PATHO-GENETIC CHARACTERIZATION OF THE
MUSCULAR DYSTROPHY GENE MYOTILIN

by

Sean Michael Garvey

University Program in Genetics & Genomics
Duke University

Date:  April 5, 2007
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Gregory A. Cox, PhD

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics & Genomics in the Graduate School of Duke University

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ABSTRACT

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Abstract

Myotilin is a muscle-specific Z-disc protein with putative roles in myofibril assembly and structural upkeep of the sarcomere. Several myotilin point mutations have been described in patients with Limb-Girdle Muscular Dystrophy Type 1A (LGMD1A), myofibrillar myopathy (MFM), spheroid body myopathy (SBM), and distal myopathy, four similar adult-onset, progressive, and autosomal dominant muscular dystrophies—collectively called the myotilinopathies. It is not yet known how myotilin mutations cause muscle disease.

To investigate myotilin’s role in the pathogenesis of muscle disease, I have created and characterized transgenic mice expressing mutant (Thr57Ile) myotilin under the control of the human skeletal alpha-actin promoter. Like LGMD1A and MFM patients, these mice develop progressive myofibrillar pathology that includes Z-disc streaming, excess myofibrillar vacuolization, and plaque-like myofibrillar aggregation. These aggregates become progressively larger and more numerous with age. I show that the mutant myotilin protein properly localizes to the Z-disc, and also heavily populates the aggregates, along with several other Z-disc associated proteins. Whole muscle physiological analysis reveals that the extensor digitorum longus (EDL) muscle of transgenic mice exhibits significantly reduced maximum specific isometric force compared to littermate controls. Intriguingly, the soleus and diaphragm muscles are spared of any abnormal myopathology and show no reductions in maximum specific
force. These data provide evidence that myotilin mutations promote aggregate-dependent contractile dysfunction.

To better understand myotilin function, I also created two separate lines of myotilin domain deletion transgenic mice: one expresses a deletion of the N-terminal domain and the second expresses a deletion of the minimal alpha-actinin binding site. Studies in these mice show that 1) the N-terminal domain of myotilin may be required for normal localization to the Z-disc; 2) interaction with alpha-actinin is not required for localization of myotilin to the Z-disc; and 3) deletion of the alpha-actinin binding site causes an aggregation phenotype similar to that of the TgT57I mouse and myotilinopathy patients.

In sum, I have established a promising patho-physiological mouse model that unifies the diverse clinical phenotypes of the myotilinopathies. This mouse model promises to be a key resource for understanding myotilin function, unraveling LGMD1A pathogenesis, and investigating therapeutics.
In dedication to my grandparents,
Thomas and Edna Garvey,
Harris and Marjorie Skinner,
contributors of The Greatest Generation
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Abbreviations

ATP  adenosine triphosphate
bp   base pair
CK   creatine kinase
DAG  dystrophin-associated glycoprotein
DM   distal myopathy
DMD  duchenne muscular dystrophy
EDL  extensor digitorum longus
EBD  evans blue dye
EM   electron microscopy
EMG  electromyography
F-(actin) filamentous actin
HSA  human skeletal actin
IgL  immunoglobulin-like
kb   kilobase pair
kD   kilodalton
LGMD limb-girdle muscular dystrophy
MD   muscular dystrophy
MDa  megadalton
MFM  myofibrillar myopathy
MGI  Mouse Genome Informatics, © The Jackson Laboratory
MIM  (online) Mendelian Inheritance of Man, ™ Johns Hopkins University
MRI  magnetic resonance imaging
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SBM</td>
<td>spheroid body myopathy</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VCPDM</td>
<td>vocal cord and pharyngeal weakness with distal myopathy</td>
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<tr>
<td>ZASP</td>
<td>z-band alternatively spliced protein</td>
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Chapter 1

1. Introduction to skeletal muscle, muscular dystrophy, and myotilinopathy

1.1 Skeletal muscle structure and function

Muscle refers to a specialized region of fibrous tissue that has the ability to contract, thus facilitating movements of body parts and maintenance of body part stance. There are three types of muscle—skeletal muscle, cardiac muscle, and smooth muscle. Cardiac muscle and smooth muscle facilitate involuntary movements. Cardiac muscle facilitates the continuous contraction, or beating, of the heart to drive blood flow. Smooth muscle facilitates several involuntary movements throughout the body, such as gut peristalsis and blood vessel constriction and dilation. In contrast, skeletal muscle drives voluntary movement, and is thus hardwired to receive signals from the central nervous system. Skeletal muscles are generally cylindrical in shape, with either ends of the cylinder attached to a bone via connective tissue. Skeletal muscles of the human body come in many sizes and serve many functions. For example, contraction of the biceps causes elbow flexion and forearm rotation. Contraction of very fine extraocular muscles
facilitates eye movement. Perhaps the most essential mammalian skeletal muscle function is to coordinate respiration and lung volume dynamics, mediated specifically by the contractile activity of the thoracic diaphragm muscle. In any case, skeletal muscles are similarly and elegantly structured at the cellular level to mediate contraction.

1.1.1 Myofiber structure

An individual skeletal muscle group is composed of many elongated muscle cells, or myofibers, arranged in parallel (Figure 1.1). During development, the myofiber is formed by the syncitial fusion of muscle progenitor cells called myocytes, resulting in a long, multinucleated cell (1;2). Nuclei are located toward the periphery of normal, healthy myofibers. The myofiber is enclosed by a specialized plasma membrane called the sarcolemma. The sarcolemma facilitates nutrient unloading from nearby blood vessels (3), transmits mechanical signals to and from adjacent myofibers (4), and interfaces with neuromuscular junctions to signal muscle contraction (5). The cytoplasmic, or sarcoplasmic, occupancy, of a myofiber is devoted to muscle contraction—housing chemical energy and signaling stores, and most importantly, the contractile apparatus.
Figure 1.1: Schematic of myofiber and sarcomere structure
1.1.2 Sarcomere structure

The individual myofiber is itself composed of many elongated tubes called myofibrils. Each myofibril is surrounded by a membrane called the sarcoplasmic reticulum, responsible for the calcium handling that regulates excitation-contraction coupling and relaxation. The average width of a myofibril is 1-3 μM. In longitudinal view, myofibrils display a repeating array of striations (Figure 1.1). A discrete subset of myofibril striations represents the fundamental unit of contraction—the sarcomere (Figure 1.1). The width of a sarcomere is 2-3 μM, and the average length of a vertebrate myofiber is in the order of centimeters. Thus, vertebrate myofibers can contain tens of thousands of adjacent sarcomeres. The sarcomere can be structurally defined by four classes of parallel filaments, and a perpendicular cross-linking structure called the Z-disc.

1.1.3 The thin and thick contractile filaments

Thin filaments are polymers of the 42 kD globular protein, actin. In vertebrate muscles, two thin filaments form a long, left-handed double-helix, each thread comprised of ~360 actin monomers (6). The actin filaments of skeletal muscle are extremely stable; the barbed end is capped by the CapZ protein at the far end of the Z-disc, and the pointed end is capped by tropomodulin toward the middle of the sarcomere (7-9). CapZ prevents dissociation of actin monomers. Tropomodulin is thought to more actively regulate thin
filament length. F-actin bundling proteins also maintain the higher order structure of parallel filaments.

Anti-parallel and bipolar thick filaments converge at a distinct zone in the center of the sarcomere called the M-line. Thick filaments are polymers containing ~300 molecules of the contractile protein, myosin, with additional nonmyosin proteins. Myosin is a hexamer containing two identical heavy chains (223 kDa each) and two pairs of lights chains (15-22 kDa each) (10). The carboxy (C-) terminal half of each heavy chain adopts a long alpha-helical structure that twists around a second heavy chain. The amino (N-) terminal halves interact with each other and with the light chains to form globular heads that bind and hydrolyze adenosine triphosphate (ATP), and also bind to actin (6). Increased ATP hydrolysis causes a conformation change in myosin, resulting in the displacement of actin toward the myosin tail.

The rapid and regulatory intermolecular sliding of thin and thick filaments generates the force of muscle contraction. In a relaxed state, the myosin-actin interaction is prevented by the interaction of actin with tropomyosin and troponin (11). A contractile event is preceded by the local release of calcium, which then makes actin available for binding to myosin. Actin binding to myosin increases hydrolysis of ATP, and the contractile ‘stroke’ occurs. The signal to release calcium is transduced by excitatory synapses at neuromuscular junctions.
1.1.4 The Z-disc

The Z-discs (also called Z lines and Z bands) are electron-dense structures that define the longitudinal boundary of the sarcomere (12). The Z-disc largely functions to anchor and link the anti-polar thin filaments of adjacent sarcomeres. The Z-disc also anchors the titin filaments of adjacent sarcomeres, and plays a role in nebulin filament stabilization. Filament anchoring at the Z-disc is achieved by a complex network of protein-protein interactions that promote F-actin cross-linking (association of filaments from adjacent sarcomeres) and bundling (association of filaments from the same sarcomere) (13).

While there are numerous actin-binding proteins at the Z-disc, alpha-actinin is the key structural molecule. Through anti-parallel homodimerization and actin binding via its N-terminal domain, alpha-actinin can efficiently cross-link actin filaments (14). The Z-disc is thus able to transmit the lateral force generated by thin-thick filament sliding along the total length of the myofibril (15).

Adjacent myofibrils also contract in unison and are kept ‘in register’ with the help of intermediate filament proteins, such as desmin and vimentin, that tether neighboring Z-discs in separate myofibrils (16;17). The Z-disc is also involved in transducing mechanical signals between whole myofibers. Z-discs transmit contractile force transversely to the sarcolemma, extracellular matrix, and neighboring fibers via a protein complex called the costamere (18;19). Both costameric and inter-myofibril cytoskeletal proteins play integral roles in the proper contractile activity of a myofiber (20;21).
1.1.5 Titin and nebulin filaments

The remaining filaments act as molecular rulers of sarcomere size and support thin and thick filament integrity. The third filament system includes the largest mammalian protein, titin, and titin-associated proteins. Titin is a ~3 MDa protein that spans half the sarcomere. One molecule of titin occupies the whole width of the Z-disc, one adjacent thin filament network, and half of the overlapping thick filament (22-24). Titin is a highly modular protein, containing tandem arrays of three peptide domains: the immunoglobulin-like (IgL), fibronectin-like (Fn-like), and PEVK domains (25). These flexible modules allow titin to act as a molecular spring and directly interact with actin and myosin throughout contraction. Myomesin is a 185 kDa protein at the M-line that can bind to both titin and myosin, serving as a link between these two filaments (26). The titin filament maintains passive tension and defines resting slack length, and also plays a fundamental role in myofibril assembly (27-29).

A fourth filament of the sarcomere is composed of the 800 kDa nebulin protein. Nebulin’s C-terminal 30 kDa inserts into the Z-disc, and the remainder of the protein extends along the length of the thin filament (30). Like titin, nebulin is a highly modular protein containing flexible superrepeats that interact with actin (31). Structural modeling of nebulin suggests that nebulin lies in the central cleft between actin filaments, thus stabilizing F-actin (32). The N-terminal end of nebulin binds to the thin filament capping protein, tropomodulin (33). Nebulin is thought to act as a molecular ruler for thin
filament length. While nebulin and titin serve to modulate logistics of sarcomere size, the power of contraction arises from actin-myosin filament dynamics.

1.1.6 ‘Fast’ and ‘slow’ myofibers

The selective expression of particular sarcomere proteins (i.e., isoforms of myosins, actins, and actinin) and the precise size of certain sarcomere components contribute to the overall contractile phenotype of a given myofiber. Indeed, no two fibers twitch at the same speed. Myofibers generally differentiate into one of three fiber types: 1) slow, oxidative type I fibers; 2) fast, oxidative type IIA fibers; or 3) fast, glycolytic type IIB fibers (34;35). Histological indentification of fiber type is typically facilitated by immuno-staining against fiber-type specific myosin heavy chains. Fast type I fibers usually have thicker Z-discs than slow type I fibers. The increased capillary, mitochondrial density, and citrate synthase activity of slow type I fibers heighten oxidative potential and promote the overall aerobic ‘endurance’ of a given muscle group (36). The increased amount of glycogen stores and increased activity of glycolytic enzymes in fast type II fibers drives decreased time to peak tension—a quicker myofiber contraction—promoting anaerobic ‘sprint’ and ‘power’ performance in a given muscle group. Different modes of innervation contribute to fiber type specificity during development, and different types of physical activity can cause fiber type switching in adult muscle. Myofiber type predominance partly dictates whole muscle group
physiology. Additional factors contributing to whole muscle physiology include the context of biomechanical stimuli and neighboring muscle groups.

1.2 Muscular Dystrophy

1.2.1 Introduction

The muscular dystrophies are a group of inherited skeletal muscle disorders characterized by both clinical signs of progressive muscle weakness and histological signs of myofiber degeneration and regeneration. Canonical muscular dystrophies are myogenic—caused by primary alteration of skeletal myofiber function rather than neurogenic denervation, which secondarily leads to myofiber atrophy and death. Muscle cell wasting in the muscular dystrophies is commonly caused by disruption or loss of proteins associated with the sarcolemma or contractile apparatus. Loss of the sarcolemma-associated protein, dystrophin (DMD; MIM 300377¹), causes the most common muscular dystrophy, Duchenne Muscular Dystrophy (DMD) (37;38). DMD accounts for 2/3 of all muscular dystrophies, occurring in approximately 1 in 3500 live births. DMD is a childhood-onset disease, and patients commonly die by 25 years of age due to respiratory complications. The common treatments for muscle weakness in DMD are

¹ Online Mendelian Inheritance in Man, OMIM™. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), Mar 8 2007. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/
corticosteroids, but these anti-inflammatories cause many side effects. There remains no known cure for muscular dystrophy.

The muscular dystrophies are a clinically and genetically heterogeneous group of disorders. Patients exhibit considerable differences in age at onset of symptoms, distribution of muscle group weakness, and involvement of cardiac abnormalities and inflammatory responses. The muscular dystrophies also show extensive genetic heterogeneity. Disruption of over 30 genes has been shown to cause primary muscle disease (39). Characterization of these genes highlights three important structures in skeletal muscle function: 1) the plasma membrane, or sarcolemma (40;41); 2) the cytoskeleton and nuclear envelope (21); and 3) the contractile apparatus, or sarcomere (42;43).

1.2.2 Sarcomeric origins of muscle disease

Mutations in genes whose gene products localize to the Z-disc or the four filaments of the sarcomere cause several inherited muscle disorders. Muscle diseases of sarcomeric origins are commonly autosomal dominant and characterized histologically by some type of sarcoplasmic protein accumulation. Intriguingly, disruption of components within each sub-structural class of the sarcomere tends to cause similar clinical phenotypes and histo-pathologies. Mutations in thin filament proteins tend to cause severe, congenital
myopathies, for example nemaline myopathy, characterized by electron-dense sarcomeric rods (44). Mutations in thick filament proteins tend to cause tubulofilamentous inclusions or inclusion bodies, and also tend to be associated with cardiomyopathy (45). Alteration of Z-disc proteins and intermediate filament proteins of the Z-disc tend to cause adult-onset myofibrillar myopathies, characterized histologically by myofibrillar protein aggregates (46). These aggregates are larger and more amorphous than those observed in the actin myopathies.

Several thin filament proteins cause muscle disease. Actin (ACTA1; MIM 102610) mutations have been described in actin myopathy (44;47), nemaline myopathy (47;48), core disease (49), and congenital fiber type disproportion (50). Immunohistochemical studies of patient tissue and in vitro studies of these actin mutations suggest that actin is abnormally folded and polymerized, resulting in actin aggregation and formation of the higher-order, multi-protein, rod-like aggregates characteristic of actin myopathy and nemaline myopathy (51). Nemaline myopathy is also caused by mutation of the thin filament-associated proteins, slow alpha-tropomyosin (TPM3; MIM 191030) (52-55), beta-tropomyosin (TPM2; MIM 190990) (56), and slow troponin-T (TNNT1; MIM 191041) (57).

Genetic studies have also demonstrated the importance of the major protein component of the thick filament, myosin. Slow beta-myosin heavy chain gene (MYH7; MIM 160760)
mutations cause Laing-type distal myopathy (58), Hyaline body, or myosin storage, myopathy (59-62), and hypertrophic cardiomyopathy with central cores in skeletal muscle (63). MYH7 gene mutations are also responsible for about 1/3 of all genetically resolved familial cases of hypertrophic cardiomyopathy (with no indication of skeletal myopathy) (64;65). The fast myosin heavy chain gene-2 (MYH2; MIM 160740) causes inclusion body myopathy-3 (66). Slow myosin light chain-3 (MYL3; MIM 160790) and slow myosin light chain-2 (MYL2; MIM 160781) mutations cause hypertrophic cardiomyopathy with skeletal myopathy (67). Functional analyses suggest that pathogenic myosin mutations can either interfere with formation of the myosin hexamer or reduce activity of the myosin globular head domains (68).

Subtle missense mutations and small deletions in the larger titin and nebulin filament proteins also cause muscle disorders. Titin (TTN; MIM 188840) mutations cause tibial muscular dystrophy and limb-girdle muscular dystrophy type 2J (69-71). Several titin mutations specifically interfere with binding to the muscle-specific protease calpain-3 at the M-line of the sarcomere. A number of calpain-3 (CAPN3; MIM 114240) mutations cause limb-girdle muscular dystrophy type 2A (72-75). Pathogenic mutations in the M-line region of titin also affect its C-terminal kinase activity, based on crystal structure modeling (76) and in vivo studies (77). Titin is presumed to phosphorylate its binding partner at the Z-disc periphery, telethonin (TCAP; MIM 604488), which causes limb-girdle muscular dystrophy type 2G (78). It is noteworthy that titin also causes a large
number of dilated cardiomyopathies due to mutations in cardiac-specific splice-forms (79). The nebulin filament is also critical to muscle function; a large number of mutations in the nebulin gene (NEB; MIM 161650) have been identified in nemaline myopathy (80-82).

Genetic studies of myofibrillar myopathy (MFM) have highlighted the importance of several Z-disc proteins in sarcomere function. MFM is a multigenic, autosomal dominant and progressive muscular dystrophy, characterized pathologically by dense patches of sarcomeric disarray and aggregation (46). Several missense mutations in the Z-disc protein myotilin (MYOT; MIM 604103) have been identified in MFM and limb-girdle muscular dystrophy type 1A (83;84). Mutation of the myotilin-binding protein, gamma-filamin (FLNC; MIM 102565) causes an MFM characterized by intrasarcoplasmic aggregates similar to those seen in myotilin-associated MFM (85). MFM is also caused by mutations in the Z-disc proteins, ZASP (z-band alternatively spliced PDZ-containing protein; LDB3; MIM 605906) (86), and desmin (DES; MIM 125660) (87-89), and the chaperone protein, alpha-beta-crystallin (CRYAB; MIM 123590) (90;91).

1.2.3 Limb-girdle muscular dystrophy

Four sarcomeric proteins—myotilin, titin, calpain-3, telethonin—are associated with limb-girdle muscular dystrophy (LGMD). LMGDs are a clinically heterogeneous group
of disorders characterized by initial weakness of proximal limb muscles of the scapular and pelvic girdles, increased serum creatine kinase levels, and absent or reduced deep-tendon reflexes (92;93). At least 20 genes cause limb-girdle muscular dystrophy; 15 have been identified (94). These genes encode many different types of proteins—sarcomeric, sarcolemmal, cytoskeletal, and enzymatic. Both autosomal dominant (LGMD1A – 1G) and autosomal recessive (LGMD2A – 2M) forms have been reported. LGMD sub-types are molecularly defined.

Among the recessive forms, the sarcomeric calpain-3 protease, telethonin, and titin cause LGMD2A, -2G, and -2J, respectively. Several sarcolemmal proteins are disrupted in autosomal recessive LGMD. LGMD2B is caused by mutations in dysferlin (DYSF; MIM 603009), which encodes a membrane repair protein that localizes to the sarcolemma and endoplasmic reticulum (41;95). LGMD-associated deficiency of four of the five sarcoglycan proteins highlights the importance of dystrophin-associated proteins at the sarcolemma: gamma-sarcoglycan (SGCG; MIM 608896) causes LGMD2C (96-98); alpha-sarcoglycan (SGCA; MIM 600119) causes LGMD2D (99;100); beta-sarcoglycan (SGCB; MIM 600900) causes LGMD2E (101;102); delta-sarcoglycan (SGCD; MIM 601411) causes LGMD2F (103;104).

Three golgi apparatus-resident glycosyl transferase genes cause autosomal recessive LGMD: fukutin-related protein gene (FKRP; MIM 606596) mutations cause LGMD2I
a protein O-mannosyltransferase-1 gene (POMT1; MIM 607423) mutation causes LGMD2K (108); and fukutin (FUK; MIM 607440) mutations give rise to LGMD2L (109). These mutations have uncovered a critical glycosylation target protein, alpha-dystroglycan, which interacts with dystrophin at the sarcolemma. Mutations in these glycosyl transferases also cause more severe congenital-type myopathies. Another enzyme, the cytosolic ubiquitin ligase, tripartite motif-containing protein-32 (TRIM32; MIM 602290) causes LGMD2H (110). The most recently identified limb-girdle muscular dystrophy, LGMD2M, has been mapped to 11p12-p13 (111).

