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Volume 642

The Sarcomere and Skeletal Muscle Disease

Edited by
Nigel G. Laing

The Sarcomere and Skeletal Muscle Disease

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DEDICATION

This book is dedicated to all the patients and families around the world affected by diseases of the skeletal muscle sarcomere and the many researchers dedicated to defeating those diseases.

"He wars on Death—for life; not men—for flags."
Wilfred Owen: "The Next War".

FOREWORD

Sarcomeric Disease: Advances and Some Unanswered Questions

The gene, and its protein product, in a serious inherited muscle disease was first identified in the case of Duchenne muscular dystrophy some twenty years ago. Dystrophin turned out to be a sarcolemmal protein. This was somewhat of a surprise because many had harboured the view up until then that most muscle diseases, if not all, might be due to defects in the sarcomere itself. After all, this was the site of muscle contraction. However, many other muscular dystrophies have subsequently been shown to be due to defects in various other sarcolemma-associated proteins.[1]

The structure of the sarcomere itself has been studied and known for many years.[2] But only in more recent times has the role of its structural and contractile proteins been detailed in specific muscle diseases. The cytoskeleton is held together by filamentous proteins, such as α-actinin and desmin, and the microtubular protein tubulin. Other proteins are nebulin and telethonin and the elastic element titin. Finally, there are the contractile proteins troponin, tropomyosin, actin and myosin. In this book experts in the field describe a variety of diseases associated with defects in these sarcomeric proteins, that are often first suspected because of a pronounced accumulation of the relevant protein in the muscle tissue.

Recently, a few relatively rare dystrophies themselves have also been shown to be associated with defects in sarcomeric proteins, for example, myotilin in LGMD1A, telethonin in LGMD2G, and titin in LGMD2J. The latter is of particular interest in the present context. Whereas homozygous mutation of the titin gene results in the autosomal recessive, severe, early onset 2J form of limb-girdle muscular dystrophy, heterozygosity for the same and other dominant mutations results in a mild, late onset form of distal (tibial) myopathy.[3,4] Furthermore, myotilin mutations have now been demonstrated in myofibrillar myopathy, or desmin-related myopathy, as well as in LGMD1A.[5]

These observations and several other examples in the text raise fundamental questions: how do we explain the very different clinical phenotypes which can result from different mutations in the *same gene*, and occasionally in different members of the *same family* with the same mutation? This problem is not unique to the sarcomeric

diseases. It is also well exemplified in the case of mutations of the LMNA gene resulting in multiple laminopathies, defects in nuclear membrane proteins, a problem which has been recognised for some time.[6]

Further detailed analysis of specific mutations in these disorders may provide some answers. But it is also possible that studying associations with single nucleotide polymorphisms or copy number variants[7-10] may be helpful though so far most of this research has centred on more common multifactorial conditions. These seem to be unexplored territories in regard to genetic forms of muscle disease. Furthermore, acute quadriplegic myopathy, due to a preferential loss of myosin and myosin-associated thick filament proteins, is acquired and *not* genetic.

It is also perhaps possible that certain common pathogens could affect disease presentation via their interaction with, for example, actin filaments and the induced filament rearrangements.[11] But again this is a field which has so far not been explored.

The results of the studies presented in this volume now pave the way for much future research into the pathogenesis of this group of fascinating conditions, leading one day to their treatment.

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PREFACE

This book arose from a request by Ron Landes of Landes Bioscience for a book on the role of the sarcomere in human skeletal muscle diseases following our article "When contractile proteins go bad: The sarcomere and skeletal muscle disease" in BioEssays in 2005 (Vol 27, pages 809-822). The sarcomere is also the basis of heart diseases, but the remit of this book is skeletal muscle diseases alone. A number of the chapters in the book provide an up-to-date review of diseases caused by mutated proteins in the different sub-compartments of the sarcomere, others document the techniques currently being used to investigate the pathobiological bases of the diseases, which remain largely unknown, and others discuss of possible routes to therapies. The book is intended to provide a snapshot of this rapidly moving field.

I thank all the authors who responded positively to my request to provide chapters in their particular areas of expertise, with many of the chapters thus written by the discoverers of the diseases being described. Hopefully, the book will generate interest in young scientists to participate in this field of research, in particular to take up the challenge of trying to develop effective treatments for these diseases caused by dysfunction of the fundamental unit of muscle contraction, the sarcomere. I thank Landes Bioscience for suggesting the book, the members of my laboratory for their support during the time of the editing of the book, particularly Dr. Kristen Nowak, and I thank the Australian National Health and Medical Research Council Fellowship Grant 403904 for support during the period of production of the book.

