replaced by fibrosis and adipose tissue. The disease is inherited as an X-linked recessive trait that leads to loss of expression or function of a protein named dystrophin. DMD affects approximately 1:3500 male births, and despite its restriction primarily to the male population it is the second most frequent monogenic disorder in Western countries (Emery and Muntoni, 2003). Clearly, DMD represents a significant burden of morbidity and mortality worldwide. Although genetic counseling has the potential to reduce the incidence of disease, approximately one third of cases are new mutations and thus not preventable (Emery and Muntoni, 2003).

In recent years improvements in clinical supportive care have successfully increased lifespan and quality of life in DMD patients. Medical management is focused on maintenance of mobility and prevention of physical deformities via exercise, bracing, orthotics, and surgical intervention (McDonald et al., 2006; Siegel and Hsu, 2006). In later stages of the disease respiratory support is often required. Standard pharmacological therapies such as angiotensin-converting enzyme inhibitors and β-blockers are used to treat cardiac dysfunction when necessary (Jefferies et al., 2005; McDonald et al., 2006). Aside from drugs aimed at improving cardiac function, the glucocorticoids prednisone, prednisolone, and deflazacort are the only pharmacological agents in widespread use for treatment of DMD. Evidence suggests that these drugs act to preserve muscle strength and function, at least in the short term (Al-Dahhak and Kissel, 2006). However, the long term benefits of these agents are not clear and there are still many questions regarding the optimal dose regimen and the age at which therapy should be initiated.

While the supportive measures described above have been important additions to the standard of care for DMD patients, the next step must come from therapeutic principles that act to ameliorate the underlying pathophysiology of DMD. These approaches require extensive knowledge of the genetic, biochemical, and cellular defects that lead to muscle wasting in DMD. The majority of our understanding in these areas comes from studies of animal models. The most frequently used animal model is the *mdx* mouse, which has a point mutation in exon 23 of the murine dystrophin gene. The mutation causes premature termination of

translation and the unstable protein does not accumulate (Hoffman et al., 1987). Despite a lack of dystrophin expression and pathologic changes in the muscle that resemble DMD, *mdx* mice live 80% of the wild-type lifespan (J.S. Chamberlain, personal communication; Lefaucheur et al., 1995). Potential reasons for this discrepancy as well as the development of other mouse models will be discussed in later chapters. Canine models of DMD (*cxmd*) have been discovered and are the most frequently studied second to the mouse. Although the dogs are thought to more closely mimic the clinical course of DMD they can be extremely affected, with death occurring shortly after birth in some individuals (Cooper et al., 1988; Shimatsu et al., 2003; Valentine et al., 1988). There is also considerable phenotypic variation both between and within litters. It is likely that mutations in the dystrophin gene can be found in the majority of animal species. However, some animal models, such as the feline, display a phenotype that is qualitatively different from that seen in DMD (Collins and Morgan, 2003).

Although the *mdx* mouse is not a perfect model of the human disease it has still been an invaluable tool to study the underlying pathophysiology of dystrophin-deficient muscular dystrophy. The ease of genetic manipulation of this model makes it particularly useful in this regard. I will review the progress that has been made in these areas and discuss the ways this knowledge has been applied to the design of several promising experimental therapies. Finally, I will present novel research examining the functional roles that dystrophin, the protein product of the causative gene in DMD, plays in skeletal muscle *in vivo*.

The dystrophin gene and isoforms

The DMD gene (*HDMD*) spans approximately 2.5 million bases located at Xp21, making it the largest gene described to date (Coffey et al., 1992; Monaco et al., 1992). It was also the first gene to be identified by positional cloning, a technique that has subsequently been used to characterize the genes involved in other inherited disorders (Koenig et al., 1987; Monaco et al., 1986). The same genetic locus is responsible for Becker muscular dystrophy (BMD), now recognized to be a milder allelic form of DMD (Kingston et al., 1983). The DMD gene contains at least 86 exons, 79 of which are present in the "full-length" 14 kilobase transcript that encodes the 427 kilodalton protein named dystrophin (Blake et al., 2002; Hoffman et al., 1987; Koenig et al., 1987). Tissue-specific promoters drive expression of three full-length isoforms in muscle, brain, and cerebellar Purkinje cells, which differ only in their first exons (reviewed by Ahn and Kunkel, 1993).

There are four more internal promoters located within introns of the *HDMD* gene that give rise to transcripts lacking variable numbers of exons at the 5' end of the gene. These are named in accordance with the molecular weight of their protein products. The largest of these is Dp260, which contains a unique first exon spliced to exon 30 and is expressed specifically in the retina (D'Souza et al., 1995). The Dp140 isoform is expressed in both brain and kidney and contains its unique first exon spliced to exon 45 (Durbeej et al., 1997; Lidov et al., 1995). However, Dp140 does not contain any unique amino acids as translation of the protein is not initiated until exon 51 (Lidov et al., 1995). Dp116 is specifically expressed in Schwann cells

that form the myelin sheath surrounding peripheral nerves. Again, it contains a unique first exon spliced to exon 56 (Byers et al., 1993). Finally, Dp71 is the shortest transcript and most ubiquitously expressed, although not in muscle (Bar et al., 1990; Rapaport et al., 1992). Dp71 contains a unique first exon that is spliced to exon 63 (Lederfein et al., 1992). Several of the exons in the 3' region of the gene are alternatively spliced, leading to an even greater complexity of isoforms (Bies et al., 1992b; Feener et al., 1989). A schematic of the dystrophin gene with the various promoters and protein products is shown in Figure 1.1.

The dystrophin protein and interactions

Dystrophin is a large cytoskeletal protein expressed in skeletal, cardiac, and smooth muscle cells as well as a subset of cells in the brain. The primary structure of dystrophin has regions that are homologous to the structural proteins α–actinin and spectrin. In striated muscles it is localized to the sarcolemma, more specifically to costameres, a lattice-like structure aligned with the Z-lines of myofibrils (reviewed in (Ervasti, 2003)). Dystrophin associates with the costameric cytoskeleton by binding to F-actin, which indirectly links it to the contractile proteins of the myofiber. It is also anchored to the sarcolemma by binding to dystroglycan, a transmembrane protein that serves as the extracellular receptor for the basement membrane protein laminin-2 (Ibraghimov-Beskrovnaya et al., 1992). Dystroglycan is one member of a large membrane-associated complex of proteins that requires dystrophin for its assembly. This complex has been termed the dystrophin-

associated protein complex (DAPC) or the dystrophin-glycoprotein complex (DGC). In skeletal muscle the molecules in this complex include the dystroglycans (α and β), sarcoglycans (α, β, δ, and γ), sarcospan, α-dystrobrevins (1, 2, and 3), and syntrophins (α, β_1, and β_2) (Ervasti, 2005). A diagram of dystrophin, the DGC, and the actin cytoskeleton is shown in Figure 1.2.

Dystrophin contains several domains, the functions of which have been elucidated *in vitro* and by deletion analysis *in vivo*. The N-terminal domain is homologous to the actin-binding domain of α-actinin and contains three separate actin-binding motifs (Bresnick et al., 1990; Levine et al., 1990; Levine et al., 1992). The adjacent region is the rod domain, which consists of 24 repeat units that are homologous to those found in spectrin (Koenig et al., 1988; Winder et al., 1995). These spectrin-like repeats are thought to each fold into a triple-helical structure, such that the rod domain is often envisioned as a spring-like structure (Cross et al., 1990; Koenig et al., 1988; Winder et al., 1995). A second actin-binding domain is located within the rod domain, formed by multiple basic repeats that make a favorable electrostatic contact with actin (Amann et al., 1998). The repeats are interrupted by four proline-rich hinges that are thought to lack significant secondary structure and could thus provide regions of flexibility (Koenig and Kunkel, 1990). The fourth hinge also contains a WW domain, which is a critical component of the dystroglycan-binding domain (Sudol et al., 1995). The dystroglycan-binding domain, also called the cysteine-rich domain, consists of the WW domain, two EF-

hand domains, and a ZZ domain which act cooperatively (Huang et al., 2000; Ishikawa-Sakurai et al., 2004; Rentschler et al., 1999). The C-terminal domain is the final functional domain of dystrophin and has homology to the dystrobrevin family. Dystrobrevins and the dystrophin C-terminal domain bind directly to each other via α-helical coiled-coil regions (Sadoulet-Puccio et al., 1997). The C-terminal domain also contains two binding sites for syntrophin, which are upstream of the coiled-coil region (Ahn and Kunkel, 1995; Newey et al., 2000). The C-terminal domain is encoded by exons 71-79, several of which are excluded in alternatively spliced isoforms (Feener et al., 1989). It is possible that alternative splicing functions to regulate the binding of various isoforms of dystrobrevin and syntrophin in specific cell types and during development (Bies et al., 1992a).

There is a group of dystrophin-related proteins that show varying degrees of conservation. The most conserved of these is utrophin, formerly called dystrophin-related protein (DRP). Utrophin has over 50% amino acid identity with dystrophin and has the same general organization of domains including an N-terminal actin-binding domain, 22 spectrin-like repeats, a dystroglycan-binding domain, and a C-terminal domain (Winder et al., 1995). In fetal and regenerating myofibers utrophin is localized around the entire sarcolemma (similar to dystrophin) but becomes specifically concentrated at the neuromuscular junction and the myotendinous junction in differentiated adult muscle (Helliwell et al., 1992; Khurana et al., 1991; Nguyen et al., 1991; Ohlendieck et al., 1991a). Utrophin differs from dystrophin in regard to some of its specific protein interactions but can compensate for much of its

function in mice (Tinsley et al., 1998). The dystrobrevins are the next member of the dystrophin-related protein family, which as discussed earlier are homologous to the carboxyl-terminal portion of dystrophin. Dystrobrevins contain a cysteine-rich domain and C-terminal domain but do not have a WW domain and thus lacks dystroglycan-binding function. However, dystrobrevins can associate with the DGC independently of dystrophin, which may reflect an interaction with one of the sarcoglycans (Crawford et al., 2000; Yoshida et al., 2000). The final member of the family is dystrophin-related protein 2 (DRP2). DRP2 is similar in size and domain organization to Dp116, but is expressed primarily in brain and not in striated muscle (Roberts et al., 1996; Roberts and Sheng, 2000).

Variability in the clinical consequences of dystrophin mutations

The clinical phenotypes associated with different dystrophin mutations can vary dramatically, which is the basis for the differential classification of Duchenne and Becker muscular dystrophies. Although they are allelic diseases resulting from mutations in the dystrophin gene, the clinical course of BMD is much less severe. The prevailing explanation for the different outcomes in these disorders is the reading frame hypothesis, which states that BMD is caused by mutations that do not disrupt the reading frame of the transcript (Monaco et al., 1988). In this case an altered dystrophin protein is produced that is less functional that the normal protein. This can occur from in frame deletions or duplications, missense mutations, or mutations that produce aberrant splicing. The converse prediction of this hypothesis

is that DMD results from mutations that lead to a frame-shift or premature termination of translation and therefore loss of dystrophin expression. This can arise from any of the types of mutations described above (other than missense mutations) as well as nonsense mutations.

In the majority of cases the predictions of the reading-frame hypothesis hold true. Diagnosis of DMD is usually confirmed by a muscle biopsy that establishes a lack of dystrophin expression, while biopsies of presumed BMD cases generally show some dystrophin expression (Emery and Muntoni, 2003). There are some exceptions, such as rare missense mutations that cause DMD despite persistent expression of dystrophin (Lenk et al., 1993; Lenk et al., 1996; Prior et al., 1993). However, these cases most likely demonstrate mutation of a critical amino acid that dramatically compromises the functionality of the protein. Deletions that remove an absolutely critical functional domain can likewise cause severe DMD even if the reading frame is not disrupted (Bies et al., 1992a). Conversely, deletions of huge stretches of the dystrophin gene that result in mild cases of BMD illustrate that other domains are not essential for dystrophin function (England et al., 1990). Further exceptions to the reading frame hypothesis occur when a mutation that predicts a change in the reading frame is associated with a clinical diagnosis of BMD (Winnard et al., 1993). In some of these cases alternate splicing patterns or start codons may still lead to expression of a truncated dystrophin (Winnard et al., 1995). Another complicating factor is the total expression level of the protein. A mutation that does not alter the reading frame may still lead to DMD if the expression level of the

truncated protein is too low. Thus, although the reading frame hypothesis may be an oversimplification, the ultimate determination of BMD versus DMD clinical phenotype depends on sufficient expression of a moderately functional protein.

Tissues other than skeletal muscle can also be affected by mutations in the dystrophin gene, which account for another aspect of clinical variability. Dysfunction in cardiac muscle and brain, the other tissues that express full-length isoforms of dystrophin, is quite common in DMD. The incidence of cardiomyopathy in DMD patients over the age of 18 approaches 100% although the age of onset shows some variability and dependence on site of mutation (Jefferies et al., 2005). Unusual mutations in the 5' region of the gene upstream of the dystrophin coding sequence are associated with X-linked dilated cardiomyopathy (Ortiz-Lopez et al., 1997; Towbin et al., 1993). Apparently these mutations cause a reduction of dystrophin expression specifically in cardiac tissue as these patients do not have muscular dystrophy. There also may be an association between sporadic dilated cardiomyopathy and dystrophin mutations or polymorphisms (Feng et al., 2002). Approximately one third of DMD patients show some degree of mental retardation. Clinical studies have shown an association between mutations that affect expression of short isoforms of dystrophin and cognitive deficits (Bardoni et al., 2000; Felisari et al., 2000; Lenk et al., 1993; Moizard et al., 2000). A single case of a DMD patient with demyelinating peripheral neuropathy was reported to have a mutation that prevented expression of Dp116 in Schwann cells (Comi et al., 1995). The importance of full-length dystrophin expression in brain is unknown.

Functional roles of dystrophin in muscle cells

The primary structure of dystrophin is immediately suggestive of a structural protein, especially considering its homology to other structural proteins. Its localization to costameres and interaction with the actin cytoskeleton also support this idea. Of particular importance is the fact that dystrophin completes a physical link between the cytoskeleton and the extracellular matrix (Ervasti and Campbell, 1993). This link is the foundation of the mechanical hypothesis of dystrophin function, which states that dystrophin is critical for maintaining the structural integrity of the sarcolemma by buffering the forces it experiences during myofiber contraction (Petrof et al., 1993; Rybakova et al., 2000). It has been suggested that one function of the costameres is to transmit forces of contraction laterally, allowing neighboring myofibers to work cooperatively (Bloch and Gonzalez-Serratos, 2003; Pardo et al., 1983). The correlate of this hypothesis is that the lack of dystrophin results in fragility of the sarcolemma, which leads to irreparable damage of the membrane during muscle contraction (Dellorusso et al., 2001; Lynch et al., 2000; Sacco et al., 1992). Dystrophin deficient myofibers accumulate molecules that normally would not be able to penetrate the cell membrane, indicating a defect in the physical barrier formed by the sarcolemma (Straub et al., 1997). The mechanical hypothesis also suggests that muscle cells that do not contract should be spared from degeneration. In fact, the intrafusal fibers of the muscle spindle rarely contract and are resistant to degeneration in DMD (Batten, 1897). However, some of the

extraocular muscles are also spared despite the fact that they are continually active, although they only produce very small forces (Kaminski et al., 1992).

Support for the mechanical hypothesis of dystrophin function also comes from studies of *mdx* mice expressing various mutant dystrophin proteins. Deletions that prevent the binding of dystrophin to either actin or dystroglycan, severing the connection between the cytoskeleton and the basal lamina, are invariably associated with severely dystrophic phenotypes. For example, dystrophins with deletions in the cysteine-rich region failed to assemble the DGC and did not improve muscle pathology (Rafael et al., 1996). Deletion of both actin binding domains resulted in severely dystrophic muscle even though the DGC was restored (Cox et al., 1994; Greenberg et al., 1994). Deletions of either the N-terminal or central actin-binding domain alone were found to produce milder phenotypes, presumably because only one of the actin-binding domains are absolutely required for some degree of mechanical function (Corrado et al., 1996; Phelps et al., 1995; Warner et al., 2002). Conversely, large deletions in either the rod domain or C-terminal domain that retain the complete N-terminal actin-binding domain and the cysteine-rich domain have been shown to be associated with extremely mild or no dystrophic phenotype (Crawford et al., 2000; Harper et al., 2002b). This result suggests that a strong connection to actin and to dystroglycan is the critical requirement for dystrophin function. However, some mutations in the central rod domain were not well tolerated, even when the critical mechanical connections were still present (Harper et

al., 2002a; Harper et al., 2002b). This result might reflect improper folding of the rod domain and thus failure to efficiently dissipate the forces of contraction.

Another possible function for dystrophin is as a scaffold for anchoring signaling molecules. Determining the precise function of dystrophin itself is complicated because the absence of dystrophin leads to failure of assembly of the entire DGC. It is possible that many of the pathological consequences of dystrophin deficiency are due to the secondary loss of these molecules. There are two primary lines of evidence suggesting that the DGC is involved in signal transduction. First, several of the proteins in the DGC, including dystrophin, can be phosphorylated by various kinases (Hasegawa et al., 1999; Madhavan and Jarrett, 1999; Sotgia et al., 2001). β-Dystroglycan and α and γ-sarcoglycan were shown to be phosphorylated in response to cellular adhesion, reminiscent of the adhesion-dependent signaling mediated by integrins (James et al., 2000; Yoshida et al., 1998). The second line of evidence comes from the observation that many of the proteins in the DGC interact with molecules known to be involved in other cellular signaling pathways. For example, dystroglycan binds to various kinases and adaptor proteins, in some cases dependent on its phosphorylation state (Russo et al., 2000; Sotgia et al., 2001; Sotgia et al., 2000; Spence et al., 2004; Yang et al., 1995a). Syntrophin is thought to be an adaptor protein that can localize a variety of signaling molecules by binding to its PDZ domain (Brenman et al., 1996; Hasegawa et al., 1999; Iwata et al., 1998; Lumeng et al., 1999; Oak et al., 2001; Oak et al., 2003).

The data described above strongly suggest that the DGC does have a role as a signal transduction complex. However, whether the loss of signaling functions is a major component of the pathophysiology of dystrophin-deficient muscular dystrophy is much less clear. Mutations in γ-sarcoglycan or α-dystrobrevin caused muscular dystrophy in mice without any evidence of mechanical damage to the myofibers (Grady et al., 1999; Hack et al., 1999). This has been interpreted as evidence for a dysfunction in signaling due to disruption of the DGC, although the mechanism is still unknown. It has been proposed that loss of homeostatic signaling through the DGC could lead to apoptotic cell death (Hack et al., 1998; Langenbach and Rando, 2002; Tidball et al., 1995). However, neither overexpression of anti-apoptotic proteins nor restoration of the DGC with a mechanically non-functional dystrophin isoform prevented pathology in *mdx* mice (Abmayr et al., 2004; Dominov et al., 2005). I will present detailed discussion as well as new evidence for the potential signaling roles of the DGC in Chapters 2-3.

Gene Therapy for DMD

Delivering a transgene to muscle

Several different genetic therapies for DMD are in development. Although technically difficult, these strategies do not necessarily require knowledge of the underlying disease mechanisms as they would directly restore expression of a functional protein. Some of the methods being employed include correction of the endogenous dystrophin gene, restoration of a reading frame in the transcript, or

delivery of an exogenous dystrophin gene (Bertoni, 2005; Gregorevic and Chamberlain, 2003; Wilton and Fletcher, 2005). The conceptually simplest method for genetic therapy of DMD involves administering a vector containing a functional dystrophin gene to a patient in a manner such that the vector delivers the transgene directly to striated muscle tissue. Such *in vivo* gene therapy could be accomplished by direct intramuscular injection of vector, or systemic administration in some form. Several different vectors have been successfully used to deliver dystrophin transgenes to skeletal muscle *in vivo* by intramuscular injection. These include plasmid DNA, adenoviral vectors, lentiviral vectors, and adeno-associated viral (AAV) vectors (DelloRusso et al., 2002; Harper et al., 2002b; Kobinger et al., 2003; Li et al., 2005; Ragot et al., 1993; Wang et al., 2000). However, the huge mass and wide distribution of skeletal muscle tissue throughout the body precludes the use of intramuscular injection as a practical method. This is particularly true considering the inaccessibility of the heart and diaphragm, two of the most critical targets to decrease mortality of the disease. Therefore, efficient systemic or regional delivery must be an important characteristic of any approach.

The obvious route for systemic delivery to muscle is via the bloodstream, as muscle tissue is highly vascularized. However, the vascular endothelium provides a barrier preventing most vectors from reaching the target tissue under physiological conditions. High pressure delivery, administration of drugs to induce vascular permeabilization, and surgical isolation of regional circulation has been successfully used to deliver various vectors intravascularly (Bridges et al., 2002; Cho et al., 2000;

Greelish et al., 1999; Su et al., 2005; Zhang et al., 2002). Each of these techniques creates a significant risk of morbidity for patients. Recently AAV vectors have been shown to effectively transduce skeletal and cardiac muscles after simple intravenous injection in mice (Gregorevic et al., 2004; Nakai et al., 2005; Wang et al., 2005). Combined with the fact that wild type AAV does not cause any known human disease (Blacklow et al., 1968) and provokes only a mild immune response (Chirmule et al., 1999) these results have quickly established AAV as the vector of choice for gene therapy targeting muscle. The remainder of this introduction will therefore focus on the progress and remaining challenges to overcome in order to translate AAV-mediated gene therapy for DMD into a clinical reality.

Recombinant adeno-associated virus vectors

AAV is a small, non-enveloped singe-stranded DNA virus of the family Parvoviridae. It is sub-classified as a Dependovirus because it requires co-infection with a second "helper" virus for replication (Atchison et al., 1965; Buller et al., 1981). The AAV genome is 4.7 kb in length and consists of two genes, Rep and Cap (Srivastava et al., 1983). The Rep gene produces several proteins necessary for viral replication and integration (Urcelay et al., 1995; Weitzman et al., 1994). The Cap gene generates the three structural proteins that form the capsid (Srivastava et al., 1983). The genome is flanked by two inverted terminal repeats (ITR) that serve as a packaging signal and origin of replication (Lusby et al., 1980; Tattersall and Ward, 1976; Xiao et al., 1997). Recombinant AAV vectors (rAAV) are generated by

deleting all of the viral genome except the ITRs, which are completely sufficient for packaging the recombinant viral genome (Berns and Linden, 1995; Xiao et al., 1997). Vector production requires three components: The rAAV genome, Rep and Cap genes supplied in trans, and helper virus functions (usually provided by adenoviral components). Standard methods for AAV production involve multiple plasmid transfection in cultured human cell lines, followed by purification and concentration of viral particles (Grimm et al., 1998; Grimm and Kleinschmidt, 1999; Xiao et al., 1998) (Figure 1.3). Although AAV2 is the serotype most commonly used as a vector, several novel serotypes of AAV have been identified and tested (Chiorini et al., 1999; Chiorini et al., 1997; Gao et al., 2002; Muramatsu et al., 1996; Rutledge et al., 1998; Xiao et al., 1999). The majority of these are produced simply by substituting the appropriate Cap gene without altering either the rAAV genome or the Rep gene (Grimm et al., 2003; Hermonat et al., 1997; Rabinowitz et al., 2002).

Scalability to large models

High titers of virus involving significant effort in production are required even to transduce the majority of muscle tissue in a small rodent. Initial efforts to scale the technique to a larger animal have shown promise (Gregorevic and Chamberlain, 2005). However, to realistically treat DMD patients will require advances in transduction efficiency and vector production. For example, creation of dedicated packaging cell lines or a baculovirus-mediated production method may prove to increase total rAAV yields (recently reviewed in (Zolotukhin, 2005)).

Ideally, increasing the transduction efficiency of lower rAAV doses would be preferred in order to reduce the load of viral protein administered to the patient. Several avenues of study have the potential to accomplish this goal.

Understanding the mechanism by which AAV exits the vascular space to reach target organs could provide the knowledge necessary to improve the process. For example, it is unclear whether the virus is crossing the endothelium by penetrating intercellular spaces or via transcytosis. Safe methods to increase vascular permeability by opening gaps between capillary endothelial cells could enhance vector uptake by muscle. It has been shown that VEGF (also known as vascular permeability factor) can enhance the transduction of skeletal muscle by lower doses of rAAV in mice, presumably through its effects on vascular permeability (Gregorevic et al., 2004). However, this method would increase vector uptake by other tissues as well. If the virus primarily crosses the endothelium by transcytosis, there may be specific cell receptor-virus interactions that facilitate the process. This could explain the variability in efficiency seen with different AAV serotypes (Wang et al., 2005). Further engineering the AAV capsid could enhance the process, along with potentially altering intracellular trafficking, tissue tropism, or the immune response. Methods to accomplish this could include identification of novel capsid types, generating chimeric vectors, or engineering specific peptide motifs into the capsid (Bowles et al., 2003; Gao et al., 2004; Gao et al., 2002; Girod et al., 1999; Hauck et al., 2003; Muller et al., 2003; Opie et al., 2003; Perabo et al., 2003; Rabinowitz et al., 2004; Warrington et al., 2004). Other procedures could

increase the fraction of vector available in the serum by blocking or removing potential inhibitors such as antibodies, platelets, or other blood cells.

Creating a minimal-sized dystrophin

Probably the most important limitation of rAAV vectors is their small genome size. Recombinant vector genomes that are significantly larger than the 4.7 kb wild type AAV genome are not packaged efficiently into virions (Hermonat et al., 1997). This size limitation is a considerable drawback for DMD gene therapy, as the dystrophin coding sequence itself is greater than 11 kb. Including the necessary regulatory elements for proper gene expression further restricts the size of the construct that can be packaged. Fortunately, studies of the allelic Becker muscular dystrophy (BMD) have provided clues that led to the development of highly functional truncated dystrophin mini-genes that can be incorporated into rAAV vectors.

BMD often this results from in-frame deletions producing an internally truncated yet partially functional protein (Koenig et al., 1989). One patient in particular demonstrated a mild phenotype resulting from a large deletion in the central rod domain (England et al., 1990). Perfect phasing of the central deletions to correspond to boundaries of the spectrin-like repeats rather than exon boundaries produced a dystrophin mini-gene that was as effective as the full-length protein in rescuing the dystrophic *mdx* mouse phenotype (Harper et al., 2002b; Phelps et al., 1995). Further studies in mice confirmed that even larger deletions in the rod

domain and COOH-terminal domain did not necessarily impair the function of the protein (Crawford et al., 2000; Harper et al., 2002b; Sakamoto et al., 2002; Wang et al., 2000) (Figure 1.4). Currently, we are using a micro-dystrophin with deletion of 20 of the 24 spectrin-like repeats as well as the entire COOH-terminal encoded by exons 71-78 (Harper et al., 2002b). This transgene has been incorporated into rAAV vectors and consistently effects an improvement in the *mdx* mouse phenotype (Abmayr et al., 2005; Gregorevic et al., 2004; Harper et al., 2002b; Liu et al., 2005; Yue et al., 2003).