Among the dominant forms of LGMD, mutations in the gene encoding the nuclear scaffold proteins, lamins A/C (LMNA; MIM 150330) have been identified in LGMD1B (112). Mutation of caveolin-3 (CAV3; MIM 601253), encoding a sarcolemmal signaling protein, causes LGMD1C (113). Four other loci have been mapped: LGMD1D to 7q (MIM 603511) (114), LGMD1E to 6q23 (MIM 602067) (115), LGMD1F to 7q32 (MIM 608423) (116), and LGMD1G to 4p21 (MIM 609115) (117). LGMD1A is caused by mutations in the myotilin gene (MYOT; MIM 604103) (118;119).

1.2.4 Myotilinopathy

Genetic alteration of the Z-disc protein myotilin is associated with four adult-onset, autosomal dominant muscular dystrophies: myofibrillar myopathy (MFM), limb-girdle
muscular dystrophy type 1A (LGMD1A), spheroid body myopathy (SBM), and a late-onset distal myopathy. These disorders are collectively referred to as the myotilinopathies (Figure 1.2). The first pathogenic myotilin mutation was discovered in a family diagnosed with limb-girdle muscular dystrophy.

*Limb-girdle muscular dystrophy type 1A*

LGMD1A is an autosomal dominant disease that presents with proximal muscle weakness becoming more severe and including distal muscles. Two independent myotilin missense mutations, Thr57Ile and Ser55Phe, cause LGMD1A in a large North American family and an Argentinian pedigree, respectively. A single, large North American family (Duke Family 39; DUK39), with over 70 affected individuals has previously been reported (120). The mean age at onset of patient-reported muscle weakness is 27 years of age. In affected members of this family, serum creatine kinase (CK) levels are increased 1.6- to 9-fold, and half of them exhibit a hypernasal speech pattern. EMG results are consistent with a primary myopathic process, and nerve conduction velocities are normal. Tight heel and reduced knee and biceps tendon reflexes are common. Preliminary analysis of a muscle biopsy from a single patient revealed several nonspecific myopathic traits, including centrally located nuclei, fiber splitting, variation in fiber size, interstitial fibrosis, fatty infiltration, rimmed vacuoles and Z-disc streaming (118). ATPase staining showed no selective myopathic
Figure 1.2: Distribution of myotilinopathy mutations

Distribution of myotilinopathy mutations (above) and schematic of myotilin protein (below). Myotilin contains two immunoglobulin-like domains at the C-terminus. The N-terminus contains a 23-residue hydrophobic stretch (solid black) and serine-rich region (red) between residues 28 and 124. The six myotilinopathy mutations are located at the myotilin N-terminus. Asterisks denote mutations that have been identified in subjects diagnosed with different MDs.
involvement of specific fiber types. Neither cardiac abnormalities nor inflammatory involvement have been reported in Family 39.

LGMD1A has also been described in an Argentinian pedigree with a strikingly similar clinical presentation (119). The four patients examined report proximal leg and arm weakness between 42 and 58 years of age. Serum CK levels are 5- to 15-fold higher than normal. Two of the four patients exhibit a hypernasal speech pattern (119).

*Spheroid body myopathy*

The myotilin Ser39Phe mutation causes spheroid body myopathy (SBM) (121), an autosomal dominant muscle disease first reported in 1978 (122). The average age at onset is in the fourth decade of life, yet onset varies from childhood up until the seventh and eighth decades (121). Like LGMD1A, affected SBM family members tend to present with proximal muscle weakness, and more than half exhibit a hypernasal speech pattern. Some subjects progress gradually whereas others require the aid of a wheelchair or mechanical respiratory care. Light microscopic analysis of biopsied patient muscle reveals trichrome-positive myofilamentous accumulations (subsarcolemmal and sarcoplasmic), centrally located nuclei, and fiber atrophy. Intriguingly, the myofibrillar accumulations primarily occur in slow type I fibers (123). Ultrastructural analysis shows Z-disc streaming-like, amorphous aggregates, or ‘nonstructured cores’ (122).
Myotilin-related myofibrillar myopathy

Several myotilin mutations—Ser55Phe, Ser60Cys, Ser60Phe, and Ser95Ile—have been identified in a subset of MFM patients (83;84). Six patients with one of the above mutations were identified in a screen of 57 MFM individuals (83). Family history has been confirmed in two of the six; no pedigrees are reported. Within the six patients, the mean age at onset was 60 years of age, ranging from 50 to 77 years. Two patients showed evidence of non-coronary cardiac involvement. Three patients presented with proximal muscle weakness greater than distal. Serum CK values ranged from normal to two-fold above normal limits. Clinical, EMG, and/or histologic features implicate peripheral nerve involvement in all six patients. Biopsied muscle shows trichrome-positive accumulations of degraded filamentous material. Streaks of electron-dense material emanating from Z-discs are the predominant ultrastructural pathology. Membrane-bound vacuoles filled with myeloid structures or degraded filaments were also observed by electron microscopy (83). Several of the aforementioned myotilin-related MFM mutations were confirmed in a Spanish cohort of individuals as well (84).

Myotilin-related distal myopathy

A Ser60Phe myotilin mutation was identified as the causative mutation in a French family with autosomal dominant late adult-onset distal leg myopathy (124;125). The age at onset ranged from 50 to 60 years of age. Origin and distribution of muscle weakness included both proximal and distal muscle groups amongst the six affected family
members. Serum CK levels ranged from normal to less than two-fold above normal limits. No abnormal speech was reported, and there is no evidence of cardiac involvement or peripheral neuropathy. Biopsied muscle shows trichrome-staining of subarcolemmal and intrasarcoplasmic masses in both fiber types. Ultrastructural pathology includes large autophagic vacuoles, large zones of myofibrillar disarray, and expansions of dark material from Z-discs. A Ser55Phe myotilin mutation was also described in a single German patient diagnosed with distal anterior leg myopathy (126). The autosomal dominant, adult-onset myotilinopathies involve sarcomeric pathology that includes abnormal accumulations of filamentous or degraded filamentous material. These disruptive aggregates manifest as small patches of Z-disc streaming in LGMD1A and larger myofibrillar plaques in the rest of the myotilinopathies. Despite myopathological similarities and recent genetic triumphs, the myotilinopathies remain clinically ill-defined muscle disorders with considerable inter- and intra-familial variation. Indeed, the Ser55Phe myotilin mutation has been reported as three different disorders. Most importantly, it remains unknown how myotilin mutations cause muscle disease.
1.3 Myotilin

1.3.1 Myotilin structure

Myotilin spans 18.7 kb of genomic DNA, contains 10 exons, and produces a 2244 bp transcript in skeletal muscle and heart (127;128). The 1560 bp coding sequence encodes a 498 amino acid protein with a predicted molecular weight of 57 kD. All myotilinopathy amino acid substitutions occur in the N-terminal domain of myotilin, a domain with no known homology or functional motifs (Figure 1.2). The N-terminal domain does contain a hydrophobic stretch of 23 amino acids buried in a serine-rich domain (27 out of 96 residues) and three potential phosphorylation motifs. The C-terminus of myotilin is homologous to the muscle proteins titin, palladin, and myopalladin and is predicted to form two immunoglobulin-like (IgL) domains with conserved key residues. These domains are most similar to the IgL domains 7 and 8 of titin that penetrate the Z-disc. Myotilin localizes to the sarcomere Z-disc, the electron-dense structure that contributes to sarcomere assembly, actin filament stabilization, and muscle force transmission (128).

1.3.2 Myotilin interactions

Myotilin interacts directly with several Z-disc proteins (Figure 1.3). Yeast two-hybrid analysis, in vitro binding studies, and cell transfection assays have been used to show
alpha-actinin:  
actin:  
gamma-filamin:  
dimerization:  
FATZ-1:  

Figure 1.3: Schematic of myotilin protein and interaction domains.
interaction and co-localization of myotilin and other proteins at the Z-disc. Myotilin binding partners include alpha-actinin, gamma-filamin, FATZ-1 (calsarcin-2; myozenin-1), FATZ-2 (calsarcin-1; myozenin-2), and both monomeric and filamentous actin.

**Alpha-actinin**

Myotilin was originally identified in a yeast two-hybrid library screen using alpha-actinin as the bait clone (128). Alpha-actinin is a well-characterized actin filament cross-linking protein at the Z-disc. Additional yeast two-hybrid and affinity precipitation studies showed that the C-terminal half of alpha-actinin (including spectrin-like repeats-3, -4, and the EF-hand) was necessary for binding myotilin. Conversely, the N-terminal 215 amino acids of myotilin were sufficient for binding to alpha-actinin. The alpha-actinin binding site of myotilin has been limited to residues 80-125 (129). Yeast two-hybrid experiments show that the Thr57Ile mutation does not significantly affect alpha-actinin binding, suggesting that LGMD1A is not associated with inability of myotilin to bind alpha-actinin (118).

**Gamma-filamin**

Myotilin interacts with gamma-filamin through either one or both of myotilin’s IgL domains at the C-terminus (130). Filamins are actin cross-linking proteins localized to the cytoskeleton in non-muscle cells. In skeletal myofibers, gamma-filamin localizes to the sarcolemma and Z-disc in cell fractionation, immunofluorescence and immuno-EM
experiments (131). Gamma-filamin interacts with myotilin through a discrete 78 amino acid insertion sequence in IgL domain 20, which is also sufficient for localization to the Z-disc.

**FATZ proteins**

Myotilin also binds and co-localizes at the Z-disc with FATZ-1 and FATZ-2 (filamin, actinin and telethonin binding protein of the Z-disc). These interactions were first studied by co-immunoprecipitation and then confirmed by yeast two-hybrid analysis (132). Yeast two-hybrid analysis also shows that several N-terminal or C-terminal myotilin truncations inhibit FATZ-1 binding, suggesting that the conformation of full-length myotilin is required for this interaction. The Ser55Phe and Thr57Ile myotilinopathy mutations do not affect FATZ-1 binding (132). The FATZ proteins bind to the calcium- and calmodulin-dependent protein phosphatase calcineurin, and are hypothesized to link Z-disc mechanics with cellular signaling (133).

**Actin**

Myotilin can also be added to a long list of actin-binding proteins. In co-sedimentation, co-immunoprecipitation, and yeast two-hybrid experiments, both actin monomers and filaments are shown to interact with the C-terminal IgL domains of myotilin (129;134).
The C-terminal IgL domains of myotilin also mediates homodimerization, as first suggested by yeast two-hybrid analysis (128). Dimerization of C-terminal fragments of myotilin was confirmed by gel filtration chromatography and mass spectrophotometric analysis (134). Through C-terminal dimerization, myotilin could cross-link alpha-actinin molecules that bind its N-terminal half. Myotilin could also theoretically link alpha-actinin to actin filaments (Figure 1.4). It is not known whether any of the myotilin interactions are regulated. Nonetheless, the multitude of IgL domain-interacting proteins suggests that myotilin is integral to Z-disc structure and function.

1.3.3 Myotilin function and the Z-disc

The Z-disc is an integral and essential component of the myofiber. A myofiber’s efficiency and survival depends on the integrity of the sarcomere, the repeating contractile unit flanked by a protein-dense structure termed the Z-disc, interspersed by long, filamentous thin actin and thick myosin filaments whose intermolecular sliding generates force. The Z-disc is a complex network of proteins serving to anchor anti-polar actin filaments from adjacent sarcomeres (13). The central F-actin cross-linking protein of the Z-disc is alpha-actinin. Alpha-actinin also binds several other Z-disc proteins—nebulin, ALP, FATZ, titin, ZASP, myopalladin, and myotilin (135-140). These proteins themselves have many binding partners at the Z-disc. These interactions create an F-actin cross-linking lattice at the Z-disc and promote overall sarcomeric integrity.
Figure 1.4: Myotilin-related protein interactions at the Z-disc

Schematic of proposed interactions amongst actin filaments (red), alpha-actinin (black), gamma-filamin (green), and myotilin (blue) at the Z-disc.
The precise function of myotilin is not yet known. The identity of several myotilin-interacting Z-disc proteins suggests that myotilin is either involved in myofibril development or stabilization of sarcomere structure in mature myofibers. The stabilization hypothesis is supported by myotilin’s role in actin filament patterning. Overexpression of myotilin in non-muscle cells causes re-patterning of parts of the actin cytoskeleton into stress fibers or actin cables (134). In \textit{in vitro} low-speed centrifugation experiments, myotilin has also been shown to \textit{directly} cross-link and bundle F-actin. These data suggest that myotilin dimerization facilitates the cross-linking of two actin filaments. This cross-linking activity is enhanced by the presence of alpha-actinin, suggesting that they act in concert to stabilize F-actin in mature myofibers. Myotilin also plays a distinct role in F-actin stabilization. Myotilin reduces latrunculin-induced filament disassembly \textit{in vitro}, and myotilin cables are resistant to latrunculin degradation in transfected cells (134). Myotilin’s role in F-actin stabilization is also supported by the predominantly sarcomeric pathology observed in patients with myotilin mutations.

Alpha-actinin and gamma-filamin are both expressed early in myofibril development, presumably working alongside or with titin and nebulin in the formation of nascent Z-discs. In developing muscle cell culture (C2C12 myotubes), myotilin is expressed days after alpha-actinin (134). Myotilin could be modulating sarcomere structure and dynamics later in myofibril development. Overexpression of either the N-terminal half of myotilin or the C-terminal half, containing the IgL domains, causes multi-protein
aggregation, stress fiber-like formation, and myofibril disruption in developing C2C12 myotubes (130;134). The lack of a phenotype in myotilin ‘knockout’ mice, though, suggests that myotilin is not an essential component for myofibrillogenesis (141).

1.4 Mouse models of muscular dystrophy

1.4.1 Introduction to mouse genetics

The experimental mouse has significantly driven the scientific community’s ability to study and integrate molecular, cellular, and physiologic mechanisms of human disease. Mouse models of human disease are also a tremendous resource for testing the efficacy and safety of novel DNA, RNA, and small molecule therapies. Not only do mice and men share similar tissues and organs, but their genomes are very similar, with many highly conserved syntenic blocks (142). Mouse models can be genetically engineered or environmentally induced. Examples of environmental models include the administration of the chemical, rotenone, to model Parkinson’s disease (143), surgical removal of the sciatic nerve to model neurogenic atrophy in hindlimb muscle, and intramuscular administration of cardiotoxin to model myofiber degeneration and regeneration (144). *Genetically* engineered mouse models add the benefits of a 1) a highly controlled experimental context from case to case; 2) specifically modeling a particular disease-associated genotype where inheritance has been determined; 3) testing for gene-gene and
gene-environment interactions; and 4) mapping out individual signal transduction pathways that may contribute to more complex, common disease. The two household varieties of ‘reverse’ genetic engineering in the mouse include targeted deletion and transgenic insertion. These approaches are powerful methodologies for determining gene function \textit{in vivo}.

\textit{Targeted deletion}

Most Mendelian forms of autosomal recessive disease can be modeled by targeted deletion, or ‘knockout,’ of a specific mouse gene (145;146). Targeted deletion can occur in embryonic stem cells (ESCs) through homologous recombination between the endogenous mouse gene and an engineered null allele flanked by homologous targeting sequences or ‘arms.’ The recombination event occurs in ESC culture after electroporation of the targeting construct. After \textit{in vitro} selection for the specific homologous targeting event, ESCs are transferred to the inner cell mass of a recipient blastocyst, or early stage embryo. Blastocysts are then implanted into pseudopregnant females. Chimeric progeny are then screened for ESC contribution to the germ cell lineage. Heterozygous mutants are then intercrossed to create a homozygous knockout mouse carrying both null alleles. Pre-existing knockout alleles can now be queried in stocks of randomly generated, mutant ESC lines maintained by both academic and industrial laboratories.
**Transgene expression**

Most autosomal dominant diseases can be modeled by expression of a mutant transgene over the endogenous, wild-type murine alleles (147;148). Transgenes are microinjected into pronuclei of one-cell embryos. The transgene commonly concatamerizes and randomly inserts into the mouse genome by *nonhomologous* recombination. Embryos are then transferred to pseudopregnant females. The progeny, termed founder mice, are then tested for transgene insertion. Each transgene-positive founder, and eventual ‘line’ of mice, represents a distinct transgene copy number and site of insertion(s). Both the copy number and insertion’s genomic context—euchromatic (transcriptionally permissive) or heterochromatic (transcriptionally silenced)—contribute to expression of total transgene product. It is standard to confirm a single site of insertion in each transgenic line, determine relative levels of transgene expression between lines, as well as confirm the disease-associated phenotypes in multiple lines to rule out the effect of endogenous gene disruption.

The contemporary transgene is a heterologous expression construct that juxtaposes the coding region of a gene of interest downstream of a well-characterized promoter, derived from a second gene. This approach facilitates expression of a transgene without exhaustive characterization of novel promoters and enhancers. The human cytomegalovirus immediate early I gene promoter has long been used to drive ubiquitous expression of transgenes in mice (149). It is best to match promoter regulatory profile
with the endogenous expression profile of the gene carrying the often gain-of-function or toxic mutation to be modeled. Transgenic mice can also be generated by the insertion of long stretches of contiguous genomic DNA selected from yeast artificial chromosome, bacterial artificial chromosome and bacteriophage-packaged libraries. By virtue of endogenous regulatory elements, these genomic constructs better capture spatio-temporal expression and RNA splicing patterns specific to a given gene. These larger vectors can be manipulated to carry disease mutations with novel ‘recombineering’ techniques (150). An alternative method of modeling autosomal dominant disease is by targeted insertion, or ‘knock-in’ of a mutant allele by homologous recombination.

A combination of targeted insertion and transgenic approaches has led to innovative conditional expression methodologies. Conditional knockouts facilitate the investigation of a gene deficiency in specific tissues and at specific embryonic or postnatal stages. Such spatio-temporal regulation is afforded by the Cre/Lox recombinase technology adapted from bacteriophage. Cre is a site-specific recombinase that deletes DNA between two 34-bp loxP element insertions that are aligned in the same direction on the same mouse chromosome (151). To create a conditional knockout, two mouse lines must be created. First, an insertional mutant carrying loxP sites flanking a specific region to be deleted is created by homologous recombination. The second line is a transgenic mouse driving Cre expression by a given spatiotemporal-specific or drug-responsive promoter. Crossing these two mouse lines promotes deletion of the targeted gene in the specific
tissues or cell types and at the specific time the Cre recombinase is expressed. Conditional approaches overcome complications imposed by constitutive expression or deletion, such as embryonic lethality and pleiotrophic phenotypes.

1.4.2 Advances specific to muscular dystrophy

Muscular dystrophy research has benefited greatly from experimental studies in genetic mouse models (152). Mutant mice provide not only a wealth of tissue, but the opportunity to study many muscle groups and pre- and post-‘clinical’ time points in those muscle groups. Physiologic testing of whole mouse muscle and single myofiber function and can be done with great statistical power. The effects of exercise training and limb immobilization are routinely determined in dystrophic models. 7-tesla magnet MRI imaging is now being fine-tuned for high-resolution analysis of whole mice. Mouse models of muscular dystrophy have facilitated testing of cell-based therapy (153), viral-based gene therapy (154), and novel pharmacologic therapeutics (155). The mouse model also serves as a great resource to directly test gene-gene interactions and query modifier loci (156).

Several key genetic resources have been developed for the analysis of muscular dystrophy genes in mice. Many skeletal and/or cardiac muscle-specific promoters heterologously expressing reporter genes such as enhanced green fluorescent protein or lacZ have been thoroughly characterized in transgenic mice. The human skeletal alpha-
actin (HSA; ACTA1) promoter drives robust, skeletal muscle-specific expression in transgenic mice (157). Both skeletal muscle and cardiac muscle transgene expression are driven by the muscle creatine kinase (MCK) promoter (158;159). The murine titin (TTN) promoter also drives dual transgene expression in skeletal and cardiac muscle (160). The use of fast or slow troponin gene promoters can drive fiber-type specific transgene expression (161;162). The myosin light chain 3f (MLC3F) enhancer also drives fiber-type specific reporter gene activity (163). Transgene expression can be limited to embryonic striated muscle with the myogenin (MYF4) promoter (164). This promoter is also activated in response to denervation of adult muscle. The promoter of the myogenic regulation factor, MyoD, drives expression in embryonic fibers and myoblasts, as well as in regenerating adult skeletal muscle (165;166).