Nigel G. Laing, PhD

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NIGEL G. LAING, PhD, is currently a Professorial Fellow in the Centre for Medical Research at the University of Western Australia, within the Western Australian Institute for Medical Research at the QEII Medical Centre in Western Australia and a Senior Medical Scientist in the Neurogenetic Laboratory at Royal Perth Hospital, Western Australia. He is originally Scottish, and completed his undergraduate studies, Honours in Pharmacology (1976), and PhD in Physiology (1979) at the University of Edinburgh. His thesis was on the effects of bungarotoxin paralysis on motor neuron death in the developing chick embryo and was supervised by Martin Prestige. He had a one-year post-doc with Professor Jan Jansen in the Department of Physiology at the University of Oslo in 1980. After that he moved to the Department of Pathology at the University of Western Australia for another post-doc position with Alan Lamb from 1981-1987. In 1987-1988, he re-trained in molecular genetics with Professor Teepu Siddique in Professor Allen Roses' laboratory at Duke University, North Carolina, returning to Western Australia in July 1988 to develop both research and diagnostic molecular neurogenetics laboratories under Professor Byron Kakulas. The research laboratory (first in the Australian Neuromuscular Research Institute and subsequently in the Western Australian Institute for Medical Research) has played a role in showing that SOD1 was a gene for familial amyotrophic lateral sclerosis. It also identified mutation of slow α-tropomyosin as the first known cause of the congenital myopathy nemaline myopathy and mutations in skeletal muscle α-actin as a significant cause of congenital myopathies (especially severe congenital myopathies), and showed that certain specific mutations in the tail of slow skeletal/β-cardiac myosin are associated with an early onset form of distal myopathy, now known as Laing distal myopathy. The Neurogenetic Laboratory at Royal Perth Hospital provides a state-wide molecular diagnostic service for neurological disorders, and an Australasian-wide service for some of these disorders and is a world reference centre for the diagnosis of skeletal muscle α-actin and slow α-tropomyosin disorders.

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Chapter 1

The Sarcomere and Sarcomerogenesis

Elisabeth Ehler* and Mathias Gautel

Abstract

Striated muscle owes its name to the microscopic appearance, caused by the longitudinal alignment of thousands of highly ordered contractile units, the sarcomeres. The assembly (and disassembly) of these multiprotein complexes (sarcomere assembly or sarcomerogenesis) follows ordered pathways, which are regulated on the transcriptional, translational and posttranslational level. Furthermore, myofibril assembly involves the participation of transient scaffolds and adaptors, notably the microtubule network. Studies in cell culture and developing embryos have revealed common pathways of sarcomere assembly in heart and skeletal muscle. Disruptions in these pathways are implicated in muscle diseases.

Introduction

The sarcomeres of striated muscles are among the most regular structures in animal cells, a prerequisite for their function as linear motors that make the highly coordinated, fast movements possible, without which the evolution of higher animals is not conceivable. Indeed, the rapid evolution of complex life forms during the Cambrian radiation around 530 million years ago led to the appearance of a large variety of mobile, rather than sessile, animals that had developed the use of locomotion for feeding, predation and flight. As such, complex locomotion can be seen not only as a result, but as a main driver of evolution. In agreement with the ancient evolutionary origin and highly conserved protein inventory, ultrastructure and function of striated muscle, the mechanisms leading to sarcomere assembly and ultimately to myofibril formation seem to be conserved between all vertebrate phyla and show significant similarities to invertebrate animals with complex locomotion.

The classical contractile filaments of the sarcomere, actin and myosin, are formed from self-assembling monomers. However, these lack the intrinsic ability for the precise termination of filament length seen in the sarcomere. The bipolar myosin filaments are precisely regulated to a length of 1.59 μm, containing 294 myosin molecules (each composed of 2 heavy and four light chains), or 147 per half thick filament.[1] Actin filaments show a narrow length distribution that varies with fibre type and generally extends beyond the 1 μm length observed for freely assembled actin filaments. Furthermore, although many reports suggest that sarcomeric proteins are rapidly incorporated into striated patterns in living cells and may rapidly exchange with a free cytoplasmic pool,[2-4] isolated myofibrils are remarkably stable and can be stored for days without disassembling spontaneously. In other words, there is no measurable equilibrium with the monomer state for key sarcomeric proteins. These observations point to two essential aspects of sarcomere assembly: the involvement of length-regulating proteins and the existence of an energetic barrier that prevents spontaneous disassembly in the absence of further cellular components. The organising proteins have been identified to be the giant molecular ruler or blueprint titin (also known as connectin),

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which organises entire half-sarcomeres from M-band to Z-disk (Fig. 1) and is intimately associated with myosin filaments; while the actin filament appears to be ordered beyond its intrinsic self-assembly by the giant protein nebulin. The involvement of ATP-dependent chaperones in sarcomere assembly may explain how folding and assembly are coordinated and why, in the absence of cellular energy and chaperones, the sarcomere is in a locked state.