However, there are still unanswered question regarding the functionality of current micro-dystrophin constructs. In mouse studies, the micro-dystrophins show mild functional deficits compared to full-length or even the larger mini-dystrophins. For instance, in our experience the ΔR4-23 transgene did not restore normal force generating capacity and also failed to localize nNOS to the sarcolemma (Chapter 2) and (Harper et al., 2002b). We have also found unusual abnormalities in the ultrastructure of myofibrils in the muscles of mice expressing micro-dystrophin (Chapter 4). These results suggest that there are defects in both signaling and contractile properties of myofibers expressing this micro-dystrophin. Apparently these defects are not severe enough to cause significant muscle cell degeneration in mice, but could be more problematic in larger animals and humans. The canine models of DMD (*cxmd*) provide an opportunity to test this concern before moving directly to clinical studies in humans, although these canine strains are severely affected and high levels of transduction may be required in order to detect a

phenotypic improvement. In short, there is still potential for improvement in the design of truncated dystrophin isoforms for gene therapy. A better understanding of the specific functional roles that dystrophin plays in muscle cells and which domains are critical for these functions would be a valuable addition to our knowledge. This is the major focus of the research presented in the following chapters.

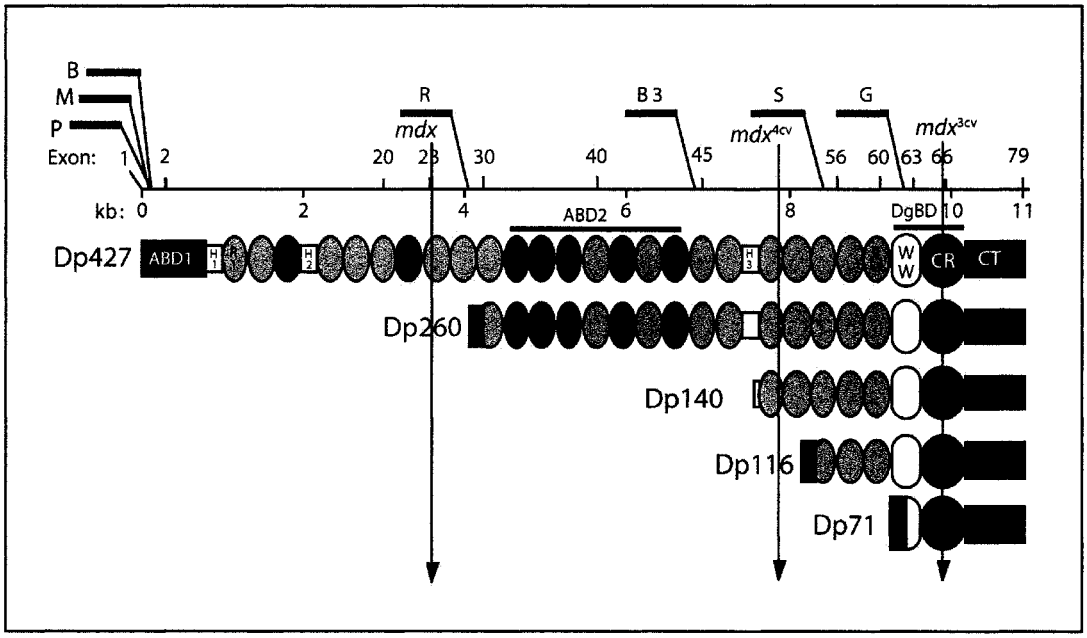


Figure 1.1: Diagram of the dystrophin gene and isoforms. At the top is shown the dystrophin mRNA with the locations of particular exons marked above the line, as well as the seven known promoters (blue rectangles) relative to their transcriptional start sites. The domain structures of the various protein isoforms are also shown below. Full-length dystrophin (Dp427) is transcribed from promoters active in muscle (M), brain (B), and Purkinje cells (P). Dp260 is transcribed from the retinal promoter (R), Dp140 from the third promoter found to be active in brain (B3), Dp116 from the Schwann cell promoter (S), and Dp71 from the ubiquitous promoter (G). The dystrophin protein is shown in color with NH_2-terminal actin-binding domain (ABD1), hinge domains (H1-3), spectrin-like repeats (orange circles = acidic repeats, blue circles = basic repeats), WW domain (hinge 4 overlaps with WW domain) cysteine-rich domain (CR), and carboxyl-terminal domain (CT). The dystroglycan-binding domain (DgBD) is formed by the WW and CR domains. The rod actin-binding domain (ABD2) is formed by the cluster of basic-repeats in the center of the protein. Amino acids that are unique to specific isoforms are shown as green rectangles where appropriate. Also shown are the sites of several of the *mdx* mutations. Intersection of the red arrows with a particular isoform indicates that expression of that isoform is affected by the corresponding mutant.

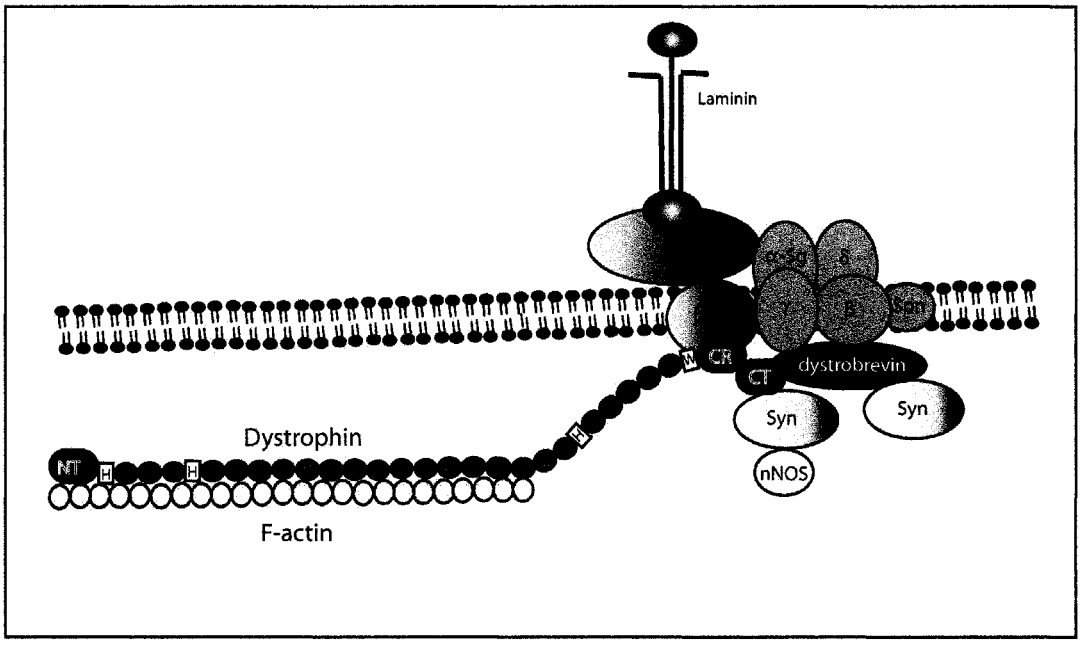


Figure 1.2: Diagram of the dystrophin-glycoprotein complex (DGC) in skeletal muscle fibers. The dystrophin protein is shown in color with NH_2-terminal actin-binding domain (NT), hinge domains (H), spectrin-like repeats (orange and blue circles), WW and cysteine-rich domains that form the dystroglycan-binding domain (W, CR), and carboxyl-terminal domain (CT). Dystrophin makes an extensive lateral connection with costameric F-actin (yellow) via its NT domain and several basic spectrin-like repeats (blue circles). The transmembrane proteins β-dystroglycan (Dg), α, β, δ, γ-sarcoglycans (Sg), and sarcospan (Spn) form the core unit of the DGC. The extracellular subunit α-dystroglycan is heavily glycosylated and binds to laminin-2 in the basement membrane. The cytoplasmic proteins dystrobrevin and syntrophin (Syn) bind to the CT domain of dystrophin while neuronal nitric oxide synthase (nNOS) interacts with syntrophin.

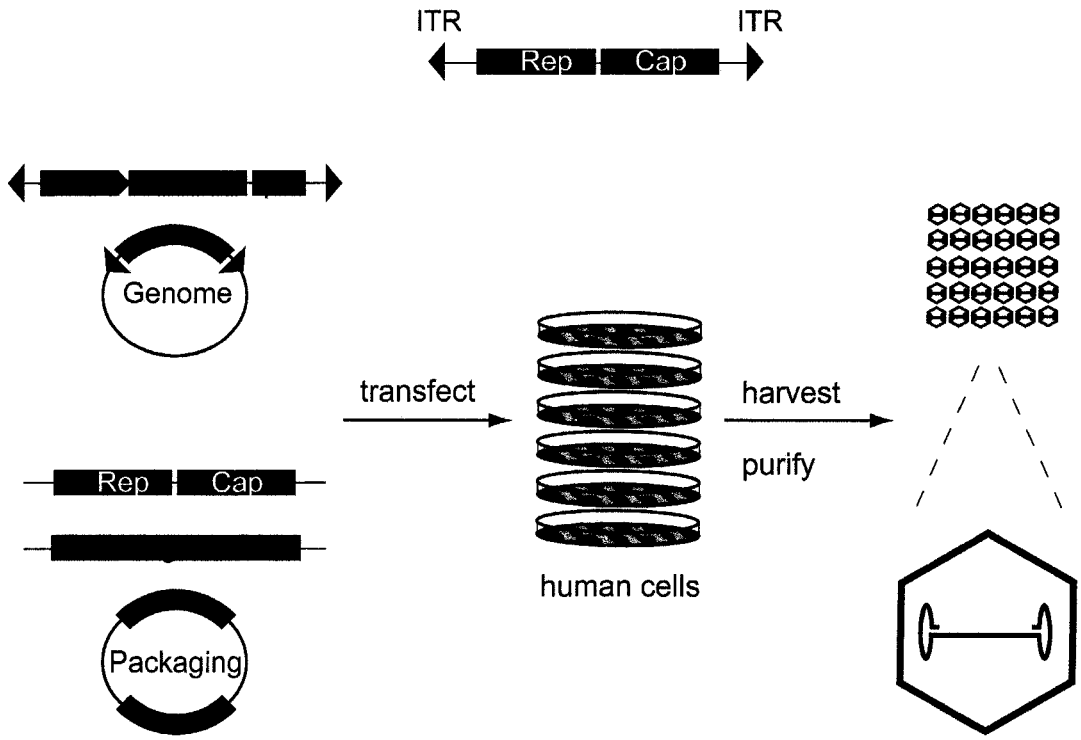


Figure 1.3: Production of recombinant AAV vectors. At the top center (black) is shown a simple schematic of the wild-type AAV genome with inverted terminal repeats (ITR) flanking the Rep and Cap genes. A recombinant AAV genome plasmid is shown on the top left (green) in which Rep and Cap have been substituted with an expression cassette. On the bottom left is shown the packaging plasmid (red and black) which contains expression cassettes for Rep, Cap, and adenoviral helper genes. A two-plasmid transfection in HEK293 cells followed by purification of vector capsid particles produces the recombinant vector.

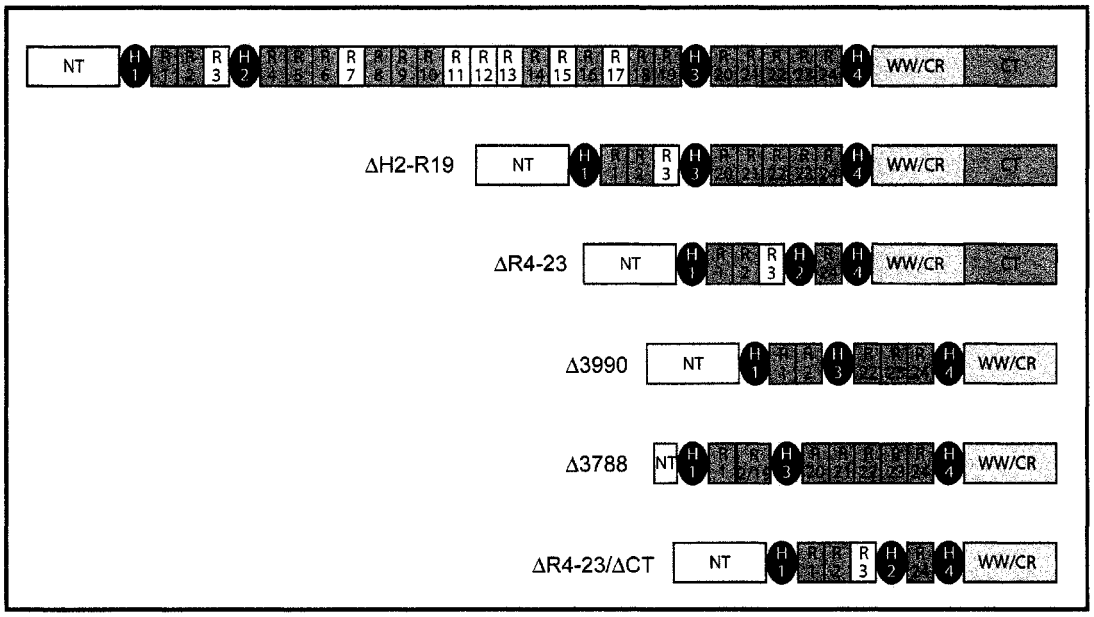


Figure 1.4: Truncated dystrophin proteins used for gene therapy. At the top is shown the complete dystrophin protein with NH_2-terminal actin-binding domain (NT), hinge domains (H1-4), spectrin-like repeats (R1-24), WW and cysteine-rich domains that form the dystroglycan-binding domain (WW/CR), and carboxyl-terminal domain (CT). The basic repeats that contribute to the rod actin-binding domain are shown in white. The ΔH2-R19 construct is referred to as a mini-dystrophin while the smaller constructs are referred to as micro-dystrophins. The functionality of ΔH2-R19 and ΔR4-23 was shown in transgenic mice (Harper et al., 2002b; Sakamoto et al., 2002). The final three constructs Δ3990, Δ3788, and ΔR4-23/ΔCT have been tested by AAV delivery in dystrophin-deficient mice (Fabb et al., 2002; Gregorevic et al., 2004; Harper et al., 2002b; Wang et al., 2000).

CHAPTER 2:

Dp116 competes with full-length dystrophin and utrophin and acts as a dominant-negative in skeletal muscles of wild-type and *mdx*^{4cv} mice

Introduction

Duchenne muscular dystrophy (DMD) is a lethal X-linked genetic disorder that affects approximately 1 in 3500 newborn males (Emery and Muntoni, 2003). The disease course is characterized by progressive wasting of the skeletal musculature with development of profound weakness, joint contractures, and kyphoscoliosis (Blake et al., 2002). Most patients die in their late teens or mid twenties, usually from respiratory or cardiac involvement. The causative gene was identified in 1986 by Kunkel and colleagues who subsequently named the protein product dystrophin (Hoffman et al., 1987; Koenig et al., 1987; Monaco et al., 1986). Mutations in the murine and canine dystrophin genes have also been identified, leading to the *mdx* and canine x-linked muscular dystrophic (*cxmd*) animal models of DMD (Bulfield et al., 1984; Cooper et al., 1988).

The dystrophin isoform in skeletal muscle is 427 kDa and is organized into four general functional domains [reviewed in (Abmayr and Chamberlain, 2005)]. First is the NH_2-terminal (NT) actin-binding domain (Ervasti and Campbell, 1993; Koenig et al., 1988). Second is the central rod region that is composed of 24 spectrin-like repeats interrupted by four proline-rich hinges. This region folds into a

series of triple helical coils that create a flexible and elastic structure (Koenig and Kunkel, 1990; Winder et al., 1995). Portions of the rod domain are also able to bind actin *via* a primarily electrostatic interaction (Amann et al., 1998). The third region has been recently named the dystroglycan-binding domain and is made up of WW, EF hand, and ZZ motifs (Ishikawa-Sakurai et al., 2004). β-dystroglycan spans the sarcolemma and binds to α-dystroglycan, which binds to laminin-α2 in the extracellular matrix (Ibraghimov-Beskrovnaya et al., 1992). Four sarcoglycans and sarcospan also interact with the dystroglycans and may facilitate this link to laminin (Araishi et al., 1999; Crosbie et al., 1999; Holt and Campbell, 1998). Finally, the COOH-terminal (CT) domain mediates interactions with dystrobrevin and syntrophin, the latter of which binds to neuronal nitric oxide synthase (nNOS) (Ahn and Kunkel, 1995; Chao et al., 1996; Sadoulet-Puccio et al., 1997; Yang et al., 1995b). The dystroglycans, sarcoglycans, sarcospan, dystrobrevins, syntrophins and nNOS collectively comprise the dystrophin-glycoprotein complex (DGC). Assembly of the DGC is dependent on specific domains of the dystrophin protein, and accordingly the DGC is absent from the sarcolemma of dystrophin deficient muscle.

Despite the wealth of knowledge about the dystrophin gene and protein, the precise molecular and cellular events by which dystrophin deficiency leads to muscle fiber degeneration are poorly known (Petrof, 2002). There are two primary hypotheses regarding the essential functions of dystrophin in skeletal muscle. The first is that dystrophin is a structural protein that completes a physical link between the extracellular matrix and the actin cytoskeleton, enabling force transduction from

within myofibers to the extracellular matrix (Bloch and Gonzalez-Serratos, 2003; Ervasti, 2003; Lynch et al., 2000). Absence of this mechanical apparatus is thought to destabilize the sarcolemma, making the myofibers more susceptible to damage during contractions (Dellorusso et al., 2001; Lynch et al., 2000; Petrof et al., 1993; Sacco et al., 1992). It is also clear that dystrophin serves as a scaffold for the assembly of a multi-component signal transduction complex, portions of which are also integral members of the DGC (Brenman et al., 1996; Madhavan et al., 1992; Yang et al., 1995a; Yoshida et al., 2000). A second hypothesis views perturbation of cellular signaling resulting from the absence of dystrophin and many of its associated proteins as a major contributor to myofiber death (Brenman et al., 1995; Grady et al., 1999; Rando, 2001a).

Previous studies in patients and in transgenic mice have examined the role of various dystrophin domains and members of the DGC in preventing the development of dystrophy (Abmayr and Chamberlain, 2005). Expression of dystrophins that lack the dystroglycan binding domain invariably lead to a severe dystrophy, illustrating the importance of the DGC in preventing muscular dystrophy (Bies et al., 1992a; Hoffman et al., 1991; Rafael et al., 1996). However, it has been difficult to test the model that restoration of the DGC in the absence of a link to actin might also rescue dystrophic muscle. We previously described lines of transgenic *mdx* mice that expressed the dystrophin isoform Dp260 in muscle. Dp260 contains much of the rod domain and can make a mechanically strong connection to costameric actin (Warner et al., 2002). While those mice expressed the DGC and displayed a mild dystrophy,

the finding that Dp260 interacts with the actin cytoskeleton prevented a clear dissection of the mechanical and signaling roles of the dystrophin/DGC network (Warner et al., 2002). In contrast, restoration of the DGC by transgenic expression of Dp71 in skeletal muscles of *mdx* mice or in dystrophin/utrophin double knockout mice failed to improve any aspect of the dystrophic pathology (Cox et al., 1994; Greenberg et al., 1994; Rafael et al., 2000). However, Dp71 lacks the entire rod domain as well as a functional WW domain (Figure 2.1), which is an integral component of the dystroglycan-binding domain in dystrophin (Huang et al., 2000; Rentschler et al., 1999; Yang et al., 1995a). In fact, Dp71 was not found in the microsomal fraction of skeletal muscle, indicating a weak association with the sarcolemma (Greenberg et al., 1994). β-dystroglycan serves as a ligand for WW domains contained in dystrophin, utrophin, and caveolin-3. Furthermore, phosphorylation of β-dystroglycan can modulate its affinity for these various binding partners (Ilsley et al., 2002). It is likely that an intact dystrophin WW domain is required for proper regulation of these interactions, which may underlie important cellular signaling processes.

In order to differentiate more carefully between the mechanical and signaling roles of dystrophin and the DGC, we generated transgenic mice that express Dp116 in skeletal muscles of *mdx*^{4cv} mice. Dp116 contains the entire WW domain but only 2 complete repeats from the rod domain (Figure 2.1). We reasoned that this protein could reconstitute the signaling functions of dystrophin and the DGC without contributing to mechanical reinforcement of the sarcolemma. Despite expression of

Dp116 and an intact DGC, *Dp116:mdx*^{4cv} mice were more severely dystrophic than their *mdx*^{4cv} littermates. The worsened phenotype was likely caused by displacement of utrophin from the sarcolemma. Analysis of nNOS expression in several transgenic lines expressing different truncated dystrophin isoforms demonstrated that specific portions of the dystrophin rod domain are necessary for localization of nNOS at the sarcolemma. However, there was no correlation between nNOS expression and phenotype in these various lines. These data clearly demonstrate that loss of mechanical function is the primary contributor to the dystrophic phenotype.

Materials and Methods

Generation of transgenic mice

The Dp116 transgene was prepared by PCR amplification from a full-length mouse dystrophin cDNA clone using the following oligonucleotides, the first of which encodes the Flag epitope tag in-frame with the unique first exon from human Dp116: 5'-cgccggccgATGGACTACAAGGACGACGACGACAAGTTACACAGGAAGACA TACCATGTAAAGGATCTCCAAGGAGAAATTGAAAC – 3' and 5' – gcgcggccgCCAAATCATCTGCCATGTGG – 3'. This PCR product was cloned into the pGEM-T Easy Vector (Promega) and sequenced. The Dp116 cDNA was subcloned into the previously described pBSX-HSAvpA expression vector (Crawford et al., 2000). The linearized expression cassette was injected into C57Bl/6 embryos and the progeny screened by PCR. Five transgene positive

founders were identified and crossed with *mdx*^{4cv} mice as independent lines. Three of the lines (2197, 2354, 2355) expressed Dp116 at high levels in skeletal muscle and were crossed onto the *mdx*^{4cv} background for a minimum of two generations. *Dp116*:wild type transgenic mice were obtained from the male offspring of *Dp116*:heterozygous *mdx*^{4cv} transgenic females. The *mdx*^{4cv} mutation was genotyped by an ARMS assay (Pearson-White, 2002). All experimental protocols involving live mice were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Western analysis

Whole tissue lysates were prepared by homogenization in extraction buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) with protease inhibitor cocktail (Roche) and cleared by centrifugation. KCl washed skeletal muscle microsomes were prepared as previously described (Ohlendieck et al., 1991b). Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce). Equivalent amounts of total protein were separated on 4-12% NuPAGE gradient gels (Invitrogen Life Technologies) and electrophoretically transferred to PVDF membranes or stained with Coomassie blue. Membranes were blocked with 5% nonfat dry milk in TBS and probed with antibodies to dystrophin COOH-terminus, β-dystroglycan, β-sarcoglycan, γ-sarcoglycan (Novocastra Laboratories Ltd.), α-dystrobrevin 2 (Peters et al., 1998), α_1-syntrophin (Peters et al., 1998), and nNOS (Zymed Laboratories). The membranes were washed with TBS containing

0.1% Tween 20 and probed with horseradish peroxidase-conjugated donkey anti-mouse or donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch). After further washing, detection of immunoblotted proteins was performed with the Enhanced Chemiluminescent Plus reagent (Amersham Biosciences) and visualization on a GeneGnome chemiluminescent imaging system (SynGene Bioimaging).

Immunofluorescence

Skeletal muscles were dissected, embedded in OCT (Sakura Finetek USA) on cork support and frozen in liquid nitrogen-cooled isopentane. Cryosections of 7-10 μm thickness were blocked in KPBS (20 mM potassium phosphate pH 7.4, 150 mM sodium chloride) containing 0.3 mg/ml BSA, 2% gelatin, and 1% Tween 20. The blocked sections were incubated with primary antibodies diluted in KPBS containing 0.2% gelatin (KPBS-G) and 2% normal goat serum. Rabbit polyclonal antibodies were used to label Flag (Sigma), dystrophin NH_2-terminus (Rafael et al., 1996), dystrophin COOH-terminus (Cox et al., 1994), nNOS (Zymed Laboratories), α_1-syntrophin (Peters et al., 1997), α-dystrobrevin 2 (Peters et al., 1998), and utrophin A (S. Froehner, unpublished). After several washes with KBPS-G the sections were incubated with goat anti-rabbit Alexafluor 488 (Molecular Probes). Monoclonal rat anti-CD11b (BD Pharmingen) was used in combination with goat anti-rat Alexafluor 594 (Molecular Probes). Monoclonal antibodies to β-dystroglycan and α, β, γ-

sarcoglycans (Novocastra Laboratories Ltd.) were used with the M.O.M. Immunodetection kit (Vector Laboratories). Double staining for utrophin-A and Flag epitope was done using a biotinylated mouse monoclonal anti-Flag antibody (BioM2, Sigma) and rabbit anti-utrophin-A. An endogenous biotin blocking step (Molecular Probes) was added to the standard blocking procedure and secondary detection was performed with streptavidin Alexafluor 594 conjugate and goat anti-rabbit Alexafluor 488 (Molecular Probes). Immunostained slides were washed repeatedly with KBPS-G and mounted with Vectashield (Vector Laboratories). All photomicrographs were obtained with a Spot II CCD camera (Diagnostic Instruments, Inc.) and Spot Advanced software connected to a Nikon Eclipse E1000 using a 20X Plan-Apochromat objective (numerical aperture = 0.75). Images for all the sections probed with a given antibody were acquired under identical exposure conditions.

Histological analysis

Quadriceps, tibialis anterior, extensor digitorum longus, soleus, and diaphragm muscles were dissected, embedded in OCT (Sakura Finetek USA) on cork support and frozen in liquid nitrogen-cooled isopentane. Cryosections of 10 μm thickness were briefly fixed in methanol and stained with Gill's hematoxylin and eosin-phyloxine. Photomicrographs were obtained with a Spot II CCD camera (Diagnostic Instruments, Inc.) and Spot Advanced software connected to a Nikon Eclipse E1000 microscope using a 20X Plan-Apochromat objective (numerical

aperture = 0.75). Images of entire muscle cross sections were obtained using the Montage Explorer software (Syncroscopy) driving a JVC 3-CCD color video camera. ImagePro software (Media Cybernetics) was used to measure muscle fiber diameters and count fibers with central nuclei. The percentage of central nuclei was determined by dividing the number of fibers with central nuclei by the total number of fibers. Muscle fiber diameter was determined by measuring the minimal diameter through the center of each fiber. Fibers from randomly located fields in the tibialis anterior muscle were scored until the total number of fibers exceeded 1000.