Several conditional Cre recombinase lines have also been created. A transgenic mouse line expressing Cre recombinase under the transcriptional control of the human skeletal muscle alpha-actin promoter [Tg(ACTA1-cre)] is available (167). The ACTA1-cre line actually drives Cre expression in both skeletal and cardiac muscle. An MCK-cre line has also been developed to drive conditional recombination in both cardiac and skeletal muscle (168). The myosin light chain 1f (MIC1F) promoter drives Cre specifically in skeletal muscle fibers (169). A MYOD-cre line is activated embryonically and in regenerating skeletal muscle fibers (170).
1.4.3 The *mdx* mouse

Perhaps no mouse mutant better exemplifies the contribution of mouse genetics to human disease research than the *mdx* model of Duchenne Muscular Dystrophy, a severe child-onset muscular disease. The *mdx* (X-chromosome-linked muscular dystrophy) mouse carries a point mutation in exon 23 of the dystrophin gene (171). Genetic engineering aside, the *mdx* mutation is a naturally occurring, spontaneous mutation that causes loss of dystrophin. Dystrophin normally localizes to the costamere, linking the contractile apparatus to the sarcolemma via interactions with cytoskeletal actin and the sarcolemmal trans-membrane protein beta-dystroglycan (172). These two proteins are part of a greater membrane-associated protein complex, the dystrophin-associated glycoprotein (DAG) complex. The sarcolemmal localization of much of the DAG complex is disrupted by the *mdx* mutation (173). The most severely affected muscle of the *mdx* mouse is the diaphragm, showing signs of progressive myofiber degeneration and fibrosis, and lowered maximum isometric force values *ex vivo* (174;175). Centrally located myofiber nuclei, a histological sign of muscle regeneration, are prevalent in *mdx* muscle. Transgenic overexpression of dystrophin restores DAG complex localization and normal diaphragm structure without toxicity (176). Transgenic rescue without toxicity often validates further experimentation of gene replacement therapies.

Overall, though, *mdx* muscle adapts well to dystrophin deficiency by enhanced muscle regeneration, leading to relatively normal life span and normal respiratory function.
compared to DMD patients. *Mdx* muscle structure and function is also thought to be bolstered by upregulation of the dystrophin-like DAG complex protein, utrophin. Expression of a truncated utrophin transgene reduces dystrophic pathology in *mdx* mice (177). Endogenous utrophin upregulation and subsequent stabilization of the DAG complex has been found to be correlated with increased calcineurin-A (CnA) activity. Expression of a constitutively active mutant CnA transgene reduces the amount of central nuclei and fibrosis in *mdx* mice (178). Utrophin knockout, *mdx* double-mutant mice develop a much more severe muscular dystrophy than caused by the *mdx* mutation alone (179;180).

Additional gene interaction crosses with *mdx* mice have led to promising therapeutic targets. For example, a nitric oxide synthase (nNOS) transgene lessens the dystrophic pathology in *mdx* mice (181). Disease progression in *mdx* mice is also slowed by administration of a molecule, HCT 1026, a derivative of the nitric oxide-releasing molecule, flurbiprofen (182). Gene interaction studies show that either transgenic expression of insulin-like growth factor-1 (IGF-1) or targeted deletion of myostatin over the *mdx* mutation increase muscle mass and strength (183;184). Myostatin inhibits myofiber growth. Following propeptide cleavage, myostatin activates the activin receptor IIB, leading to inhibition of myofiber growth through activation of Smad-type transcription factors (185). Intraperitoneal injection of a myostatin blocking antibody or myostatin propeptide cause improvements in muscle mass, size, and strength in *mdx* mice.
Intriguingly, induction of the myostatin antagonist protein, follistatin, in myofiber progenitor cells with deacetylase inhibitors also increases \textit{mdx} myofiber size (188). The trichostatin A deacetylase inhibitor also ameliorates pathology in alpha-sarcoglycan deficient mice (188). Genetic studies of dystrophinopathy in the \textit{mdx} mouse have clearly paved the way toward both a molecular understanding of DMD disease pathogenesis and validation of promising therapeutics relevant to DMD.
1.5 Specific aims of the research study

*In vitro* binding studies and cell culture overexpression studies show that myotilin is an active component of Z-disc structure and contributor to sarcomere function. Unfortunately, current muscle cell culture models do not capture three fundamental aspects of skeletal muscle biology: vascularization, innervation, and contraction. The lack of faithful contraction in myotubes is an especially huge obstacle to studying sarcomere protein function. Development of an LGMD1A mouse model will help to elucidate myotilin function, detail pathogenesis, and promote therapeutic development. *In vivo* analysis of myotilin and the myotilinopathies will highlight and perhaps converge on mechanisms of MD caused by dysfunctional sarcomere protein expression or activity, while also contributing to our knowledge of normal muscle cell structure and function. Indeed, it is our lack of knowledge concerning normal muscle biology that has hindered efforts to treat MD.

**Specific Aim 1. Extend the histological and immunohistochemical analysis of LGMD1A tissue.**

I will further examine histology and ultrastructure of LGMD1A muscle from the large North American family, Duke Family 39 (DUK39). I will also compare clinical outcomes and pathology in two LGMD1A subjects—one moderately and one severely affected.
Specific Aim 2. Construct and analyze a transgenic mouse model of myotilinopathy.

To further address the pathogenesis of myotilinopathy, I will create a transgenic mouse model expressing the Thr57Ile myotilin mutation causing LGMD1A in Duke Family 39. Analysis of transgenic mice will include microscopic evaluation, determination of myotilin transgene product and sarcomeric protein abundance and localization, and physiologic analysis of whole, intact muscles.

Specific Aim 3. Characterize in vivo effects of myotilin domain deletions.

I will examine the effects of two additional myotilin transgenes: one deleted for the N-terminal domain (amino acid residues 1-79; TgΔNT) and a second deleted for the minimal alpha-actinin binding site (amino acid residues 80-124; TgΔABS). A TgΔNT mouse will address whether dominant effects result from loss of the entire N-terminal domain, and whether these effects are similar to myotilinopathy missense mutations that occur in this domain. A TgΔABS mouse line will help determine the significance of the myotilin-actinin interaction. Alpha-actinin is expressed early during myofiber differentiation and may help recruit myotilin to the Z-disc. The TgΔABS mouse will also address the importance of myotilin and alpha-actinin’s cooperativity in cross-linking and stabilizing F-actin bundles at the Z-disc.
Chapter 2

2. Further characterization of the LGMD1A phenotype: clinical, microscopic, and immunohistochemical analyses

2.1 Introduction

Myotilin mutations have been reported in four autosomal dominant muscle diseases: limb-girdle muscular dystrophy type 1A (LGMD1A), spheroid body myopathy (SBM), myofibrillar myopathy (MFM), and distal myopathy, collectively termed the myotilinopathies. The myotilinopathies are all caused by missense mutations that affect the N-terminal domain of myotilin. SBM and MFM are defined histologically by the presence of large myofibrillar aggregates (46;122). Aggregation is also observed in muscle of affected members of a family with myotilin-related distal myopathy (124). Gross intramyofiber aggregation has not been observed in LGMD1A muscle. The myotilinopathies also exhibit substantial clinical heterogeneity, evidenced by significant differences in age at onset, severity and progression of disease, muscle group origin of weakness, and muscle group distribution of muscle wasting. The involvement of
hypernasal speech, cardiomyopathy, inflammation, and neuropathy is also varied in the myotilinopathies.

Both the clinical heterogeneity of the myotilinopathies and the intrafamilial variation of symptoms in LGMD1A Duke Family 39 prompted a more detailed phenotypic analysis. In this chapter, I present clinical, ultrastructural and immunohistochemical findings from muscle biopsies of two individuals from Family 39 expressing the Thr57Ile myotilin mutation.

2.2 Results

2.2.1 Light Microscopy

Muscle biopsies were obtained from two affected members of LGMD1A Family 39: quadriceps muscle biopsy from a 24-year old moderately affected individual (subject I), and quadriceps and deltoid muscle biopsies from a 36-year old, severely affected individual (subject II). Relative affection status was based on clinical data presented in Table 2.1. Subject I muscle contains rimmed vacuoles and small intrasarcoplasmic aggregates (Figure 2.1). Subject II deltoid muscle shows evidence of more numerous rimmed vacuoles and aggregates (Figure 2.1). Myofibers containing aggregates were
### Table 2.1: Clinical comparison of two LGMD1A subjects

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<td>Site of biopsy</td>
<td>Quadriceps</td>
<td>Deltoid &amp; Quadriceps&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup>3-fold above upper normal

<sup>b</sup>Muscle strength scores are based on the British Medical Research Council grading scale of six categories, 0 thru 5; 5=normal muscle strength, 4=active movement against gravity and resistance, 3=active movement against gravity alone.

<sup>c</sup>Quadriceps muscle biopsy revealed 100% adipose replacement.

nd=not determined
originally identified as target cells. Subject II quadriceps muscle had been fully replaced by adipose tissue (data not shown).

2.2.2 Electron Microscopy

Myofibrillar disarray comprises much of the LGMD1A ultrastructural pathology. Analysis of longitudinal sections by electron microscopy reveals two prominent pathologies—Z-disc streaming and autophagic vesicles (Figure 2.1 & 2.2). Muscle from the more severely affected LGMD1A subject displays remarkably increased areas of Z-disc streaming compared to muscle from the moderately affected subject (Figure 2.1). Z-disc streaming bodies are made up of amorphous, electron-dense granules and lack normal sarcomeric architecture. Z-disc streaming occurs in numerous patches of variable size in LGMD1A muscle. Misaligned myofibrils are common and often occur near patches of Z-disc streaming in LGMD1A muscle. LGMD1A ultrastructure also includes larger areas of myofibrillar aggregation commonly observed in the other myotilinopathies (Figure 2.2).

LGMD1A muscle demonstrates large numbers of autophagic vesicles (Figure 2.2). These vacuoles contain lysosomes and myeloid bodies with little evidence of filamentous inclusions. Autophagic vesicles are present in both the sarcoplasmic and subsarcolemmal zones of LGMD1A muscle. Near the membrane, vacuoles contain mitochondrial and
Figure 2.1: Microscopic evaluation of LGMD1A muscle

Light microscopic and ultrastructural evaluation of two LGMD1A muscle biopsies. (A) Haematoxylin and eosin (H & E)-stained cross-section of quadriceps muscle from the moderately affected subject I shows the presence of rimmed vacuoles (white arrow) and intrasarcoplasmic aggregation (white arrowheads). (B) H & E-stained cross-section of deltoid muscle from the more severely affected subject II shows more vacuolation (white arrows) and aggregation (white arrowheads). (C) Gomori trichrome-stained deltoid muscle from subject II demonstrates the prevalence of aggregation in one myofiber. (D) Z-disc streaming and myofibrillar vesicles are present in muscle from subject I. (E) Z-disc streaming is more prevalent in muscle from subject II. Autophagic vesicles are also present in greater numbers. Scale bars: 50-μm (A—C); 2-μm (D, E).
Figure 2.2: Ultrastructural pathology of LGMD1A muscle

Myofibrillar pathology underlies LGMD1A muscle ultrastructure. Longitudinal thin sections were prepared from subject II deltoïd muscle biopsy. (A, B) Large tracts of myofibrillar aggregation (ma) can be found in addition to Z-disc streaming (zs). The aggregates are amorphous and lack membranous structures. (C) LGMD1A muscle also contains myeloid-type inclusion vesicles and often irregular arrays of membranous material. (D) Just beneath the sarcolemma (s), mitochondria (m) are depleted by autophagic vesicles (v). Scale bars: 2-μm.
other organellar remnants. Subsarcolemmal mitochondria adopt many irregular shapes and sizes in LGMD1A muscle. Myofibrillar vacuoles located within the areas of Z-disc streaming pathology often contain the amorphous material common to Z-disc streaming. Intact single Z-discs can also be found in these vacuoles.

2.2.3 Immuno-EM

Myotilin localizes abundantly to the dense patches of Z-disc streaming in LGMD1A muscle (Figure 2.3). Myotilin also localizes properly to the Z-disc, yet staining is appreciably reduced near areas of Z-disc streaming. Minimal staining of I-band components is not uncommon. Myotilin also localizes to the costamere, the myofiber component that links the Z-disc to the sarcolemma (Figure 2.3). Myotilin staining excludes M-line, sarcoplasmic reticulum, and transverse tubules. The heavy myotilin staining of the Z-disc streaming pathology further implicates Z-disc abnormalities in the primary disease mechanism of LGMD1A.

2.2.4 Immunohistochemistry

The dystrophin-associated glycoprotein (DAG) complex is unaffected in LGMD1A muscle. Several DAG complex constituents, including dystrophin, alpha-sarcoglycan, gamma-sarcoglycan, and beta-dystroglycan are properly expressed in LGMD1A muscle, as determined by multiplex western blotting (Figure 2.4). Dysferlin, the LGMD2B gene
Figure 2.3: Immuno-EM localization of myotilin

Immuno-gold electron microscopy of myotilin localization in LGMD1A muscle. (A,B) Myotilin localizes heavily to the Z-disc streaming pathology. (C) Myotilin also localizes to the costamere (c), the protein-rich complex that links the Z-disc (z) to the sarcolemma (s). Normal Z-disc staining is also observed. Scale bars: 1-μm.
that encodes a muscle membrane repair protein, and calpain-3, the LGMD2A gene that encodes a muscle-specific protease, are expressed at normal levels in LGMD1A muscle. LGMD1A muscle does show reduced levels of the laminin-alpha-2 chain, as determined by western blot; however, indirect peroxidase labeling of transverse sections of LGMD1A muscle demonstrates normal localization of the laminin-alpha-2 chain at the sarcolemma (Figure 2.5). Normal immuno-labeling is also observed for dystrophin, alpha-, beta-, gamma-, and delta-sarcoglycans, beta-dystroglycan, beta-spectrin, dysferlin, caveolin-3, and emerin (data not shown). The laminin-gamma-1 chain, however, is severely and uniformly reduced in LGMD1A sections compared to control sections. Labeling of the beta-1-chain also appears weak and variable in LGMD1A muscle. These data demonstrate that the DAG complex is intact in LGMD1A muscle.

2.3 Summary and discussion

LGMD1A muscle displays extensive myofibrillar disarray, Z-disc streaming, and an abundance of autophagic vesicles. The extent of myofibrillar pathology correlates with increased muscle weakness. I also show evidence of intrasarcoplasmic aggregation—a phenotype common to the myotilinopathies. Immuno-EM shows that myotilin localizes to the Z-disc streaming bodies in LGMD1A muscle. While the levels of several laminin chains are reduced in LGMD1A muscle, the expression of multiple members of the
Figure 2.4: Abundance of MD-related proteins in LGMD1A muscle

Multiplex western blot analysis of LGMD1A deltoid muscle lysate from subject II (1A) and control muscle lysates (C). Normal bands are seen for dystrophin, dysferlin, calpain-3, laminin-alpha-2, alpha-sarcoglycan, beta-dystroglycan, and gamma-sarcoglycan. Multiple, fainter bands are observed for laminin-alpha-2 chain in the LGMD1A subject, but this is a non-specific result commonly seen in patients with active muscle pathology.
Immunohistochemical labeling of laminin chains in transverse sections of control and LGMD1A muscle sections. Control muscle (left panels) shows clear, uniform labeling of the alpha-2-, gamma-1-, and beta-1-laminin chains. Muscle from subject II with LGMD1A (right) demonstrates normal labeling for laminin-alpha-2 chain, but expression of laminin-gamma-1 chain is severely and uniformly reduced. Labeling of the laminin-beta-1 chain appears weak and variable when compared to the control section. Scale bars, 100-μm.

Figure 2.5: Immunolocalization of laminin chains
dystrophin-associated glycoprotein (DAG) complex is normal. These data confirm that myofibrillar aggregation is the pathological hallmark of the myotilinopathies, and suggest that sarcomeric dysfunction may be the cause of muscle weakness in the myotilinopathies.

2.3.1 Myofibrillar aggregation in LGMD1A

Ultrastructural analysis of LGMD1A muscle suggests a primary defect in Z-disc function. Decreased actin tethering at the Z-disc could lead to the abundance of streaming bodies and larger bodies of myofibrillar aggregation. It has been proposed that Z-disc streaming also reflects primary disease processes in additional muscle disorders. The nemaline myopathies, characterized by streaming-like rods, are caused by mutations in five thin filament-associated proteins of the sarcomere: alpha-tropomyosin-3 (52), alpha-actin (47), nebulin (81), troponin TI (57), and beta-tropomyosin (56). Myotilin localizes heavily to the Z-disc streaming bodies (Figure 2.3) and to nemaline rods (189). Nemaline-like rods can be discerned in LGMD1A muscle, but the amorphous streaming of Z-discs in LGMD1A muscle lacks the internal structure of nemaline rods. Nonetheless, the abundance of these myofibrillar defects in nemaline and LGMD1A muscle suggests that they are not merely non-specific pathologies.
An equally prominent pathological feature of LGMD1A muscle is the presence of extensive sarcoplasmic and subsarcolemmal vacuoles. Similar vacuoles are found in patients with inclusion body myopathies, and myotilin testing is suggested in such cases of suspected, yet unknown genetic etiology. Rimmed vacuoles may result from the sequestration and clearing of the myofibrillar aggregates. Intramyofibrillar vacuoles are likely to contribute to muscle weakness.

2.3.2 The LGMD1A sarcolemma

Despite the myofibrillar pathology, LGMD1A muscle exhibits intact and normal plasma membranes, or sarcolemma. The expression and localization of multiple muscle membrane proteins, including the DAG complex, is normal in LGMD1A tissue, suggesting that the basic mechanism of myotilinopathy is different from many of the autosomal recessive LGMDs and Duchenne muscular dystrophy. The reduced sarcolemmal staining of the laminin-gamma-1 and laminin-beta-1 chains in transverse sections of LGMD1A muscle is likely a non-specific phenotype. Reduced laminin staining has also been reported in LGMD2I, Bethlem myopathy, facioscapulohumeral muscular dystrophy, and Emery-Dreifuss muscular dystrophy (190;191). Altogether, these data suggest that therapeutics designed to promote sarcolemmal maintenance and repair may not be useful in treating myotilinopathies.
Myotilin mutations may act at the interface of the sarcolemma and the Z-disc—the costamere. The costamere functions to tether the myofibrillar apparatus to the sarcolemma, to disperse the force of contraction throughout the sarcolemma, and possibly to transduce signals (19). Myotilin localizes to the costamere by immuno-EM. Previous reports of patchy staining of myotilin at the sarcolemma of human muscle by peroxidase staining (128) and indirect immunofluorescence (134) may reflect its localization at the precisely spaced costameres along the sarcolemma. Two of myotilin’s binding partners are associated with sub-sarcolemmal or sarcolemmal proteins: FATZ-1 interacts with the transmembrane protein, beta-1 integrin; gamma-filamin interacts with the subsarcolemmal proteins, gamma- and delta- sarcoglycan (131;132). In complex with these proteins, myotilin could influence cytoskeletal or cosmeenersic actin dynamics. Because proper costamere function depends on interactions with actin, myotilin mutations could weaken the attachment of Z-discs to the sarcolemma. While no gross pathology was identified at costameres in LGMD1A muscle, the possibility of costameric dysfunction remains.

This hypothesis is supported by the fact that mutations in desmin, an intermediate filament protein of the costamere, cytoskeleton, and Z-disc, cause muscle disease in a subset of myofibrillar myopathy (MFM) patients (87;89). MFM is defined histologically by myofibrillar dissolution and extensive aggregation of sarcomeric proteins. A total of six MFM patients have been described with mutations in the N-terminal domain of
myotilin (83). One MFM patient carries the same myotilin mutation (Ser55Phe) identified in the Argentinian LGMD1A family. Clinical overlap between MFM and LGMD is extensive, hence the need for mutation and histological analyses to test for myotilinopathy. I show here, for the first time, MFM- and SBM-like aggregates in LGMD1A muscle.

2.3.3 Phenotypic heterogeneity

Clinical data highlight the intrafamilial variation and clinical complexity in the myotilinopathies. I have presented a detailed study of muscle biopsies from two affected family members in LGMD1A Duke Family 39—one moderately and one severely affected. Vacuolation, myofibrillar aggregation and Z-disc streaming are more prevalent in the subject showing greater muscle weakness and hypernasal speech. Despite an earlier age of onset, subject I has had a slower rate of progression of muscle weakness, less severe muscle pathology, and no hypernasal speech. Therefore, early age at onset may not necessarily indicate faster progression of severity in LGMD1A.

Interfamilial variation is also striking in the myotilinopathies. It is surprising that the six MFM patients with myotilin mutations also show signs of cardiomyopathy, neuropathy, and inflammation—symptoms not observed in LGMD1A. Modifier genes within the LGMD1A families may prevent onset of cardiomyopathy or severe neuropathy. While
the cardiomyopathy is absent in LGMD1A patients, the myopathy is heightened—onset is decades earlier than in MFM patients, and the number of rimmed vacuoles and extent of Z-disc streaming is greater. These data suggest that modifying genes or environmental factors and lifestyle lessen the impact of myotilin mutations on skeletal muscle in MFM patients reported to date. Clinical heterogeneity may also result from the varying effects of the separate, yet similar myotilin missense mutations.