In the following, we will discuss the current understanding of how sarcomeric proteins, the giant rulers and transiently associated factors cooperate in the formation of sarcomeres. The disruption of these mechanisms emerges as a key contributor to muscle diseases.

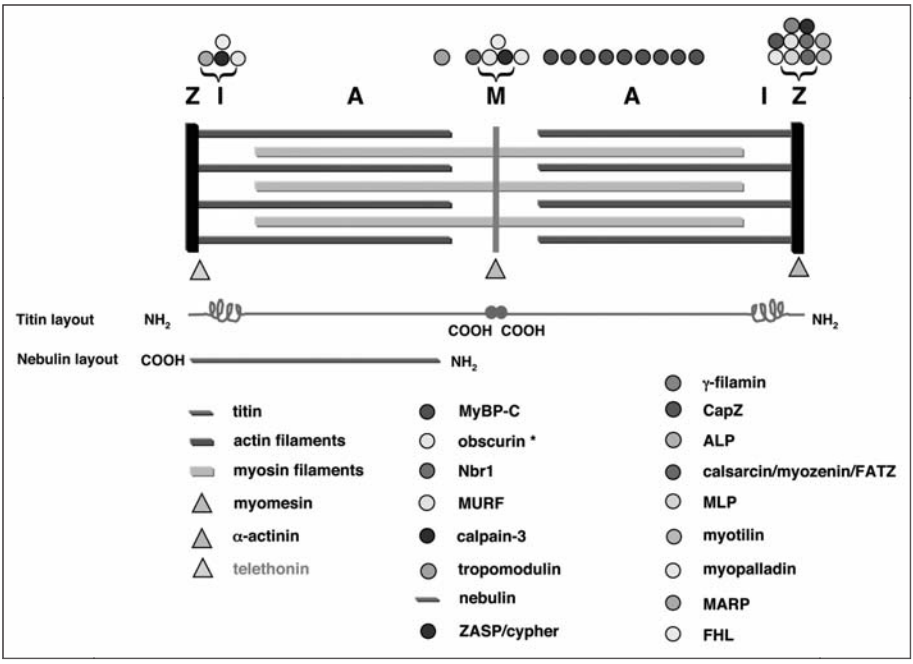

Figure 1. Overview of the sarcomere and key constituent proteins. The interdigitating actin and myosin filaments are composed of hundreds of subunits and contain accessory proteins like the actin-bound troponin-tropomyosin complex (not shown) and myosin-bound MyBP-C that are involved in contraction regulation. Myosin filaments are crosslinked by M-band proteins, notably the constitutively expressed myomesin, at the M-band. Actin filaments are crosslinked by alpha-actinin at the Z-disk and additionally (at the Z-disk periphery) by gamma-filamin. The giant ruler protein titin is absolutely required for sarcomere assembly, as are myomesin, alpha-actinin, actin, myosin and possibly proteins like cypher and telethonin/TCAP. The giant ruler protein nebulin is dispensable for primary sarcomere assembly. Multiple accessory proteins are associated with the sarcomere, though many of these are not essential for sarcomere assembly (like MyBP-C, myotilin, cypher, MARP, or MURFs). Combined evidence suggests that sarcomeric myosin, actin, titin, alpha-actinin and myomesin are essential components. In the case of telethonin, knockout mouse data are still required.
Symbols: accessory proteins shown as circles. Constitutive components essential for primary sarcomere assembly are shown as triangles in addition to titin, actin and myosin filaments.
Abbreviations: MyBP-C: myosin-binding protein-C; ALP: actinin-associated PDZ-LIM protein; MURF: muscle-upregulated RING finger protein; MARP: muscle ankyrin repeat proteins; FHL: four-and-one-half LIM protein; Nbr1: next-to-BRCA1; MLP: muscle LIM protein.