Results

Generation of transgenic mice

A full-length *Dp116* cDNA carrying an N-terminal Flag tag was generated by PCR and cloned downstream of the human skeletal α-actin promoter prior to injecting into C57Bl/6 mouse embryos. Figure 2.1 shows the domain structure of Dp116 compared with full length dystrophin, Dp260, Dp71, and mini- and micro-dystrophins with internal deletions of the rod domain. All of these latter constructs have been previously tested in transgenic mice with a variety of phenotypic consequences (Cox et al., 1993; Cox et al., 1994; Greenberg et al., 1994; Harper et al., 2002b; Warner et al., 2002). Dp116 is unique among these various dystrophin proteins because it contains the complete dystroglycan binding domain but lacks both the NH_2-terminal and rod domain actin-binding domains.

Transgene-positive F_0 mice were identified by a PCR screen and backcrossed onto the *mdx*^{4cv} background, which has a nonsense mutation in exon 53 of the murine dystrophin gene (Im et al., 1996). The *mdx*^{4cv} mutation does not affect normal expression of Dp116 or Dp71, but it does eliminate expression of all of the larger dystrophin isoforms (Im et al., 1996). Three independent lines of *Dp116* transgenic mice were backcrossed to *mdx*^{4cv} for multiple generations. Skeletal muscles from all three of these transgenic lines expressed Dp116 protein at high levels as visualized by Western blot (Figure 2.1B). Immunofluorescent labeling using antibodies to the Flag epitope and the dystrophin COOH-terminal domain demonstrated uniform staining of the muscle fiber sarcolemma throughout the quadriceps and diaphragm (Figure 2.1C) as well as the tibialis anterior and soleus muscles (data not shown).

Histopathology of Dp116 transgenic muscle

Dp116:mdx^{4cv} transgenic mice and *mdx*^{4cv} littermates from line 2197 were sacrificed at 3, 4, 12, and 28 weeks of age along with age-matched C57Bl/6 controls. Mice from lines 2354 and 2355 were also sacrificed at 12 weeks age to verify a consistent phenotype. Hematoxylin and eosin stained cross sections from quadriceps, tibialis anterior, extensor digitorum longus, soleus, and diaphragm muscles were examined at each age to evaluate the extent of dystrophic pathology. In muscles from *mdx*^{4cv} mice the dystrophic pathology developed significantly between 3 and 4 weeks of age (Figure 2.2 and data not shown). At 3 weeks, relatively small areas of focal necrosis and infiltration of mononuclear cells were

apparent (Figure 2.2). Regenerating fibers, marked by the presence of centrally located nuclei, were rare or absent. By 4 weeks of age there was continued evidence of degeneration and inflammation, but large numbers of regenerating fibers with central nuclei were now present (Figures 2.2, 2.3). Surprisingly, *Dp116:mdx*^{4cv} muscle displayed an earlier onset of a more severe dystrophy. Degeneration and inflammation were much more extensive in transgenic muscle at 3 and 4 weeks of age, particularly in the diaphragm and soleus (Figures 2.2, 2.3). Unlike in *mdx*^{4cv} muscles, clusters of small regenerating fibers were already visible at 3 weeks in *Dp116* transgenic muscles.

At 12 weeks of age the quadriceps, tibialis anterior, and extensor digitorum longus muscles appeared qualitatively similar between *Dp116:mdx*^{4cv} and *mdx*^{4cv} mice. However, the diaphragm and soleus muscles continued to be more severely affected in the *Dp116:mdx*^{4cv} mice (Figure 2.3). The *mdx*^{4cv} soleus showed a majority of relatively normal, successfully regenerated muscle fibers at this age, with focal areas of continued necrosis and inflammation. In contrast, *Dp116:mdx*^{4cv} soleus muscles showed widespread inflammation and loss of normal muscle architecture. Immunostaining against CD11b, a surface marker for the monocyte/macrophage lineage, demonstrated focal immunoreactivity clustered around necrotic fibers in *mdx*^{4cv} muscles, but diffuse staining surrounding even apparently normal fibers in *Dp116:mdx*^{4cv} muscles (Figure 2.3). Histopathology was very similar between all three transgenic lines at 12 weeks of age. At 28 weeks of age both *Dp116:mdx*^{4cv} and *mdx*^{4cv} muscles appeared very similar to those at 12

weeks, but with less inflammatory infiltrate and more development of fibrotic tissue (data not shown).

Our breeding scheme also produced *Dp116*:C57 transgenic mice with a wild-type dystrophin allele, some of which were sacrificed at 12 weeks of age. Overexpression of Dp116 on the wild type background had dominant negative effects in the quadriceps, tibialis anterior, and soleus muscles. *Dp116:*C57 muscles displayed increased numbers of fibers with central nuclei without overt evidence of necrosis or inflammation (Figure 2.4). Immunostaining with the anti-Flag antibody demonstrated strong expression of Dp116 at the sarcolemma, while immunostaining with an antibody to the dystrophin NH_2-terminal domain revealed decreased expression of the full-length dystrophin isoform compared with controls (Figure 2.4A). Western analysis confirmed a decrease in the total amount of full-length dystrophin protein in *Dp116*:C57 skeletal muscles (Figure 2.4B).

Two standard histological parameters used to quantitatively asses the extent of dystrophic pathology in mouse skeletal muscle are the percentage of fibers with central nuclei and the variance in fiber cross-sectional diameter (Briguet et al., 2004; Harper et al., 2002b). Dystrophic muscle is characterized by a high percentage of centrally nucleated fibers and a large variance in fiber cross sectional size. Transgenic expression of Dp116 on the wild type background caused an increase in the percentage of myofibers with central nuclei to over 20% compared to < 1% in C57Bl/6 (Figure 2.5). Transgenic expression of Dp116 on the mdx^{4cv} background resulted in a slight, but statistically significant, decrease in the percentage of

myofibers with central nuclei (64%) compared with *mdx*^{4cv} (74%). This difference likely stems from the large increase in small caliber, regenerating myotubes and myofibers in the *Dp116:mdx*^{4cv} muscles, in which it is very difficult to determine whether the cell has central or peripherally located myonuclei. The analysis of muscle fiber diameter demonstrated a striking difference between *Dp116:mdx*^{4cv} transgenic and *mdx*^{4cv} tibialis anterior muscles. The *mdx*^{4cv} muscles showed greater variation in muscle fiber diameter and a smaller median fiber diameter (34.1 μm) compared with wild type (40.9 μm). The *Dp116:mdx*^{4cv} transgenic muscles showed less variation in fiber diameter than *mdx*^{4cv} muscles but both the median (21.3 μm) and overall distribution were markedly skewed in favor of fibers with small diameters.

Dp116 assembles the dystrophin-glycoprotein complex and displaces utrophin

To explore the mechanism by which overexpression of Dp116 led to a severe phenotype on the *mdx*^{4cv} background, we examined the dystrophin-glycoprotein complex in Dp116/*mdx*^{4cv} skeletal muscle. Endogenous Dp116 expressed in Schwann cells is associated with a glycoprotein complex that differs from that found in skeletal muscle. In Schwann cells, ε-sarcoglycan is highly expressed while α-sarcoglycan and γ-sarcoglycan are not (Imamura et al., 2000). Immunofluorescent analysis showed that Dp116 transgenic mice express the normal skeletal muscle complex on the sarcolemma, including β-dystroglycan as well as α, β, and γ-

sarcoglycans (Figure 2.6A). Overexpression of Dp116 resulted in upregulation of members of the glycoprotein complex on the sarcolemma, as seen by Western blots of KCl-washed skeletal muscle microsomes (Figure 2.6B). The sub-sarcolemmal proteins α_1-syntrophin and α-dystrobrevin 2 were restored to levels similar to wild-type (Figure 2.6A,B).

In normal muscle, the PDZ domain of α_1-syntrophin binds to nNOS, localizing it to the inner face of the sarcolemma (Adams et al., 2001; Brenman et al., 1996). In dystrophin deficient muscle nNOS expression is lost from the membrane along with other members of the DGC (Brenman et al., 1995). However, no nNOS immunoreactivity was found on the sarcolemma of *Dp116:mdx*^{4cv} muscle despite the presence of α_1-syntrophin (Figure 2.7). This observation is consistent with previous data indicating that various dystrophin mutations are associated with a loss of nNOS from the sarcolemma despite persistence of α_1-syntrophin expression (Chao et al., 1996). We also examined nNOS expression in several transgenic mouse lines expressing various truncated dystrophin proteins in an attempt to identify the portion of dystrophin that is required for nNOS expression and localization (Figure 2.7). Immunofluorescent staining showed that transgenic *mdx* mice expressing mini-dystrophin (ΔH2-R19) or micro-dystrophin (ΔR4-R23) had minimal amounts of membrane-associated nNOS, even though these mice do not show signs of dystrophic pathology (Harper et al., 2002b). Transgenic *mdx* mice expressing Dp260, which results in a phenotype intermediate between wild type and *mdx*

(Warner et al., 2002), showed greater nNOS immunoreactivity at the sarcolemma, although still less than wild type.

Western blots of both the pellet (membrane fraction) and supernatant (cytosolic fraction) from muscle microsome preparations showed nNOS protein in both cellular compartments in C57Bl/6, but nNOS was nearly undetectable in both fractions from *mdx*^{4cv} and *Dp116:mdx*^{4cv} (Figure 2.7B). Microsome pellets from *ΔR4-23:mdx* muscles had barely detectable nNOS, confirming the immunofluorescence data. Interestingly, the soluble fraction from the *ΔR4-23:mdx* preparation did contain significant levels of nNOS, although less than wild type. It was previously shown that nNOS was present in *Dp260:mdx* muscle microsomes (Warner et al., 2002). This analysis indicates that expression of α_1-syntrophin and the complete DGC is not sufficient for localization of nNOS to the sarcolemma, but that portions of the central rod domain that are present in Dp260 but lacking in the micro- and mini-dystrophins are also important. However, nNOS expression was not correlated with the extent of muscle pathophysiology in these various transgenic lines.

Utrophin-A, normally restricted to the neuromuscular junction in mouse skeletal muscle, is broadly distributed on the sarcolemma in *mdx* muscle (Weir et al., 2002). Upregulation of utrophin may partially compensate for dystrophin deficiency, so we investigated whether overexpression of Dp116 could displace utrophin from the sarcolemma of *mdx*^{4cv} muscle in a manner similar to its

competition with dystrophin in wild type muscle. Immunostaining with an antibody specific to the Utrophin-A isoform demonstrated increased immunoreactivity at the sarcolemma in *mdx*^{4cv} quadriceps muscle compared with wild type controls. This upregulation of utrophin was mosaic in nature, with some fibers showing strong staining of the sarcolemma and others showing little (Figure 2.8A). *Dp116:mdx*^{4cv} quadriceps showed similar mosaic staining for utrophin, making comparison difficult. In contrast, the *mdx*^{4cv} soleus muscle showed a dramatic and uniform increase in utrophin immunoreactivity at the sarcolemma, which was almost completely absent in *Dp116:mdx*^{4cv} soleus muscle. This is consistent with previous data demonstrating greater upregulation of utrophin in slow soleus compared to fast extensor digitorum longus muscles (Gramolini et al., 2001). Transgenic *mdx* mice expressing the highly functional ΔR4-R23 micro-dystrophin protein displayed utrophin immunostaining that was very similar to that of wild type mice in both quadriceps and soleus (Figure 2.8A). It is notable that when comparing *mdx*^{4cv} and *Dp116:mdx*^{4cv} skeletal muscles, the soleus showed the greatest difference in both histopathology and utrophin expression.

Double labeling with antibodies against the Flag epitope and utrophin-A showed an inverse correlation between Dp116/Dp260 and utrophin-A expression at the sarcolemma of individual myofibers (Figure 2.8B). In general, larger caliber myofibers expressed high levels of the transgene while small caliber myofibers expressed high levels of utrophin at the sarcolemma. Utrophin is known to be upregulated in regenerating myofibers (Galvagni et al., 2002; Helliwell et al., 1992;

Miura et al., 2005). Thus, it is likely that the smaller, utrophin-positive fibers in this analysis have recently regenerated.

Discussion

Increasing evidence suggests that dystrophin and the DGC act as a scaffold that anchors signaling proteins to the sarcolemma (Rando, 2001a). Dystroglycan, sarcoglycans, and short isoforms of dystrophin are expressed in a wide variety of tissues that do not have the unique contractile properties of muscle. Thus, non-structural functions of the DGC in other cell types are likely to be also active in muscle cells. In cultured myoblasts, dystroglycan binding to laminin in the extracellular matrix activates the phosphatidylinositide-3'-kinase/Akt pathway, which regulates cell growth and survival (Langenbach and Rando, 2002). Abnormal activity of various kinases in this and other signaling pathways have been also been reported in dystrophic muscle in vivo (Kolodziejczyk et al., 2001; Kumar and Boriek, 2003; Kumar et al., 2004; Lang et al., 2004). However, it is not clear whether these abnormalities are primary contributors to the dystrophic phenotype or if they are secondary consequences. In some cases they may be adaptive responses that reflect increased regeneration and hypertrophy of muscle fibers.

We hypothesized that transgenic expression of a dystrophin isoform containing the entire dystroglycan binding domain, including the complete WW domain, could restore not only expression of the DGC, but also normal regulation of the interactions within the complex. Our results clearly show that expression of

Dp116 in skeletal muscle actually exacerbated the dystrophic phenotype, particularly in muscles such as the diaphragm and soleus. Two explanations for this result are possible. First, instead of restoring normal signaling through the DGC we may have merely pushed the balance too far in the opposite direction. We did observe significant overexpression not only of Dp116, but also of β-dystroglycan and the sarcoglycan complex (which may contain a clue to the regulation and assembly of this complex). However, this explanation is incompatible with previous studies showing that marked overexpression of full-length or mechanically functional mini-dystrophins fully corrected the dystrophic phenotype (Cox et al., 1993; Crawford et al., 2000; Harper et al., 2002b). These latter studies showed no negative consequences resulting from increased levels of β-dystroglycan and the sarcoglycan complex. Furthermore, overexpression of Dp116 in the wild type background only caused a very mild phenotype. Such dominant-negative phenotypes have previously been observed only in mice expressing functionally impaired dystrophins (Crawford et al., 2001; Leibovitz et al., 2002; Warner et al., 2002). Our data support the second explanation, that overexpression of Dp116 competes with utrophin and displaces it from the sarcolemma, further destabilizing the mechanical connection between the DGC and the cytoskeleton.

Utrophin is normally restricted to the neuromuscular junction, but is expressed throughout the sarcolemma in dystrophin-deficient muscle (Weir et al., 2002). Mice that are deficient for both utrophin and dystrophin (*mdx:utrn*$^{-/-}$) have a much more severe dystrophy than *mdx* mice (Deconinck et al., 1997; Grady et al.,

1997b). Further, overexpression of utrophin in either *mdx* or *mdx:utrn*$^{-/-}$ mice can effectively compensate for the lack of dystrophin (Tinsley et al., 1998; Wakefield et al., 2000). In sum, these data highlight the fact that utrophin and dystrophin are functionally redundant to some degree and that upregulation of utrophin partially compensates for the lack of dystrophin in *mdx* mice. These observations further underscore the importance of the mechanical functions of these proteins in that low level expression of full-length utrophin was more effective at maintaining myofiber integrity than high level expression of Dp116 (Figures 2.3, 2.8). Differences in utrophin expression may also explain the fact that the soleus and diaphragm muscles were most notably affected by expression of Dp116. These two muscles have a high proportion of slow fibers, which contain the highest levels of utrophin in *mdx* mice (Gramolini et al., 2001). Interestingly, this significant upregulation of utrophin in the *mdx*^{4cv} soleus was almost completely reversed by Dp116 expression. Conversely, larger muscles such as the quadriceps showed greater variability in both utrophin expression and dystrophic pathology. It may also be that the mechanical properties of the soleus and diaphragm predispose them to dystrophic injury, as they are two of the most severely affected muscles in *mdx* mice.

The best studied signaling protein linked to the DGC is neuronal nitric oxide synthase (nNOS). This enzyme produces the second messenger nitric oxide, which can have diverse effects including the activation of guanylate cyclase and inhibition of cytochrome-*c* oxidase (Stamler and Meissner, 2001). Transgenic overexpression of nNOS in *mdx* skeletal muscle ameliorated the dystrophic phenotype without

restoring expression of other DGC members, apparently by attenuating the inflammatory response in the dystrophic muscles (Tidball and Wehling-Henricks, 2004; Wehling et al., 2001). Another function of nNOS in skeletal muscle is to regulate blood flow during exercise. DMD patients and mice with primary deficiencies of either dystrophin or nNOS are unable to attenuate the vasoconstriction produced by adrenergic stimulation during muscle contraction (Sander et al., 2000; Thomas et al., 1998). Recent data also showed the same defect in regulation of blood flow in mice lacking α_1-syntrophin or expressing a transgenic syntrophin with the PDZ domain deleted (Thomas et al., 2003). These results indicate that nNOS must be localized to the sarcolemma to serve this particular function, as association with the PDZ domain of α_1-syntrophin is necessary to target nNOS to the inner face of the sarcolemma (Adams et al., 2001).

It is also clear that expression of α_1-syntrophin alone is not sufficient to localize nNOS to the sarcolemma. Transgenic *mdx* mice and Becker muscular dystrophy (BMD) patients expressing dystrophin with deletions of the rod domain display loss of sarcolemma-associated nNOS despite normal expression of other DGC components, including α_1-syntrophin (Chao et al., 1996; Crosbie et al., 1998; Torelli et al., 2004; Wells et al., 2003). Our data support these results, demonstrating that deletion of the central rod domain region in the *ΔH2-R19* or *ΔR4-23* transgenic mice results in the loss of nNOS from the sarcolemma. Dp116 also failed to restore nNOS expression in either the membrane-associated or cytoplasmic fractions. The

Dp260 transgene was the most successful at restoring nNOS to the sarcolemma in our studies, although nNOS levels were still reduced compared to wild type. These observations confirm that the central rod domain has some role in localizing nNOS to the sarcolemma. Direct binding of nNOS to dystrophin has not been detected, so the mechanism of this effect remains uncertain.

The majority of data suggest that the loss of nNOS from the sarcolemma is not by itself a primary contributor to the dystrophic phenotype. Disruption of either the nNOS or α_1-syntrophin genes in mice did not produce myopathy (Adams et al., 2000; Chao et al., 1998). Analysis of BMD patients with expression of various internally truncated dystrophin proteins did not show a correlation between sarcolemmal nNOS expression and the severity of phenotype (Chao et al., 1996). Similarly, our data show that nNOS expression at the sarcolemma is not correlated with the presence, or degree of the dystrophic phenotype in mice expressing various types of dystrophin. For example, *mdx* mice expressing ΔH2-R19 display no phenotypic differences from wild type mice despite dramatic reduction of nNOS at the sarcolemma (Figure 2.7) and (Harper et al., 2002b). However, examination of all of the previous data reveals one potential pattern. Mutations in dystrophin that result in a severely dystrophic phenotype are invariably associated with the loss of nNOS from the sarcolemma, independent of the extent of mutant dystrophin protein or DGC expression [e.g. transgenic mice expressing either Dp116 (Figure 2.7) or dystrophins lacking various portions of the dystroglycan-binding domain (Rafael et al., 1996)]. Conversely, mutations that result in a very mild or no detectable

phenotype can be associated with either the loss or persistence of nNOS, depending on the exact nature of the mutant protein. It is possible that two phenomena are being observed. First, there may be abnormalities of dystrophic muscle that promote the degradation of nNOS or inhibit its production. Second, regions of the rod domain appear to be necessary, in addition to α_1-syntrophin, to localize nNOS to the sarcolemma. The combination of these hypotheses could explain our observation that the *ΔR4-23* transgenic mice do express some nNOS in the cytoplasm of their skeletal muscles. Prevention of the dystrophic phenotype by expression of micro-dystrophin apparently partially corrects the defect in overall nNOS expression. However, these mice maintain a defect in nNOS localization to the sarcolemma, presumably because of a requirement for portions of the rod domain.

In summary, we found that overexpression of Dp116 in skeletal muscle of *mdx*^{4cv} mice did not ameliorate the dystrophic phenotype. Our results support previous data suggesting that a mechanically functional dystrophin is absolutely required to effect a significant improvement in the *mdx* muscle phenotype. Our results do not rule out the possibility that subtle signaling defects contribute to the phenotype to a lesser degree. For example, a transgenic dystrophin construct containing a deletion in the cysteine-rich domain partially corrected abnormalities in fiber type composition and neuromuscular junction topology when tested in *mdx:utrn*$^{-/-}$ mice (Rafael et al., 2000). The effect in these studies was presumably due to restored signaling, because the deletion included critical portions of the dystroglycan-binding domain, rendering the protein mechanically non-functional.

Nonetheless, the myofibers in these transgenic *mdx:utrn*$^{-/-}$ mice remained dystrophic.

Finally, it is still not clear if nNOS plays a role in the pathophysiology of muscular dystrophy. An ideal strategy for gene replacement of dystrophin in DMD patients would result in restoration of nNOS, given its importance in maintaining perfusion of the exercising muscle. However, current micro-dystrophins being tested for delivery to skeletal muscle by adeno-associated viral vectors do not restore nNOS. Further understanding of the factors that regulate the expression, localization, and activity of this enzyme may help us to design more effective strategies for gene replacement.

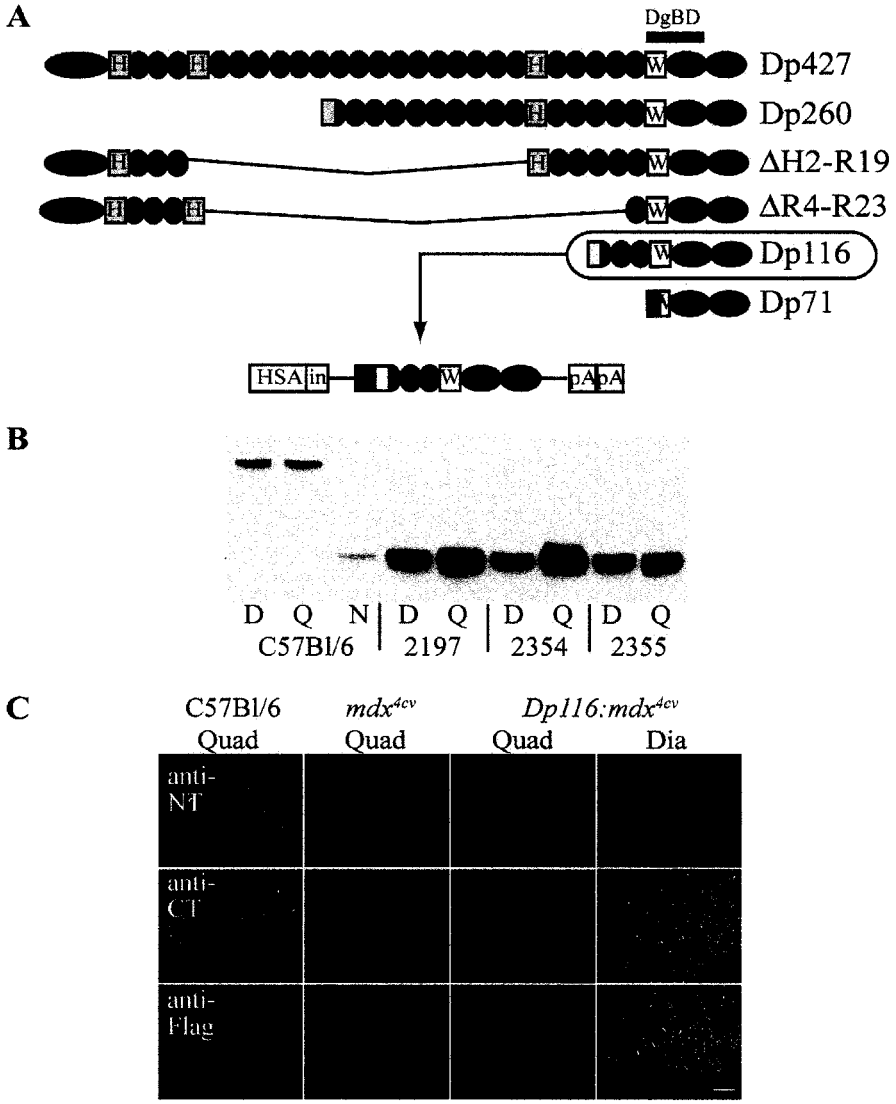


Figure 2.1: Generation of *Dp116:mdx*^{4cv} transgenic mice. *A)* Design of the Dp116 expression construct with the domain structure of Dp116 compared with other dystrophin isoforms and mini-genes. NT = NH_2-terminal domain, H = hinge, W = WW domain, CR = cysteine rich domain, CT = COOH-terminal domain, DgBD = dystroglycan binding domain, HSA = human skeletal α-actin promoter, in = hybrid HSA/SV40 vp1 intron, F = Flag epitope tag, pA = SV40 polyadenylation sequence. *B)* Western analysis using a monoclonal antibody specific for the dystrophin C-terminal domain (Dys-2) shows expression of Dp116 in diaphragm (D) and quadriceps (Q) muscles from three independent lines of *Dp116:mdx*^{4cv} transgenic mice. Samples from control C57Bl/6 mice show full length dystrophin in diaphragm and quadriceps, and Dp116 in peripheral nerve (N).
C) Immunofluorescent staining with antisera to the dystrophin N-terminal domain detects full-length dystrophin only in control C57Bl/6 muscles. Polyclonal antibodies to the dystrophin C-terminal domain or the Flag epitope demonstrate uniform expression of the *Dp116* transgene in quadriceps and diaphragm muscles (line 2197). Scale bar: 100 μm.

Figure 2.2: Soleus muscle is dramatically affected at an early age in *Dp116:mdx*^{4cv} transgenic mice. Complete cross sections of hematoxylin and eosin stained soleus muscles from *Dp116:mdx*^{4cv} (A' and B') and *mdx*^{4cv} (A and B) mice at both 3 weeks (A and A') and 4 weeks (B and B') of age are shown. At 3 weeks the *mdx*^{4cv} muscle has small areas of degeneration but minimal numbers of fibers with central nuclei. At this age *Dp116* transgenic muscle shows extensive degeneration and inflammation with clusters of regenerated fibers containing central nuclei (arrow). At 4 weeks the *mdx*^{4cv} muscle has many successfully regenerated fibers (arrow) and large areas of normal tissue. In contrast, the transgenic muscle at 4 weeks has little normal muscle tissue remaining. Scale bar: 500 μm.