It is likely that genetic modifiers are contributing to phenotypic heterogeneity in Family 39 and in other MD pedigrees with extensive clinical variability. One promising candidate genetic modifier is the alpha-actinin isoform, alpha-actinin-3, which localizes to the Z-disc and likely cross-links actin filaments. Alpha-actinin-3 does not itself cause MD, but this gene is not expressed in 18% of normal, healthy Caucasians due to homozygosity for the Arg577Stop allele (192). The functional allele is significantly associated with power performance in Olympic athletes, and the null allele is associated with endurance performance (193). Could either allele also be associated with a more severe clinical metric in MD patients? Preliminary screening of 72 LGMD1A subjects in Family 39 shows that the alpha-actinin-3 null allele is segregating, and 35% are homozygous for the null allele (data not shown). The next step forward will be to establish heritability of clinical severity and test for association.
I conclude that myofibrillar aggregation is the leading contributor to muscle weakness in the myotilinopathies. This same pathology is the defining feature of MFM. MFM is also caused by mutations in desmin, alpha-beta-crystallin, ZASP, and gamma-filamin. Several lines of research suggest that the mechanism of aggregation in desmin- and gamma-filamin-related MFM is different than in the myotilinopathies (126). The desmin and gamma-filamin mutants oligomerize and aggregate in vitro, suggesting a primary defect in protein folding and deregulated self-association (85;194). Myotilin mutations do not cause such heightened self-association in vitro, and most likely promote F-actin destabilization at the Z-disc. However, the resultant aggregate pathology in all MFMs is both constitutionally and spatially similar in the diseased muscle cell. Thus, intervention aimed at reducing non-specific myofibrillar aggregation may be successful in all myofibrillar myopathies. Testing the efficacy of different types of exercise and experimentally validated anti-aggregation drugs in a myotilin transgenic mouse model would be invaluable.

2.4 Material and Methods

Both subjects in this study are affected members from LGMD1A Duke Family 39. Clinical characteristics of these subjects are shown in Table 2.1. This study was
conducted with oversight from the Duke University Medical Center Institutional Review Board.

2.4.1 Subject I

Subject I is a Caucasian male who was 24 years of age at the time of biopsy. He had been active in sports when in high school but noticed a waddling quality to his gait 3 years prior to biopsy. He next encountered difficulty carrying heavy objects, especially up a flight of stairs. His neurological exam revealed no evidence of dysarthria or nasal speech. Examination of cranial nerves and facial muscles was normal. Motor exam revealed tight heel cords which could be passively stretched to neutral. No weakness of the proximal or distal upper extremities was noted. In the lower extremities there was 4 out of 5 weakness of the hip flexors, extensors, and hamstrings (British Medical Research Council scale). All other lower extremity muscles had normal strength. The subject could perform 5 deep squats but had difficulty on the 4th and 5th. He was able to do 5 sit-ups. Sensory exam was normal. Deep tendon reflexes were 2+ (where 2=normal and 3=brisk but not necessarily pathological) in the upper extremities and at the knees, and trace at the ankles. I have classified subject I as moderately affected.
2.4.2 Subject II

Subject II is a Caucasian female who was 36 years of age at the time of muscle biopsy. She reported the onset of weakness, pain, and easy fatigue of her back muscles beginning at age 32. She reported difficulty climbing stairs and standing from a seated position. She stated that she could bend over to pick up an object from the floor but was unable to stand from a squatting position. She reported no swallowing difficulties or arm weakness. Her neurological exam revealed occasional nasal speech. Examination of the cranial nerves was normal. Motor exam revealed no scapular winging. Her heel cords could be passively stretched to neutral. Deltoids, quadriceps, and tibialis anterior muscles showed the greatest weakness, all graded 3 out of 5 on the BMRC scale. The sensory exam was normal to all modalities. Deep tendon reflexes were 2+ in the upper extremities, 1+ (where 1=hypoactive reflex and 2=normal) at the knees, and absent at the ankles. She displayed both a Trendelenberg gait and bilateral foot drop. I have classified subject II as severely affected.

2.4.3 Electron microscopy

Biopsied quadriceps muscle (subject I) and both quadriceps and deltoid muscle (subject II) specimens were fixed with 4% glutaraldehyde in 0.1M cacodylate buffer for one hour. They were washed in 0.1M cacodylate buffer containing 7.5% sucrose, 3 X 20 min each wash. Specimens were post-fixed in 1% osmium tetroxide in cacodylate buffer,
dehydrated through a graded series of ethanol, followed by propylene oxide, and then embedded in PolyBed 812 epoxy resin. Specimens were baked overnight at 60 °C. Ultra-thin sections (60-90 nm) were cut with a diamond knife and post-stained with uranyl acetate and lead citrate. Sections were examined in a Philips EM 400 or CM 12 electron microscope.

2.4.4 Immuno-EM

Fresh deltoid muscle (subject II) specimens were fixed in 4% paraformaldehyde for four hours, embedded in LR white, and baked overnight at 50 °C. Thin sections were blocked with 5% fetal bovine serum in phosphate-buffered saline (PBS), and then incubated with a myotilin peptide antibody for 60 min (1:100, rabbit polyclonal, against myotilin residues 473-488, CVKQAFNPEGFQRLLAQ). Sections were then washed extensively in PBS and incubated for 30 min with 10 nm colloidal gold-conjugated goat anti-rabbit antibody. After washing with PBS and then water, sections were post-stained with uranyl acetate, and examined in a Philips EM 400 or CM 12 electron microscope.

2.4.5 Immunohistochemistry

Multiplex western blot detection of muscle disease proteins has been described previously (195-197).
Note: Clinical testing was performed in collaboration with Jeffrey M. Stajich, Dr. James M. Gilchrist, P. Craig Gaskell, and Dr. Marcy C. Speer (Duke University Medical Center, Durham, North Carolina). Multiplex western blotting was performed in collaboration with Dr. Louise V.B. Anderson (University of Newcastle upon Tyne, United Kingdom).
Chapter 3

3. Patho-physiological characterization of an LGMD1A mouse model expressing a Thr57Ile myotilin transgene

3.1 Introduction

Several lines of *in vivo* evidence suggest that myotilin mutations do not act through a loss-of-function mechanism. Comparison of LGMD1A and control muscle sections stained with myotilin antibody shows no difference in the level of expression at the Z-disc. Western blot also confirms no reduction in the expression of myotilin in LGMD1A muscle (118). Cloning and sequencing of myotilin exon 2 reverse transcription-PCR (RT-PCR) products from LGMD1A muscle shows that wild-type and mutant transcripts are equally abundant. Therefore, the Thr57Ile myotilin mutation has no dominant-negative effect on total myotilin (mutant + wild-type) expression.

Loss-of-function is also ruled out by studies of a myotilin knockout mouse. Cre/Lox-mediated deletion of myotilin in mice does not affect muscle structure or function (141).
LoxP sites were designed to flank myotilin exon 3 at the endogenous locus in ESCs. Exon 3 was removed by crossing chimeric mice with a transgenic EIIa-cre mouse ubiquitously expressing Cre. Homozygous deletion of exon 3 results in a valid knockout—myotilin cannot be detected by RT-PCR, northern blot, or western blot. Myotilin knockout muscle morphometry and ultrastructure are normal. Grip strength testing of 10-month and older myotilin knockout mice showed that forelimb peak force measurements are comparable to controls. Voluntary running activity and endurance was also unaffected. Myotilin loss may be compensated for by the functional redundancy or upregulation of similar Z-disc proteins (141). Alpha-actinin and gamma-filamin are two additional F-actin bundling proteins that are highly expressed at the Z-disc and may be capable of compensating for loss of myotilin. Gamma-filamin has been shown to be upregulated in several MDs, including DMD, LGMD2C, and in mdx, gamma-sarcoglycan knockout, and delta-sarcoglycan knockout mice (130).

I hypothesize that transgenic expression of mutant myotilin in mice will recapitulate symptoms of muscular dystrophy. LGMD1A and the myotilinopathies are autosomal dominant disorders. Autosomal dominant diseases are commonly modeled in the mouse by transgenic expression of a mutant or toxic cDNA. In a transgenic mouse, the wild-type allele on the homologous chromosome in humans is analogous to the wild-type, endogenous gene on homologous chromosomes in the mouse.
A thorough molecular dissection of LGMD1A and myotilinopathy disease etiology has largely been limited by the availability of human tissue and the lack of an experimental model system. To address these needs, I created a transgenic mouse model by expressing the human myotilin cDNA bearing the LGMD1A Thr57Ile mutation on a wild-type murine myotilin background. These transgenic mice reproduce many of the symptoms and pathology associated with the myotilinopathies—Z-disc streaming, myofibrillar aggregation, and muscle weakness. This mouse model not only unifies the diverse phenotypes of the human myotilinopathies, but also promises to be a key resource for understanding myotilin function, unraveling LGMD1A pathogenesis, and investigating possible therapeutics.

3.2 Results

3.2.1 Generation of myotilin transgenic mice

I modeled the autosomal dominant disease LGMD1A by co-expressing a mutant human myotilin transgene in the presence of normal levels of endogenous wild-type murine myotilin. The mutant transgene contains the human myotilin cDNA with the LGMD1A Thr57Ile point mutation, cloned downstream of the skeletal-muscle specific human skeletal alpha-actin (HSA) promoter (Figure 3.1). The human cDNA was used because there are sequence differences between murine and human myotilin near the mutation.
Figure 3.1: Transgene design and expression. (A) The human myotilin cDNA was cloned downstream of the skeletal-muscle specific human skeletal actin (HSA) promoter and the VP1 splice donor and acceptor sites. The human myotilin cDNA includes the full-length coding region (gray box) and 5′- and 3′-UTRs (white boxes). A c-myc epitope tag (red line) was appended to the myotilin N-terminus. Both wild-type (TgWT) and Thr57Ile point mutant (TgT57I) myotilin transgenic mice were generated. (B) The transgene was genotyped by PCR using a forward HSA primer and a reverse myotilin 5′-UTR primer. +/Tg, transgene-positive heterozygote; +/+, control littermate. (C) RT-PCR of total RNA from brain (B), heart (H), kidney (K), liver (L), spinal cord (SC), and skeletal muscle (SM) shows that the TgT57I transcript is only observed in skeletal muscle. (D) Western blot survey of several soluble fractions of muscle lysates from 3-month old TgT57I mice. The myotilin C-term peptide antibody reacts to both murine and transgenic human myotilin peptides; the c-myc antibody is specific to the transgene products. Ab, abdominal wall muscle; Bi, bicep; Di, diaphragm; Ed, extensor digitorum longus; Ga, gastrocnemius; Qu, quadricep; So, soleus; Ti, tibialis. (E) Soluble fractions of quadriceps lysates from 3-month old transgenic mice were analyzed by western blot to show relative expression levels. The c-myc antibody shows that the TgWT product is expressed at 2.3-fold higher levels than the TgT57I product. The myotilin antibody shows that the TgWT levels are 7.7-fold higher than endogenous myotilin levels. TgT57I levels are 2.6-fold higher than endogenous levels, as determined by optical scanning densitometry on diluted samples. (F,G) A Cy3-conjugated c-myc antibody was used to specifically immunolocalize transgene products in soleus muscle cross-sections. TgWT (F) staining is stronger than TgT57I (G) staining. (H,I) Double-labeling of TgT57I quadriceps muscle transverse sections with a slow isoform-specific myosin antibody (H) and the c-myc antibody (I) shows that the transgene is expressed in both slow type I and fast type II fibers.
site. I wanted to avoid the possibility that these differences would alter the effects of the human mutation. Because myotilin antibodies recognize both human and murine proteins, I incorporated a c-myc epitope tag at the N-terminus of the human transgene product, thus enabling transgene-specific quantification and localization. Transgenes were genotyped by PCR amplification (Figure 3.1), and a single site of insertion was verified by Southern blot (data not shown).

Transgene expression and transgene-associated pathology were similar in three distinct Thr57Ile mutant myotilin lines. I chose to thoroughly characterize line 71, Tg(HSA-MYOT)71Mah, hereafter referred to as TgT57I. I also generated a line of mice expressing a similar transgene containing the wild-type human myotilin cDNA, Tg(HSA-MYOT)12Mah, hereafter referred to as TgWT. These two transgenic constructs differ only by the single base pair that causes the Thr57Ile amino acid substitution. Transgenic mice were obtained at the expected Mendelian ratio, and neither TgT57I nor the TgWT mice displayed gross abnormalities or reduced survival. RT-PCR analysis shows that transgene expression is limited to skeletal muscle in both TgT57I (Figure 3.1) and TgWT mice (data not shown). Western blotting with a c-myc antibody specific to the transgene product shows that the expected 57 kD TgT57I protein is appropriately expressed in multiple muscles (Figure 3.1). This c-myc antibody also allows a direct comparison of transgene expression levels: TgWT mice express their transgene product at 2- to 3-fold higher levels than do TgT57I mice, as determined by analysis of soluble fractions of
quadriiceps muscle lysates (Figure 3.1). The abundance of TgWT expression is further confirmed using an antibody raised to a peptide that is identical in human and murine myotilin (Figure 3.1). Scanning densitometry was used to determine that TgWT levels are 7.7-fold higher than endogenous levels, while TgT57I levels are 2.6-fold higher than endogenous levels. Immunostaining of frozen muscle cross-sections shows that transgene expression is uniform across myofibers within a given muscle group and also similar between slow type I and fast type II fibers (Figure 3.1).

There is no overt toxicity associated with overexpression of the human N-terminally epitope-tagged myotilin transgene product (TgWT) in mouse skeletal muscle, even at two years of age. While I do observe age-related pathology common to transgene-negative littermate controls (i.e., tubular aggregates and rimmed vacuoles), minimal transgene-specific pathology is observed. 18-month TgWT tricep, quadricep, EDL, and soleus muscle histology is comparable to 12-month control muscle (Figure 3.2). I observe small eosinophilic aggregates beneath the sarcolemma in quadriceps and triceps muscles of older TgWT mice as result of significant myotilin overexpression. Because the TgWT protein is expressed at several-fold higher levels than the TgT57I product, any pathology in TgT57I mice must be caused solely by the Thr57Ile point mutation.
Figure 3.2: TgT57I mice develop myofibrillar aggregates. (A,B) Light microscopic evaluation of TgT57I muscle. (A) Masson’s trichrome-stained cross-section of a 12-month old TgT57I quadriceps myofiber. The sarcoplasmic aggregate is dense and amorphous, and stains dark red or bluish-green. (B) The amorphous structure of the aggregate and the presence of associated vacuoles are further revealed in this toluidine blue-stained 0.5-μm epoxy cross-section. (C-N) Masson’s trichrome-stained cross-sections of quadriceps, triceps, EDL, and soleus muscles from TgT57I (12-month), TgWT (18-month), and control littermate (+/+, 12-month) mice. (C, F, I) Myofibrillar aggregates (arrows) are prevalent in TgT57I quadriceps, triceps, and EDL muscles. Adipose infiltration (asterisks) and an increase in the number of tubular aggregates (white circles) are also common in older TgT57I muscle. (L) TgT57I soleus muscle exhibits no myopathology. (D, G, J, M) TgWT muscle does not develop myofibrillar aggregates. (E, H, K, N) Cross-sections of wild-type muscle. Scale bars: 10-μm (A,B); 50-μm (C-N).
Figure 3.2: TgT57I mice develop myofibrillar aggregates
3.2.2 TgT57I mice develop progressive myofibrillar pathology

Expression of Thr57Ile mutant myotilin results in a myofibrillar muscle pathology remarkably similar to that seen in the human myotilinopathies. TgT57I mice develop dense and irregular myofibrillar aggregates (Figure 3.2). A survey of muscles from a 12-month old TgT57I mouse shows that the myofibrillar aggregates are prevalent in two proximal muscle groups, the quadriceps and triceps, and also in the EDL muscle (Figure 3.2). Fibrosis, adipose infiltration, and increased tubular aggregation also occur in older TgT57I muscle (Figure 3.3).

Muscle pathology in the TgT57I mouse is progressive, with both the size and number of aggregates increasing with age. At two weeks of age, aggregates are small, focal points, that subsequently expand up to 40-μm in diameter, often occupying the entire cross-sectional areas of myofibers in older mice (Figure 3.3). The number of affected fibers also increases with age: survey of at least 800 myofibers from the quadriceps shows that the proportion containing aggregates increases from 11.7% at 6 months to 17.8% at 12 months. During the same time period, the number of affected fibers in the triceps muscle increases from 11.6% to 18.2%.

Different muscle groups show different levels of myopathic involvement (Table 3.1). Quadriceps, triceps, and hamstring muscles exhibit a higher proportion of myofibers with aggregates than EDL, gastrocnemius, abdominal wall, and tibialis muscles. Interestingly,
Figure 3.3: Myopathology is progressive in TgT57I mice

(A) Masson’s trichrome-stained cross-section of quadriceps muscle from a 2-week old TgT57I mouse. Myofibrillar aggregates are small and focal (arrows). (B) Cross-section of quadriceps muscle from a 12-month old TgT57I mouse. Larger aggregates (arrows) populate many of the myofibers. Pathology also includes a tract of fibrosis (white asterisk) and adipose infiltration (black asterisk). Scale bars 25-μm.
Table 3.1: Myofibrillar aggregation is muscle-group dependent in TgT57I mice.

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<tr>
<td>Hamstring</td>
<td>+++</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>+++</td>
</tr>
<tr>
<td>EDL</td>
<td>++</td>
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<tr>
<td>Abdomenal</td>
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<tr>
<td>Tibialis</td>
<td>+</td>
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<tr>
<td>Soleus</td>
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<td>Diaphragm</td>
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<td>Ulnar</td>
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<tr>
<td>Bicep</td>
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The relative degree of myofibrillar aggregation (MA) in various TgT57I muscle groups at 12 months of age was scored by counting at least 800 representative myofibers from each quadricep and tricep muscle cross-section; 400 myofibers for hamstring, gastrocnemius, and bicep; 50 myofibers for EDL, soleus, diaphragm, and ulnar muscles. +++ >15% aggregate-containing myofibers; ++, 5-10%; +, <5%; -, none.
the diaphragm, soleus, bicep, and ulnar muscles show no evidence of myofibrillar aggregation. This selectivity of muscle group involvement is also seen in human patients: magnetic resonance imaging of myotilin-related MFM patients has revealed selective muscle wasting in the leg (84). The three most severely affected muscles in TgT57I mice are upper forelimb and hindlimb muscles—precisely the same muscle groups that show initial weakness in LGMD1A patients.

3.2.3 Ultrastructural analysis of TgT57I muscle

TgT57I muscle recapitulates the ultrastructural defects observed in human myotilinopathy patients. Broadening of isolated Z-discs is observed in both single LGMD1A sarcomeres and in TgT57I muscle (Figure 3.4). Disrupted Z-discs may subsequently merge into streaming bodies involving multiple Z-discs. Z-disc streaming in TgT57I mice is indistinguishable from the pathology seen in human LGMD1A patients (Figure 3.4). TgT57I muscle also develops striking patches of sarcomeric deterioration and aggregation. These myofibrillar aggregates are irregular, amorphous, and devoid of any peripheral or internal membranous material. Myofibrillar aggregates are often associated with autophagic vesicles (Figure 3.4), a vacuolar pathology that is prevalent in LGMD1A muscle. Myofibrillar aggregates are also sometimes associated with tubular aggregates, a non-specific pathology observed in older inbred male mice (198). These tubular aggregates are more prevalent in TgT57I mice than male littermate controls (data
Figure 3.4: **Ultrastructural pathology of myotilin transgenic muscle.** Electron microscopy was performed on quadriceps muscle dissected from a 12-month old male +/TgT57I mouse (A-F) and a 12-month old female +/TgWT mouse (G-I). (A) An electron micrograph of a longitudinal section of TgT57I skeletal muscle shows that Z-discs are often irregular and broadened (bracket), as is also seen in LGMD1A patient muscle (inset). (B) The Z-disc streaming (asterisk) in TgT57I muscle is reminiscent of Z-disc streaming in LGMD1A patient muscle (inset). (C) Expanded regions of sarcomeric dissolution and myofibrillar aggregation are prevalent in TgT57I muscle. (D) The myofibrillar aggregates are irregular and amorphous, and expand up to 25 to 40-μm in both length and diameter. (E) Myofibrillar aggregates are often associated with vacuoles (arrows). (F) Tubular aggregation (TA) is often associated with the transgene-induced myofibrillar aggregates (MA). TgWT quadriceps muscle ultrastructure. (G) Longitudinal section of TgWT muscle shows normal sarcomeric structure. (H) Subsarcolemmal aggregates with internal vesicles develop in older TgWT muscle. (I) Sarcolemmal ultrastructure is normal in TgWT muscle. Scale bars: 0.5 μm (A); 1.0 μm (B,G,H,I); 5.0 μm (C-F).
Figure 3.4: Ultrastructural pathology of myotilin transgenic muscle
Tubular aggregates have been reported to be associated with the intrasarcoplasmic aggregates in SBM patient muscle (122). Centrally located nuclei are observed in TgT57I thin sections, but not regularly. Sarcolemmal, mitochondrial, and nuclear structures all appear normal by electron microscopy.

TgWT muscle exhibits normal sarcomeric ultrastructure, however, small aggregates can be found beneath the sarcolemma in older mice (Figure 3.4). These sub-sarcolemmal aggregates are commonly less than 5-μm in diameter and contain embedded, often fragmented, vesicles. The appearance of these aggregates contrasts sharply with that of larger myofibrillar aggregates in TgT57I mice that are devoid of any membranous deposits. Ultrastructural analysis of TgT57I muscle further highlights the largely myofibrillar pathology that is common to the human myotilinopathies.