Insights in Sarcomere Assembly from Cell Culture Studies

The possibility of differentiating primary myoblasts or myogenic cell lines in vitro allows studying the mechanisms governing transcriptional regulation of muscle differentiation and its actual functional manifestation, the formation of ordered contractile myofibrils. Insight from studies using chick, quail, or rodent myoblasts has revealed a wealth of information on the temporal dynamics of myofibril assembly. The intermediates of myofibrillogenesis are hard to study and still incompletely characterised,[5] partly due to the unclear role of the increasing number of recently identified additional components.[6] As in vitro myofibrillogenesis is generally slower than in vivo, many steps can, however, be observed that may be hard to capture in sufficient detail in embryonic muscle, although the basic mechanisms are likely to be very similar (Fig. 2).

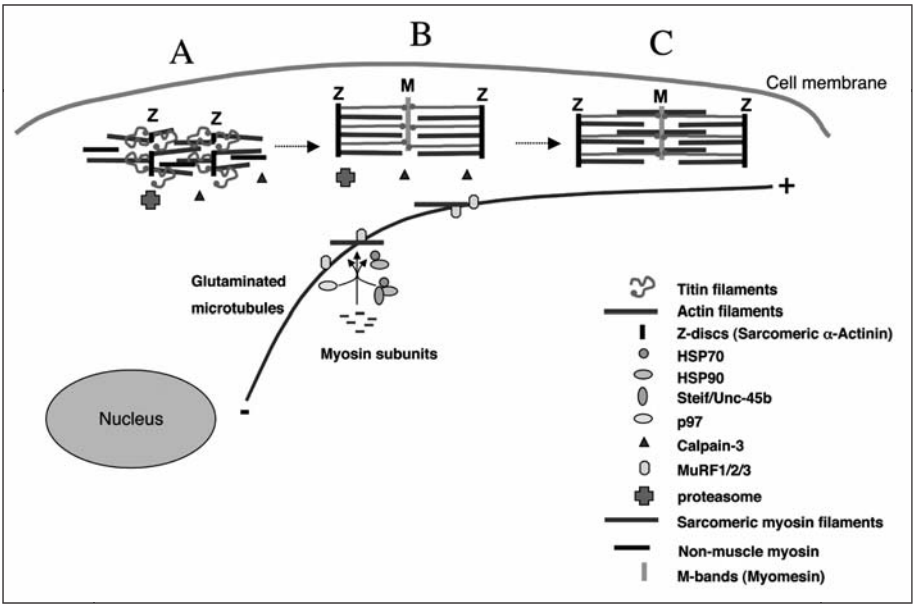

Figure 2. The assembly of myofibrils follows an ordered sequence of intermediates. A) Initial assembly occurs on actin stress-fibre like structures that accumulate sarcomeric alpha-actinin. At least in cultured skeletal myocytes, these primordial structures (premyofibrils) also contain nonmuscle myosin-II that may serve as a substitute for sarcomeric myosin. The giant sarcomere ruler titin is first incorporated at its N-terminal Z-disk portion, presumably via interactions with alpha-actinin. At this stage, C-terminal epitopes of titin are not resolved or colocalize with Z-disk titin, suggesting that titin at this stage is coiled up. Primitive Z-spots are spaced about 1 µm apart. B) Premyofibrils undergo elongation involving possibly the action of motor proteins and accessory proteins. This leads to the formation of an initial Z-M-Z scaffold with clearly separated titin Z- and M epitopes. These structures have not yet accumulated all sarcomeric proteins, notably myosin filaments. The synthesis of myosin subunits and their assembly into filaments involves and requires the participation of the chaperones HSP70 and HSP90 in cooperation with Steif/Unc45 and the ubiquitin-dependent chaperone p97. The ubiquitin E3-ligases MURF, as well as the proteasome participate in yet incompletely understood ways in sarcomere assembly. In skeletal muscle, the cysteine protease calpain-3 is also involved. One of their roles may be the removal of misfolded sarcomere constituents. Plus-end directed microtubule transport brings myosin to sites of sarcomere assembly. After incorporation of myosin, the nascent myofibril undergoes further restructuring and microtubules disappear. C) The mature myofibril has a Z-Z spacing of about 2 µm and now accumulates additional proteins like myotilin. Not drawn to scale.