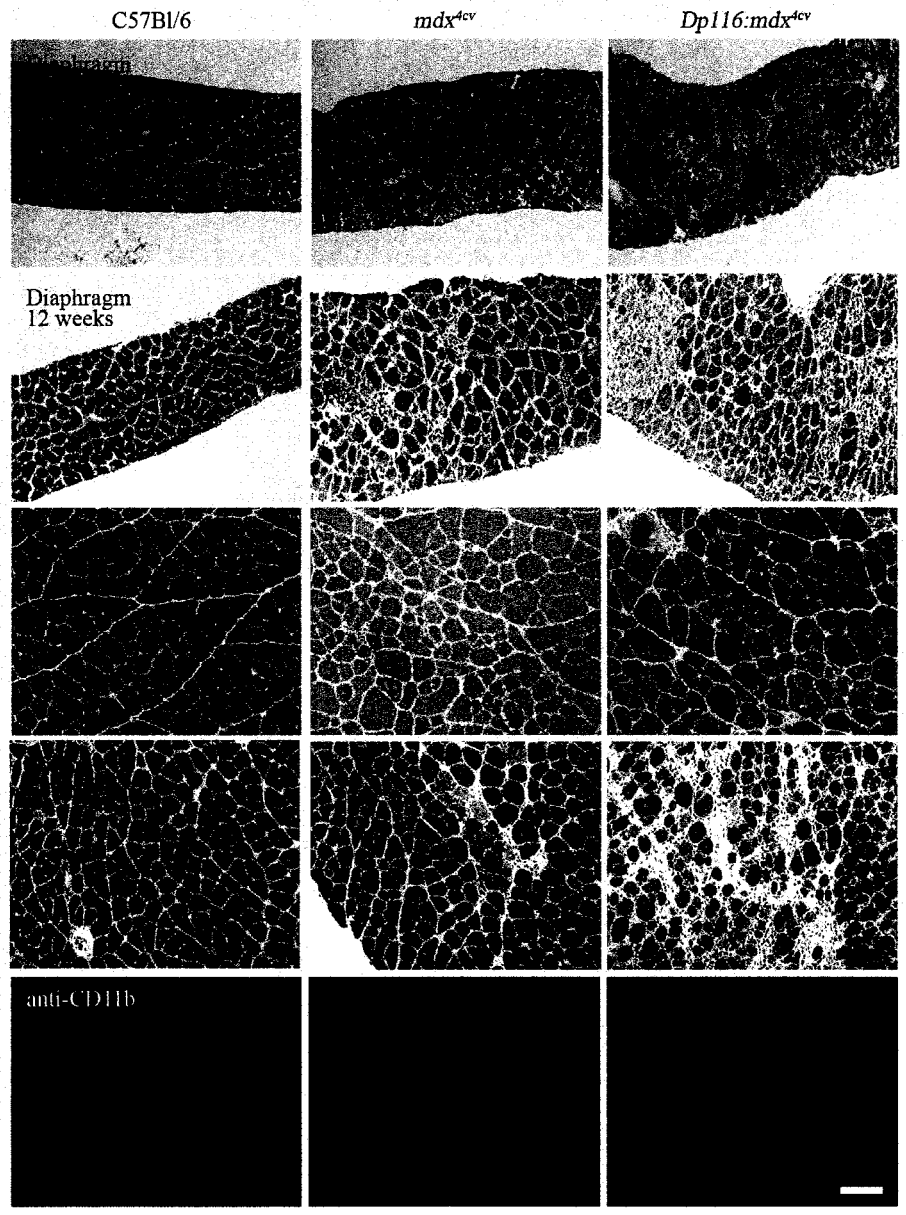


Figure 2.3: Histopathology of *Dp116:mdx*^{4cv} transgenic muscle is more severe than that of *mdx*^{4cv}. Hematoxylin and eosin stained sections from the diaphragm muscle at 4 weeks of age and diaphragm, quadriceps, and soleus muscles at 12 weeks age are shown for C57Bl/6, *mdx*^{4cv}, and *Dp116:mdx*^{4cv} mice. Widespread necrosis of muscle fibers and mononuclear cell infiltrates are more prominent in the diaphragm and soleus muscles of *Dp116:mdx*^{4cv} transgenic mice at all ages compared with *mdx*^{4cv}. The quadriceps muscles from *Dp116:mdx*^{cv} and *mdx*^{4cv} mice were not noticeably different at 12 weeks. Immunofluorescent staining of serial sections of 12 week soleus muscles with anti-CD11b antibody demonstrates infiltration of inflammatory cells in *mdx*^{4cv} and *Dp116:mdx*^{4cv} muscles. Inflammation in the *Dp116:mdx*^{4cv} soleus muscle was widespread and diffuse, in contrast to the focal pattern in *mdx*^{4cv} soleus muscle. Scale bars: 100 μm.

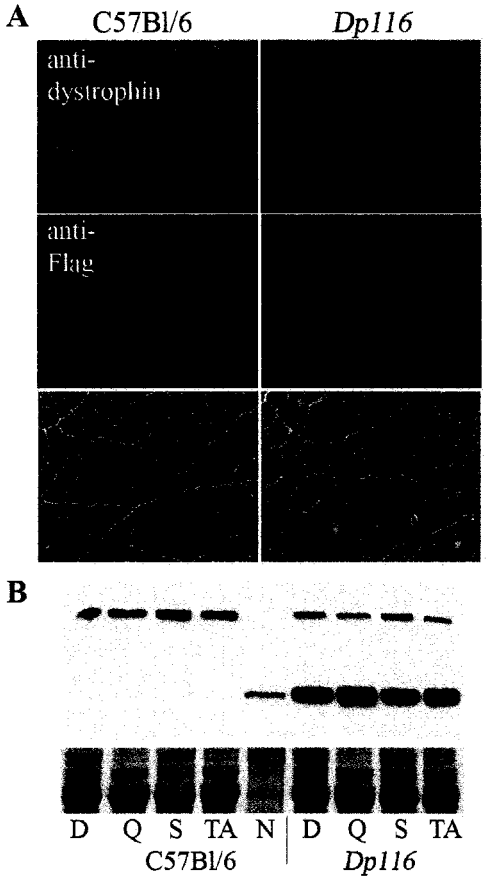


Figure 2.4: Overexpression of Dp116 on a wild type background has dominant negative effects. *A)* Cross sections of quadriceps muscle from *Dp116* transgenic mice and C57Bl/6 controls. Immunofluorescent staining using antibodies specific to the dystrophin N-terminal domain and the Flag epitope demonstrate decreased expression of full-length dystrophin but high levels of Dp116 on the sarcolemma of transgenic muscles. Hematoxylin and eosin staining shows increased numbers of muscle fibers with central nuclei in the transgenic quadriceps. Scale bar: 100 μm. *B)* Western analysis using a monoclonal antibody specific for the dystrophin C-terminus confirms reduced amounts of full-length dystrophin protein in various skeletal muscles of transgenic mice compared with controls. An identical gel stained in parallel with coomassie blue is shown as a loading control. D = diaphragm, Q = quadriceps, S = soleus, TA = tibialis anterior, N = peripheral nerve.

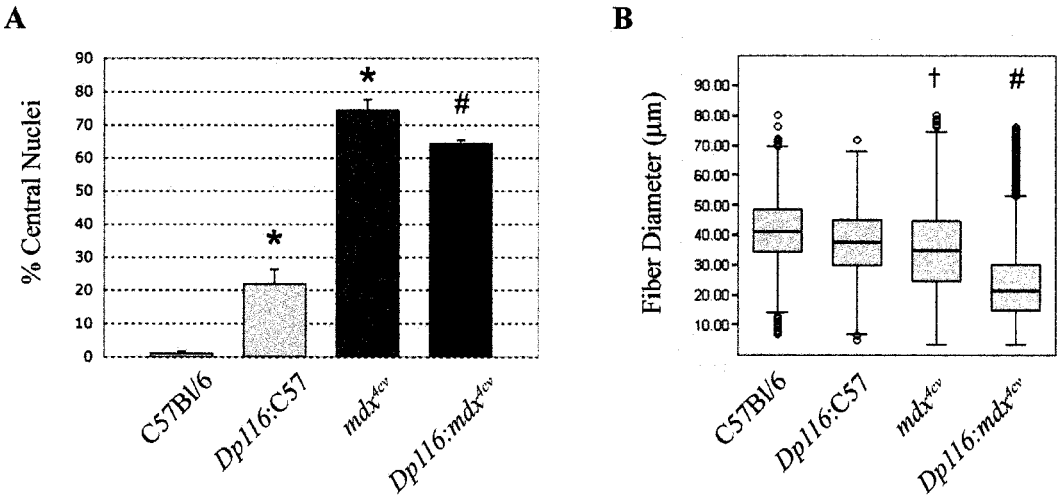


Figure 2.5: Quantitative assessment of histopathology in tibialis anterior muscles of 12 week old mice. *A)* Percentage of fibers with central nuclei. *B)* Box plots showing variance in the muscle fiber diameter. Boxes represent the middle quartiles from the 25th to 75th percentiles, bar represent the high and low values (statistical outliers are shown as circles). Each data set is composed of >1000 fibers from each of n = 3-6 mice. ANOVA statistical tests were performed on measurements of % central nuclei and the median fiber diameters. * indicates statistical difference compared to C57Bl/6, $p < 0.01$. # indicates statistical difference compared to *mdx*^{4cv}, $p < 0.01$. † indicates statistical difference compared to C57Bl/6, $p < 0.05$.

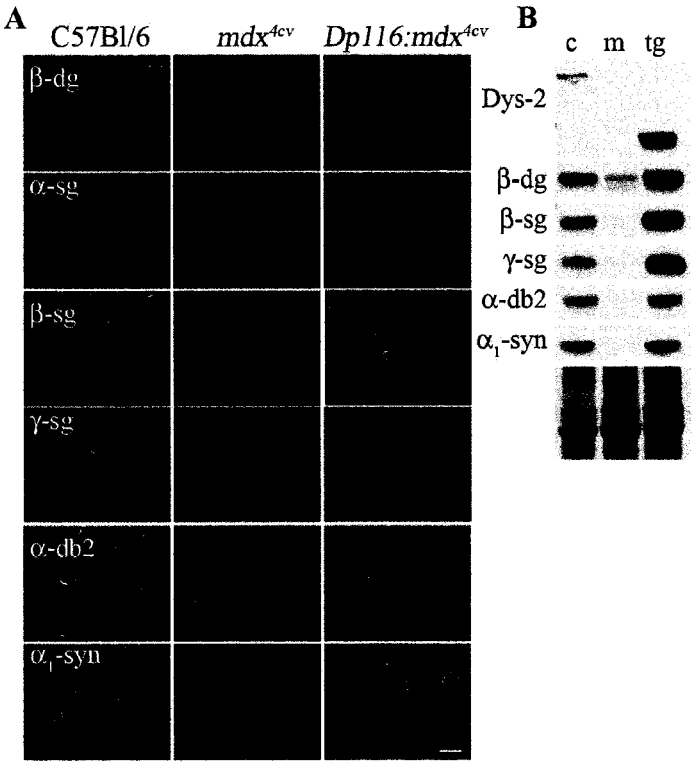


Figure 2.6: Expression of dystrophin-associated proteins is restored in *Dp116:mdx*^{4cv} transgenic muscle. *A)* Immunofluorescent staining of quadriceps muscles with antibodies specific for β-dystroglycan, α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, α-dystrobrevin 2, and α_1-syntrophin. Scale bar: 100 μm. *B)* Western Blots from KCl-washed microsomes using antibodies to the dystrophin C-terminus (Dys-2), β-dystroglycan, β-sarcoglycan, γ-sarcoglycan, α-dystrobrevin 2, and α_1-syntrophin. An identical gel stained in parallel with coomassie blue is shown as a loading control. c = C57Bl/6, m = *mdx*^{4cv}, tg = *Dp116:mdx*^{4cv}.

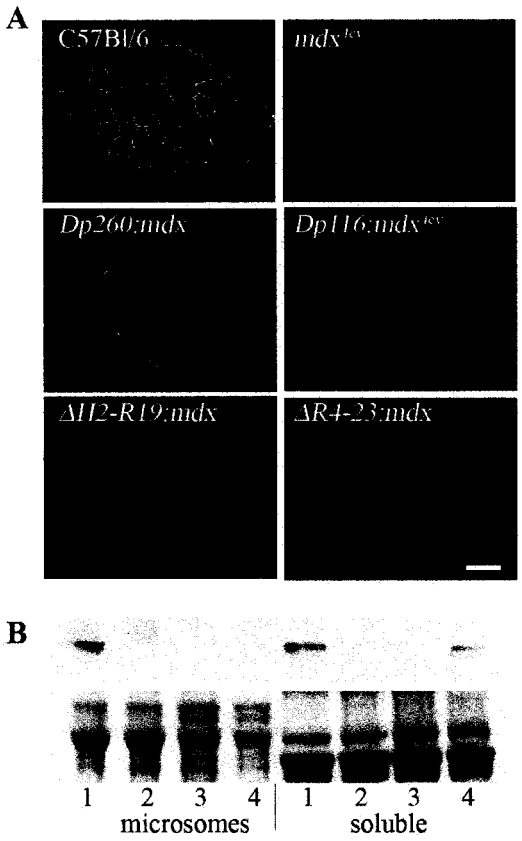
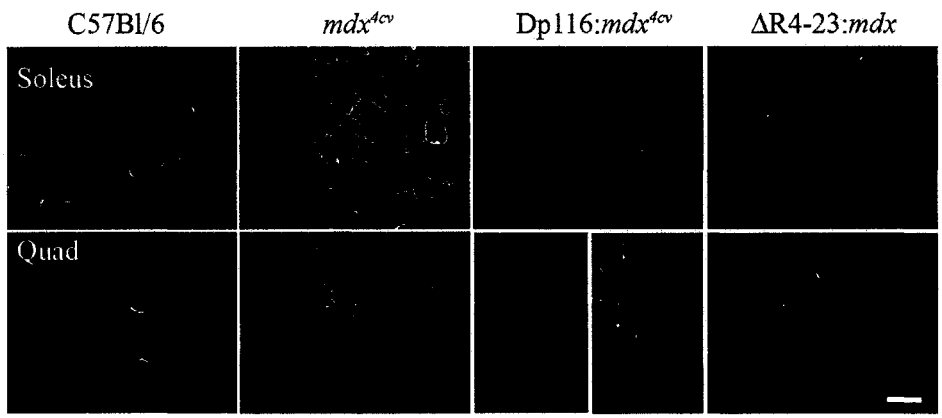


Figure 2.7: Expression of nNOS is not restored by expression of Dp116.
A) Immunofluorescence staining of quadriceps muscles using a polyclonal antibody against nNOS. *Dp116:mdx*^{4cv} and transgenic *mdx* muscles expressing Dp260, ΔH2-R19 (mini-dystrophin), and ΔR4-23 (micro-dystrophin) are shown. Scale bar: 100 μm. *B)* Western analysis of nNOS from pellet (microsomal) and supernatant (soluble) fractions of KCl-washed microsome preparations from C57Bl/6 (1), *mdx*^{4cv} (2), *Dp116:mdx*^{4cv} (3), and *ΔR4-23/mdx* (4) skeletal muscles. An identical gel stained in parallel with coomassie blue is shown as a loading control.

A



B

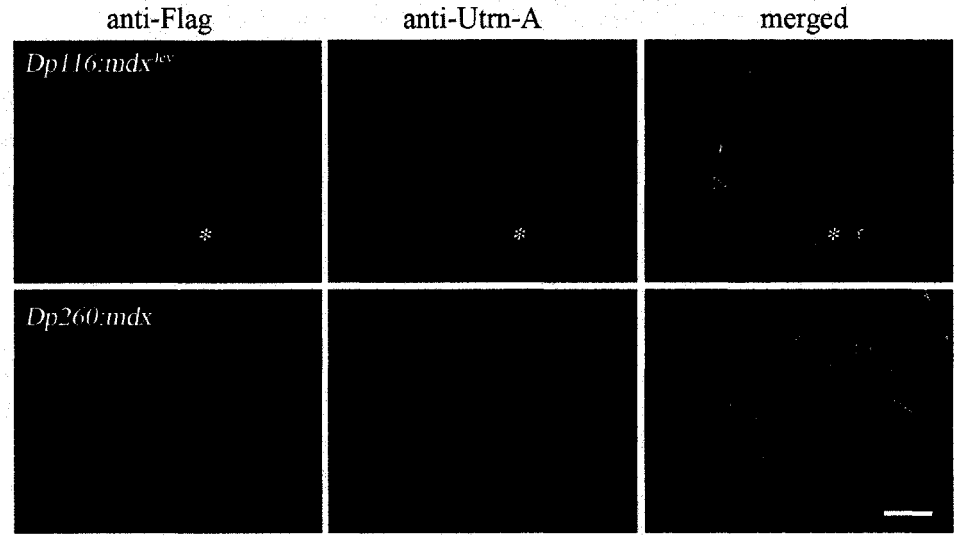


Figure 2.8: Dp116 expression displaces utrophin from the sarcolemma. *A)* Immunofluorescent staining of quadriceps and soleus muscles using an antibody specific for the utrophin-A isoform. The most significant upregulation of utrophin was found in the soleus muscles of *mdx*^{4cv} mice, which was greatly reduced by expression of Dp116. Split panels shown for *Dp116:mdx*^{4cv} quadriceps muscle represent extremes in utrophin staining. *B)* Sections of quadriceps muscles from *Dp116:mdx*^{4cv} and *Dp260:mdx* mice were immunostained with antibodies to both Flag and utrophin-A. Most myofibers express either the transgene or utrophin at high levels, but not both (one exception to this pattern is marked *). Scale bars: 100 μm.

CHAPTER 3:

Expression of Dp116 in skeletal muscles partially compensates for lack of utrophin in *mdx:utrn*$^{-/-}$ double mutant mice

Introduction

Duchenne muscular dystrophy is a severe muscle-wasting disease caused by mutations in the dystrophin gene, which is located on the X-chromosome. The disease is relatively common, affecting 1:3500 males, and has no effective treatment (Emery and Muntoni, 2003). Investigation of several potential avenues of therapy, including gene replacement, gene correction, and regulation of compensatory genes show promise (Chamberlain and Rando, 2006). However, our understanding of the fundamental mechanisms of disease pathogenesis in dystrophin-deficient muscle remains incomplete. More knowledge about the molecular and biochemical events that influence muscle cell death in the absence of dystrophin is critical for the rational development of genetic and pharmacological therapies.

The majority of studies of disease pathogenesis have been performed using the *mdx* mouse model, a naturally occurring dystrophin-deficient strain (Bulfield et al., 1984; Ryder-Cook et al., 1988; Sicinski et al., 1989). Muscles in *mdx* mice shows many of the histological characteristics of early DMD muscles, including necrosis, inflammation, and regenerating myofibers (Carnwath and Shotton, 1987; Coulton et al., 1988b). Despite being an exact genetic model of DMD, the *mdx*

mouse has been criticized for a failure to recapitulate the severe progressive nature of muscular dystrophy in humans. Unlike humans, *mdx* mice do not have a dramatically shortened lifespan, and display muscle hypertrophy and retention of muscle function throughout the majority of their life (Coulton et al., 1988a; Lefaucheur et al., 1995). Chemical mutagenesis and targeted deletion have been used to generate additional mutations in the dystrophin gene but all produced similar phenotypes (Araki et al., 1997; Chapman et al., 1989; Im et al., 1996; Kudoh et al., 2005). In contrast, mice deficient in both dystrophin and its autosomal homologue utrophin (*mdx:utrn*$^{-/-}$), display many of the "clinical" features of DMD including progressive muscle wasting, skeletal deformities, and premature death (Deconinck et al., 1997; Grady et al., 1997b). This observation has supported the hypothesis that the upregulation of utrophin observed in *mdx* muscles compensates for dystrophin deficiency, leading to the mild phenotype of these mice. Overexpression of utrophin at even greater levels prevented muscular dystrophy in *mdx* mice, proving that utrophin and dystrophin are indeed functionally redundant to some degree (Tinsley et al., 1998).

Surprisingly little data is available elucidating the mechanism underlying the extreme phenotype of *mdx:utrn*$^{-/-}$ mice. As presented in Chapter 1, dystrophin is thought to have a mechanical role in protecting the sarcolemma from stresses during contraction and transmission of force laterally, as well as serving as a scaffold for anchoring signaling molecules. Dystrophin is required for complete assembly of the dystrophin-glycoprotein complex (DGC), which consists of the dystroglycan,

sarcoglycan-sarcospan, and dystrobrevin-syntrophin sub-complexes. Many of the proteins in these complexes bind to or are substrates for known signaling molecules. However, restoration of the DGC without restoring the mechanical function of dystrophin does not improve the phenotype of *mdx* mice (Cox et al., 1994; Greenberg et al., 1994; Judge et al., 2006). These results support the hypothesis that loss of the mechanical function of dystrophin is the primary basis of pathogenesis of muscular dystrophy in *mdx* mice. The increased severity of *mdx:utrn*$^{-/-}$ pathology compared to *mdx* could similarly be explained by a further weakening of the mechanical connection between the cytoskeleton and the extracellular matrix, caused by the additional loss of utrophin. Another possible explanation is that utrophin upregulation in *mdx* muscles is adequate for maintenance of signaling through the DGC, thus the severe consequences of the double mutant are due to the loss of both the mechanical and signaling functions (in contrast to loss of primarily the mechanical function in *mdx*).

Several transgenic mouse models have succeeded in ameliorating *mdx:utrn*$^{-/-}$ muscle pathology to varying degrees. These include exogenous expression of a dystrophin mini-gene, utrophin mini-gene, the Dp260 isoform of dystrophin, and α7 integrin (Burkin et al., 2001; Gaedigk et al., 2006; Rafael et al., 1998; Rafael et al., 2000). Unfortunately, none of these studies can be differentiated by the basis of their positive effect. Dystrophin, utrophin, Dp260, and integrin all link the muscle cytoskeleton to extracellular laminin, so a mechanical role cannot be ruled out for any of the four (Belkin et al., 1997; Rybakova et al., 2002; Rybakova et al., 2000;

Song et al., 1992; Song et al., 1993; Warner et al., 2002). Likewise, exogenous dystrophin, utrophin, and Dp260 assemble the DGC at the sarcolemma and thus have the potential to restore the signaling functions of the complex (Cox et al., 1993; Tinsley et al., 1998; Warner et al., 2002). Although α7β1 integrin is not known to directly interact with components of the DGC, many of the signaling pathways that are proposed to involve the DGC are also common to integrin-mediated signaling events in cell adhesion (Rando, 2001a; Winder, 2005; Yoshida et al., 1998). It is possible that a combination of mechanical resistance to damage and enhanced signaling leading to increased cell survival are present in all of these models.

Conversely, transgenic mice expressing the dystrophin isoform Dp71 and/or a dystrophin mutant with a deletion in the cysteine-rich domain (Δcys) failed to improve dystrophic pathology in *mdx:utrn*$^{-/-}$ mice (Gardner et al., 2005; Rafael et al., 2000). This was a predictable result as neither of these transgenes had a positive effect on the phenotype of *mdx* mice (Cox et al., 1994; Rafael et al., 1996). Neither Dp71 nor Δcys are mechanically functional; Dp71 lacks both actin-binding domains and Δcys is defective in binding to β-dystroglycan. Moreover, Δcys does not restore the DGC to the sarcolemma. Although Dp71 does assemble the DGC, it has an incomplete WW domain, which is critical for dystrophin binding to β-dystroglycan (Huang et al., 2000). The dystrophin-dystroglycan interaction forms the central axis of the DGC and is likely to be in dynamic equilibrium with other protein-protein interactions (Galbiati et al., 2000; Ilsley et al., 2002; James et al., 2000; Sotgia et al.,

2001; Sotgia et al., 2000). Thus, a full physiological interaction between dystrophin and dystroglycan is likely to be necessary for regulation of the signaling functions of the DGC.

In the previous chapter we described transgenic lines that express the dystrophin isoform Dp116 in skeletal muscle. Dp116 is normally expressed in Schwann cells of the peripheral nervous system where it associates with a DGC-like complex that, like in skeletal muscle, binds to laminin-2 in the extracellular matrix (Imamura et al., 2000). Furthermore, Dp116 contains the complete dystroglycan-binding domain of dystrophin but does not have any of the actin-binding motifs, allowing us to distinguish between the mechanical and signaling properties of the DGC. Expression of Dp116 in skeletal muscles of *mdx*^{4cv} mice displaced utrophin from the sarcolemma and exacerbated the dystrophic pathology. Based on this result, we hypothesized that expression of Dp116 in *mdx:utrn*$^{-/-}$ mice would allow a clearer dissection of the signaling and mechanical roles of the DGC, as residual utrophin in *mdx* muscle may maintain critical signaling processes. Young transgenic *Dp116:mdx:utrn*$^{-/-}$ mice had increased muscle mass compared to *mdx:utrn*$^{-/-}$controls, although their muscles were similarly dystrophic. Unexpectedly, expression of Dp116 prevented the most severe aspects of the *mdx:utrn*$^{-/-}$ phenotype, including growth retardation, joint contractures, kyphosis, and premature death. We propose that expression of Dp116 and restoration of the DGC does not prevent myofiber necrosis but compensates for muscle damage by maintaining muscle mass and regenerative capacity.

Materials and Methods

Animals

To generate *Dp116:mdx:utrn*$^{-/-}$ mice, male *Dp116:mdx*^{4cv} mice hemizygous for the *Dp116* transgene were mated with *mdx:utrn*$^{-/+}$ females (Grady et al., 1997b). The *Dp116* positive *mdx:utrn*$^{-/+}$ male progeny of this first cross were mated for a second generation with *mdx:utrn*$^{-/+}$ females. The second cross produced six possible genotypes that were used in experiments: *mdx*, *mdx:utrn*$^{-/+}$, and *mdx:utrn*$^{-/-}$, each either positive or negative for the *Dp116* transgene. This breeding scheme was carried out independently with two different lines of *Dp116* transgenic mice (2197 and 2354). All mice were genotyped by PCR at weaning age for both the utrophin mutation (Grady et al., 1997a) and the *Dp116* transgene. Selected mice were genotyped for the *mdx* mutation by an ARMS assay (Amalfitano and Chamberlain, 1996). As expected, all mice tested were positive for the *mdx* mutation. Mice were weighed weekly after weaning and assignment of genotype. All experimental protocols involving live mice were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Immunofluorescence

Selected tissues were dissected, embedded in OCT (Sakura Finetek USA) and frozen in liquid nitrogen-cooled isopentane. Cryosections of 7-10 μm thickness were blocked in KPBS (20 mM potassium phosphate pH 7.4, 150 mM sodium chloride) containing 0.3 mg/ml BSA, 2% gelatin, and 1% Tween 20. The blocked sections

were incubated with primary antibodies diluted in KPBS containing 0.2% gelatin (KPBS-G) and 2% normal goat serum. Rabbit polyclonal antibodies were used to label Flag (Sigma), dystrophin NH_2-terminus (Rafael et al., 1996), and utrophin A (S. Froehner, unpublished). After several washes with KBPS-G the sections were incubated with goat anti-rabbit Alexafluor 488 (Molecular Probes). Immunostained slides were washed repeatedly with KBPS-G and mounted with Vectashield (Vector Laboratories). All photomicrographs were obtained with a Spot II CCD camera (Diagnostic Instruments, Inc.) and Spot Advanced software connected to a Nikon Eclipse E1000 using a 20X Plan-Apochromat objective (numerical aperture = 0.75). Images for all the sections probed with a given antibody were acquired under identical exposure conditions.