### 3.2.4 Mutant myotilin and Z-disc proteins localize to the aggregates

The myofibrillar aggregates in TgT57I muscle harbor several Z-disc proteins, including the mutant transgene product (Figure 3.5). Immunostaining for the c-myc epitope tag allows for specific localization of the transgene products. Both the TgWT and TgT57I products localize normally to the Z-disc, but the T57I mutant protein is also found in the myofibrillar aggregates (Figure 3.5). Overexpression of wild-type myotilin also drives sub-sarcolemmal accumulation. I investigated the localization of several other Z-disc-
Figure 3.5: Immunolocalization of Z-disc proteins in myotilin transgenic muscle. Proteins were immunolocalized in longitudinal sections of quadriceps muscle from 6-month old +/TgT57I, +/TgWT, and control littermate (+/+) mice. (A-B) A Cy3-conjugated c-myc antibody was used to localize the transgene products. (A) TgT57I localizes to the Z-disc and heavily at the myofibrillar aggregates. (B) TgWT localizes to the Z-disc and occasionally accumulates near the sarcolemmal membrane. (C) No background staining is observed in control littermate muscle. (D-F) A myotilin peptide antibody cross-reacts with both the human transgene product and murine myotilin. The myotilin antibody decorates the Z-disc and aggregates in TgT57I mice (D), and the Z-discs of both TgWT and control mice (E,F). alpha-actinin (ACTN2) (G-I), gamma-filamin (FLNC) (J-L), and desmin (M-O) also localize to the Z-disc in each muscle shown and to the aggregates of TgT57I mice. (P) A mono-ubiquitin antibody stains the aggregates of TgT57I muscle. Scale bars: 10 μm (A-C); 20 μm (D-R).
Figure 3.5: Immunolocalization of Z-disc proteins in myotilin Tg muscle
associated proteins. While alpha-actinin, gamma-filamin, and desmin all localize to the Z-disc in muscles from TgT57I, TgWT, and control littermates, these proteins also localize heavily to the aggregates in TgT57I muscle. Slight accumulation of gamma-filamin and desmin can be observed in TgWT muscle. Sarcomeric proteins titin and myosin localize to the aggregates as well, however vinculin does not (data not shown). In addition, the aggregates contain ubiquitinated protein (Figure 3.5). Similar patterns of ectopic expression of sarcomeric components and ubiquitinated proteins have been reported in MFM and SBM muscle (46;83;84;123).

In order to quantitate the level of accumulation of proteins found in the aggregates, I conducted immunoblot analysis of the insoluble fractions of muscle lysates from 12-month old mice. The aggregates are not represented in the soluble fractions shown in Figure 3.1. I specifically examined the soleus, which is spared, and the quadriceps muscles, which are severely affected. Soleus muscle shows minimal accumulation of the TgT57I product, while this protein is present at high levels in the quadriceps (Figure 3.6). This is most likely a reflection of the difference in aggregate burden between the two muscles. Total myotilin abundance has also been shown to be greater in the detergent-insoluble fraction of SBM muscle compared to control lysates (121). Gamma-filamin and desmin levels are increased in the quadriceps, but not in the soleus, while alpha-actinin levels are unchanged (Figure 3.6). These differences reflect the levels of these proteins in the myofibrillar aggregates (Figure 3.5). An increase in total levels of
Figure 3.6: Abundance of Z-disc proteins in myotilin Tg muscle

Immunoblot analysis of myofibrillar fractions of myotilin transgenic muscle lysates. The insoluble fractions of soleus and quadriceps muscle lysates were prepared from 12-month old +/TgT57I, +/TgWT, and control littermate (+/+ ) mice. (A) c-myc immunoreactivity shows the relative abundance of myotilin transgene products. TgWT abundance is greater than TgT57I in quadriceps. The myotilin antibody (MYOT) shows 9- and 21-fold greater myotilin abundance in TgT57I and Tg WT quadriceps, respectively, compared to the control. Immunoblot analysis shows comparable levels of ACTN2, FLNC, and desmin (DES) in the soleus muscles of TgT57I, TgWT, and control mice. ACTN2 is also present at similar levels in the quadriceps muscles of transgenic and control mice. FLNC and desmin abundance, however, are higher in myotilin transgenic quadriceps. Desmin levels are significantly elevated in TgT57I quadriceps. (B) Coomassie blue staining of myosin and actin bands confirms comparable loading of samples.
ubiquitinated protein and the heat-shock protein, HSP70, is also observed in TgT57I muscle lysates by western blot (data not shown).

3.2.5 Maintenance of sarcolemmal integrity in myotilin transgenic mice

Vital staining with Evans blue dye (EBD) was used to evaluate plasma membrane permeability in myotilin transgenic mice. EBD is a small molecular weight tracer that binds to serum albumin; the EBD-albumin complex can easily pass into the sarcoplasm of myofibers with a damaged sarcolemma. Macroscopic evaluation of forelimbs and hindlimbs showed little EBD uptake in either 12-month old TgWT mice and control littermate, or in 9-month old TgT57I mice (Figure 3.7). EBD uptake results in red fluorescence of myofiber sarcoplasm when viewed under a microscope with a rhodamine filter. Cross-sections of quadriceps muscle from TgT57I, TgWT, and littermate control mice show little fluorescence. In contrast, large amounts of internalized EBD are evident by macroscopic and fluorescent evaluation in dystrophin-deficient mdx muscle, a positive control for sarcolemmal damage. Similar patterns of EBD uptake in mdx limbs have been shown previously (199;200). These data indicate that the sub-sarcolemmal mini-aggregates do not disrupt sarcolemmal integrity in TgWT muscle. Moreover, maintenance of sarcolemmal integrity in TgT57I muscle further distinguishes this mouse model from others that have been associated with membrane defects.
Examination of sarcolemmal integrity by vital staining with EBD. Muscle was evaluated 18 hours after intraperitoneal injection with EBD. TgWT and control littermate (+/+), TgT57I mice were 9-months old; mdx mice were 2.5-months old. The mdx mouse serves as a positive control for sarcolemmal damage. (A-D) Macroscopic analysis shows minimal EBD uptake in wild-type, TgWT, and TgT57I forelimbs. EBD uptake occurs in the triceps of mdx mice. (E-H). Macroscopic analysis of hindlimbs also reveals minimal EBD uptake in control littermate, TgWT, and TgT57I mice. EBD uptake is prominent in mdx quadriceps femoris and gastrocnemius. (I-L). EBD vital-stained quadriceps were prepared for cryotomy. 7-μm cross-sections were analyzed by fluorescence microscopy with a rhodamine filter. Dye uptake results in bright red emission in muscle cytoplasm. Dye uptake is limited to mdx muscle by fluorescent microscopy.
3.2.6 TgT57I muscle exhibits contractile dysfunction

Significant physiological dysfunction was detected in whole, intact EDL muscles of TgT57I mice. TgT57I EDL muscle mass is reduced by 33% as compared to littermate controls (8.8 mg in TgT57I, 13.2 mg in controls, Figure 3.8). Similarly, cross-sectional area is reduced by 30% (1.52 mm² in TgT57I, 2.16 mm² in controls), and specific maximum force is reduced by 24% (153 kN/mm² in TgT57I, 200 kN/mm² in controls, Fig. 8). This reduced specific force and reduced muscle size combine to lower the maximum isometric force generated by the EDL by 46% (230 mN in TgT57I, 426 mN in controls). In striking contrast, overexpression of wild-type human myotilin does not cause such a contractile dysfunction. TgWT EDL and soleus muscles are statistically indistinguishable from littermate controls with respect to mass, mean cross-sectional area, and specific maximum force (Figure 3.8). Intriguingly, TgT57I soleus mass, cross-sectional area, and specific maximum force are comparable to transgene-negative littermate controls (Figure 3.8). TgT57I diaphragm also generates specific maximum force similar to controls. Thus the TgT57I mouse model displays the same variable presentation of different muscle groups that is seen in the human myotilinopathies.

EDL and diaphragm muscles from TgT57I mice are less vulnerable to contraction-induced injury than those from littermate controls. Susceptibility to injury is measured by stimulating a muscle to contract while simultaneously stretching it. Maximum isometric force generation before and after the stretch is used to calculate a force
Whole EDL, soleus (SOL), and diaphragm (DI) muscles were dissected from 8-10 month old male TgWT and littermate control mice [+/+ (I)], and from 6-8 month old male TgT57I and littermate control mice [+/+ (II)]. (A) TgT57I EDL muscle’s mass is reduced compared to TgWT and wild-type muscle (p=0.001). Soleus muscles from TgWT and TgT57I mice show no differences in mass. (B) The cross-sectional area of TgT57I EDL muscles is also smaller (p=0.0005). (C) The maximum force generated by whole EDL muscles from TgT57I mice is lower than TgWT and littermate controls (p=0.001). The maximum force of TgWT and TgT57I soleus muscles are similar to controls. (D) The specific maximum force of EDL muscles from TgT57I mice is also decreased (p=0.044). (E) Muscles were subject to lengthening contractions (2 X 30%) to determine force deficit, a function of contraction-induced injury. TgT57I EDL (p=0.007) and diaphragm (p=0.0006) show reduced force deficit values (i.e. resistance to injury) following lengthening contractions. Asterisks denote statistically significant differences (p<0.05) between transgenic data sets and the respective littermate control data sets. P-values were determined by two-tailed t-test for two samples assuming equal variance.
deficit—muscles susceptible to damage will show a large force deficit. After a lengthening contraction protocol, EDL and diaphragm of TgT57I show less of a force deficit than muscles from littermate controls (Figure 3.8). Lengthening contraction-induced force deficit values for TgWT EDL and soleus are statistically indistinguishable from littermate controls.

In addition to the intact, whole muscle physiology, TgT57I EDL myofibers were prepared for permeabilized single-fiber studies (data not shown). These maximum specific force and force deficit data are comparable to single-fiber data from littermate controls, only when zero values are discarded. The zero values result from fiber tearing during the lengthening contraction. These data suggest that a significant population of TgT57I myofibers may indeed be susceptible to either stretch- or contraction-induced injury.

3.3 Discussion

I have generated a transgenic mouse model that successfully recapitulates the pathological features of human myotilinopathies. The myotilinopathies, including LGMD1A, SBM, and a subset of MFM and distal myopathy, comprise an autosomal dominant class of muscle diseases, all caused by missense mutations in exon 2 of the
myotilin gene. Despite the proximity and similarity of these mutations, the reported clinical features of LGMD1A, SBM, MFM, and DM are somewhat divergent. LGMD1A patients typically present with proximal muscle weakness, while distal muscle weakness is common in myotilin-related MFM (83). The defining features of MFM and SBM are dense sarcoplasmic bodies, yet this pathology had not yet been reported in LGMD1A.

The transgenic mouse model presented in this chapter unites the disparate phenotypes of the myotilinopathies. The TgT57I mouse displays expansive myofibrillar aggregation very similar to the hallmark pathologies observed in MFM and SBM. At the same time, these mice display the abundant autophagic vesicles and Z-disc streaming that are characteristic of LGMD1A patients. This strongly suggests that the Thr57Ile myotilin mutation can induce all of the skeletal muscle pathologies observed in the human myotilinopathies, and raises the possibility that variations in the presentation of human patients are the result of modifier loci.

3.3.1 The myotilin Thr57Ile mutation promotes myofibrillar aggregation

The myofibrillar aggregates and Z-disc streaming found in the TgT57I mouse model reflect dramatic disruptions in muscle structure—it is surprising that such profound changes can arise from a seemingly conservative amino acid replacement in the myotilin protein. I propose two potential mechanisms: first, mutant myotilin could act directly to
nucleate protein aggregates, and second, mutant myotilin could act indirectly by disrupting actin anchoring at the Z-disc.

In the direct model, myotilin mutations could nucleate myofibrillar aggregation by promoting heightened intermolecular associations. Myotilin binds promiscuously, homodimerizing as well as interacting with multiple muscle proteins. Many of these interactions are mediated by its two C-terminal IgL domains. Proteins containing tandem or serially arrayed IgL domains have previously been shown to be aggregate-prone (201). Myotilin mutations could promote oligomeric self-association, leading to pathogenic aggregation. This kind of single-molecule dependent nucleation and aggregation model is supported by *in vitro* cross-linking experiments in filamin-related MFM (85). The gamma-filamin Trp2710Stop mutation causes a truncation at the C-terminal IgL domain 24, a domain that mediates dimerization of the wild-type protein. The truncation may promote misfolding and increase intra-molecular association of gamma-filamin IgL domains, as could also be the case for myotilin mutations. Myotilin mutations could also promote inter-molecular aggregation with other Z-disc proteins that harbor similar IgL domains, including palladin, myopalladin, and titin (202). The abundance of IgL domain-containing proteins at the Z-disc may well mediate rapid association of proteins to promote Z-disc assembly. In the TgT57I mouse, it does not appear that Z-disc assembly is adversely affected, since aggregates are not observed in younger mice. Direct nucleation mechanisms such as these imply that the missense substitution in the N-
terminal domain of myotilin is able to alter the binding properties of the C-terminal IgL domains.

Alternatively, myotilin mutations could indirectly lead to myofibrillar aggregation by destabilizing actin anchoring at the Z-disc. The myofiber Z-disc houses a dense network of proteins that anchors actin filaments, thus facilitating the production and transmission of actin-myosin-initiated force. The chief F-actin cross-linking protein of the Z-disc is alpha-actinin, which interacts with myotilin (128) and a number of other Z-disc proteins, including nebulin (135), the alpha-actinin-associated LIM-protein (ALP) (136), the FATZ proteins (137), titin (138;139), ZASP (140), and myopalladin (202). Myotilin also associates directly with F-actin, thereby bundling and stabilizing actin filaments. Through possible combinatorial interactions with additional actin and alpha-actinin binding proteins that localize to the Z-disc (i.e., alpha-actinin, gamma-filamin, FATZ-1 and -2), myotilin may be even more intimately involved with actin stabilization and Z-disc function. Clearly, myotilin mutations could easily interfere with the total actin-tethering capacity of the Z-disc.

This indirect mechanism of aggregate formation is strongly supported by the pathology in TgT57I muscle. The spectrum of Z-disc pathology, from discrete broadening to streaming to myofibrillar aggregation, suggests that initial Z-disc destabilization leads to the stochastic increase in protein aggregation. Several transfection studies show that
overexpression of the C-terminal half of myotilin leads to multi-protein aggregation in C2C12 myotubes, but this aggregation is accompanied by a complete block in any sarcomere formation or myofibril development (130;134). *In vitro* work in COS-7 cells suggests that the Thr57Ile mutation has no effect on actin binding or bundling, nor does it induce formation of aggregates (129). The dramatic effect of this Thr57Ile mutation in live, contracting murine muscle suggests that additional functional testing of mutant myotilin is required.

### 3.3.2 TgT57I muscle pathophysiology is muscle group dependent

In the TgT57I mouse, muscle groups with differences in fiber composition displayed significant variation in pathological involvement. The skeletal muscles of adult wild-type mice are composed of three different fiber types, one slow type I fiber type and two fast type II fiber types, IIA and IIB, also termed slow-oxidative (SO), fast oxidative-glycolytic (FOG) and fast glycolytic (FG) fibers (203). The three fiber types have unique physiologic characteristics and genomic expression profiles (35;203). The EDL muscle of the mouse is composed completely of fast fibers with ~87% fast type IIB fibers (204). Gastrocnemius also contains predominantly type IIB fibers (79%) (204). Adult murine diaphragm muscle largely contains type IIA fibers, totaling ~78% (204). In contrast, the soleus muscle is composed of approximately equal percentages of slow type I and fast type IIA fibers (204). These ratios are not significantly altered in the TgT57I mouse.
The EDL and gastrocnemius muscles of the TgT57I mouse are heavily populated with myofibrillar aggregates, and the EDL displays physiologic deficits. The soleus and diaphragm muscles, however, are completely spared of any pathology or physiologic deficits. The fast type IIB fibers prevalent in the affected EDL and gastrocnemius muscles have few mitochondria, and consequently fatigue more rapidly than either of the other two fiber types. In addition, fast type II fibers have thinner Z-discs than slow type I fibers (205). Type II fibers have the highest velocity of shortening and are the most powerful fibers per unit mass (206-208), but the difference in specific forces developed by type IIB fibers is not sufficient to account for the increased pathological involvement. Despite this, the predominance of fast type IIB fibers in the EDL muscle may be responsible for the severe contractile dysfunction observed as a result of myotilin mutation. Fiber typing of the broken fibers encountered during single-fibers studies will shed light on the contribution of the fast type IIB physiological profile to pathology and weakness.

The phenotypic differences between TgT57I muscles could be explained by fiber type- and muscle-specific gene expression profiles. Hierarchical clustering analysis of gene expression patterns can be used to easily distinguish EDL and soleus muscles of the mouse (209). The presence or absence of a specific protein in fast type IIB fibers in muscles of TgT57I mice may increase their risk to damage. For example, alpha-actinin-3, a structural protein of the Z-disc, is expressed exclusively in fast type IIB fibers (210).
A major difference between fast type II and slow type I fibers and muscles is their susceptibility to contraction-induced injury. The fast type II fibers, and muscles largely composed of these fibers, display a three- to four-fold greater force deficit for a given strain, or for a given amount of work done to stretch a fiber (211;212). Surprisingly, the EDL and diaphragm muscles in the TgT57I mice do not display the high force deficits expected. Similar observations of low values for maximum force and for force deficits have been made in fatigued muscles and in the muscles of desmin-deficient mice (213;214). This resistance to contraction-induced injury may be secondary to the reduced force-generating capacity of the EDL muscle. This interpretation is supported further by the data on the soleus muscles of TgT57I mice compared with littermate controls, which did not differ in either force-generating capacity or in susceptibility to contraction-induced injury. The magnitude of the force deficit correlates highly with the work required to stretch the muscle, which is the product of the displacement and the average force developed by the muscle during the strain (212;215;216). Consequently, the EDL muscle of the TgT57I mouse may have a very low force deficit because it is damaged and only able to generate reduced force. The same explanation may hold true for the reduced force deficit in the diaphragm, although the decrease in specific force for this muscle does not reach the level of statistical significance.

The response to lengthening contractions illustrates that myotilin mutations give rise to a dramatically different myopathy than mutations in the dystrophin-associated glycoprotein
(DAG) complex. The muscles of mdx mice display a significant reduction in specific force (217); however, they are highly sensitive to contraction-induced injury (218). With the absence of dystrophin and the DAG complex in the mdx mouse, the large force deficits of the EDL and soleus muscles arise from disruptions of the sarcolemma and of mechanical properties of the cytoskeleton (212). Myotilin mutations do not interfere with the link between the DAG complex and the contractile elements. Consequently, TgT57I muscles are not exposed to this acute injury response to lengthening contractions. Rather, the myotilin mutations directly impair the development of force. Further investigations of the contractility and susceptibility to contraction-induced damage in the TgT57I mouse model are necessary for a more complete understanding of the pathophysiology of mutations in Z-disc proteins. In particular, physiological measurements of single fast type IIB fibers may shed additional light on their role in the myotilinopathies.

3.3.3 The TgT57I mouse complements current MFM models

Mutations in multiple genes other than myotilin have been shown to cause MFM—mouse models incorporating several of these genes have been reported. Adult desmin knock-out mice show signs of muscle degeneration, Z-disc streaming, and myofibril misalignment, but myofibrillar aggregation does not occur (20;219). In contrast to normal TgT57I diaphragm and soleus muscles, desmin-null diaphragm exhibits increased degeneration,
and desmin-null soleus muscle generates 90% less maximum isometric force (20). These pathophysiological differences can be explained both by desmin’s additional role at myofiber costameres, structures that connect Z-discs to the sarcolemma, and by the approach of targeted inactivation of desmin, rather than transgenic expression. Indeed, transgenic expression of the MFM desmin allele, D7-des (deletion of 7 amino acids, 173-179) causes intrasarcoplasmic aggregation in cardiac myocytes (220). Cardiac-restricted expression by the alpha-myosin heavy-chain promoter prevents the opportunity to study skeletal myopathy in this model.

Targeted inactivation of another MFM gene, alpha-beta-crystallin (CRYAB), causes non-specific histological features of muscular dystrophy and small patches of amorphous granules in older mice, but no significant myofilbrillar aggregation (221). In the same way as the transgenic desmin allele, cardiac-restricted transgenic expression of the CRYAB Arg120Gly MFM mutation causes eosinophilic aggregation in cardiac myocytes (222). Targeted inactivation of cypher, the murine ortholog of ZASP, causes perinatal lethality, right and left ventricular dilation, and disorganization of skeletal muscle Z-discs, and no aggregation is observed (223). Targeted inactivation of gamma-filamin causes perinatal lethality due to respiratory failure, and suggests that gamma-filamin is critical to primary embryonic myogenesis (224).
The desmin, CRYAB, and ZASP mouse mutants have proven very successful in the study of cardiac failure associated with MFM. The transgenic myotilin Thr57Ile mouse, however, best captures the skeletal muscle phenotype of MFM. The desmin/CRYAB and myotilin transgenic mouse models complement each other well in the effort to identify the causes and functional impacts of myofibrillar aggregation in cardiac and skeletal muscle.