Histological analysis

Muscles were dissected, embedded in OCT (Sakura Finetek USA) and frozen in liquid nitrogen-cooled isopentane. Cryosections of 10 μm thickness were briefly fixed in methanol and stained with Gill's hematoxylin and eosin-phyloxine. The sections were washed, dehydrated, and cleared in xylene before mounting with Permount. NADH staining was performed by incubating unfixed sections in 0.2 M Tris pH 7.4, 1.5 mM β-NADH-reduced form disodium salt (Sigma), and 1.5 mM nitro-blue tetrazolium (Sigma) at 37 °C for 30 minutes. The stained sections were washed for 2 minutes each in 30, 60, 90, 60, and 30% acetone and mounted in Vectashield (Vector Laboratories). Photomicrographs were obtained with a QICAM

Fast cooled digital CCD camera and QCapture Pro software (QImaging) connected to a Nikon Eclipse E1000 microscope using a 20X Plan-Apochromat objective (numerical aperture = 0.75). Images of entire muscle cross sections were obtained using SyncroscanRT software (Syncroscopy).

Results

Generation of Dp116:mdx:utrn$^{-/-}$ mice

Dp116 transgenic mice were crossed with *mdx:utrn*$^{-/+}$mice (Grady et al., 1997b) for two generations to obtain *Dp116:mdx:utrn*$^{-/-}$ mice (see materials and methods). From nearly 300 pups we obtained 10.9% of genotype *mdx:utrn*$^{-/-}$ and 7.4% of genotype *Dp116:mdx:utrn*$^{-/-}$. This was less than the expected Mendellian ratio of 12.5% for each. To verify that the genotypes accurately reflected expression of the three gene products of interest we performed immunofluorescence staining of skeletal muscles using polyclonal antibodies against dystrophin N-terminus, utrophin-A, and the Flag epitope (Figure 3.1). Dystrophin was only detected in wild-type muscles and occasional revertant fibers in the various *mdx* muscles. Utrophin-A was upregulated in *mdx* and *Dp116:mdx* but absent in *mdx:utrn*$^{-/-}$ and *Dp116:mdx:utrn*$^{-/-}$ muscles as expected. Dp116 was found to be localized to the sarcolemma in all transgenic muscles examined, although expression in the diaphragm was consistently much lower than in the limb muscles.

Global signs of muscle weakness are reduced in Dp116:mdx:utrn$^{-/-}$ mice

As expected, *mdx:utrn*$^{-/-}$ mice were smaller than their littermates at weaning and showed significant growth retardation throughout their lifespan. Although *Dp116:mdx:utrn*$^{-/-}$ mice were slightly smaller on average than control *Dp116* transgenic mice with a functional utrophin gene (*Dp116:mdx:utrn*$^{-/+}$), they continued to gain body mass at a similar rate until at least 18 weeks of age (Figure 3.2). Control *mdx:utrn*$^{-/-}$ mice developed severe kyphosis of the spine, joint contractures, and reduced mobility between 8 – 10 weeks of age. In contrast, *Dp116:mdx:utrn*$^{-/-}$ mice did not develop any of these overt signs of muscle weakness and were essentially indistinguishable from their other littermates (Figure 3.3).

Consistent with their improved physical appearance, *Dp116:mdx:utrn*$^{-/-}$ mice had a longer lifespan than the non-transgenic controls (Figure 3.4). The median lifespan of *mdx:utrn*$^{-/-}$ mice in this study was 12 weeks (n = 21, males and females) with the longest lived mouse reaching 20 weeks of age. This is consistent with previously published studies of this genetic model as well as separate studies with these mice in our own laboratory (Burkin et al., 2001; Grady et al., 1999; Grady et al., 1997b; Gregorevic et al., 2006). Although we could not calculate the median lifespan of the *Dp116:mdx:utrn*$^{-/-}$ transgenic animals, at the time of writing all had lived longer than 20 weeks (n = 15, males and females), the oldest of which had reached 44 weeks of age. Thus far none of the mice in this study have died other than *mdx:utrn*$^{-/-}$ controls.

Histopathology of mdx:utrn$^{-/-}$ muscle is not altered by expression of Dp116

The finding that Dp116 prevented the development of severe muscle wasting and premature death in *mdx:utrn*$^{-/-}$ mice was extremely surprising, particularly considering the negative effect of Dp116 expression in *mdx*^{4cv} mice (Chapter 2). Therefore, we sacrificed C57Bl/6, *mdx*, *mdx:utrn*$^{-/-}$, and *Dp116: mdx:utrn*$^{-/-}$ mice at 8 weeks of age (the large majority of *mdx:utrn*$^{-/-}$ mice live to at least this age) to examine their skeletal muscles more closely. The mass of both the tibialis anterior (TA) and quadriceps muscles were increased in *Dp116:mdx:utrn*$^{-/-}$ mice relative to *mdx:utrn*$^{-/-}$ controls, although not to the extent of corresponding *mdx* muscles (Table 3.1).

Examination of hematoxylin and eosin stained cross sections showed no improvement in dystrophic pathology in quadriceps muscles from *Dp116:mdx:utrn*$^{-/-}$ mice (Figure 3.5). This was not unexpected as our previous experiments demonstrated that Dp116 did not prevent myofiber necrosis in *mdx*^{4cv} muscles (Chapter 2). The pathology in *mdx* and *mdx:utrn*$^{-/-}$ muscles at 8 weeks of age was qualitatively similar although *mdx:utrn*$^{-/-}$ muscles had larger and more numerous patches of necrosis and inflammation. Similar pathology was found in the tibialis anterior, soleus, diaphragm, and intercostal muscles.

Abnormalities in fiber type and post-synaptic structure are not corrected by Dp116

Finding that *Dp116:mdx:utrn*$^{-/-}$ muscles were equally prone to dystrophic injury, we sought an alternative explanation for the improved phenotype of these

mice. We hypothesized that the result was likely due to a restored signaling function of the DGC. Two established characteristics of *mdx:utrn*$^{-/-}$ muscles are an abnormal fiber type distribution and highly fragmented post-synaptic structure at the neuromuscular junction (Gardner et al., 2005; Grady et al., 2000; Rafael et al., 2000). Both of these defects were at least partially corrected by expression of the dystrophin Δcys mutant (Rafael et al., 2000). The Δcys construct is not mechanically functional, suggesting that the effect was due to an unknown signaling mechanism.

We performed NADH staining on sections of quadriceps and tibialis anterior muscles from wild-type, *mdx*, *mdx:utrn*$^{-/-}$, and *Dp116:mdx:utrn*$^{-/-}$ mice to determine the oxidative potential of myofibers. Wild-type tibialis anterior and quadriceps muscles showed a mosaic pattern of darkly stained oxidative and lightly stained glycolytic fibers. The corresponding *mdx:utrn*$^{-/-}$ muscles had more dark and intermediate staining showing an increased population of oxidative fibers. The latter pattern was reproduced nearly identically in *Dp116:mdx:utrn*$^{-/-}$ muscles (Figure 3.6). Cursory examination of teased whole myofibers from *Dp116:mdx:utrn*$^{-/-}$ gastrocnemius muscles stained with fluorescently labeled α-bungarotoxin showed a highly fragmented pattern of acetylcholine receptors in the post-synaptic membrane (Figure 3.7). Whatever signaling processes were responsible for the effects of the Δcys mutant do not appear to be activated by Dp116. However, these results do not rule out a signaling pathway as the potential mechanism for the increased viability of *Dp116:mdx:utrn*$^{-/-}$ mice.

Discussion

The above data clearly demonstrate that restoration of the DGC by skeletal muscle-specific expression of the Dp116 isoform of dystrophin modifies the phenotype of *mdx:utrn*$^{-/-}$ mice to resemble a milder *mdx*-like phenotype. This result was quite unexpected, because expression of Dp116 in skeletal muscles of mice deficient only in dystrophin actually exacerbated the dystrophic pathology in that model. Although it is possible that a mechanical connection to the cytoskeleton is made through dystrobrevin (Ervasti, 2003), the fact that Dp116 cannot restore the normal link to F-actin suggests that cell signaling pathways may be responsible for the maintenance of muscle mass in our model. Other groups have proposed that perturbation of signaling pathways is an important mechanism of cell death in muscular dystrophies caused by defects in the DGC (reviewed in (Rando, 2001a; Winder, 2005)). These pathways could regulate cell survival, apoptosis, hypertrophy, and other defense mechanisms. Below we discuss several of these proposed pathways and whether activation of each would be consistent with our results.

The signaling pathway associated with the DGC that has been the most thoroughly investigated involves the second messenger nitric oxide. Isoforms of neuronal nitric oxide synthase (nNOS) are expressed in skeletal muscle and associate with the DGC (Brenman et al., 1995). Muscular dystrophy caused by mutations in dystrophin or sarcoglycans results in the loss of nNOS from the sarcolemma (Brenman et al., 1995; Crosbie et al., 2002). One of the classic effects of nitric oxide

is relaxation of vascular smooth muscle via regulation of cyclic GMP levels (Ignarro et al., 1987; Rapoport et al., 1983). This is an important role of nitric oxide production in skeletal muscle as both DMD patients and *mdx* mice have impaired vasomodulation during exercise, most likely caused by the mislocalization of nNOS (Sander et al., 2000; Thomas et al., 1998; Thomas et al., 2003; Thomas and Victor, 1998). This defect could lead to inappropriate vasoconstriction and subsequent ischemia in muscle fibers (Rando, 2001b). Overexpression of nNOS in skeletal muscle does ameliorate pathology in *mdx* mice, although the effect in this case has been attributed to protection from inflammatory damage (Nguyen and Tidball, 2003; Wehling et al., 2001). While evidence is accumulating that specific parts of the dystrophin rod domain are required for nNOS to associate with the DGC, there is no correlation with absence of nNOS at the sarcolemma and severity of pathology (Chapter 2) and (Chao et al., 1996; Wells et al., 2003). Most importantly, nNOS is not associated with the DGC in muscles of transgenic mice expressing Dp116 (Figure 2.7) or other short isoforms of dystrophin (Crawford et al., 2000). Thus, although increased nitric oxide mediated signaling in dystrophic muscle may be protective, it is not a likely mechanism for the results in this study.

Nitric oxide is one of several potential signaling molecules that associate with the DGC through interaction with syntrophin (Adams et al., 2001; Chockalingam et al., 1999; Hasegawa et al., 1999; Iwata et al., 1998; Lumeng et al., 1999; Madhavan and Jarrett, 1999; Oak et al., 2001). Dystrobrevin has also been proposed to couple signaling processes to the DGC (Blake, 2002; Grady et al., 1999; Yoshida et al.,

2000). However, both Dp116 and Dp71 restore dystrobrevin and syntrophin expression to the sarcolemma, while only Dp116 restored viability to *mdx:utrn*$^{-/-}$ mice (Cox et al., 1994; Greenberg et al., 1994; Rafael et al., 2000). For this reason, we feel that the signaling pathway responsible for the effect mediated by Dp116 does not depend primarily on an interaction with syntrophin and/or dystrobrevin. It would be more logical to consider a pathway that works through dynamic interaction with dystroglycan, as the crucial difference between Dp116 and Dp71 is the presence of a complete dystroglycan binding domain in the former. Previous data suggest several such pathways that could be investigated in this regard.

Dystroglycan consists of two subunits, the transmembrane β-dystroglycan and the extracellular α-dystroglycan. Together they constitute a cellular receptor for the α2 chain of laminin-2, which is a major component of the basement membrane in skeletal muscle (Ervasti and Campbell, 1993; Ibraghimov-Beskrovnaya et al., 1992). β-dystroglycan is known to undergo adhesion dependent phosphorylation of tyrosine residues at its cytoplasmic carboxyl-terminus, which contains a PPxY motif that interacts with the WW domains in dystrophin, utrophin, and caveolin-3 (Huang et al., 2000; James et al., 2000; Sotgia et al., 2000). Phosphorylation of the tyrosine residue in the same PPxY motif inhibits binding of dystrophin and utrophin but recruits various SH2 domain-containing proteins including tyrosine kinases Src and Fyn (Ilsley et al., 2001; James et al., 2000; Sotgia et al., 2001). In contrast, caveolin-3 interacts with β-dystroglycan irrespective of its phosphorylation state (Sotgia et al.,

2000). β-dystroglycan also binds Grb-2, an adapter protein involved in signal transduction through mitogen activated protein kinase (MAP kinase) cascades (Cavaldesi et al., 1999; Chardin et al., 1995; Russo et al., 2000; Yang et al., 1995a). It was recently reported that β-dystroglycan can directly interact with extracellular signal-regulated kinase (ERK) and MAP kinase kinase 2 (MEK) (Spence et al., 2004). All of these examples serve to illustrate the fact that β-dystroglycan has many properties of a cellular receptor, with a multitude of intracellular interactions regulated by binding of its extracellular ligand. In fact, two studies have shown *in vitro* that laminin binding activates MAP kinase and phosphatidylinositol-3 kinase (PI-3 kinase)/Akt pathways in muscle cells (Langenbach and Rando, 2002; Oak et al., 2003).

What cellular processes might these signaling pathways influence? Both PI-3 kinase and MAP kinases are involved in downstream signaling from growth receptors. Therefore, possibilities for the potentially similar signaling functions of the DGC include regulation of apoptosis/survival, atrophy/hypertrophy, and regeneration. It is a well established hypothesis that the balance between pro-apoptosis and pro-survival signals could be perturbed in dystrophin-deficient muscle (Abmayr et al., 2004; Tidball et al., 1995). In one study, blocking the interaction between laminin and dystroglycan in cultured myoblasts caused an increase in apoptotic cell death (Langenbach and Rando, 2002). Another particularly interesting study in this regard tested overexpression of the anti-apoptotic protein BCL2 in

skeletal muscles of both *mdx* and *Lama-2* null mice (a laminin-2 deficient model of congenital muscular dystrophy type 1A). The authors found that BCL2 had no effect on *mdx* pathology but did increase viability of *Lama-2* null mice (Dominov et al., 2005). Similarly, overexpression of ARC (apoptosis repressor interacting with caspase recruitment domain) in skeletal muscles of *mdx* mice had no beneficial effects (Abmayr et al., 2004). This could suggest completely different pathogenic mechanisms in these two disease models. Alternatively, it is possible that the difference is more quantitative than qualitative. Deficiency of the extracellular ligand (laminin-2) might inactivate the protective signaling pathway completely, while deficiency of dystrophin could cause only partial inactivation, because upregulation of utrophin might allow for sufficient levels of the receptor (dystroglycan) to persist. If so, *mdx:utrn*$^{-/-}$ mice would be expected to have a complete deficiency of the receptor and complete inactivation of the signaling pathway similar to *lama-2* null mice. It would be interesting to test this hypothesis by expressing ARC or BCL2 in muscles of *mdx:utrn*$^{-/-}$ mice.

Another possibility is that Dp116 and the DGC activate signaling pathways that favor hypertrophy. It is clear that muscles from *mdx* mice are capable of substantial hypertrophy, which is not the case in *mdx:utrn*$^{-/-}$ mice (Table 3.1). This could simply reflect an increased rate of muscle damage, but there is evidence of an association between muscle atrophy and disruption of the DGC in other animal models (Acharyya et al., 2005; Chockalingam et al., 2002). In one case maintenance of high levels of dystrophin expression prevented muscle atrophy in a model of

cancer cachexia by suppressing expression of the muscle-specific E3-ubiquitin ligases *MuRF-1* and *MAFbx* (Acharyya et al., 2005). Signaling through NF-κB activates expression of *MuRF-1* and *MAFbx* while signaling through PI-3 kinase/Akt blocks their expression (Bodine et al., 2001; Ladner et al., 2003; Sandri et al., 2004). If the DGC does participate directly in signaling through PI-3 kinase/Akt it may act to oppose apoptosis and promote hypertrophy.

Another cellular mechanism that is likely to be defective in *mdx:utrn*$^{-/-}$ mice is regeneration. Until late in their lifespan, *mdx* mice show very efficient regeneration of skeletal muscle that maintains muscle mass despite the ongoing damage and necrosis of myofibers. Regeneration of skeletal muscle is primarily dependent on satellite cells, the resident stem cell population. Damage to myofibers by various mechanisms activates satellite cells, which proliferate and then differentiate into myoblasts that can fuse with damaged myofibers to repair them, or fuse with each other to generate new myofibers (Charge and Rudnicki, 2004). Irradiation of *mdx* muscles prevents satellite cell proliferation and results in muscle pathology that more closely resembles DMD (Wakeford et al., 1991). Inhibition of regeneration by concomitant knockout of the muscle regulatory gene *myoD* in *mdx* mice produces a phenotype very much like that of *mdx:utrn*$^{-/-}$ mice, although the *mdx:myoD*$^{-/-}$ mice have a longer lifespan (Megeney et al., 1996).

Maintenance of regeneration has been proposed to explain the relatively mild phenotype of mice in which dystroglycan was disrupted specifically in skeletal muscle. These mice were generated using a muscle creatine kinase (MCK) promoter

driven Cre-loxP system to delete the dystroglycan gene in skeletal muscle cells (Cohn et al., 2002). This cross resulted in the complete disruption of the DGC in mature myofibers, but not in satellite cells or regenerating fibers. The authors concluded that expression of dystroglycan in muscle precursor cells is critical for continued regeneration, which quite efficiently compensated for muscle damage in their mice. It is tempting to speculate that regeneration is maintained in muscles of *Dp116:mdx:utrn*$^{-/-}$ mice, which would explain their increased muscle mass despite no apparent protection from damage.

The effects of the signaling processes that we have discussed are ultimately mediated by alterations in gene transcription. Therefore, if inactivation of critical signaling pathways is a major component of the cellular defect in *mdx:utrn*$^{-/-}$ muscles, there should be measurable changes in gene expression. Baker et al. performed the logical experiment of comparing gene expression in muscles of *mdx* and *mdx:utrn*$^{-/-}$ mice by microarray (Baker et al., 2006). They found surprisingly few genes in which expression differed by more than two-fold between the two genotypes, none of which were clearly involved in any signal transduction pathways. The most significant differences were found in genes which correlate with slow-twitch fiber phenotype. Interestingly, we did not observe any change in the proportion of slow oxidative fibers by NADH staining of *Dp116:mdx:utrn*$^{-/-}$ *and* *mdx:utrn*$^{-/-}$ muscles. This suggests that the increased expression of slow muscle genes may not be a causative factor. Regardless, it is difficult to imagine why these

modest changes in gene expression are correlated with such disparate phenotypes in these two animal models.

To our knowledge, this is the first time in which a transgene produced opposite effects when tested in both the *mdx* and *mdx:utrn*$^{-/-}$ backgrounds. Such a result provides the opportunity to investigate the underlying mechanisms that lead to the divergent phenotypes of these two models of DMD. Not only would this provide insights into fundamental processes in cell biology, extension of this knowledge to a deeper understanding of which of the models better mimics the biochemical and cellular abnormalities in DMD patients would provide a crucial advantage in the development of effective therapies.

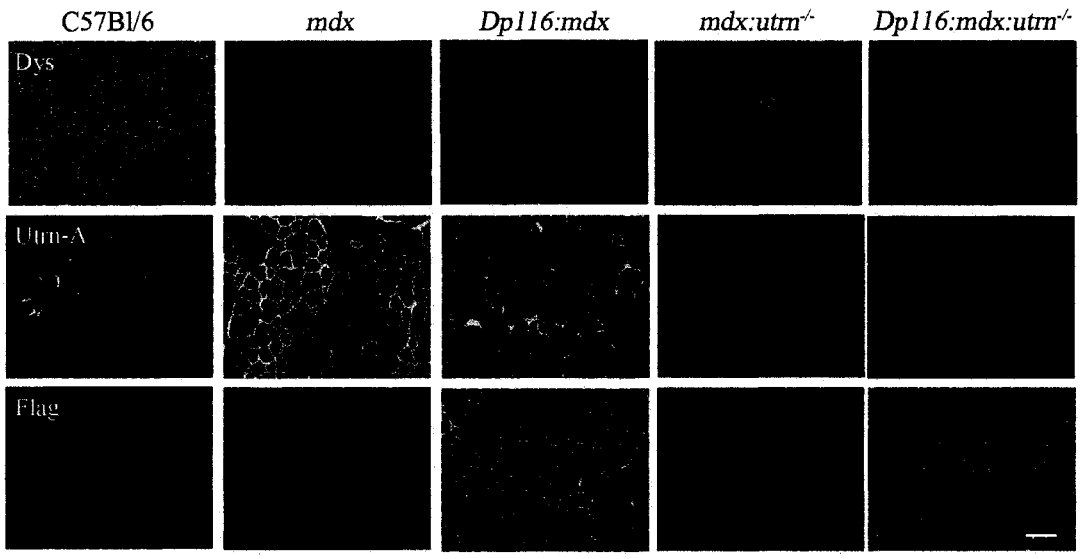
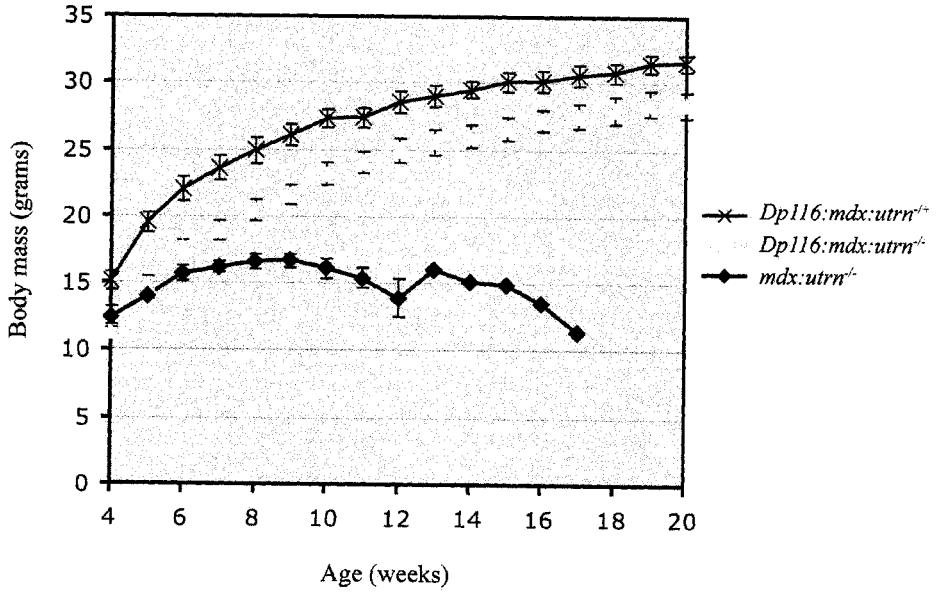


Figure 3.1: Immunofluorescent staining of quadriceps muscles for full-length dystrophin, utrophin-A, and Dp116. Dystrophin (Dys) is localized to the sarcolemma of wild-type C57Bl/6 myofibers and rare revertant fibers in *mdx* muscles (for example see *mdx:utrn*$^{-/-}$ top panel). Utrophin-A (Utrn-A) is restricted to the neuromuscular junctions in wild-type myofibers but surrounds the entire sarcolemma in the majority of *mdx* myofibers. Flag epitope-tagged Dp116 is localized to the sarcolemma in transgenic myofibers. Scale bar: 100 μm.

A



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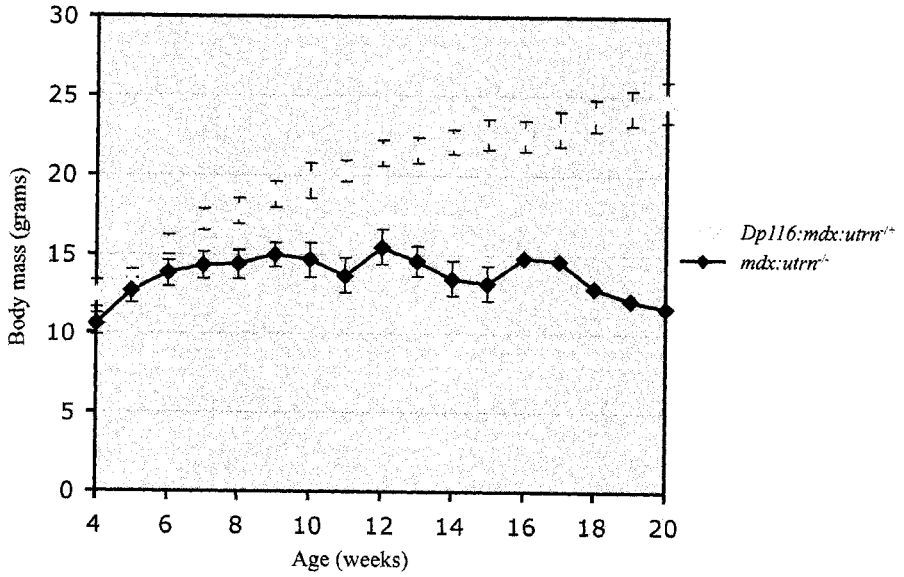


Figure 3.2: Expression of Dp116 prevents growth retardation in *mdx:utrn*$^{-/-}$ mice. Both male (*A*) and female (*B*) transgenic *Dp116:mdx:utrn*$^{-/-}$ mice had similar body mass to *mdx:utrn*$^{-/-}$ controls from 4-6 weeks of age, but the transgenic animals continued to increase in body mass with age while the majority of control mice actually began to decrease in body mass after 10 weeks of age. The transgenic *Dp116:mdx:utrn*$^{-/-}$ mice were consistently smaller than control mice with one functional utrophin allele. Error bars signify ± s.e.m. and are not shown for time points where only one surviving mouse remained in the cohort.

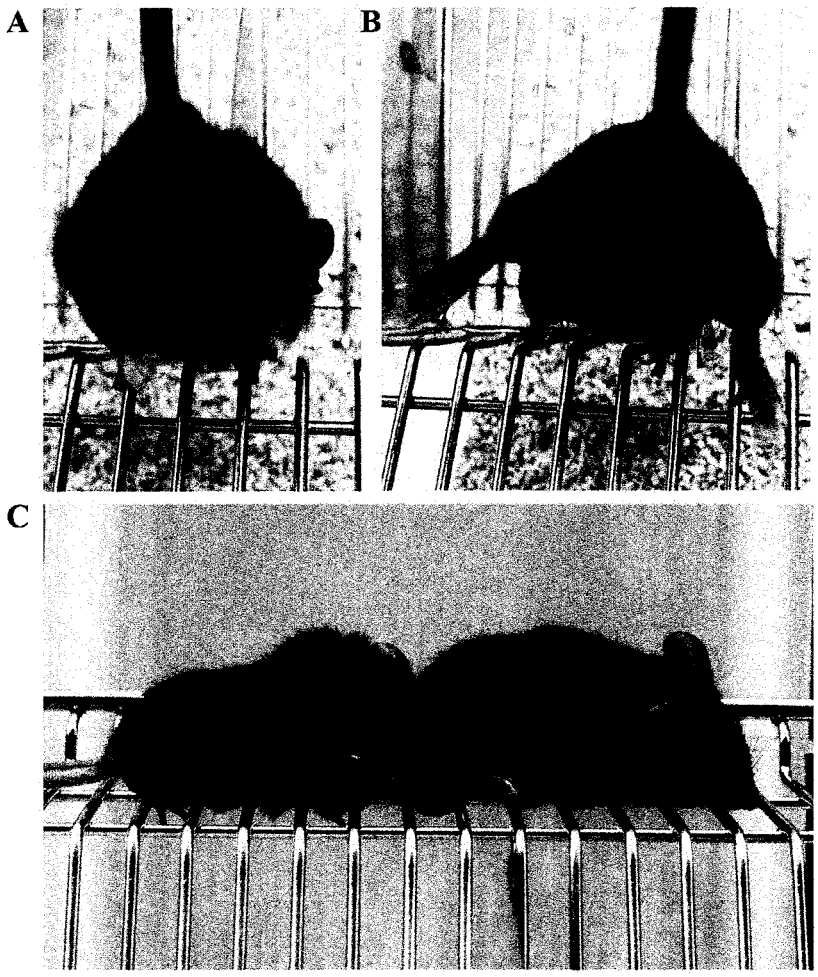


Figure 3.3: Physical characteristics of *mdx:utrn*$^{-/-}$ and *Dp116:mdx:utrn*$^{-/-}$ littermates. *A)* 11 week old *mdx:utrn*$^{-/-}$ male demonstrating joint contractures of the hind limbs. *B) Dp116:mdx:utrn*$^{-/-}$ male littermate does not suffer from joint contractures and is able to extend its hind limbs. *C)* The same littermates are shown to demonstrate that spinal kyphosis and wasting of musculature in the *mdx:utrn*$^{-/-}$ control (*left*) are absent in the *Dp116* transgenic mouse (*right*).

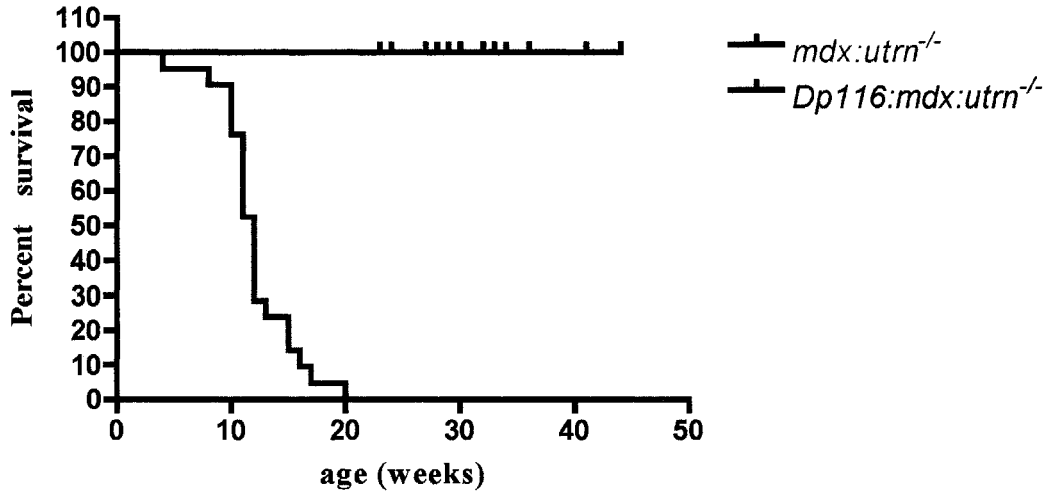
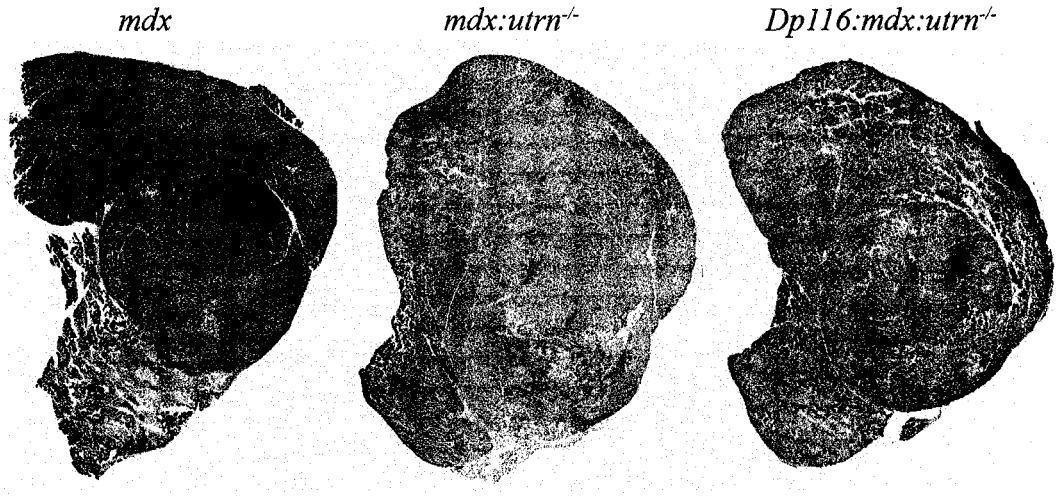


Figure 3.4: Expression of Dp116 in *mdx:utrn*$^{-/-}$ mice increases lifespan. Kaplan-Meier analysis demonstrated a significant increase in survival ($p < 0.0001$) of *Dp116:mdx:utrn*$^{-/-}$ mice ($n = 15$) compared to control *mdx:utrn*$^{-/-}$ mice ($n = 21$). Each tick-mark in the transgenic cohort indicates the age of a surviving mouse at the time of analysis. Both male and female mice were included in this analysis.

Table 3.1: Comparison of muscle mass in *Dp116:mdx:utrn*$^{-/-}$ and control mice (8 weeks age).

	C57Bl/6	*mdx*	*mdx:utrn*$^{-/-}$	*Dp116:mdx:utrn*$^{-/-}$
Body mass	22.7 ± 0.38 g	27.1 ± 0.85 g	15.7 ± 1.6 g	19.8 ± 0.57 g
TA mass	36.7 ± 2.0 mg	65.9 ± 1.6 mg	31.9 ± 2.6 mg	41.1 ± 0.90 mg
TA mass/ body mass	0.00161 ± 8.0e-5	0.00243 ± 4.1e-5	0.00201 ± 1.0e-4	0.00208 ± 6.2e-5
Quad mass	165.2 ± 1.5 mg	233.7 ± 3.2 mg	132.0 ± 7.4 mg	158.5 ± 4.5 mg
Quad mass/ body mass	0.00727 ± 8.5e-5	0.00863 ± 1.6e-4	0.00838 ± 1.9e-4	0.00803 ± 3.0e-4

A



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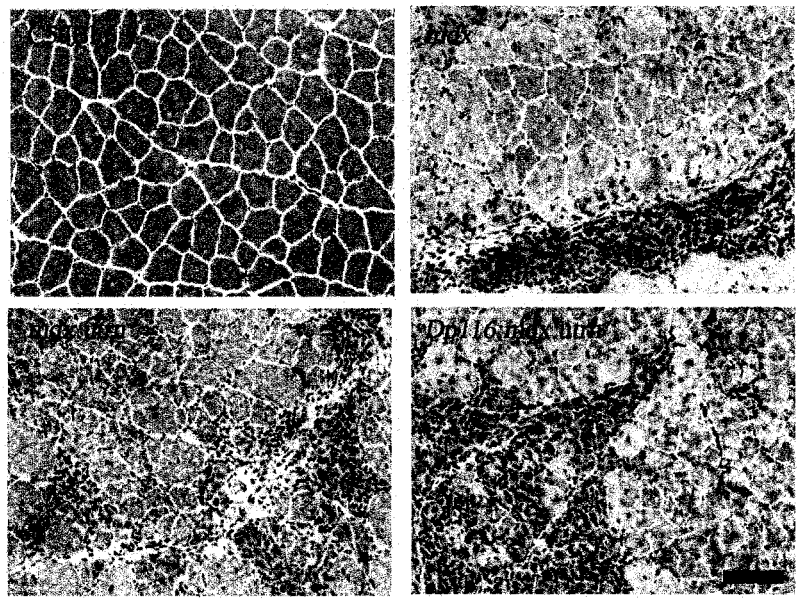


Figure 3.5: Histology of quadriceps muscles from 8 week-old transgenic and control mice. *A)* Entire cross-sections of quadriceps muscles indicate extensive degeneration in both *mdx:utrn*$^{-/-}$ and transgenic *Dp116:mdx:utrn*$^{-/-}$ muscles compared to *mdx*. Images are shown at unequal scale for ease of comparison. *B)* High power (20X) fields of the muscles shown above along with wild-type control. Pathology of *mdx* and *mdx:utrn*$^{-/-}$ skeletal muscles are qualitatively similar at this age, with areas of extensive necrosis, inflammatory infiltrate, and large numbers of myofiber containing centrally located nuclei. Similar pathologic changes are seen in the *Dp116* transgenic muscles. Scale bar: 50 μm.

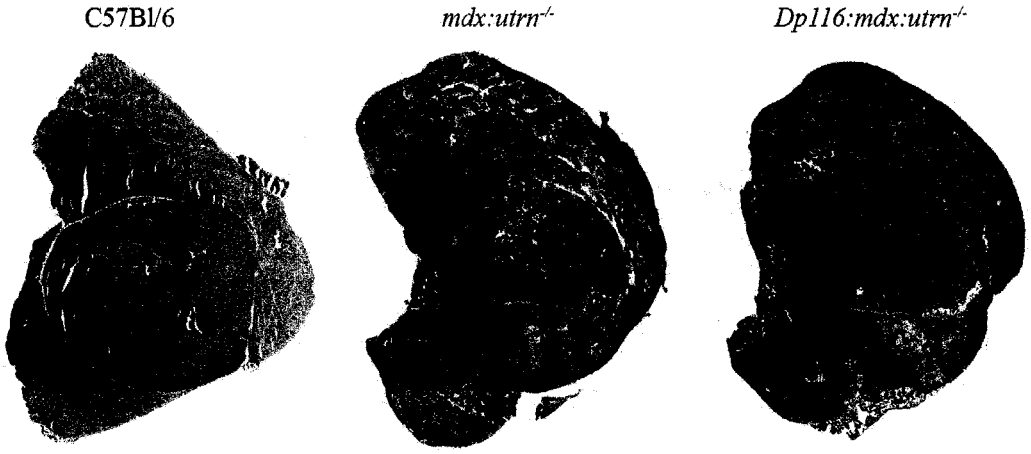


Figure 3.6: Expression of Dp116 does not prevent the fiber type abnormality in *mdx:utrn*$^{-/-}$ muscles. Shown are entire cross sections of quadriceps muscles stained with NADH. Dark staining indicates oxidative fibers while light staining indicates glycolytic fibers. Wild-type C57Bl/6 quadriceps shows a mosaic pattern of oxidative and glycolytic fibers while both the *mdx:utrn*$^{-/-}$ and transgenic quadriceps show a much higher proportion of oxidative fibers as well as an increase in intermediately stained fibers. Images are shown at unequal scale for ease of comparison.

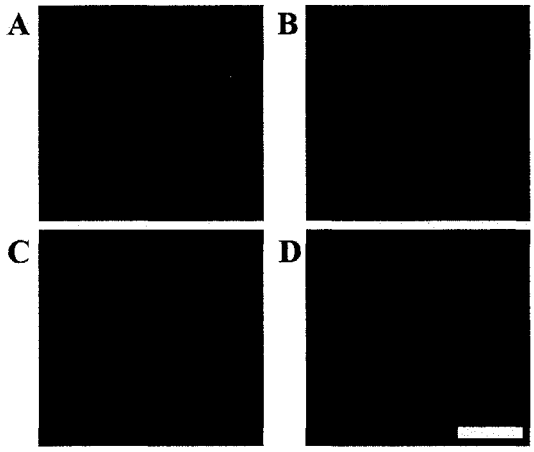


Figure 3.7: Post-synaptic structure of neuromuscular junctions in *Dp116:mdx:utrn*$^{-/-}$ muscles is discontinuous. *A)* A rare example of a myofiber with a relatively continuous staining pattern of acetylcholine receptors is shown. *B – D)* The vast majority of myofibers show a discontinuous staining pattern of acetylcholine receptors as shown in these examples. Scale bar: 50 μm.

CHAPTER 4:

The dystrophin rod domain influences both signaling and contractile properties of myofibers

Introduction

Dystrophin binds to costameric F-actin at its N-terminus and to the transmembrane laminin receptor dystroglycan at its C-terminus (Ervasti and Campbell, 1993). This apparatus creates a robust physical link between the contractile elements inside the myofiber and the basement membrane surrounding it (Rybakova et al., 2000). The central rod domain of the protein is made up of a series of spectrin-like repeats that fold into a triple helical structure that has properties resembling a spring (Koenig and Kunkel, 1990; Winder et al., 1995). Dystrophin can thus be envisioned as "molecular shock absorber" that transmits forces generated by the contractile apparatus inside myofibers out to the extracellular matrix (Bloch and Gonzalez-Serratos, 2003; Petrof et al., 1993).

Large portions of the rod domain can be removed from the protein without dramatically compromising its function (England et al., 1990; Harper et al., 2002b; Phelps et al., 1995). This property has allowed the creation of miniaturized dystrophin genes that can be delivered with viral vectors for gene therapy (Gregorevic et al., 2004; Harper et al., 2002b; Wang et al., 2000). In essence, these constructs are designed to truncate the "spring" while maintaining the critical

connections at each end. However, the description of the rod domain of dystrophin as a simple spring is overly simplistic. For example, there is a second actin-binding domain located within the central region of the rod domain (Amann et al., 1998; Warner et al., 2002). This activity is generated by a group of several basic spectrin-like repeats which make an electrostatic interaction with actin (Amann et al., 1998). This rod actin-binding domain can be deleted without a major loss of function, but different deletions can have drastically different effects even when they are of comparable size (Harper et al., 2002b). Replacing spectrin-like repeats in dystrophin with homologous repeats from α–actinin also generated a non-functional protein (Harper et al., 2002a). These observations demonstrate that the repeats do not all have identical functionality.

Dystrophin also serves as a scaffold for various signaling molecules, which primarily interact with its C-terminus directly or indirectly via dystrobrevin and syntrophin (Rando, 2001a). In dystrophin-deficient muscles most of the members of these complexes are lost from the sarcolemma or greatly reduced. Two of these are neuronal nitric oxide synthase (nNOS) and aquaporin-4, which require the PDZ domain of α_1-syntrophin to localize to the sarcolemma (Adams et al., 2000; Adams et al., 2001; Brenman et al., 1996). Although the C-terminal domain of dystrophin contains binding sites for both dystrobrevin and syntrophin, deletion of this domain did not prevent assembly of the complete DGC, including nNOS (Crawford et al., 2000). This result suggests that dystrobrevin, which also contains syntrophin binding sites, has an alternate mode of anchoring to the sarcolemma, perhaps by

binding to the sarcoglycan complex (Newey et al., 2000; Peters et al., 1997; Yoshida et al., 2000).

Surprisingly, the dystrophin rod domain appears to have a role in localizing nNOS to the sarcolemma. Mini- and micro-dystrophin genes with large deletions of the central portion of the rod domain fail to completely restore nNOS to the sarcolemma (Chapter 2) and (Chao et al., 1996; Wells et al., 2003). It is unlikely that this observation is a reflection of underlying dystrophic pathology, as transgenic *mdx* mice expressing these transgenes throughout development had very healthy muscle by all parameters tested (Harper et al., 2002b). Also, expression of micro-dystrophin did increase expression of nNOS compared to *mdx* controls, but it was primarily found in the soluble fraction (Figure 2.7). We hypothesized that specific parts of the dystrophin rod domain somehow influence nNOS localization and that we could identify those domains experimentally. Studies of BMD patients expressing internally truncated dystrophin support this idea. In this population nNOS is variably localized to the sarcolemma depending on the location of the deletion (Chao et al., 1996; Torelli et al., 2004; Wells et al., 2003). More supporting data come from studies of revertant fibers in *mdx* mice. Revertant fibers arise from alternative splicing skipping exons surrounding the nonsense mutation in exon 23, restoring an open reading frame. Localization of nNOS to the sarcolemma in revertant fibers is also variable, depending on which exons are skipped (Wells et al., 2003).

The existing data from BMD patients and revertant fibers demonstrated an association between deletion of exons 45 – 51 and the loss of nNOS from the

sarcolemma. We used viral vectors to quickly test truncated dystrophin constructs containing various spectrin-like repeats encoded by these exons. Our goal was to identify the minimum-sized dystrophin rod domain that could successfully localize nNOS to the sarcolemma. This approach would allow us to improve the design of gene therapy vectors for DMD in order to restore both the mechanical and the signaling functions of dystrophin and the DGC. However, we were not able to identify a truncated dystrophin construct that restored normal nNOS expression. Interestingly, we found that unlike the previously designed ΔR4-23/ΔCT micro-dystrophin, the novel constructs used in this study did not form abnormal arrangements of myofibrils. These results further demonstrate the unique specialization of the dystrophin rod domain.

Materials and Methods

Mice

We utilized C57Bl/6 wild-type control mice, dystrophin-deficient *mdx* and *mdx*^{4cv} mice, and the following transgenic mice: dystrophinΔCT:*mdx*, ΔH2-R19ΔCT-GFP:*mdx*^{4cv}, ΔR4-23:*mdx*, and Dp116/*mdx*^{4cv}. Mice were genotyped as previously described (Crawford et al., 2000; Harper et al., 2002b; Li et al., 2006). All experimental protocols involving live mice were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Preparation of viral constructs and production of viral vectors

AAV transfer plasmids expressing novel micro-dystrophins were prepared in the following manner. Fragments encoding specific parts of the rod domain from human dystrophin were amplified from full-length human dystrophin using recombinant PCR and cloned into a TOPO 2.1 vector (Invitrogen). The complete sequence of each cloned PCR product was verified before proceeding. A *Bsu36I/XhoI* fragment containing the sequence for part of repeat 1, repeat 18, repeat 19, hinge 3, and part of repeat 24 was ligated into the corresponding fragment of pAAVCMVμDys (Gregorevic et al., 2006) to generate μDys2. A *Bsu36I/PshAI* fragment containing the sequence for part of repeat 1, repeat 18, repeat 19, hinge 3, repeat 20, and part of hinge 4 was ligated into the corresponding fragment of pAAVCMVμDys to generate μDys3. A *NsiI/PshAI* fragment containing the sequence for part of the NT domain, hinge 1, repeat 17, repeat 18, repeat 19, hinge 3, repeat 20, and part of hinge 4 was ligated into the corresponding fragment of pAAVCMVμDys to generate μDys4. A *Bsu36I/PshAI* fragment containing the sequence for part of repeat 1, repeat 2, repeat 3, and part of hinge 4 was ligated into the corresponding fragment of pAAVCMVμDys to generate μDys5. Transient transfection of HEK293 cells followed by Western blot of cell lysates probed with anti-dystrophin monoclonal antibody (Dys-3, Novocastra) verified expression of dystrophin proteins displaying the expected size.

Lentivirus transfer plasmids expressing novel mini-dystrophins were prepared in the following manner. An *EagI* fragment containing human mini-dystrophin (ΔH2-R19ΔCT) was ligated into pGEM T-Easy (Promega) linearized with *NotI*. The *NheI/Acc65I* fragment from pAAVCMVμDys3 was ligated into the corresponding restriction fragment of ΔH2-R19ΔCT to generate ΔR2-17ΔCT. Recombinant PCR was used to amplify a fragment from human dystrophin containing the sequence for part of repeat 2, repeat 17, repeat 18, repeat 19, hinge 3, repeat 20, and part of repeat 21. The PCR product was cloned into pGEM T-Easy (Promega) and the sequence verified. A *ClaI/BlpI* fragment of the cloned PCR product was ligated into the corresponding restriction fragment of ΔH2-R19ΔCT to generate ΔR3-16ΔCT. All three mini-dystrophin variants were then subcloned into a lentivirus transfer plasmid downstream of the MSCV promoter (Li et al., 2005). Adeno-associated virus, lentivirus, and gutted adenovirus vectors were prepared as previously described (Blankinship et al., 2004; DelloRusso et al., 2002; Li et al., 2005; Scott et al., 2002). Table 4.1 shows the sequences of all primers used to generate the constructs in these experiments.

Injection of viral vectors

Young *mdx* and *mdx*^{4cv} mice were anesthetized with isoflurane, while neonatal *mdx*^{4cv} mice were anesthetized by hypothermia prior to intramuscular injection. Gutted adenoviral vectors (4 x 10^{10} viral genomes) expressing full-length

mouse utrophin, mouse dystrophin, or human dystrophin driven by the 6.5 kb MCK promoter were diluted in phosphate-buffered saline and injected into tibialis anterior muscles of 6 week-old *mdx* mice in a total volume of 25 μL. Adeno-associated viral vectors (5 x 10^{10} viral genomes) expressing human micro-dystrophin driven by the CMV promoter were diluted in Ringer's solution and injected into tibialis anterior muscles of 2-3 week-old *mdx*^{4cv} mice in a total volume of 25 μL. Lentiviral vectors (~0.5-1.0 x 10^6 infectious units) expressing human mini-dystrophin driven by the MSCV promoter were diluted in tris-buffered saline. Five minutes prior to injection polybrene was added to a final concentration of 8 μg/mL and the vector was injected into tibialis anterior muscles of 2-day old *mdx*^{4cv} mice in a total volume of 5-10 μL.

Immunofluorescence

Injected and control tibialis anterior muscles were dissected, embedded in OCT (Sakura Finetek USA) on cork support and frozen in liquid nitrogen-cooled isopentane. Cryosections of 7-10 μm thickness were blocked in KPBS (20 mM potassium phosphate pH 7.4, 150 mM sodium chloride) containing 0.3 mg/ml BSA, 2% gelatin, and 1% Tween 20. The blocked sections were incubated with primary antibodies diluted in KPBS containing 0.2% gelatin (KPBS-G) and 2% normal goat serum. Rabbit polyclonal antibodies were used to dystrophin NH_2-terminus (Rafael et al., 1996), nNOS (Zymed Laboratories), aquaporin-4 (Chemicon), α_1-syntrophin (Peters et al., 1997), α-dystrobrevin 2 (Peters et al., 1998), and utrophin A (S.

Froehner, unpublished). After several washes with KBPS-G the sections were incubated with goat anti-rabbit Alexafluor 488 (Molecular Probes). Monoclonal rat anti-laminin β2 chain (Chemicon) was used in combination with goat anti-rat Alexafluor 594 (Molecular Probes). Immunostained slides were washed repeatedly with KBPS-G and mounted with Vectashield with or without DAPI (Vector Laboratories). All photomicrographs were obtained with a Spot II CCD camera (Diagnostic Instruments, Inc.) and Spot Advanced software connected to a Nikon Eclipse E1000 using a 20X Plan-Apochromat objective (numerical aperture = 0.75). Images for all the sections probed with a given antibody were acquired under identical exposure conditions.

Electron Microscopy

Small (approximately $2mm^3$) pieces of skeletal muscles were fixed in half-strength Karnovsky's fixative for at least 24 hours at 4 °C. The fixed muscles were washed with 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide/cacodylate buffer for 2-3 hours. They were washed again with 0.1 M cacodylate buffer, dehydrated through ethanol, embedded in Epon (Resolution Performance Products), and polymerized at 60 °C overnight. Thick (1 μm) sections were cut and stained with toluidine blue. Ultrathin sections (70-100 nm) were cut and stained with saturated aqueous uranyl acetate and Reynold's lead citrate. Sections were imaged with a JEOL 1010 Transmission Electron Microscope (JEOL

USA, Inc.). Photographs were obtained wide angle 1024x1024 Gatan 792 Multiscan 600W CCD camera (Gatan, Inc.).

Results

Full-length dystrophin, but not utrophin, restores nNOS localization after delivery by viral vectors

In order to establish that delivery of dystrophin to *mdx* muscle in post-natal animals could restore nNOS expression we tested injection of gutted adenovirus vectors expressing mouse or human full-length dystrophin. These are vectors in which all of the endogenous adenoviral genes have been deleted and replaced with the experimental transgene. One month after intramuscular injection of gutted adenovirus vectors we saw good expression of both mouse and human dystrophin. Furthermore, expression of nNOS associated with the sarcolemma was restored to a similar extent in both cases (Figure 4.1). This result validated our approach of using viral vectors to study the functional domains of dystrophin responsible for assembling the signaling components of the DGC, including nNOS. Consistent with previous reports, we also observed that some *mdx* myofibers that stained positive with the dystrophin N-terminal antibody (revertant fibers) also expressed nNOS at the sarcolemma (Figure 4.1). Other revertant fibers were negative for nNOS expression, indicating a lack of the specific domain of dystrophin required for nNOS localization.

We also tested a gutted adenovirus vector expressing full-length mouse utrophin. Utrophin is highly homologous to dystrophin in both domain structure and amino acid sequence, although there are significant differences in the rod domain. For example, utrophin has only 22 spectrin-like repeats compared to the 24 found in dystrophin. Utrophin is upregulated in normal *mdx* muscle and surrounds the circumference of the sarcolemma in many *mdx* myofibers. Intramuscular injection of the utrophin vector further increased the expression of utrophin in *mdx* tibialis anterior muscles, but we did not observe any nNOS immunoreactivity at the sarcolemma (Figure 4.2A). This was unexpected, as transgenic mice overexpressing utrophin were previously found to have restored expression of α-dystrobrevin and syntrophin at the sarcolemma, although nNOS was not examined in those studies (Tinsley et al., 1998). Although multiple members of the syntrophin family can bind nNOS *in vitro*, only α_1-syntrophin is able to localize nNOS to the sarcolemma *in vivo* (Adams et al., 2001). Therefore, we verified that myofibers from muscle injected with the utrophin vector co-expressed utrophin-A and α_1-syntrophin at the sarcolemma (Figure 4.2B). This result further demonstrated that nNOS requires specific regions of the dystrophin protein in order to associate with the DGC.