3.3.4 Therapeutic potential of myotilin transgenic mouse models

Analysis of the TgWT mouse demonstrates that in vivo expression of high levels of wild-type myotilin protein results in small sub-sarcolemmal aggregates and slight accumulation of gamma-filamin and desmin in TgWT mice (Figure 3.6). However, these minor abnormalities do not induce any changes in sarcolemmal integrity (Figure 3.7) or physiologic function of the muscle (Figure 3.8). The lack of toxicity of high levels of myotilin protein raises the possibility that myotilin overexpression could be used therapeutically to treat muscle diseases caused by intermyofibrillar or sarcomeric dysfunction, especially those characterized by destabilization of F-actin tethering. The TgWT mouse serves as a promising genetic resource for testing the therapeutic effects of myotilin overexpression in other established mouse models.
The TgT57I mouse also provides a useful system in which to test disease therapeutics such as RNA-directed approaches to correct the mutant myotilin transcript or the oral administration of anti-aggregation drugs. Doxycycline and trehalose have proven effective at reducing nuclear aggregation and muscle weakness in a mouse model of oculopharyngeal muscular dystrophy (225;226), and rapamycin has also been shown to have potent anti-aggregation activity \textit{in vivo} (227). Rapamycin is effective at reducing cellular pathology in a cell culture model of dominant dysferlinopathy. The Leu1341Pro dysferlin mutation causes intracellular aggregation (228). This dysferlin protein aggregation in the endoplasmic reticulum is reduced by rapamycin treatment, in cell culture (229).

The TgT57I mouse is an excellent murine model that unites the pathological and physiological phenotypes of the human myotilinopathies. This mouse model recapitulates many of the features of human patients, including the selective pattern of muscle group weakness. Further characterization of the TgT57I mouse promises to reveal the mechanistic roots and impacts of myofibrillar aggregation in muscle disease, and allows for the development and testing of therapeutic approaches to all the myotilinopathies.
3.4 Material and Methods

*Note:* Further myotilin gene detail and results of future experimentation on the myotilin transgenic mice presented in this chapter can be queried online at The Jackson Laboratory Mouse Genome Informatics database, http://www.informatics.jax.org/ (TgT57I, MGI ID: 3640478; TgWT, MGI ID: 3640477).

3.4.1 Generation and characterization of myotilin transgenic mice

The wild-type myotilin cDNA was isolated from a pTriplEx human skeletal muscle cDNA library (BD Clontech), and cloned into a modified pBluescript vector (Stratagene) containing an enhanced HSA promoter cassette and two SV40 polyadenylation signals. This HSA promoter fragment, -2139 to +239, has previously been shown to drive skeletal muscle-specific expression (157), as well as the enhanced HSA cassette (HSA-VP1) containing the 400 bp SV40 VP1 intron (181;230;231). The myotilin cDNA includes the 1530 bp coding region, 281 bp of 5’-UTR and 485 bp of 3’-UTR. Recombinant PCR was used to incorporate the c-myc epitope tag and the Thr57Ile point mutation; sub-cloning was facilitated by *EcoRI* and *SphI* double digestion of recombinant products and subsequent ligation into the wild-type clone. The 5.9 kb linear transgenic constructs were released by *KpnI/NaeI* digestion, purified with gel extraction columns (QiaGen), sterilized by ethanol precipitation, and microinjected into B6SJLF2 one-cell embryos at
the Duke University Transgenic Mouse Facility. Transgene-positive founder mice (F0, also B6SJLF2) were subsequently backcrossed to C57BL/6. Data reported in this chapter were gathered from progeny that were backcrossed at least 5 times to C57BL/6 [N5(B6SJLF2XB6)], with the exception of F2N3 animals used to assess TgWT and control littermate muscle physiology. Because the SJL strain develops myopathy due to a null mutation in the dysferlin gene (232), homozygosity of the wild-type C57BL/6 allele was tested and confirmed in all transgenic F2N1 breeders. Myotilin transgene genotyping was done by PCR amplification of a 394 bp fragment from tail clip DNA with a forward HSA-VP1 primer (5′-GCTCCTGTAAATTGGTATAAC-3′) and a reverse myotilin 5′-UTR primer (5′-AACCCACTATTGAAGGGAAG-3′). C57BL/6 breeders were purchased from Harlan. Male C57BL/10ScSn-Dmdmdx/J mice were purchased from The Jackson Laboratory. Mice were bred under standard conditions in the Genome Sciences Research Bldg. II animal facility at Duke University.

### 3.4.2 Analysis of myotilin transgene expression

Total RNA was prepared from dissected and homogenized brain, kidney, heart, liver, spinal cord, and quadriceps tissues (SV Total RNA Isolation System, Promega). For RT-PCR, 1 µg of total RNA was reverse transcribed using random hexamers (Promega). A transgene-specific 481 bp product was amplified with a forward HSA exon 1 primer (5′-GAGTAGCAGTTGTAGCTACC-3′) and a reverse myotilin exon 2 primer (5′-
AGGAGGCTGCAATCTGGAGC-3\'). Analysis of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as a positive control for cDNA synthesis and RT-PCR (forward: 5\'-TTAGCACCCCCTGGCCAAGG-3\'; reverse: 5\'-CTTACTCCTTGAGGCCATG-3\'). Muscle tissue for downstream western blotting was snap-frozen in liquid nitrogen, ground in a mortar and pestle, and resuspended (200 μL/100 mg tissue) in 1% SDS, supplemented with a mammalian protease inhibitor cocktail (Sigma). For crude soluble fraction isolation, lysates were vortexed for 30 s, boiled for 5 min, vortexed for 30 s, boiled for 60 s, and then spun at 12,000 g for 60 s. The supernatant is the soluble fraction. To obtain the insoluble fraction, muscle lysates were spun at 12,000 g for 20 min. The pellet was resuspended in 1X SDS buffer (62.5mM Tris pH 8.8, 6% SDS, 50mM DTT), vortexed for 1 min, boiled for 5 min, vortexed for 1 min, then further resuspended with an electric homogenizer. Prior to electrophoresis, aliquots of lysates were mixed 1:1 with 2X Laemmli buffer, boiled for 1 min, spun for 60 s, and loaded onto denaturing 4-15% polyacrylamide Tris-HCl gels (Bio-Rad). Proteins were separated at 15 mA for 3 hours and mobilized by tank transfer (40 V for 4 hours) to a 0.45 um nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% non-fat dry milk + 1% donkey serum + 0.1% Tween 20 in TBS, and stained with primary antibody for 60 min at room temperature in blocking buffer. Membranes were washed 1 X 15 min, then 3 X 5 min each in TBS + 0.1% Tween 20 (TBST), stained with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000, Jackson Immunoresearch) for 45 min at room temperature in
blocking buffer, and washed 1 X 15 min then 3 X 5 min each in TBST.

Immunodetection was carried out with the enhanced chemiluminescence (ECL) Plus Western Blotting Detection System (Amersham Biosciences). Primary antibodies for western blot included: myotilin peptide antibody (1:4000, rabbit polyclonal, generated by Bethyl Laboratories using the peptide corresponding to myotilin residues 473-488, CVKQAFAFNPEGEFQRLAAQ); anti-cmyc (1:10000, rabbit polyclonal, RDI Division of Fitzgerald Industries); anti-ACTN (sarcomeric), clone EA-53 (1:4000, Sigma); rabbit anti-FLNC-A1 (1:1500, Dr. Louis Kunkel) (131); anti-desmin, clone DE-R-11 (1:400, Novocastra). Optical scanning densitometry was performed with Image-Pro Plus software (MediaCybernetics).

3.4.3 Histology

Mice were transcardially perfusion-fixed with 4% paraformaldehyde in PBS. Dissected muscle was then fixed at least overnight at room temperature, also in 4% paraformaldehyde in PBS. Tissue was then dehydrated in 70% ethanol and processed via manufacturer’s instructions in an automatic processor (Tissue-Tek VIP). Specimens were embedded in paraffin, sectioned at 7-μm, and stained with Masson’s trichrome stain (233).
3.4.4 Electron microscopy

Mice were transcardially perfusion-fixed with 4% paraformaldehyde and 0.25% glutaraldehyde fixative in HEPES buffer. Whole muscles were dissected, diced into 1-2 mm$^3$ cubes, and further fixed with 4% glutaraldehyde in 0.1M cacodylate buffer for at least an hour. They were washed in 0.1M cacodylate buffer containing 7.5% sucrose, 3 X 20 min each wash. Specimens were then post-fixed in 1% osmium tetroxide in cacodylate buffer, dehydrated through a graded series of ethanol, followed by propylene oxide, and embedded in PolyBed 812 epoxy resin (Polysciences). Specimens were baked overnight at 60 ºC. Semi-thin sections (0.5-μm) were cut and stained with toluidine blue. Ultra-thin sections (60-90 nm) were cut with a diamond knife and post-stained with uranyl acetate and lead citrate. Sections were examined in a Philips EM 400 or CM 12 electron microscope.

3.4.5 Immunofluorescence

All immunofluorescence experiments were performed on fresh, frozen muscle sections. Muscle specimens were saturated overnight at 4 ºC in PBS + 20% sucrose. Tissue was then embedded in optimal cutting temperature (OCT) compound (Tissue-Tek) over liquid nitrogen-cooled isopentane. Frozen sections were cut at 7-μm, washed for 15 min in PBS, blocked for 60 min in 2% bovine serum albumin (BSA), washed 3 X 10 min each in PBS, and incubated for 60 min at room temperature with primary antibody diluted in PBS
+1% BSA +1% goat serum (Sigma). Sections were washed 3 X 10 min each with PBS, stained for 60 min at room temperature with the appropriate fluorophore-conjugated secondary antibody diluted in PBS +1% BSA, washed 3 X 10 min each in PBS, and mounted with ProLong Gold antifade reagent (Invitrogen). The following primary antibodies were used for immunofluorescence: Cy3-conjugated anti-cmyc, clone 9E10 (1:200, Sigma); myotilin peptide antibody (1:800); anti-ACTN (sarcomeric), clone EA-53 (1:500, Sigma); rabbit anti-FLNC-A2 (1:200, L. Kunkel) (131), anti-desmin, clone DE-R-11 (1:50, Novocastra), anti-myosin (FAST), clone MY-32 (1:400, Sigma), anti-myosin (SLOW), clone NOQ7.5.4D (1:400, Sigma), and rabbit anti-ubiquitin (1:80, Sigma). Secondary antibodies included Alexa Fluor 488- and Alexa Fluor 594-conjugated chicken anti-rabbit, goat anti-rabbit, and rabbit anti-mouse IgG antibodies (1:1000, Invitrogen).

3.4.6 Evans blue dye uptake assay

Mice were administered an intraperitoneal injection of EBD (Sigma) (1 mg/10 g body weight). After 16-18 hours, mice were euthanized and skinned. Whole limbs were fixed in 4% paraformaldehyde for 6 hours and examined macroscopically for dye uptake. For microscopic analysis, fresh quadriceps muscles were embedded in OCT over liquid nitrogen-cooled isopentane. 7-μm sections were cut and examined for dye uptake by fluorescence microscopy using a rhodamine filter.
3.4.7 Muscle physiology

EDL and soleus muscles were isolated, and the nerves to the muscles were dissected and cut. Ties were placed around the proximal and distal tendons of the muscle, and the muscle was removed. The tissue was placed in buffered mammalian Ringer's solution. The solution was maintained at 25 °C and gassed continuously with a mixture of 95% O₂ and 5% CO₂. One tendon was tied to a servo-motor (Aurora Scientific Inc.) and the other to a force transducer (Kulite model BG-50). The muscle was stimulated directly by an electrical field generated between two large platinum electrodes situated in the bath on either side of the muscle. Square-wave pulses 0.2 ms in duration were amplified to increase current intensity to a sufficient level to produce a maximum response. Muscle length was adjusted for maximum isometric twitch force. The entire diaphragm, including adjacent ribs, was also removed and immersed in an oxygenated bath containing mammalian Ringer solution at 25 °C. Diaphragm strips, 1-2 mm wide, were carefully dissected from the central region of the lateral costal hemi-diaphragm. The connections of the strip of fibers to a small section of a single rib and part of the central tendon were preserved. The rib and the tendon were attached to the force transducer and servo-motor. Diaphragm strips were stimulated directly by an electric field as described for EDL and soleus. To measure force deficits following lengthening contractions, two stretches of magnitude 0.3 fiber-lengths and velocity 0.5 fiber-lengths/s were initiated from the plateau of an isometric contraction. Lengthening ramps were separated by 10 s and stimulation was terminated at the end of the second lengthening ramp. Force deficit
was assessed during a final isometric tetanic contraction one minute after the second lengthening contraction ended. Further details on these physiological protocols can be found elsewhere (234-237). Physiological experiments were performed in collaboration with Dr. John Faulkner at the University of Michigan Muscle Contractility Core.

*Note:* This work was published in part in *Human Molecular Genetics* (Garvey et al., 2006). Copyright © 2006, The Author. Used by permission of Oxford University Press.
Chapter 4

4. Myotilin overexpression enhances aggregate pathology in LGMD1A mouse model

4.1 Introduction

I wanted to further test the model that myotilin indirectly causes myofibrillar aggregation by first disrupting actin interactions at the Z-disc. This model depends on the progressive de-tethering effect of muscle contraction in adult TgT57I mice, ‘sensitized’ by the myotilin mutation. If myotilin were acting by an indirect mechanism, adding significantly more wild-type myotilin would increase the size of aggregates. The ectopic myotilin would add to the sarcomeric pool of aggregating proteins. Alternatively, if TgT57I myotilin molecules are directly nucleating by self-oligomerization, the addition of wild-type myotilin might not modify the TgT57I phenotype.

To test this hypothesis, TgT57I; TgWT double-transgenics were generated. These double-transgenic mice will also suggest whether or not therapies that supplement myotilin levels, or have an effect on myotilin levels, would ameliorate TgT57I pathology.
4.2 Results

4.2.1 Generation of double-transgenic mice

Heterozygote +/-TgT57I and +/-TgWT mice were bred to generate double-transgenic +/-TgT57I;+/-TgWT mice, hereafter referred to as double-transgenic (DTg) mice. Two separate heterozygote matings generated a total of 28 progeny. The Mendelian ratio of DTg’s was maintained: 7 of the 28 mice carried the two separate myotilin transgenes. The DTg genotype was confirmed by two separate allele-specific PCR reactions (see section 4.4.2). DTg mice develop overt, ambulatory symptoms of muscular dystrophy between 6-8 months of age. These overt symptoms are preceded by histological signs of severe myopathy.

4.2.2 Myotilin overexpression exacerbates myopathy

Overexpression of the wild-type transgene causes increased severity of muscle disease in DTg mice. Light microscopic analysis of quadriceps muscle from 6-month old DTg mice shows stronger staining of large, myofibrillar aggregates compared to littermate control single-transgenic TgT57I muscle (Figure 4.1). The more intense aggregate pathology in DTg muscle accompanies histological signs of muscle hypertrophy, centrally located nuclei, variations in fiber size, necrosis, and fibrosis. Some muscles hardly affected in TgT57I become affected in the DTg’s. For example, tibialis muscle from a 2 month old
A TgT57I mouse shows a single myofibrillar aggregate (arrowhead) in tibialis muscle at 2-months of age. Control littermate, double-transgenic (DTg) tibialis muscle contains widespread myofibrillar aggregation. DTg muscle also shows variations in fiber size, fibrosis, necrosis, and adipose replacement. Quadriceps from 6-month TgT57I mice shows multiple myofibrillar aggregates. Control littermate DTg mice show denser and more prevalent aggregation, as well as necrosis, centrally located nuclei, and variations in fiber size.

Figure 4.1: Myopathy is more severe in double-transgenic mice
DTg exhibits gross myofibrillar aggregation, variations in fiber size, fibrosis, necrosis, and adipose infiltration (Figure 4.1). Tibialis muscle from the littermate control TgT57I mouse shows just one aggregate (Figure 4.1).

Exacerbation of the myofibrillar phenotype is also seen by electron microscopy (Figure 4.2). DTg aggregates (Figure 4.2) are larger and more electron-dense than TgT57I aggregates (Figure 3.4). DTg ultrastructure exemplifies the full spectrum of aggregate severity. In the left panel, dense myofibrillar aggregation in the upper right corner is circumscribed by an arc of Z-disc streaming, which is further circumscribed by an arc of broadening of single Z-discs (Figure 4.2). DTg ultrastructural pathology also includes intermyofibrillar tracts of enlarged mitochondria (data not shown).

4.2.3 Nuclei are associated with myofibrillar aggregates

Both TgT57I and DTg myofibrillar aggregates are associated with nuclei (Figure 4.3). This interaction is seen by both light microscopic analysis of H & E-stained cross-sections of TgT57I muscle and ultrastructural analysis of longitudinal sections of DTg muscle (Figure 4.3). Intermyofibrillar nuclei can also be observed in areas of normal muscle (data not shown). These round nuclei may be in transit to areas of aggregation and degeneration. Their shape starkly contrasts the long, slender, immobile nuclei at the myofiber periphery.
Figure 4.2: Ultrastructural analysis of myotilin double-transgenic muscle

Longitudinal sections of quadriceps muscle from a 6-month female DTg mouse shows a gradient of aggregation pathology (left) and a zone of dense myofibrillar aggregation (right). Scale bars: 2-μM.
4.3 Discussion

4.3.1 Total myotilin correlates with disease severity

I have generated myotilin double-transgenic mice (expressing both the TgWT and TgT57I myotilin transgenes; DTg) in order to test the effect of overall myotilin protein levels on the severity and progression of disease. DTg mice exhibit significantly more severe myopathic symptoms than the single-transgenic TgT57I mouse. DTg mice demonstrate considerably more abundant and expansive myofibrillar aggregation, as well as earlier onset of myopathology, and greater involvement of muscles much less affected in single-transgenic TgT57I mice (Figure 4.1). The increased severity of symptoms in the DTg mice is not due to a higher dosage of mutant myotilin, but rather to a higher level of normal myotilin. This observation is consistent with our model in which myotilinopathy missense mutations initiate actin de-tethering and Z-disc streaming. Maturation to full-fledged myofibrillar aggregates depends on the total amount of myotilin, including wild type protein.

4.3.2 Focal repair in myotilin Thr57Ile transgenic mice

There is evidence for focal repair of myofibrillar aggregates in myotilin transgenic mice. Light and electron microscopic analysis of TgT57I single-transgenic and DTg muscle
Figure 4.3: Aggregate-associated nuclei in myotilin transgenic muscle

(A,B) H & E-stained cross-sections of quadriceps muscle from a TgT57I mouse. Haemotoxylin-staining blue nuclei are associated with the dense myofibrillar aggregates. (C,D) Ultra-thin, longitudinal sections of quadriceps muscle from a 6-month old DTg mouse. (C) A long, slender peripheral nucleus of a healthy myofiber adjacent to a myofiber with a rounded-up nucleus in an area of myofibrillar degeneration. (D) Central nucleus embedded in a degenerative zone. Scale bars: 20-μM (A,B); 5-μM (C,D).
reveals a high number of central nuclei that track with and penetrate myofibrillar aggregates. Central nuclei are typically considered pathological hallmarks of muscle degeneration and regeneration. The central nuclei in TgT57I mice suggest otherwise. These nuclei have likely been recruited by mechano-transductive signals from the periphery, migrating in between myofibrils, to target aggregates for degradation. The nucleus could then be programmed to specifically express proteins for aggregate clearance, protein degradation, or protection from oxidative stress. In such a way, a particular myofibril could be focally repaired.

This type of focal repair has previously been suggested to occur in a mouse model of nemaline myopathy (238). HSA-directed transgenic expression of the Met9Arg tropomyosin-3 mutation (TgM9R) recapitulates much of phenotype of TPM3-associated nemaline myopathy (239). Z-disc-associated rod pathology is progressive and likely contributes to the observed muscle weakness in the TgM9R mice. Central nuclei are shown embedded within zones of sickly mitochondria and near an area of myofibrillar degeneration. The increase in the number of satellite cells in TgM9R mice could be in response to repair stimuli from degenerating myofibers. Both the nemaline rod-associated nuclei of TgM9R mice and the myofibrillar aggregate-associated nuclei of TgT57I mice provide a new binary context for centrally located myonuclei: regeneration or repair.
4.3.3 Implications for treatment of myotilinopathy

The observations in DTg mice suggest that a ‘knockdown’ approach, rather than an additive or replacement strategy, would be the most effective therapeutic method aimed at modulating myotilin gene expression or protein levels. A myotilin knockdown will reduce the amount of both mutant and normal myotilin. I hypothesize that the reduction of mutant myotilin will reduce the number of nucleation events, while the reduction of normal myotilin will slow progression of these lesions to full-blown myofibrillar aggregates. I do not expect any toxicity associated with myotilin knockdown, as the myotilin knockout mouse displays no adverse phenotype (141).