Truncated dystrophins containing repeats 17-21 do not restore nNOS localization

Previous studies with transgenic mice expressing various dystrophin deletion mutants in our lab led us to believe that there is a specific region of the rod domain

that influences nNOS localization at the sarcolemma (Chapter 2). This hypothesis is supported by experiments that correlated the dystrophin exons expressed in revertant fibers with localization of nNOS (Wells et al., 2003). The authors concluded that expression of exons 45-48 was necessary for localization of nNOS at the sarcolemma. These exons encode parts of repeats 17-19. Examples of BMD patients expressing internally truncated dystrophin also implicate exons 48-51, which encode parts of repeats 18-20 and hinge 3 (Chao et al., 1996; Torelli et al., 2004; Wells et al., 2003). To test experimentally if expression of a truncated dystrophin containing these specific domains could localize nNOS, we created several novel micro-dystrophin (μDys) constructs and packaged them in adeno-associated virus (AAV) vectors (Figure 4.3A). The vectors were injected into tibialis anterior muscles of young *mdx*^{4cv} mice. One month after injection we used immunofluorescence staining of cross sections of the injected muscles to examine expression of components of the DGC.

We saw expression of all three novel micro-dystrophins localized at the sarcolemma along with α-dystrobrevin-2 and α_1-syntrophin (Figure 4.3B). However, we did not observe any nNOS immunoreactivity at the sarcolemma. In our experience transgenes delivered by AAV vectors take longer to reach peak expression that those delivered by adenovirus vectors. Thus, it was possible that while nNOS expression was restored only one month after injection with gutted adenoviral vectors expressing dystrophin, it might take longer in the case of an AAV

vector. We repeated the analysis two months after injection of the various micro-dystrophin AAV vectors but obtained the same result (data not shown).

We examined two possible explanations for the failure of all three novel micro-dystrophins to restore nNOS localization. First, the functionality of these constructs may be reduced compared to the original micro-dystrophin. In this case the transduced myofibers would remain dystrophic, which would likely prevent nNOS from associating with the sarcolemma as we have previously observed that total nNOS expression is greatly reduced in dystrophic muscle even when the rest of the DGC is restored (Chapter 2). To evaluate the ability of the new micro-dystrophin constructs to prevent dystrophic pathology we injected young *mdx*^{4cv} mice (2-3 weeks old) with 5 x 10^{10} genomes of each AAV vector in both tibialis anterior muscles. At 10 weeks post injection the injected muscles were dissected and cross sections were triple-labeled with antibodies to dystrophin N-terminus, laminin β2 chain, and DAPI. This protocol allowed us to simultaneously visualize all myofibers by staining of laminin in the basement membrane, successfully transduced fibers by the dystrophin staining, and the localization of nuclei with DAPI (Figure 4.4). We found that with all four constructs the dystrophin positive fibers in the injected muscles were larger and had fewer centrally located nuclei than the non-transduced myofibers within the same muscle. This indicated that all of the constructs were able to prevent myofiber degeneration to some extent over the time course of this experiment.

The second explanation we considered for the failure of the micro-dystrophin vectors to restore nNOS expression was that the deletions of the rod domain were too extensive to allow proper folding of the protein and interactions within the DGC. To test this possibility we designed two mini-dystrophins with a smaller deletion in the central rod domain (along with deletion of the C-terminal domain). These constructs were similar in size and organization to the highly functional ΔH2-R19 mini-dystrophin (Figure 4.5A). However, the region of deletion was modified to closely mimic those in BMD patients with deletions of exons 13-41 or 13-44 that retained moderate expression of nNOS at the sarcolemma (Chao et al., 1996; Wells et al., 2003). These mini-dystrophins were too large to package in AAV vectors so we cloned each into a lentiviral vector driven by the MSCV promoter. Lentiviral vectors can stably transduce muscle cells in vivo when injected intramuscularly in newborn mice (Li et al., 2005). We injected each vector into the tibialis anterior muscles of 2-day old *mdx*^{4cv} mice and analyzed expression of dystrophin and nNOS at 8-10 weeks of age (Figure 4.5B). Clusters of dystrophin-positive myofibers were observed in the majority of injected muscles by immunofluorescent staining with an antibody to the dystrophin N-terminus, although the overall transduction efficiency of the lentiviral vectors was much lower than that of the adenoviral or AAV vectors. Serial sections immunostained for nNOS showed that occasional dystrophin-positive fibers also expressed nNOS at the sarcolemma (Figure 4.5B). However, these fibers were a significant minority as most of the successfully transduced cells did not show nNOS immunoreactivity. We also stained serial sections with an antibody to the

dystrophin C-terminus, which is deleted in the lentiviral constructs but would be present in revertant fibers. We found that in each case the fibers that were positive for nNOS were also stained by the dystrophin C-terminal antibody, suggesting that they were likely to be revertant fibers (Figure 4.5B).

Annular formation of myofibrils is dependent on specific regions of the dystrophin rod domain

In normal muscle the myofibrils are oriented in parallel with the long axis of the myofiber. We observed that a subset of myofibers in muscles from ΔR4-23/*mdx* transgenic mice show an unusual ring-like phenotype. Electron microscopy demonstrated that this phenotype was caused by a peripheral layer of myofibrils near the sarcolemma forming concentric rings, while the myofibrils in the center of the cell were oriented normally (Figure 4.6). This ring formation was never observed in wild-type or *mdx* mice, suggesting that it might be caused specifically by expression of micro-dystrophin. Furthermore, it was not observed in transgenic mice expressing a larger mini-dystrophin-GFP fusion or dystrophin with deletion of only the carboxyl-terminal domain (Figure 4.6). These results suggested that the phenotype was caused by the large deletion of the rod domain in the micro-dystrophin. Finally, it was not observed in mice expressing Dp116, indicating that a connection to the actin cytoskeleton was also required to produce the ring structure, which will be referred to as annular myofibril formation.

We hypothesized that the small size of the rod domain in micro-dystrophin was causing the annular myofibril formation, which would explain why it was not observed with the larger mini-dystrophin. To confirm this hypothesis we injected 4 week old *mdx*^{4cv} mice with rAAV vectors expressing the various micro-dystrophins, one of which contained an even larger deletion of the rod domain (Figure 4.7A). After two months we analyzed the injected muscles for expression of dystrophin and annular myofibril formation. Surprisingly, only the muscles injected with the control micro-dystrophin vector showed annular myofibrils. The novel constructs μDys2 and μDys5 did not produce annular myofibrils despite being a similar size (μDys2) or even smaller (μDys5) compared to the control μDys (Figure 4.7B). In fact, none of the novel micro-dystrophin constructs formed annular myofibrils despite similar levels of expression compared to the control construct (data not shown for μDys3 and μDys4). This result indicates that the composition rather than the size of the rod domain is responsible for formation of annular myofibrils. Intriguingly, one characteristic of the novel micro-dystrophins tested in this experiment is that unlike the original construct they all lack hinge 2. This difference is also present in the mini-dystrophin-GFP construct that did not cause annular myofibril formation.

Discussion

It is widely accepted that nNOS is lost from the sarcolemma in dystrophin-deficient muscle (Brenman et al., 1995; Chang et al., 1996). However, there is

disagreement in the literature regarding the overall expression levels of nNOS, levels of soluble nNOS, and its enzymatic activity in dystrophic muscle cells. Most studies have concluded that both total and soluble nNOS expression levels are reduced in dystrophin-deficient muscle, although calculations of the extent of the reduction vary widely (Brenman et al., 1995; Chang et al., 1996; Dudley et al., 2006; Kameya et al., 1999). It is less clear whether enzymatic production of nitric oxide in *mdx* muscles is reduced or increased, as both results have been reported (Brenman et al., 1995; Dudley et al., 2006; Wehling et al., 2001). One might assume that a decrease in nNOS protein levels should correlate with a decrease in NO concentration, but there are two reasons why this may not be true. First, other isoforms of NOS could account for increased production of nitric oxide in dystrophic muscle. Endothelial NOS (eNOS) and inducible NOS (iNOS) are only expressed at very low levels in rodent myofibers, but are expressed in endothelial cells and macrophages within the muscle tissue (Kobzik et al., 1995; Stamler and Meissner, 2001; Thompson et al., 1996). Macrophages infiltrate dystrophic muscles during the inflammatory response to dystrophic injury, which could be associated with increased iNOS activity. Second, association with the DGC could be a mechanism for negative regulation of nNOS activity. Caveolin-3 can interact with the DGC by binding to β-dystroglycan or nNOS and can inhibit nNOS activity (Garcia-Cardena et al., 1997; Sotgia et al., 2000; Venema et al., 1997). In this case, loss of the regulatory interaction with the DGC could cause increased nNOS activity despite the overall reduction in its expression levels.

It is a long-standing hypothesis that oxidative damage is an important component in the pathophysiology of muscle necrosis in various types of muscular dystrophy (reviewed in (Rando, 2002)). An increase in production of nitric oxide could exacerbate oxidative damage in dystrophic muscle by the production of toxic free radicals (Beckman and Koppenol, 1996; Brenman et al., 1995; Rando, 2001b). A recent study demonstrated such a propensity to increased oxidative damage in *mdx* muscles, which could be reduced by pharmacological inhibition of NOS (Dudley et al., 2006). However, the effects of NO-induced damage in this study only occurred after artificial induction of severe ischemia/reperfusion in the muscle. Genetic disruption of the α-dystrobrevin gene caused a mild myopathy, and the only abnormality that was found other than the absence of dystrobrevin was a displacement of nNOS to the soluble fraction of muscle cells (Grady et al., 1999). The authors measured a corresponding decrease in levels of cyclic GMP, but did not attempt to directly measure NO concentration or oxidative byproducts.

A significant amount of data is inconsistent with the hypothesis that muscle cell necrosis results from oxidative damage due to mislocalization of nNOS and a corresponding loss of regulatory control over the enzyme. Either complete knockout of the α-syntrophin gene ($\alpha syn^{-/-}$) or deletion of its PDZ domain (αsynΔPDZ) resulted in loss of nNOS from the sarcolemma that was more complete than that seen with the dystrobrevin knockout, but did not cause muscle necrosis (Adams et al., 2000; Adams et al., 2001; Kameya et al., 1999). Deletion of the nNOS gene itself did not alter the phenotype of *mdx* muscle (Chao et al., 1998; Crosbie et al., 1998).

NO synthesis was also found to be unrelated to the susceptibility of muscle cells to oxidative stress *in vitro* (Zhuang et al., 2001). Furthermore, overexpression of nNOS in *mdx* skeletal muscles caused an increase in NO production but actually ameliorated muscle pathology (Wehling et al., 2001). Interestingly, the excess nNOS was localized to both the soluble and membrane fractions of skeletal muscle even in the absence of the DGC (Tidball and Wehling-Henricks, 2004).

These latter results support the contrary hypothesis that NO is actually protective against muscle necrosis. Mislocalization and/or reduction of nNOS levels could thus cause a detrimental reduction in NO mediated signaling. Functional ischemia has been proposed as the potential mechanism for myofiber necrosis due to decreased NO signaling. Nitric oxide (identified as the molecule originally called endothelial derived relaxation factor) is known for inducing relaxation of vascular smooth muscle. Nitric oxide production in metabolically active skeletal muscle counteracts sympathetic vasoconstriction during exercise (Thomas and Victor, 1998). This mechanism is defective in both *mdx* mice and DMD patients, leading to muscle ischemia during exercise (Sander et al., 2000; Thomas et al., 1998). The same phenotype was observed in $\alpha syn^{-/-}$ and $\alpha syn\Delta PDZ$ mice, which have normal nNOS activity in the cytoplasm but loss of activity at the sarcolemma (Thomas et al., 2003). This result suggests that only nNOS associated with the DGC is sufficient for attenuation of sympathetic vasoconstriction. Wehling and colleagues argued that overexpression of nNOS in *mdx* skeletal muscles decreased pathology by reducing

inflammation , but it would be interesting to see whether the restored NO production also corrected the defect in vasomodulation (Wehling et al., 2001).

These results have revived interest in the finding that ischemia alone could reproduce the same pattern of myofiber necrosis seen in DMD muscle (Mendell et al., 1971). However, as neither nNOS deficient mice, $\alpha syn^{-/-}$ mice, nor $\alpha syn\Delta PDZ$ mice show myofiber necrosis this mechanism alone is not sufficient to cause the pathology seen in dystrophin-deficient muscle. It is possible that functional ischemia exacerbates muscle necrosis when combined with sarcolemmal instability due to deficiency of dystrophin and the DGC (Dudley et al., 2006; Rando, 2001b). The truncated dystrophin transgenes that have been developed for viral gene therapy will likely have reduced function compared to the full length protein, producing a BMD-like phenotype in humans. These constructs also fail to restore nNOS to the sarcolemma, raising the concern that the treated myofibers may be vulnerable to damage from functional ischemia. For this reason we sought to identify a region of the dystrophin rod domain that is responsible for localization of nNOS in order to improve current gene therapy vectors. Advances in rAAV technology have demonstrated the possibility of increasing the packaging capacity by splitting the therapeutic gene into two separate vectors which can recombine to generate the complete construct (Halbert et al., 2002; Lai et al., 2005). This generates the option of designing slightly larger dystrophin constructs that could have increased functionality, such as restoration of nNOS. Unfortunately, even our larger mini-dystrophin constructs did not fully localize nNOS to the sarcolemma.

There are conflicting reports in the literature regarding the localization of nNOS in myofibers expressing the mini-dystrophin based on deletion of exons 17-48. Data from both transgenic mice and mice injected intramuscularly with plasmid vectors have shown both the absence and the presence of nNOS at the sarcolemma following expression of this protein (Chao et al., 1996; Crosbie et al., 1998; Decrouy et al., 1998; Wells et al., 2003). However, the reports that showed the presence of nNOS at the sarcolemma in muscles expressing this dystrophin mutant showed either low level expression or expression in a very limited number of myofibers. Furthermore, they did not rule out the possibility that revertant fibers contributed to the cases where only a small number of myofibers were analyzed.

We found that transgenic mice expressing the ΔH2-R19 (very similar to the deletion of exons 17-48) or ΔR4-23 dystrophin mutants did have low level nNOS at the sarcolemma but this was much reduced compared to wild-type (Figure 2.7). When introduced via viral vectors the analogous constructs (with the additional deletion of the C-terminal domain) did not show any restoration of nNOS at the sarcolemma compared to dystrophic control muscles. Similarly, we found that our novel micro- and mini-dystrophin constructs did not increase the levels of nNOS expression at the sarcolemma in dystrophin-deficient mice following viral vector administration (Figures 4.3, 4.5). This suggests that there is not a discrete domain that is responsible for association of nNOS to the DGC, but that a large portion of the rod domain may fold to create a binding site for nNOS. It is also possible that the interaction is indirect, with dystrophin binding to a third protein that is required to

localize nNOS to the DGC. Alternatively, there could be secondary processes that influence nNOS expression and trafficking that do not require a direct interaction with dystrophin.

Previous studies of the rod domain have focused on its proposed structural functions rather than signaling functions. One well accepted hypothesis postulates that costameres function to transmit forces from contracting myofibrils laterally to the sarcolemma and therefore to adjacent myofibers (Pardo et al., 1983). The dystrophin rod domain forms an integral component of this apparatus, serving as a strong, flexible, and elastic linker directly connected to the sarcolemma through β-dystroglycan (Rybakova et al., 2000). The observation that deletion of specific regions of the dystrophin rod domain caused an alteration in myofibrillar structure is further *in vivo* evidence for a functional connection between dystrophin and contractile myofibrils. Annular myofibril formation, also known as ringbinden, has been observed in various diseases of muscle but most prominently in myotonic dystrophy (Banker and Engel, 1994). Although it is unknown what causes this phenomenon, the pathogenesis of myotonic dystrophy is associated with abnormal RNA splicing that could affect structural proteins such as dystrophin (Nakamori et al., 2006).

It is unclear why the ΔR4-23 deletion of the rod domain caused annular myofibril formation while other deletions of similar size did not. It seems most likely that either the rod domain assumes an unusual secondary structure or has decreased flexibility in that particular context. It is notable that one unique feature of

the ΔR4-23 protein is the location of hinge 2 in between the third and fourth spectrin-like repeats. The ΔH2-R19 construct and our novel four-repeat micro-dystrophins have hinge 3 in this location. It would be relatively simple to alter the ΔR4-23 construct to replace hinge 2 with hinge 3 to test if that domain arrangement is responsible for this phenomenon. In any case, the high prevalence of annular myofibrils may explain why limb muscles from transgenic mice expressing ΔR4-23 have reduced specific force. The myofibrils that form the concentric rings are oriented essentially perpendicular to the majority of the myofibrils, which are oriented longitudinally. This arrangement would clearly result in sub-optimal contractile properties. It will be interesting to see if muscles expressing micro-dystrophins that do not cause annular myofibril formation will have improved contractile properties as well.

Table 4.1: Sequences of oligonucleotides used to generate novel dystrophin constructs.

Construct	Forward/ Reverse	Sequence
μDys2	F1	5'- GGA ATG CCT CAG GGT AGC TAG CAT GGA AAA ACA AAG CAA TTT ACA TAA GAA TAT CTT GTC AGA ATT TC -3'
μDys2	R1	5'- GAG TCT TTC AAG CTC CAA CAT CAA GGA AGA TGG -3'
μDys2	F2	5'- GAT GTT GGA GCT TGA AAG ACT CCA GGA ACT TC -3'
μDys2	R2	5'- TGT TTG GCG AGA TGG CTC -3'
μDys3	F	5'- GGA ATG CCT CAG GGT AGC TAG CAT GGA AAA ACA AAG CAA TTT ACA TAA GAA TAT CTT GTC AGA ATT TC -3'
μDys3	R	5'- GAT GCT GGA CCA AAG TCC CTG TGG GCC ATT TCA TTC AAC TGT TGC C -3'
μDys4	F1	5'- GAA CAA TGT CAA CAA GGC AC -3'
μDys4	R1	5'- CCA TTT CTC AAC ACT CTC CAT CAA TGA ACT GCC -3'
μDys4	F2	5'- GAT GGA GAG TGT TGA GAA ATG GCG GCG -3'
μDys4	R2	5'- GAT GCT GGA CCA AAG TCC CTG TGG GCC ATT TCA TTC AAC TGT TGC C -3'
μDys5	F	5'- GAA CAA TGT CAA CAA GGC AC -3'
μDys5	R	5'- GAT GCT GGA CCA AAG TCC CTG TGG GCA GCC TGT GAA ATC TGT GC -3'
miniDys3	F	5'- GAT CGA TGG GCA AAC ATC TGT AGA TGG ACA GAA GAC CGC TGG GTT CTT TTA CAA GAC ATC GTT GAG AAA TGG CGG CG -3'
miniDys3	R	5'- AGT CGC CTC CAA TAG GTG CC -3'

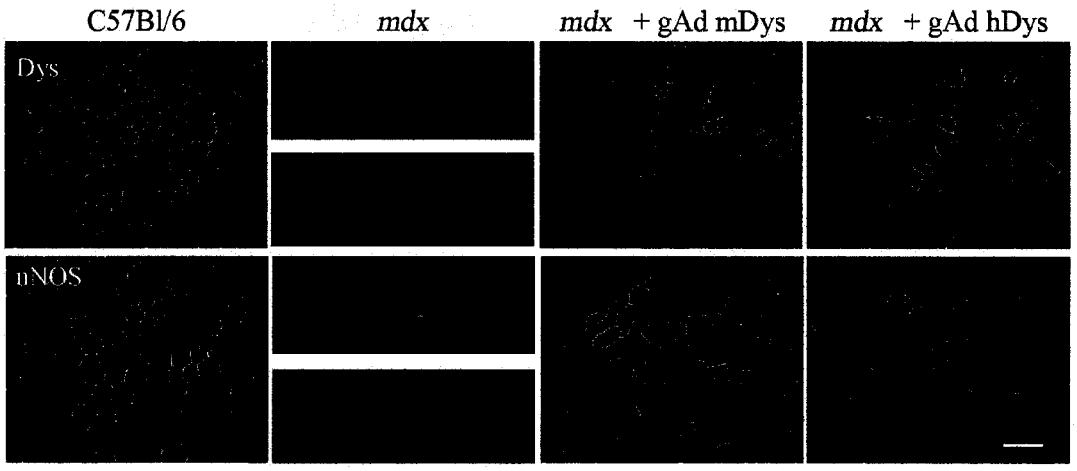


Figure 4.1: Full-length dystrophin delivered by gutted-adenovirus vector restores nNOS expression at the sarcolemma to near wild-type levels. Sections of tibialis anterior muscle were stained one month after vector injection. Two dystrophin-positive revertant fibers are shown from control *mdx* muscle. One of the fibers is also positive for nNOS (top) while the other is negative (bottom). gAd = gutted adenovirus, mDys = mouse dystrophin, hDys = human dystrophin. Scale bar: 100 μm.

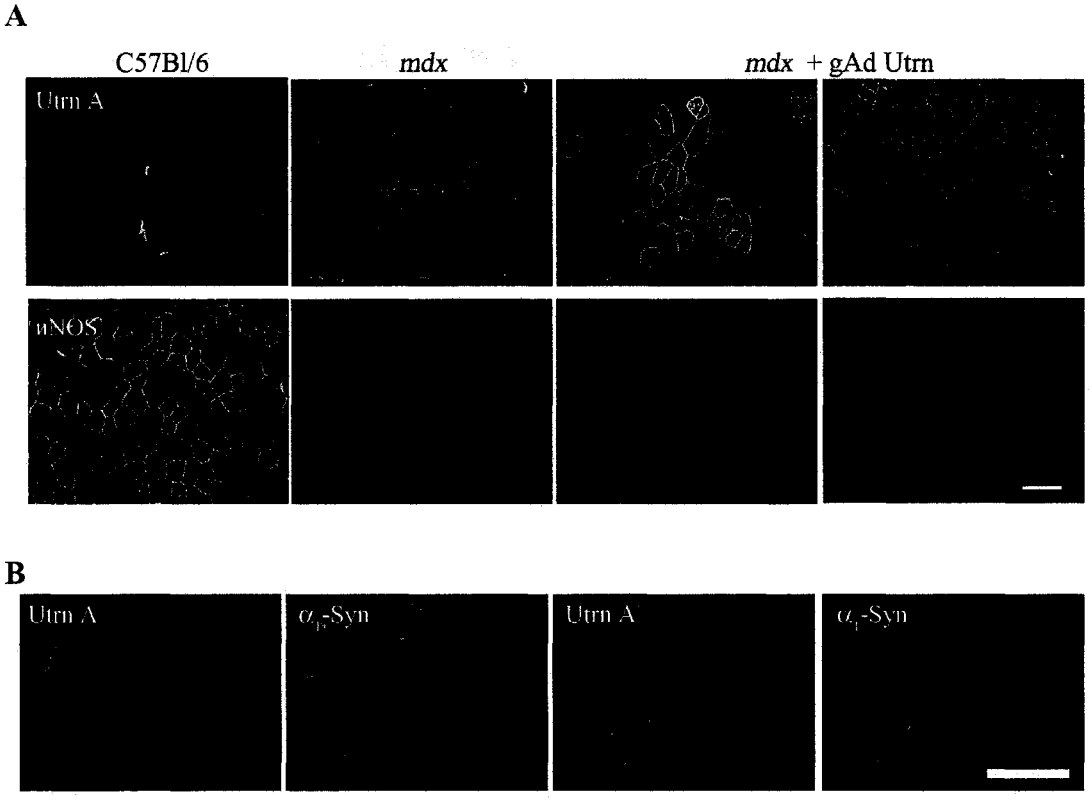


Figure 4.2: Full-length utrophin delivered by gutted-adenovirus vector fails to localize nNOS to the sarcolemma. *A)* Sections of tibialis anterior muscle were stained one month after vector injection. Two examples of injected muscle are shown, one with a small patch of fibers with extreme overexpression of utrophin *(left)* and another with a larger area of uniformly moderate overexpression *(right)*. The uninjected *mdx* muscle also shows upregulation of utrophin but neither the injected or control *mdx* myofibers have nNOS expression at the membrane. gAd = gutted adenovirus, Utrn = utrophin. *B)* Two examples of serial sections from injected muscles stained with antibodies to utrophin-A or α_1-syntrophin. Serial sections show that individual myofibers expressing high levels of utrophin at the sarcolemma also express α_1-syntrophin. Scale bars: 100 μm.