RNA interference is a naturally occurring pathway that utilizes small RNAs to reduce, or knockdown, gene expression. Approaches based on RNA interference have been applied to the treatment of numerous diseases caused by overexpression of toxic genes and proteins. Advances in the understanding of small interfering RNA biology show that short hairpin RNAs targeting a specific transcript can effectively knockdown gene expression. Therapeutic knockdown could be tested in TgT57I mice by systemic delivery of a short hairpin RNA against myotilin (shMYOT) by pseudotyped adeno-associated viral vectors. Such a short hairpin RNA could also be studied in transgenic mice. Transgenic mice driving shMYOT expression with a drug-responsive transgene regulatory element could subsequently be crossed to TgT57I mice. Physiological
analysis of TgT57I muscle suggests that the extent of myofibrillar aggregation would be a good clinical indicator for effective therapy.

4.4 Materials and Methods

4.4.1 Generation of myotilin double-transgenic mice

The TgT57I and TgWT mice were described in chapter 3. Myotilin transgenic mice were maintained by back-crossing to C57BL/6 mice (Harlan). Heterozygote TgT57I [N7(B6SJLF2XB6)] and TgWT [N11(B6SJLF2XB6)] mice were bred to generate double-transgenic +/TgT57I;+/TgWT (DTg) mice. Mice were bred under standard conditions in the Genome Sciences Research Bldg. II animal facility at Duke University.

4.4.2 Genotyping of double-transgenic mice

Two allele-specific PCR reactions were developed to distinguish the T57I and WT transgenes from tail clip DNA. DTg genomic DNA generates amplicons from both reactions. Both PCR reactions amplify a 371 bp product using the same forward myotilin 5′-UTR primer (5′-CTCAACAAGGAAGACAGAC-3′) and a separate reverse mismatch-designed 19mer. The reverse myotilin exon 2 primer, 5′-GTGATGTGAGCAGCTCAGTA-3′, specifically amplifies transgenic DNA containing the
T57I nucleotide 450 thymidine mutation. The reverse myotilin exon 2 primer, 5′-GTGATGTGAGAGCTCAAATG-3′, amplifies DNA containing the wild-type nucleotide 450 cytosine allele. The very 3′ base of each reverse primer is complementary to either the wild-type C or mutant T nucleotide, and the next 5′ adenosine creates a destabilizing mismatch in the reverse WT primer. The annealing conditions during thermo-cycling for both allele-specific PCRs are identical: eight-cycle ‘touchdown’ from 60º to 56º, reducing the annealing temperature 0.5º per cycle, followed by 25 cycles of annealing at 56º.

4.4.3 Histology and electron microscopy

Mice were transcardially perfusion-fixed with 4% paraformaldehyde, 0.25% glutaraldehyde in HEPES buffer. Whole muscles were dissected and processed as described in sections 3.4.3 and 3.4.4.
Chapter 5

5. Patho-physiological characterization of myotilin domain deletions

5.1 Introduction

The myotilinopathies are a group of adult-onset, progressive, muscle disorders caused by mutations in the myotilin gene. Myotilin is a 498 amino acid residue cardiac and skeletal muscle-abundant protein involved with actin cross-linking and bundling at the myofiber Z-disc. Six independent myotilin missense mutations have been reported, and five of the mutations occur within the 79 amino acid N-terminal domain, a region with unknown function and no homology. A sixth myotilinopathy mutation, Ser95Phe, occurs within the minimal alpha-actinin binding site, amino acid residues 80-124 (129).

\textit{In vitro} binding studies have shed light on two possible functions of the N-terminal half of myotilin: binding to alpha-actinin and enhancing interactions associated with full-length myotilin or the C-terminal half of myotilin. Yeast two-hybrid analysis originally showed that the N-terminal 214 residues are required for alpha-actinin binding (128).
The minimal alpha-actinin binding site has further been described as between myotilin residues 80 and 124 (129). The C-terminal half of myotilin interacts with F-actin and facilitates homodimerization. Several recombinant myotilin peptides largely composed of C-terminal sequence have been shown to bind actin by yeast two-hybrid analysis (129). Yeast two-hybrid experiments show that the N-terminal half is not required for homodimerization (128) or actin binding (129), but full-length myotilin does enhance homodimerization. Yeast two-hybrid work shows that full-length myotilin binds to the Z-disc protein, FATZ-1 (132). A number of myotilin truncations, including deletion of the N-terminal 79 amino acids, block FATZ-1 binding. Therefore, the N-terminal half of myotilin may enhance homodimerization and FATZ-1 binding.

*In vitro* studies show that myotilin plays a role in actin cross-linking, stabilization, and bundling (134). Bundling can be assayed by determining the relative number of induced phalloidin-staining actin cytoskeletal thick cables in yeast or mammalian cells in culture. Intriguingly, myotilin amino acids 185-214, which precede the IgL domains, are particularly important for actin bundling. Consistent with this finding, actin filament cosedimentation assays showed that a truncated myotilin peptide (res. 80-462) strongly binds F-actin (134). Via a ternary complex of myotilin, alpha-actinin, and F-actin at the Z-disc, myotilin’s presence enhances F-actin-associated functions of alpha-actinin (134). Thus, it would be interesting to determine the effect of deleting the alpha-actinin binding site on sarcomere structure and overall muscle structure and function in a transgenic
mouse. Deletion of the functionally elusive N-terminal 79 amino acids of myotilin would also shed light on the pathogenic mechanism associated with myotilinopathy mutations.

To better understand myotilin function \textit{in vivo}, two separate lines of myotilin domain deletion transgenic mice were created: one expresses a deletion of the N-terminal domain, amino acids 1-79 (TgΔNT), and the second expresses a deletion of the minimal alpha-actinin binding site, amino acids 80-124 (TgΔABS). Rather than express whole C-terminal or C-terminal halves of myotilin, I have designed these discrete deletions to avoid the prospect of embryonic lethality. Overexpression of the N-terminal 251 amino acids or the C-terminal 247 amino acids in cultured muscle cells causes protein aggregations and complete obstruction of striated myotube development (134). The TgΔNT and TgΔABS mice serve as invaluable resources for testing myotilin domain function in live, contracting skeletal muscle. These mice will also answer whether loss of either myotilin domain causes the myofibrillar aggregation pathology observed in the point mutant TgT57I mouse presented in the previous two chapters.
5.2 Results

5.2.1 Generation of myotilin domain deletion transgenic mice

The mutant transgenes contain the human myotilin cDNA with either domain deletion, cloned downstream of the skeletal muscle-specific human skeletal actin (HSA, or ACTA1) promoter (Figure 5.1). The first 79 amino acids are removed from the myotilin N-terminal domain deletion product, hereafter referred to as TgΔNT. Three similar TgΔNT-expressing lines were generated, and data from line 1 is reported here [Tg(HSA-MYOT)1Mah]. The second myotilin deletion removes amino acids 80-124, the minimal alpha-actinin binding site, hereafter referred to as TgΔABS. One TgΔABS-expressing line was obtained [Tg(HSA-MYOT)6Mah]. In the previous two chapters, I reported a control line of mice expressing a similar transgene containing the wild-type human myotilin cDNA, referred to as TgWT. Because myotilin antibodies recognize both human and murine proteins, I incorporated a c-myc epitope tag at the N-terminus of the human transgene products, thus enabling transgene-specific quantification and localization. Transgenes were genotyped by PCR amplification (Figure 5.1). The genotyping primer pair flanks both deletions, thus generating smaller amplicons in TgΔNT and TgΔABS genomic DNA compared to TgWT. All transgenic mice were obtained at the expected Mendelian ratio, and neither TgΔNT nor the TgΔABS mice
Figure 5.1: Design and expression of TgΔNT and TgΔABS myotilin transgenes

(A) All transgenes are driven by the HSA promoter and contain the c-myc epitope tag (red) at the N-terminus. TgΔNT contains a deletion of the N-terminal 79 amino acids. TgΔABS contains a deletion of amino acids 80-124, the minimal alpha-actinin binding site. (B) The Tg-specific genotyping PCR shows the appropriate deletions. (C) RT-PCR shows abundant expression of transgenes in quadriceps (Qu) and soleus (So) muscles compared to brain (B), heart (heart), and spinal cord (Sc). (D) Tg-specific western blot using the anti-cmyc antibody shows that the TgΔNT and TgΔABS peptides are expressed and stable. TgΔABS is expressed at levels comparable to the highly expressed TgWT transgene (7.7-fold more than endogenous myotilin). A myotilin peptide antibody shows expression of the 57 kD endogenous myotilin peptide, and the smaller TgΔNT and TgΔABS transgenes.
displayed gross abnormalities, reduced size, or reduced survival compared to littermate controls.

The TgΔNT and TgΔABS transgenes faithfully produce transcripts and predictably smaller recombinant myotilin peptides. RT-PCR analysis shows that transgene expression is largely restricted to skeletal muscle (quadriceps and soleus) in TgWT, TgΔNT, and TgΔABS lines. Western blot analysis using the c-myc and myotilin peptide antibodies shows that the myotilin deletion transgenes are stable and appropriately translated (Figure 5.1). TgΔABS expression approximates that of the wild-type transgene (~ 7-8 fold greater than endogenous myotilin expression), whereas TgΔNT expression is approximately four-fold less.

5.2.2 TgΔABS muscle develops progressive myofibrillar pathology

Transgenic expression of the alpha-actinin binding site deletion causes progressive myopathy. Light microscopy of quadriceps and triceps muscles from 9-month old TgΔABS mice reveals increased central nuclei, necrotic fibers, and adipose cell infiltration compared to littermate controls (data not shown). TgΔABS muscle also develops the subsarcolemmal eosinophilic deposits seen in TgWT muscle. Histological analysis of TgΔABS muscle at two and three months is normal. The TgΔABS pathology observed at 9 months is subtle compared to the TgT57I point mutant mice. Several
Figure 5.2: Ultrastructure of myotilin domain deletion transgenic muscle

(A) Overexpression of wild-type myotilin causes no abnormal sarcomeric ultrastructure in muscle from 12-month old TgWT mice. (B) TgΔNT mice also show no abnormal sarcomeric ultrastructure. (C) TgΔABS muscle develops Z-disc streaming. The Z-disc streaming in TgΔABS muscle expands in both (D) myofibrillar and (E) sub-sarcolemmal zones. (F) High-resolution micrograph of TgΔABS streaming aggregate shows amorphous and irregular structure.
muscle groups of TgΔNT mice up to 12 months of age have been analyzed. TgΔNT muscle develops no abnormal myopathology.

The most striking myopathology of TgΔABS muscle is observed at the ultrastructural level. The electron-dense Z-disc streaming pathology pervades longitudinal sections of quadriceps muscle from a 9-month old male mouse (Figure 5.2). The electron-dense aggregates clearly emanate from Z-discs and Z-disc remains and match the density of intact Z-discs. High-resolution imaging of streaming bodies shows that they are amorphous and devoid of membranous material. The majority of large zones of streaming occur just under the sarolemma (Figure 5.2), as predicted by the eosinophilic deposits seen under the light microscope. Additional ultrastructural pathology includes mis-registered intermyofibrillar sarcomeres and both rimmed and non-rimmed vacuoles. These vesicles contain either amyloid-like membranous material or amorphous sarcomeric remnants. TgWT and TgΔNT muscle shows normal sarcomeric ultrastructure (Figure 5.2).

**5.2.3 Expression of Z-disc proteins in TgΔNT and TgΔABS muscle**

The Z-disc streaming aggregates of TgΔABS muscle contain several Z-disc proteins, including the transgene product. The transgene product can be specifically localized with a c-myc antibody (Figure 5.3). Both the wild-type myotilin and TgΔABS transgene
Figure 5.3: Immunolocalization of Z-disc proteins in TgANT and TgΔABS muscle

Immunofluorescent localization of transgene products and other Z-disc proteins in myotilin domain deletion mice; myotilin (MYOT), alpha-actinin (ACTN2), gamma-filamin (FLNC), desmin (DES), and titin (TTN).
products localize properly to the Z-disc, and the TgΔABS product also stains the mini-aggregates. The TgΔNT product, however, does not localize to the Z-disc and cannot be immunolocalized with the c-myc antibody in both fresh, frozen and partially fixed, frozen sections.

The TgΔNT and TgΔABS products do not affect the Z-disc localization or abundance of several Z-disc proteins, including myotilin interacting proteins, alpha-actinin and gamma-filamin. The myotilin peptide antibody and titin monoclonal antibody show normal staining of the Z-disc and ectopic staining at the streaming aggregates in TgΔABS muscle (Figure 5.3). Myotilin, alpha-actinin, gamma-filamin, desmin, and titin all localize normally to the Z-disc in TgWT, TgΔNT, and TgΔABS muscle. Fluorescent signal strengths are all comparable to control littermate staining. Western blot analysis with antibodies targeting alpha-actinin and gamma-filamin shows normal sized bands and abundance in TgΔNT and TgΔABS muscle lysates compared to control littermate muscle lysate (data not shown).

5.2.4 TgΔNT and TgΔABS muscle display subtle contractile aberrations

In section 3.2.6, I showed that significant physiological dysfunction was specific to whole, intact EDL muscles of the point mutant TgT57I mice. Overexpression of wild-type myotilin in TgWT mice causes no muscle morphometric or contractile dysfunction.
Like the wild-type overexpressors, EDL and soleus muscles from both TgΔNT and TgΔABS mice exhibit muscle mass and cross-sectional area values comparable to littermate controls (Figure 5.4). Maximum specific force values of EDL and soleus, normalized for differences in size, are also comparable to littermate controls. The maximum specific force of TgΔNT diaphragm is also similar to controls. TgΔABS diaphragm was not tested. Subtle changes in whole muscle morphometry likely contribute to statistically significant differences (p<0.05) in the maximum isometric force generated by TgΔNT and TgΔABS EDL muscles. The TgΔNT EDL maximum isometric force is increased 13% compared to littermate controls (491 mN in TgΔNT, 433 mN in controls, p=0.044, Figure 5.4). The TgΔABS EDL maximum isometric force is decreased 23% compared to littermate controls (409 mN in TgΔABS, 529 mN in controls, p=0.029, Figure 5.4). TgΔNT and TgΔABS soleus muscles exhibit maximum force values indistinguishable from controls.

5.3 Discussion

TgΔNT mice develop no abnormal myopathy. Intriguingly, the truncated TgΔNT product does not properly localize to the Z-disc. TgΔABS myopathy includes increased central nuclei, necrotic fiber death, adipose infiltration, sub-sarcolemmal
Muscle was tested from 7- to 8-month old TgΔNT mice and control littermates [+/+ (I)], and from 8- to 9-month old TgΔABS mice and their respective control littermates [+/+ (II)]. Asterisks denote statistically significant (p<0.05) differences between Tg’s and respective control littermates. P-values were determined by two-tailed t-test for two samples assuming equal variance.
aggregation, and large patches of Z-disc streaming. The TgΔABS product localizes to both normal Z-discs and the streaming pathology. Despite the pathology, TgΔABS EDL muscle generates maximum specific force values comparable to littermate controls. These data show that 1) the N-terminal domain of myotilin may be required for normal localization to the Z-disc; 2) interaction with alpha-actinin is not required for localization of myotilin to the Z-disc; and 3) deletion of the alpha-actinin binding site causes an aggregation phenotype similar to that of the TgT57I mouse and myotilinopathy patients.

5.3.1 The myotilin N-terminal domain and Z-disc targeting

Immunofluorescence and western blot data demonstrate that the TgΔNT product does not localize properly to the Z-disc, suggesting that amino acids 1-79 are required for Z-disc targeting. The molecular composition of the N-terminal domain suggests two possible explanations for this observation: 1) a chaperone protein interacts with myotilin to promote Z-disc localization and 2) myotilin adopts a specific intramolecular structure critical for Z-disc localization.

The N-terminal domain of myotilin may bind to an as of yet undiscovered chaperone protein. The N-terminal domain of myotilin contains many serine residues. Serine contains the polar hydroxyl (-OH) side chain group, which, in series, could serve as a noncovalent docking pad for a novel myotilin binding partner. This binding partner
could be expressed early in myofiber differentiation and help direct myotilin to the presumptive Z-disc, where the sarcomere length ruler titin has begun to lay down the blueprint for F-actin anchoring. If such a chaperone protein exists for myotilin, it has evaded many yeast two-hybrid and immunoprecipitation-based screens.

Alternatively, the N-terminal domain may be critical for the intramolecular folding of myotilin. The N-terminal domain contains a stretch of hydrophobic amino acids which could embed this domain at the core of a globular myotilin protein. Loss of this hydrophobic stretch would cause misfolding, inhibition of quaternary protein interactions, and/or protein instability. Indeed, western blot shows that the TgΔNT product is several-fold less abundant than all other myotilin transgenes tested. A caveat to this analysis is that the N-terminal domain deletion could cause instability or a conformation change that obstructs antibody detection of the c-myc epitope tag. The TgΔNT product is immunolocalized on denaturing gels, but this result does not guarantee epitope accessibility in situ, even in fresh, frozen tissue sections.

Expression of the TgΔNT product does not cause muscle impairment. Histological analysis of several muscle groups and physiologic analysis of EDL, soleus, and diaphragm show that TgΔNT mice are normal. These data suggest that the mechanism of TgΔNT product mislocalization is distinct from molecular events leading to myofibrillar
aggregation caused by the myotilinopathy missense mutations in the N-terminal domain. Myotilinopathy mutations thus give rise to a very specific gain-of-function phenotype.

5.3.2 Myotilinopathy-like effects of alpha-actinin binding site deletion

Like the TgT57I LGMD1A mouse model, TgΔABS mice develop progressive myofibrillar pathology and show normal localization of the transgene product to the myofiber Z-disc. Deletion of the alpha-actinin binding site in a myotilin transgene does not affect Z-disc localization. Alpha-actinin can be ruled out as a putative Z-disc-targeting chaperone for myotilin. Alpha-actinin abundance and localization at the Z-disc are also not affected in TgΔABS muscle. The ΔABS mutation does not exert a dominant effect on alpha-actinin localization, but pathology does suggest a dominant effect on actin anchoring at the Z-disc.

TgΔABS muscle develops Z-disc streaming aggregates. These aggregates likely result from F-actin de-tethering at the Z-disc, followed by super-association of sarcomeric proteins. The TgΔABS mutation could cause F-actin de-tethering by either reducing structural functions of alpha-actinin or by displacing other alpha-actinin and F-actin binding proteins. Consider the first explanation: streaming pathology may directly result from the inability of myotilin to simultaneously interact with both alpha-actinin and F-actin. *In vitro* studies suggest that this ternary complex heightens F-actin cross-linking
compared to a binary complex of alpha-actinin and F-actin. Long-term contraction could thus wear on this sensitized Z-disc, leading to F-actin de-tethering. Alternatively, or in conjunction with the above hypothesis, the TgΔABS product may simply displace the critical mass of precisely tuned alpha-actinin and F-actin binding proteins at the Z-disc.

The pathology of TgΔABS muscle is distinct from the large, contiguous areas of myofibrillar aggregation in TgT57I muscle. TgΔABS-associated aggregation occurs at a later age and is limited to zones of smaller Z-disc streaming aggregation. TgΔABS muscle contains greater numbers of central nuclei, suggesting that degeneration is occurring. Central nuclei in TgT57I muscle appear to be more repair-oriented. Alternatively, TgT57I muscle may be deficient in myofiber regeneration. Another big difference is that TgΔABS myopathy does not lead to the significant physiological impairment observed in TgT57I EDL muscle. The effect of myotilin dosage does not explain these results; the TgΔABS product is expressed several-fold more than the TgT57I product.

Taking these data as a whole, the most likely explanation may be that myotilinopathy mutations indeed affect alpha-actinin binding. The enhanced aggregation phenotype in TgT57I muscle could be explained by additional effects of the Thr57Ile mutation, such as preventing interactions with other Z-disc structural proteins such as gamma-filamin and the FATZ proteins.
5.3.3 Myotilin turnover

The $\Delta$NT, $\Delta$ABS, and Thr57Ile mutations may have differential effects on myotilin protein stability and turnover. A larger population of stable myotilin peptides could easily enhance myofibrillar aggregation. Myotilin contains an SDEL endoplasmic reticulum (ER) targeting sequence at its C-terminus. ER targeting is associated with clearance of proteins through lysosome fusion and subsequent autophagy. The different myotilin mutations could affect the availability or function of the SDEL sequence.

Intriguingly, myotilin was retrieved in a yeast two-hybrid screen to uncover binding partners of the muscle-specific RING finger protein, MURF-1 (240). A MURF-1 ‘bait’ clone uncovered eight additional myofibrillar ‘prey’ proteins, many of which also interact with MURF-2. The MURFs are ubiquitin ligases that target specific muscle proteins for degradation by the proteasome (241). Regulated ubiquitination can also modulate protein function without protein degradation (242). Specific degradation of sarcomeric proteins could serve to modulate muscle energetics and contractility. Regardless of regulation, skeletal muscle is a highly oxidative tissue that demands stringent clearance of damaged proteins. Identification of the specific myotilin residues required for both MURF binding and ubiquitination may explain the extent of aggregation observed in myotilin transgenic mice.
5.4 Materials and Methods

5.4.1 Generation of myotilin domain deletion transgenic mice

The wild-type myotilin cDNA was isolated from a pTriplEx human skeletal muscle cDNA library (BD Clontech), and cloned into a modified pBluescript vector (Stratagene) containing the enhanced HSA promoter cassette described in section 3.4.1. Recombinant PCR was used to incorporate the c-myc epitope tag directly 3’ of the start codon. This clone was used as template for subsequent recombinant PCR to create each of the TgΔNT and TgΔABS deletions. 5’-ΔNT and 3’-ΔNT amplicons, flanking the deletion, were generated with dHPLC-purified, tailed complementary reverse and forward primers, respectively, to facilitate subsequent annealing and amplification of the linked amplicons. This now single amplicon containing the deletion was then sub-cloned into the pCR4TOPO vector (Invitrogen), purified after EcoRI/BglII double-digestion, and cloned back into the HSA-myotilin plasmid construct. The ΔABS amplicon was similarly generated and sub-cloned. Primers used to create the deletion-flanking sub-amplicons are listed 5’→3’:

5’-ΔNT, forward: GATCGATCCCTCGAGTCTAG
5’-ΔNT, reverse: GTAACCCTTTGGCCTGGGTTCAGGTCTTCCTCGCTGATCAGC
3′-ΔNT, forward:
GCTGATCAGCGAGGAAGACCTGAACCCAGGCCAAAGGGTTACAAC
3′-ΔNT, reverse: AGCTTTGGTGAGGTATAG
5′-ΔABS, forward: GATCGATCCCTCGAGTCTAG (same as 5′-ΔNT, forward)
5′-ΔABS, reverse: CATTATAGGTTGGCCGGAGCAGCATGCTGCTGGGAG
3′-ΔABS, forward: GCAGCATGCTGGCTCCGGCCAACCTATAAATGCAAAGCCA
3′-ΔABS, reverse: AGCTTTGGTGAGGTATAG (same as 3′-ΔNT, reverse)

The linear transgenic constructs were released by KpnI/NaeI double-digestion, purified with gel extraction columns (QiaGen), sterilized by ethanol precipitation, and microinjected into C57BL/6 (TgΔNT) or B6SJLF2 (TgΔABS) one-cell embryos at the Duke University Transgenic Mouse Facility. Transgene-positive founder mice were subsequently backcrossed to C57BL/6. Data reported in this chapter were gathered from progeny that were backcrossed at least 4 times to C57BL/6. Myotilin transgene genotyping was done by PCR amplification of a 639 bp (wild-type) fragment from tail clip DNA with a forward 5′-UTR primer (5′-CTCAACAAGGAAGGACAGAC-3′) and a reverse myotilin exon 3 primer (5′-TCCTTGTATTTTCATGATCAGGAG-3′). This primer pair, distinct from that used in section 3.2.1, was designed to flank the TgΔNT and TgΔABS deletions. C57BL/6 breeders were purchased from Harlan. Mice were bred under standard conditions in the Genome Sciences Research Bldg. II animal facility at Duke University.
5.4.2 Analysis of myotilin transgene expression

Total RNA was prepared from dissected and homogenized brain, heart, spinal cord, quadriceps, and soleus tissues (SV Total RNA Isolation System, Promega). For RT-PCR, 1 μg of total RNA was reverse transcribed using random hexamers (Promega). A transgene-specific 246 bp product was amplified with a forward HSA exon 1 primer (5′-GAGTAGCAGTTGTAGCTACC-3′) and a reverse myotilin 5′-UTR primer (5′-GTCTGCTCTCCTTGTTGAG-3′). Muscle tissue for downstream western blotting was snap-frozen in liquid nitrogen, ground in a mortar and pestle, and resuspended (200 μL/100 mg tissue) in 1% SDS, supplemented with a mammalian protease inhibitor cocktail (Sigma). Western blotting was performed as described in section 3.4.2. Primary antibodies for western blot included: myotilin peptide antibody (1:4000, rabbit polyclonal, generated by Bethyl Laboratories using the peptide corresponding to myotilin residues 473-488, CVKQAFNPEGFQRRLAQ); anti-c-myc (1:10000, rabbit polyclonal, RDI Division of Fitzgerald Industries)

5.4.3 Electron microscopy

Mice were transcardially perfusion-fixed with 4% paraformaldehyde, 0.25% glutaraldehyde in HEPES buffer. Whole muscles were dissected and processed as described in section 3.4.4.
5.4.4 Immunofluorescence

All immunostaining was performed on sections of fresh, frozen quadriceps muscle. The titin antibody was used at a dilution of 1:750 (anti-titin, clone 9 D10, developed by Dr. Marion Greaser and obtained from the Developmental Studies Hybridoma Bank at U. Iowa) (243). Detailed immunofluorescence protocol and antibody information is available in section 3.4.5.

5.4.5 Muscle Physiology

Described in section 3.4.7

Note: Muscle physiology was performed in collaboration with Dr. John A. Faulkner (University of Michigan, Ann Arbor, Michigan).
Chapter 6

6. Conclusions and Future Directions

I created an LGMD1A transgenic mouse model by expressing the human myotilin cDNA bearing the DUK39 Thr57Ile mutation on a wild-type murine myotilin background. These transgenic mice (TgT57I) reproduce many of the symptoms and pathology associated with the myotilinopathies—Z-disc streaming, myofibrillar aggregation, and muscle weakness. This mouse model not only unifies the diverse phenotypes of the human myotilinopathies, but also promises to be a key resource for understanding myotilin function, unraveling LGMD1A pathogenesis, and investigating possible therapeutics.

I also studied two myotilin deletion transgenes *in vivo*. These data show that 1) the N-terminal domain of myotilin may be required for normal localization to the Z-disc; 2) interaction with alpha-actinin is not required for localization of myotilin to the Z-disc; and 3) deletion of the alpha-actinin binding site causes an aggregation phenotype similar to that of the TgT57I mouse and myotilinopathy patients.
6.1 The spectrum of sarcomeric aggregation in myotilin transgenic mice

The myotilin transgenic mice delineate a spectrum of pathological severity that can be
generalized by a model of ‘4 degrees of aggregation.’ While ages are given below, this
model is essentially based on histology and ultrastructure of aggregates. Variations in
myofibrillar aggregation are present in all myotilin transgenic mice, with the exception of
the TgΔNT mice expressing the N-terminal domain deletion. TgΔNT muscle
demonstrates no aggregate pathology, which I shall call ‘zero-order’ aggregation.

TgWT mice overexpressing wild-type myotilin exhibit ‘first-degree’ aggregation: small
~ 5-μM aggregates develop at the sarcolemma by 10-12 months of age. These
subsarcolemmal aggregates appear as focal eosinophilic deposits by light microscopy.
TgΔABS mice expressing a deletion of the alpha-actinin binding site demonstrate
‘second-degree’ aggregation: 5- to 20-μM subsarcolemmal aggregation coupled with
inter-myofibrillar aggregation. The myofibrillar aggregation occurs in the form of 3- to
20-μM diameter zones of Z-disc streaming by 6 months of age. Functional testing shows
that first- and second-degree aggregations are not associated with significant muscle
weakness in TgWT and TgΔABS mice.

The ‘second-degree’ Z-disc streaming presumably leads to the continuously dense ‘third-
degree’ aggregation observed in muscle from the TgT57I mouse model. TgT57I muscle
develops up to 40-μM electron- and protein- dense myofibrillar aggregates by 6 months
of age. TgT57I muscle also demonstrates subsarcolemmal aggregation and Z-disc streaming. Third-degree aggregation in TgT57I mice is associated with decreased specific force generation, but may protect against contraction-induced injury. TgT57I; TgWT double-transgenic mice (DTg) exhibit ‘fourth-degree’ aggregation—aggregates that consume approximately the whole cross-sectional area of a myofiber, leading to signs of myofiber degeneration, necrosis, fibrosis, and overt dystrophy by 6 months of age.

6.2 Resolving LGMD1A phenotypic heterogeneity

TgT57I expression induces all of the skeletal muscle pathologies observed in the human myotilinopathies, and raises the possibility that variations in the presentation of human patients are the result of modifier loci. It is likely that genetic modifiers are contributing to phenotypic heterogeneity in Family 39 and in other MD pedigrees with extensive clinical variability. One promising candidate genetic modifier is the alpha-actinin isoform, alpha-actinin-3, which localizes to the Z-disc and likely cross-links actin filaments. Alpha-actinin-3 does not itself cause MD, but this gene is not expressed in 18% of normal, healthy Caucasians due to homozygosity for the Arg577Stop allele (192). The functional allele is significantly associated with power performance in Olympic athletes, and the null allele is associated with endurance performance (193). Could either allele also be associated with a more severe clinical metric in MD patients? Preliminary
screening of 72 LGMD1A subjects in Family 39 shows that the alpha-actinin-3 null allele is segregating, and 35% are homozygous for the null allele. Crossing the TgT57I mouse to an alpha-actinin-3 knockout mouse will prove invaluable for confirming any effect of the R577X allele in human MD families.

6.3 Implications for treatment of myotilinopathies

The TgT57I mouse serves as an invaluable resource for testing gene-specific, pharmacologic, and exercise treatments and therapies. The sensitized DTg mouse also serves as a means to test treatments on pathology in younger mice. Physiological analysis of TgT57I EDL v. soleus muscles (Figure 3.8) shows force deficits specific to the EDL—a muscle that develops myofibrillar aggregation. TgT57I soleus does not develop myopathology. These observations suggest that myofibrillar aggregation is associated with muscle weakness. Targeted reduction of aggregate burden is likely to improve muscle strength in TgT57I mice and LGMD1A patients.

The DTg mice show that total myotilin levels (mutant + wild-type) are positively correlated with myofibrillar pathology. The observations in DTg mice suggest that a ‘knockdown’ approach, rather than an additive or replacement strategy, will be an effective therapeutic method aimed at modulating myotilin gene expression or protein levels to ameliorate dystrophic symptoms. I have proposed using RNA interference,
specifically with the use of short hairpin RNAs against myotilin transcript, to specifically downregulate myotilin in TgT57I mice. Myotilin knockdown could reduce the initial stages of actin de-tethering that lead to myofibrillar aggregation. After the Z-disc has ‘streamed’, further knockdown would downregulate the amount of wild-type myotilin accumulating at the aggregates. RNAi therapy is still at its infancy, though, and safe and effective modes of skeletal muscle-specific delivery have yet to be fine-tuned.

Alternatively, the indirect result of myotilin mutation, the myofibrillar aggregate, could be pharmacologically targeted. Testing of aggregate-reducing pharmacologics in the TgT57I mouse model is advised. Rapamycin, proline, trehalose, and doxycycline all have potent anti-aggregation effects in both cell and whole organism models. In particular, doxycycline and trehalose have proven effective at reducing nuclear aggregation and muscle weakness in a mouse model of oculopharyngeal muscular dystrophy (225;226). Rapamycin, also known as sirolimus, an FDA-approved antifungal and anti-inflammatory, has been shown to induce autophagy of protein aggregations (244). Subsequent fusion of autophagic vesicles to lysosomes leads to the degradation of luminal aggregates. The prevalence of autophagic vesicles in LGMD1A and TgT57I muscles further suggests that myofibrillar aggregates are actively being cleared through this mechanism. This focal repair process could be enhanced by rapamycin.
6.4 TgT57I: a model for nuclei-mediated focal repair

Focal myofibrillar repair appears to also be aided by direct homing of nuclei to regions of myofiber degeneration and aggregation in TgT57I and DTg mice (Figure 4.3). A similar phenomenon is observed in the TgM9R nemaline myopathy mouse model (238). Genomic expression analysis of whole muscles from TgM9R mice v. controls suggests that hypertrophic signaling factors are upregulated—these signals are likely generated by neighboring satellite cells. The satellite cells are also contributing to focal repair, but their genomic signatures may be drowning out the gene expression patterns of these fascinating repair nuclei. I propose to use laser-capture microdissection to precisely harvest RNA from both the central repair nuclei and normal, peripheral nuclei of affected TgT57I muscle. Differential gene expression may highlight autophagosome, heat-shock, proteosome, or even nuclear cytoskeletal remodeling pathways. The peripheral nucleus must remodel its cytoskeleton in order to dislodge from its peripheral cavity and slither between myofibrils to a damaged area. In such a way, study of the TgT57I mouse may even shed light on the myopathies involving nuclear envelope proteins.
Appendix

Appendix A: Myotilin is not the causative VCPDM gene

A.1 Introduction

Vocal Cord and Pharyngeal Weakness with Distal Myopathy (VCPDM, also called MPD2; MIM 606070) is an adult-onset, autosomal dominant muscular dystrophy of unknown genetic and molecular origin. VCPDM patients initially present with muscle weakness in the feet and hands, frequently accompanied by vocal or swallowing dysfunction (245). Analysis of seven muscle biopsies consistently demonstrated rimmed vacuoles and central nuclei. Linkage analysis in a single large North American pedigree revealed that the VCPDM gene maps to a 12-cM interval on chromosome 5q between markers D5S1995 and D5S436 (245). A recombination within the disease haplotype of a 49-year-old healthy individual suggests a smaller 7-cM interval between markers D5S1995 and D5S658. Within this interval lies the attractive candidate gene, myotilin.

Myotilin mutations have been shown to cause the autosomal dominant disorder, Limb-Girdle Muscular Dystrophy Type 1A, in two independent pedigrees (118;119). LGMD1A patients present with proximal muscle weakness at a mean age at onset of 27
years. Half of LGMD1A patients exhibit dysfunction and paralysis of the vocal cords leading to a dysarthric and nasal speech pattern. Ultrastructural analysis of muscle biopsies reveals rimmed vacuoles and extensive Z-disc streaming. The two myotilin point mutations identified in LGMD1A cause amino acid replacements (Ser55Phe and Thr57Ile) in the N-terminus of myotilin, a region with no known function or homology with other proteins. Expressed in skeletal and cardiac muscle, myotilin localizes to the Z-disc of the sarcomere (128). Myotilin may contribute to sarcomere assembly and promote actin cross-linking and sarcomeric integrity through proven interactions with alpha-actinin, gamma-filamin, and F-actin (134). Additional missense mutations in the same region of myotilin have recently been identified in patients with a similar muscle disease, myofibrillar myopathy (MFM), including one individual with the same Ser55Phe missense mutation previously reported in a LGMD1A patient (83). It is unclear how these subtle mutations alter myotilin function to cause LGMD1A and MFM.

Myotilin is an attractive functional and positional candidate for VCPDM. LGMD1A and VCPDM share several features. Both are adult-onset, autosomal dominant, progressive myopathies that involve altered speech patterns, and both exhibit rimmed vacuoles and central nuclei. Although VCPDM presents in distal muscles, shoulder weakness was also observed in 4 of 12 patients examined (245). Moreover, half of the 6 MFM patients with myotilin mutations show primary distal involvement, highlighting the clinical
heterogeneity of myotilinopathies. Neither LGMD1A nor VCPDM patients display any
signs of inflammatory involvement, cardiac disease, or ophthalmoplegia.

A.2 Results

I analyzed the myotilin coding region for sequence variants in two VCPDM patients.
Patient samples were obtained after informed consent in accordance with IRB oversight.
Previously described intronic primers were used to amplify each of the 10 exons of
myotilin, including approximately 50bp of flanking intronic sequence (118). Sequence
analysis of these genomic PCR products reveals no variants in VCPDM. To further
examine the possibility of myotilin as the VCPDM gene, I examined the myotilin
transcript in one VCPDM patient by RT-PCR. Total RNA was prepared from biopsied
gastrocnemius muscle of a VCPDM patient. Two primer pairs were used to amplify
overlapping RT-PCR products spanning the myotilin cDNA, and a third product spans
the entire transcript. RT-PCR shows normal splicing of myotilin in VCPDM (Figure
A1). All known myotilin mutations map to exon 2. Southern blot analysis using a
myotilin exon 2 probe on EcoRI- and HindIII-digested control and VCPDM genomic
DNA reveals bands of comparable intensity and size (Figure A1). Additional PCR
analyses confirm that there is no difference in the size of the HindIII-fragment.
Additional EcoRI (5.7 kb), NarI (6.9 kb), and Xmal (7.5 kb) restriction fragments were
also probed by Southern blot in order to screen the myotilin locus from 4.9 kb upstream
Figure A.1: Myotilin appears unaltered in a patient with VCPDM. (A) There is no evidence of myotilin splicing defects in VCPDM muscle. RT-PCR products spanning all splice junctions of the myotilin gene were amplified from control (C) and VCPDM patient (V) skeletal muscle total RNA. Exons 1 to 7 (ex. 1-7), exons 7 to 10 (ex. 7-10) and the full-length transcript (ex. 1-10) were amplified in the three reactions shown. The smaller products in the ex. 1-7 and ex. 1-10 reactions represent alternative splice events common to both control and VCPDM muscle. Slightly decreased intensity of VCPDM bands is consistent with the GAPDH control. 1 kb DNA ladder was loaded in the first lane. (B) Southern blot analysis shows that the 5’ region of myotilin, including the mutation hot spot, exon 2, is intact in VCPDM. 7-ug of genomic control and VCPDM DNA was digested with EcoRI and HindIII, separated on an agarose gel, transferred to nitrocellulose, and probed with an exon 2 digoxigenin-labeled PCR product. Both the EcoRI fragment, 10.7 kb, and the HindIII fragment, 6.0 kb, contain partial promoter, exon 1, intron 1, exon 2, and part of intron 2 of myotilin. (C) Myotilin protein is expressed at wild-type levels in VCPDM muscle. VCPDM muscle lysate was probed with a peptide antibody specific to amino acids 473 to 488 of the myotilin protein. (D) A VCPDM patient is heterozygous for two adjacent A/T SNPs (rs3777122, rs3777121) in myotilin intron 2.
of exon 1 to 1.5 kb downstream of exon 10. There were no differences between control and VCPDM DNA (data not shown). Two adjacent A/T single nucleotide polymorphisms (SNPs) in myotilin’s second intron are heterozygous in a VCPDM patient (Figure A1). Sequencing of the putative myotilin promoter, 2 kb of genomic sequence directly upstream of exon 1, yielded one C/T SNP at position -1010, which did not segregate with the phenotype. VCPDM samples are also heterozygous for two novel dinucleotide short tandem repeat markers, one located within intron 1 (build 35 pos 137,233,876) and the other 31 kb 3’ of the myotilin gene (build 35, position 137,282,533). These data exclude the possibility of a large heterozygous deletion of the myotilin locus in VCPDM. I also show that the 57 kD myotilin protein is unaltered in VCPDM by western blotting, using gastrocnemius extracts and an antibody specific to a C-terminal myotilin peptide (Figure A1).

A.3 Conclusions

These data provide strong evidence that LGMD1A and VCPDM are not allelic disorders. One major pathological difference supports these molecular data. While VCPDM, LGMD1A, and MFM share several clinical features, VCPDM muscle does not show the ultrastructural hallmarks of LGMD1A and MFM—Z-disc streaming and myofibrillar aggregation. Further study of LGMD1A and VCPDM promises to reveal novel mechanisms of muscle disease. While several neuropathies also involve vocal cord
dysfunction and paralysis, LGMD1A and VCPDM share this clinical feature, which is unusual in muscular dystrophies. Discovery of the VCPDM gene will contribute to our understanding of the myogenic origin of vocal cord dysfunction and perhaps shed light on the mechanism of LGMD1A.

*Note:* This work was published in the *Annals of Human Genetics* (Garvey et al., 2006). Copyright © 2005, The Authors; © 2005, University College London. Used with permission.
Appendix B: Myotilin Gln74Lys is non-pathogenic

Sequencing of myotilin exon 2 in non-dystrophic controls revealed two SNPs, A429G and C500A, that convert lysine 36 to glutamic acid and glutamine 74 to lysine, respectively. Follow-up analysis has shown that these missense SNPs are non-causative, ethnicity-associated SNPs that occur with allele frequency ~ 5% in African-Americans. The C500A SNP (rs6890689) was included in the HapMap Project dataset (www.hapmap.org). The 500A allele occurred at a frequency of 5.0% in the Yoruba population sampled in Ibadan, Nigeria. Each allele, 429G and 500A, was speculated to be a pathogenic mutation in two separate subjects presenting with sporadic muscular dystrophy (84). The authors also report MRI data showing muscle degeneration in the sporadic case with the Gln74Lys mutation. These patients most likely do not have myotilinopathy.
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Biography

Sean Garvey was born in Burlington, Vermont on November 9th, 1977. He was raised in nearby Essex Junction and graduated salutatorian of his class at Essex Junction High School in 1995. Sean attended the University of Vermont and graduated magna cum laude and Phi Beta Kappa in 1999 with a Bachelor of Science degree in Microbiology and Molecular Genetics and minors in Chemistry and Philosophy. For the next two years, Sean worked with Dr. Gregory Cox at The Jackson Laboratory in Bar Harbor, Maine. In 2001, Sean enrolled in the University Program in Genetics at Duke University, Durham, North Carolina, where he was awarded a Ruth L. Kirschstein National Research Service Award pre-doctoral fellowship.

List of publications