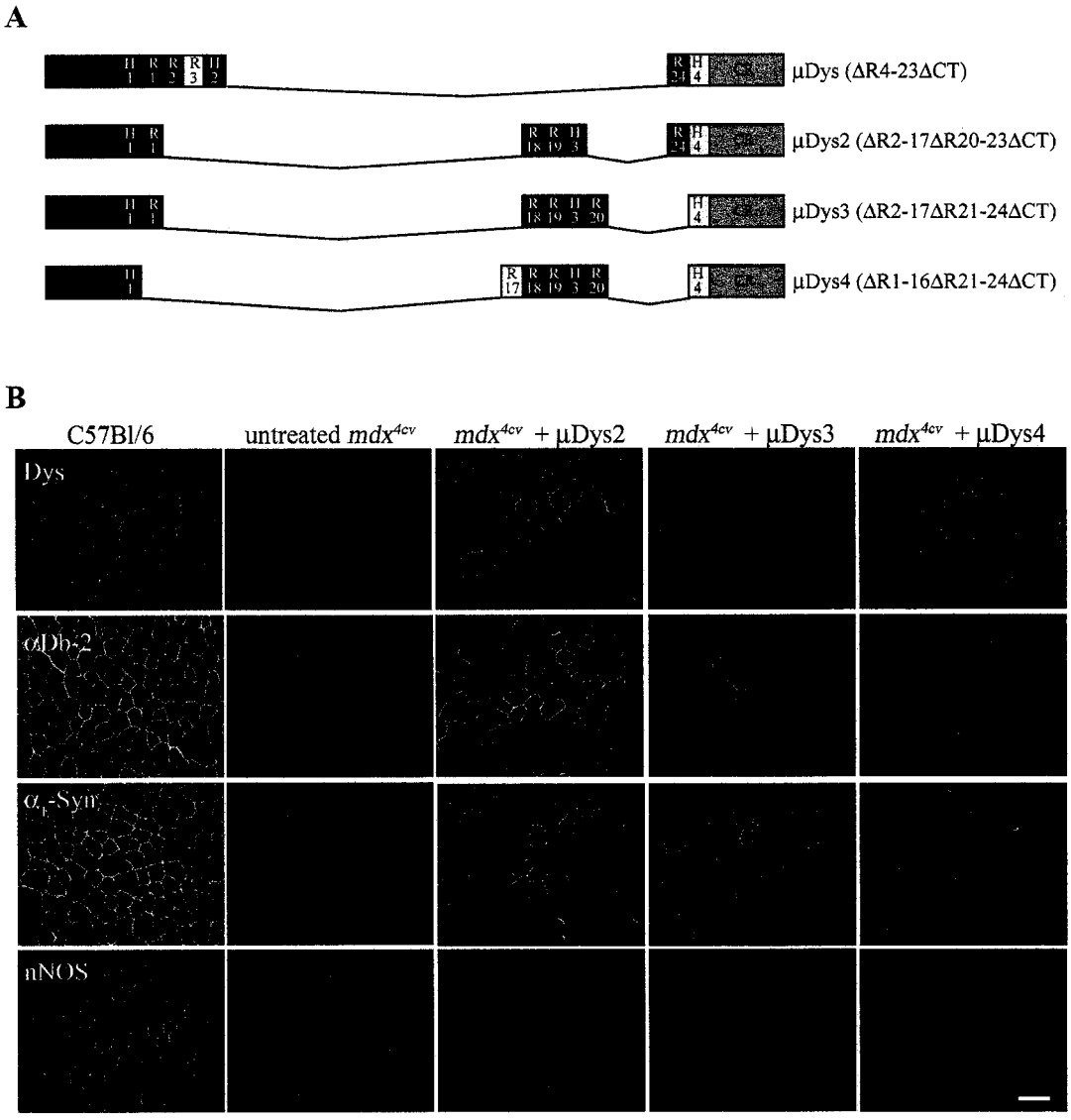


Figure 4.3: Micro-dystrophins containing repeats 17-20 do not localize nNOS to the sarcolemma. *A)* Schematic of the domain structure of the original micro-dystrophin construct (μDys) and three novel micro-dystrophins with different deletions of the rod domain. All four were packaged in AAV vectors driven by the CMV promoter. *B)* Expression of components of the DGC following injection of AAV vectors expressing novel micro-dystrophins. Although dystrophin, α-dystrobrevin-2, and α_1-syntrophin were all detected at the sarcolemma in the injected muscles, no nNOS staining was observed. Scale bar: 100 μm.

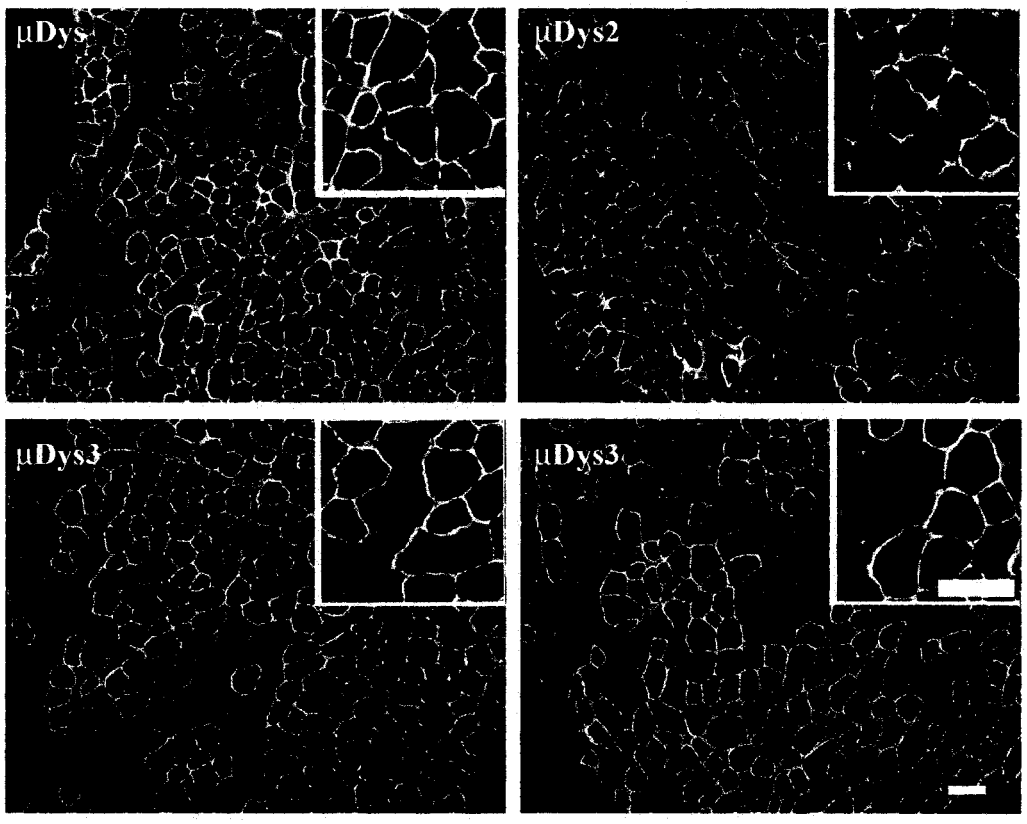
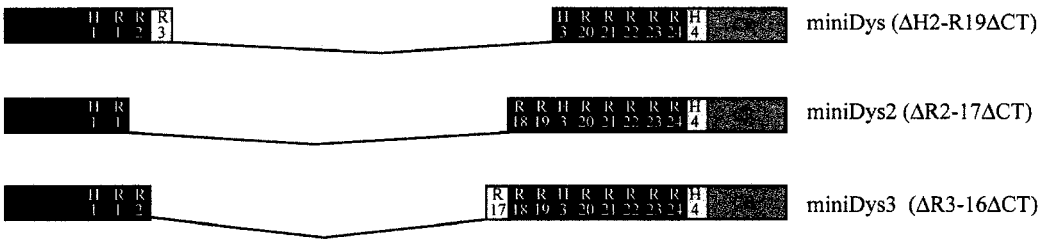


Figure 4.4: Novel micro-dystrophin vectors protect myofibers from degeneration. Tibialis anterior muscles from *mdx*^{4cv} mice injected with µDys, µDys2, µDys3, and µDys4 stained with anti-dystrophin N-terminus (green), anti-laminin β2 chain (red), and DAPI (blue). The staining demonstrates that in each case the myofibers that stain positive for dystrophin are larger and have mostly peripherally located nuclei while the dystrophin-negative myofibers are smaller and frequently have centrally located nuclei. Scale bars: 100 µm.

A



B

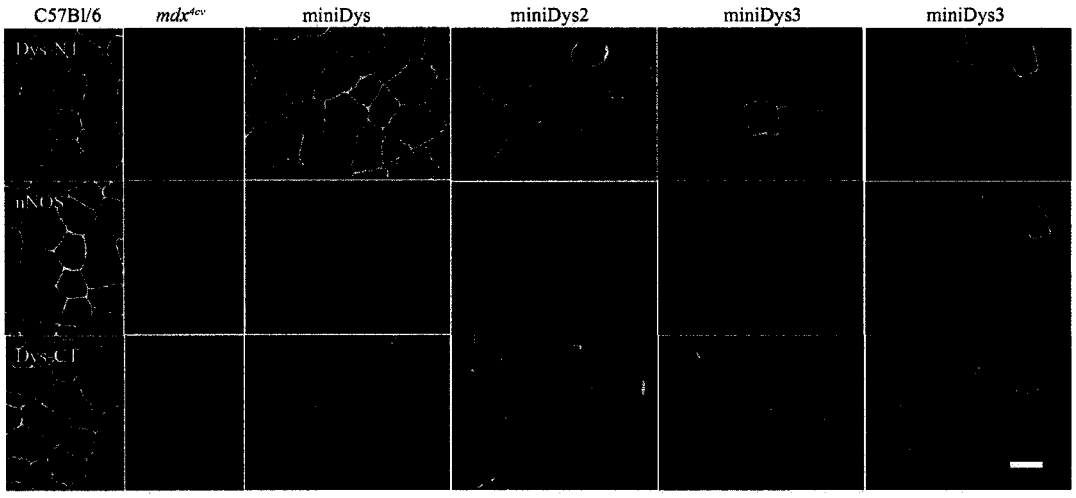


Figure 4.5: Mini-dystrophins containing repeats 17-19 do not localize nNOS to the sarcolemma. *A)* Schematic of the domain structure of the original mini-dystrophin construct (miniDys) compared with two novel mini-dystrophins with different deletions of the rod domain. The novel constructs were packaged in lentiviral vectors driven by the MSCV promoter. *B)* Muscles from *mdx*^{4cv} mice injected with lentiviral vectors show patches of dystrophin expression from each construct (top row). However, the large majority of dystrophin-positive clusters were not positive for nNOS immunoreactivity (middle row). A small minority of fibers that stained positive with the dystrophin N-terminal antibody were positive for nNOS (example in right column). However, these fibers consistently stained positive with the dystrophin C-terminal antibody (bottom row) indicating that they are likely to be revertant fibers. Scale bar: 50 μm.

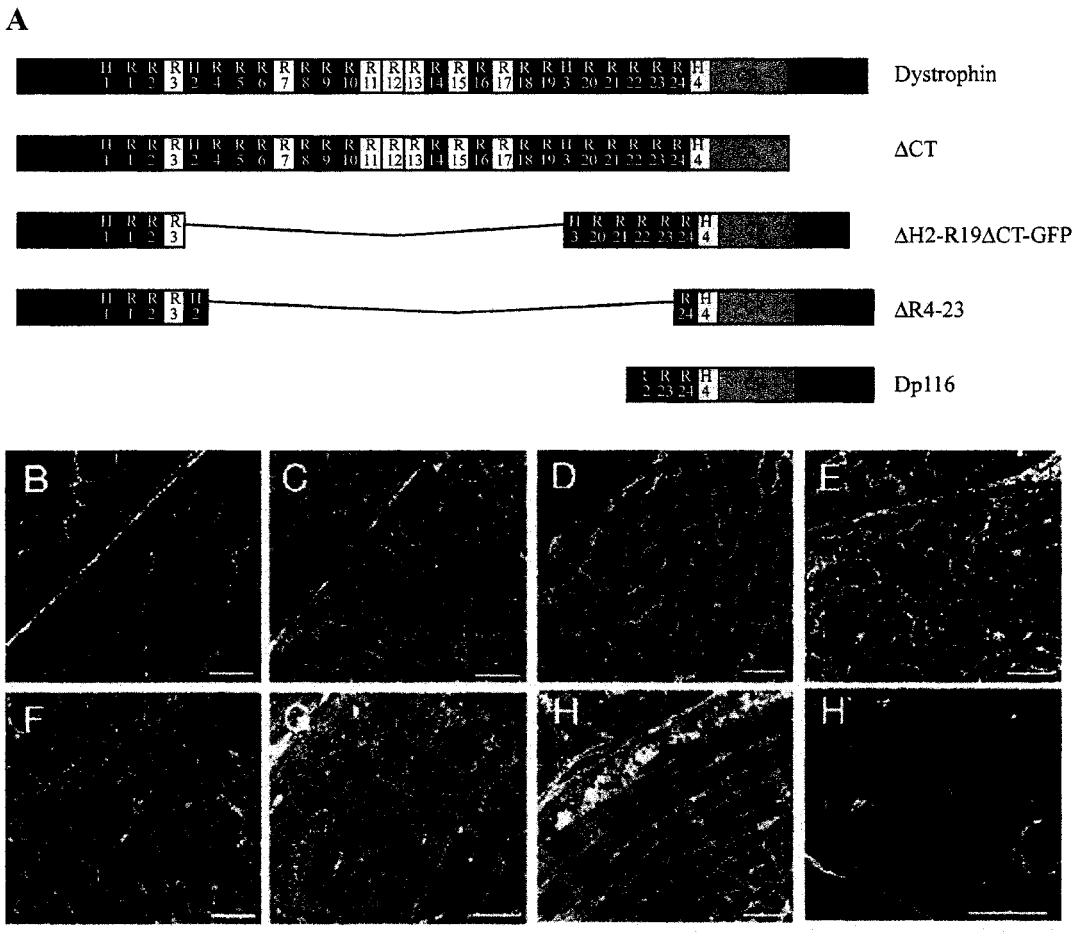


Figure 4.6: Expression of micro-dystrophin ΔR4-23 causes annular myofibril formation. *A)* Domain structure of dystrophin transgenes used in this experiment. Electron micrographs of gastrocnemius muscles from *B)* wild-type, *C) mdx, D) ΔCT:mdx, E) ΔH2-R19ΔCT-GFP:mdx^{4cv}, F) Dp116:mdx^{4cv}, G) ΔR4-23:mdx* 2 weeks old, *H)* ΔR4-23:*mdx*. Muscles were from 3-month old mice unless otherwise specified. *H')* Myofibers from *ΔR4-23:mdx* gastrocnemius muscles stained with toluidine blue show the ring phenotype. Electron micrographs show two adjacent myofibers in cross section to demonstrate the orientation of peripheral myofibrils near the sarcolemma. The myofibrils from normal muscles are oriented parallel to the long axis of the myofiber, as seen in *B-F*. In contrast, the peripheral myofibrils from *ΔR4-23:mdx* muscles are often oriented in concentric rings as seen in *G-H*, generating the ring phenotype seen in the toluidine blue stain. Scale bars: 2 μm (*B-F,H*), 1 μm (*G*), 50 μm (*H'*)

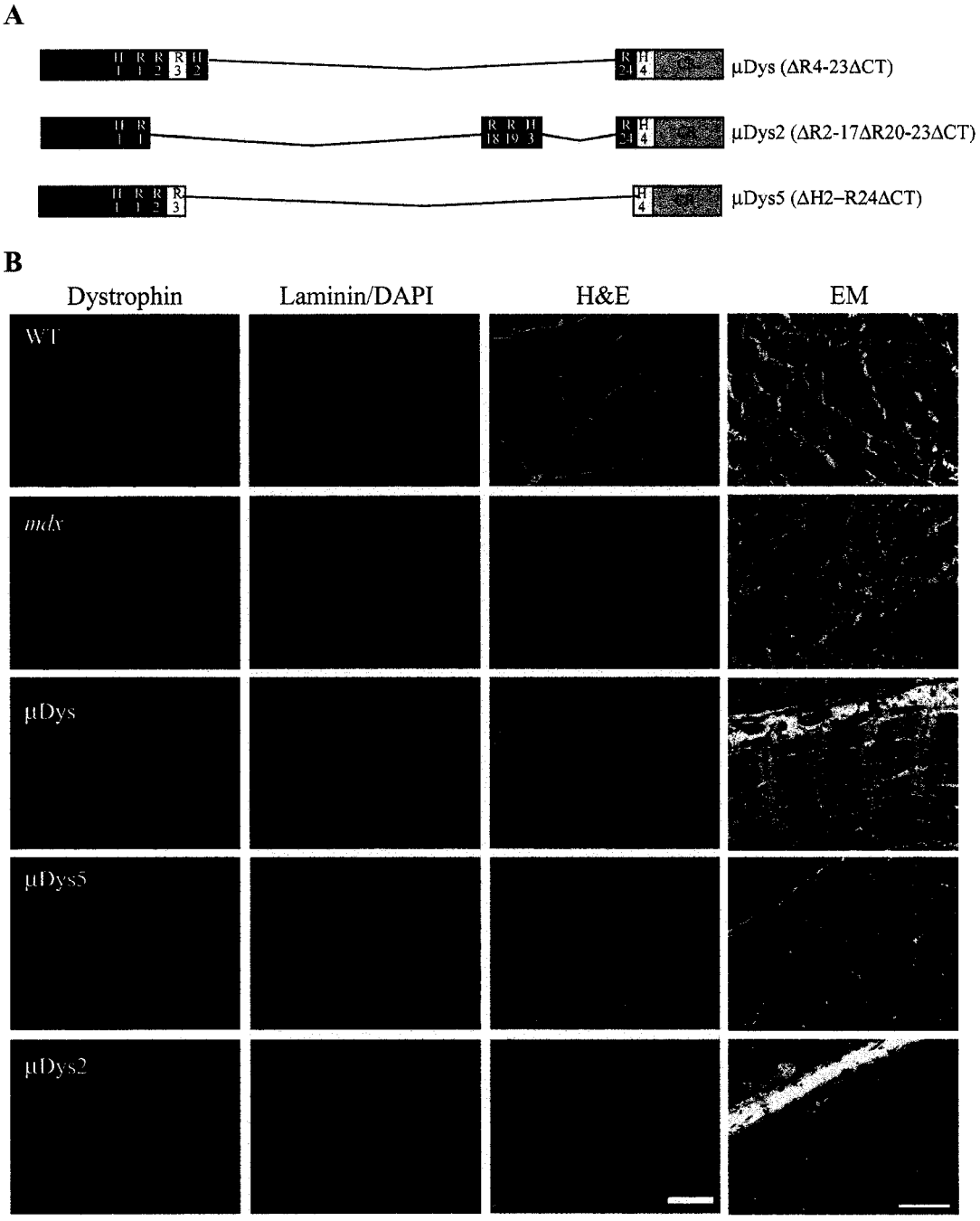


Figure 4.7: Formation of annular myofibrils depends on specific regions of the dystrophin rod-domain. *A)* Domain structure of micro-dystrophin constructs shown in this experiment. *B)* Immunofluorescence staining with polyclonal anti-dystrophin N-terminus and monoclonal anti-laminin β2 chain demonstrate similar expression in all injected muscles. Electron micrographs show annular myofibrils in gastrocnemius muscles injected with control μDys vector but not with novel μDys2 or μDys5 vectors. Scale bars: 50 μM for photomicrographs, 2 μm for electron micrographs.

CHAPTER 5:

Perspectives and future directions

Understanding the pathologic mechanisms that contribute to muscle cell death in the various muscular dystrophies is critical for developing rational therapeutic strategies. It would be particularly important to identify underlying signaling defects that contribute to muscle cell death as these processes could be amenable to traditional pharmacological drug discovery. Many of the different muscular dystrophies are caused by mutations that affect the synthesis or processing of proteins within the DGC (Cohn and Campbell, 2000; Martin, 2005). It is thus attractive to hypothesize that this group of disorders share a common disease mechanism that could be targeted by small molecule therapies. In this ideal case, one could identify a therapeutic target that would be effective for all of these diseases. However, if the mechanism of cell death in DMD is primarily due to a structural defect in dystrophin-deficient myofibers then traditional drug discovery is unlikely to provide an effective treatment. In this case the structural defect would have to be corrected by replacing the defective protein. This goal could be accomplished by genetic replacement or correction, protein replacement, cell therapy, or upregulation of a compensatory protein such as utrophin. All of these possibilities are areas of active research.

A main focus of research in the Chamberlain laboratory is development of gene therapy technologies for DMD. As described in Chapter 1, a major hurdle for the success of this strategy is the development of efficient vectors that can target muscle tissue in a systemic manner without triggering a deleterious immune response. An advantage of this type of therapy is that it does not necessarily require a complete understanding of the mechanism of disease as long as the underlying genetic defect can be corrected. However, the vectors that show the most promise in this regard are not large enough to package the entire dystrophin coding sequence. This limitation has necessitated the development of truncated dystrophin proteins that retain a high degree of functionality. The use of truncated dystrophins necessitates an understanding of the basic structure-function relationships in this protein, so that informed choices can be made in regards to which functional domains should be retained. Thus, we feel that the results presented in Chapters 2 – 4 have implications for basic muscle biology, pathophysiology of muscular dystrophy, and gene therapy.

The development of the Dp116 transgene may prove to be a good model for studying the basic biology of skeletal muscle signaling pathways through the DGC. One of the problems with studies that have looked for signaling abnormalities by comparing normal and dystrophic cells is that one cannot determine if the changes are due to perturbation of signaling associated with dystrophin or if they are secondary consequences of dystrophic pathology. Many signaling processes are likely to be activated in dystrophic muscle as an attempt to maintain homeostasis and

compensate for ongoing muscle damage. In contrast, comparison of *mdx* muscle cells to *Dp116:mdx* muscle cells, which are both dystrophic, could identify signaling that requires specific interaction with the DGC. There is even more potential for discovery using the Dp116 transgene in the dystrophin/utrophin deficient background, as it will be fascinating to elucidate the mechanism by which Dp116 modifies the phenotype of this model.

The fact that expression of Dp116 dramatically extended the lifespan of *mdx:utrn*$^{-/-}$ mice generates the question of the therapeutic potential of this protein. The Dp116 promoter and unique exon lie within intron 55 of the dystrophin gene. Interestingly, the majority of identifiable dystrophin mutations that lead to DMD are located in the central region of the gene and should not affect the expression of Dp116 (Emery and Muntoni, 2003). It would be theoretically possible to target the Dp116 promoter to activate expression of this isoform in skeletal muscle similar to the attempts to identify molecules that can increase expression of utrophin. Alternatively, Dp116 is small enough to be packaged into an AAV vector in a gene delivery approach. Clearly Dp116 would not be expected to be as functional as micro-dystrophin, but would have the advantage of being non-immunogenic in patients with normal expression of Dp116 in Schwann cells. It might also be interesting to test systemic delivery of Dp116 to *mdx:utrn*$^{-/-}$ mice via AAV for purely academic reasons. If our hypothesis is correct that Dp116 does not protect muscle cells from damage, then any positive effect from Dp116 expression would quickly be attenuated as the vector would be lost during myofiber turnover.

More realistically, the results presented in Chapter 3 should provide motivation for further examination of the pathophysiology of combined dystrophin and utrophin deficiency. The fact that expression of Dp116 produced a negative effect on the phenotype of *mdx* mice but a positive effect in *mdx:utrn*$^{-/-}$ mice suggests that the severe pathology of the latter model is due to a distinct mechanism. If true, that mechanism should also be investigated in humans as *mdx:utrn*$^{-/-}$ mice more accurately model the severe course of disease in DMD. Like *mdx* mice, DMD patients have upregulation of utrophin at the sarcolemma, and the degree of upregulation is associated with clinical outcome (Kleopa et al., 2006). For this reason it is unlikely that *mdx:utrn*$^{-/-}$ mice represent a true model of DMD. However, they could still serve as a useful tool if they are found to recapitulate a particular cellular defect, such as a failure of regeneration. If Dp116 restores a signaling pathway that can maintain regeneration this could lead to the identification of an important drug target.

The finding that *mdx:utrn*$^{-/-}$ muscle did not show significant changes in transcript levels involved in known signaling pathways relative to *mdx* could suggest that the worsened phenotype is merely due to a further weakening of the mechanical connection between the sarcolemma and costameres. It is possible that either Dp116 or dystrobrevin makes a mechanical connection to the cytoskeleton that is moderately functional, at a level similar to the utrophin upregulation seen in *mdx* muscle. Functional tests of muscle physiology in *Dp116: mdx:utrn*$^{-/-}$ mice could rule out that possibility if they show no increase in specific force or protection from

contraction induced injury compared to control *mdx:utrn*$^{-/-}$. Another explanation for the paucity of changes in transcript levels could be that the important changes in transcription only take place in the minority of cells within the muscle tissue. As described in Chapter 3, one hypothesis is that restoration of the DGC by Dp116 increased muscle mass via maintenance of regenerative capacity. In this case, signaling through the DGC may lead to changes in transcription specifically in satellite cells and myoblasts, which may be difficult to detect from extracts of whole muscle tissue.

Although nitric oxide has been the most often studied and discussed signaling pathway related to the DGC, our results argue that nNOS is not the most critical signaling component of the complex, at least in mice. For example, truncated mini- and micro-dystrophin constructs that protect muscle from dystrophic damage had greatly reduced nNOS expression at the sarcolemma. We did find an increase in cytoplasmic nNOS in muscles expressing these proteins, so we cannot completely rule out a protective role for nNOS in the prevention of dystrophy in those muscles. However, the cytoplasmic nNOS would presumably function independently of the DGC. Furthermore, Dp116 dramatically ameliorated the phenotype of *mdx:utrn*$^{-/-}$ mice despite failure to restore either cytoplasmic or membrane-associated nNOS expression. This is not to say that nNOS does not have an important function in skeletal muscle. It clearly functions to maintain blood flow to exercising muscle, which raises the point that laboratory mice in cages are not very active. Functional

ischemia due to loss of nNOS-mediated vasomodulation could contribute more to muscle degeneration in a larger, more active animal.

Thomas Rando has proposed the "two-hit" model of muscle cell necrosis, in which functional ischemia combined with an underlying sensitivity to various forms of damage combine to cause myofiber death in dystrophic muscle (Rando, 2001b). The best case scenario for gene therapy with micro-dystrophin is often described as converting a DMD clinical outcome into one resembling a mild BMD. The failure of micro-dystrophin to localize nNOS is a potential concern if it means that the transduced cells, which are likely to be more susceptible to injury than normal healthy cells, are continually subjected to functional ischemia. Although there are definitely certain regions of the rod domain that are required for complete localization of nNOS to the sarcolemma, we were not able to identify a small region that could be substituted to restore this function of the rod domain. It appears that only small deletions of the dystrophin rod domain can be tolerated for nNOS to remain associated with the DGC. It is also possible that the combination of deletions in the rod domain and C-terminal domain precluded nNOS localization in our experiments. Deletion of the C-terminal domain alone did not reduce the expression of sarcolemma-associated nNOS, but could be a factor when combined with deletions in the rod domain. However, we did not feel this was a serious concern as transgenic mice expressing ΔH2-R19ΔCT-GFP, where a GFP fusion replaced the C-terminal domain (Figure 4.6A), still had low level nNOS expression at the sarcolemma (G. Banks, personal communication). This result was similar to what

we showed with transgenic mice expressing ΔH2-R19 with an intact C-terminus. Also, it was reported that viral delivery of ΔH2-R19 with an intact C-terminus also failed to restore nNOS expression in *mdx* mice (Lai et al., 2005).

Finally, the observation of annular myofibrils specifically in the mice expressing ΔR4-23 micro-dystrophin is very interesting. It will be important to understand the basis of this structural deformity and if it has a negative impact on muscle physiology. It seems safe to assume that the annular myofibrils would not contribute to optimal muscle contraction, but they could be a reaction that protects the myofibers from damage. Therefore, the micro-dystrophin constructs that we designed that did not form annular myofibrils should be tested carefully alongside the ΔR4-23 micro-dystrophin. If they are able to protect equally against dystrophic damage but do not form annular myofibrils we speculate that they could generate an increase in specific force.

We are currently witnessing the undertaking of several clinical trials for genetic therapies and new pharmacological strategies to treat DMD. These new experiments are the start of a new phase of therapeutic advances based on the foundation of basic research in animal models of DMD. As our knowledge continues to expand we are optimistic that some combination of these approaches will someday lead to an effective treatment.

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