Temporal and Spatial Organisation of Myofibril Assembly

One of the most consistent observations about myofibril assembly in cultured skeletal muscle cells is the existence of a sequential programme of both gene expression and subsequent integration of muscle proteins into sarcomeres,[7] where the expression of proteins of the initial actin-associated complex, including actin, is regulated differentially from myosin and other thick filament components. Transcriptional control via the myogenic basic helix-loop-helix factor Mef2 seems to play a role in this sequential and ordered onset of gene expression after terminal differentiation.[8] The thick filament proteins, myosin heavy and light chains and myosin-binding protein C, require Mef2 for normal expression, whereas the thin filament proteins actin, tropomyosin and troponin do not. This sequential activation of muscle genes is topologically ordered and commences with proteins of the Z-disk and the Z-disk associated intermediate filaments. Thus, the muscle-specific intermediate filament protein desmin, muscle-specific alpha-actinin and the Z-disk portion of titin are expressed first and assemble on stress-fibre actin filaments in both skeletal and cardiac myocytes.[9-11] The expression of sarcomeric actin, sarcomeric myosin and the proteins of the M-band follows with some delay, suggesting that the initial assemblies, called variously stress-fibre like structures (SFLS)[10] or premyofibrils,[9,12] are essentially modifications of the pre-existing actin cytoskeleton. During these early stages of myofibrillogenesis in cultured cells, titin colocalises with alpha-actinin in the Z-bodies of nascent myofibrils and is thought to be responsible for the anchorage of alpha-actinin (Fig. 2).[13-17] These resulting assemblies of primordial Z-disks and actin filaments, termed IZI bodies, still show a spacing of the presumptive Z-disks of only around 1 μm and do not yet contain sarcomeric myosin. In cultured cells, early stress fibre-like structures/premyofibrils contain nonmuscle myosin 2 (NMM2)[5,11] in a punctuate pattern alternating with alpha-actinin dots, reminiscent of mini-sarcomeres. Although the premyofibril model predicts the involvement of NMM 2B,[10,12] a mouse knockout model of this protein[18] showed that sarcomere and myofibril formation progress to normal-appearing myofibrils (although some myofibril disarray was observed) and that defects are more in agreement with cell migratory defects. Similarly, knockdown of NMM 2A and 2B in cultured myoblasts showed changes in cell-motility driven alignment and fusion, but muscle sarcomeres in the knockdown myotubes could still be observed.[19] These observations may be reconciled if we assume some degree of redundancy between NMM 2A, B and C, although it is important to note that the cardiac cytokinesis defects in NMM 2B knockout animals cannot be rescued by NMM 2A.[20]

During the transitions from premyofibrils to nonstriated myofibrils, the spacing between alpha-actinin-containing Z-bodies increases from less than 1 μm in SFLS and nascent myofibrils/premyofibrils to more than 2 μm in mature myofibrils. Titin epitopes that initially colocalise at Z-bodies are separated during this process,[21,22] indicating that stretching of the titin molecule and possibly exposure of binding sites for other myofibrillar proteins, is an essential process for the assembly of sarcomeres (Fig. 2). Exposure of titin M-band epitopes coincides with the assembly of myomesin in M-bands.[23,24] Finally, sarcomeric myosin filaments integrate into sarcomeric A-bands in the already-assembled cytoskeletal scaffold (Fig. 2) consisting of Z-discs, actin filaments, titin and M-bands,[23,25] suggesting that titin plays a major role in integrating myosin filaments within the pre-existing I-Z-I brushes.[26] The pattern of myosin changes considerably during myofibrillogenesis, from the diffuse or continuous staining of SFLS, to the periodic staining that is characteristic for mature myofibrils.

The concept that titin is a molecular ruler for myofibrillogenesis is supported by the observation that complete ablation of the titin gene disrupts myofibril formation despite persisting expression of other sarcomeric proteins.[27] Sarcomeric myosin fails to assemble into regularly arranged filaments and adopts a diffuse[28] or random distribution.[27]

For many sarcomeric proteins, especially the giant proteins with transcripts of over 80,000 bp, the transport of mRNA from the perinuclear region to the sites of translation and assembly seems inconceivable without active transport mechanisms. Indeed, for myosin heavy chain mRNA, microtubule-mediated transport has been shown.[29] At the nascent myofibril, protein translation and assembly appear to be coupled in a cotranslation mechanism, explaining possibly why some C-terminal titin epitopes are undetectable when N-terminal epitopes are already integrated into I-Z-I brushes.[30,31]

Associated Proteins, Scaffolds and Cofactors

The Giant Blueprints of Sarcomere Assembly, Titin and Nebulin

The spontaneous assembly of actin and myosin monomers into filamentous structures closely reminiscent of native filaments suggests that the self-assembly properties of sarcomeric proteins contribute importantly to sarcomere assembly. However, mixtures of such proteins, like alpha-actinin and actin, never assemble into ordered sarcomere-like structures in vitro, suggesting that additional organising components are missing. As stated in the introduction, the two proteins titin and nebulin provide a general blueprint function for sarcomere assembly due to their outstanding size and modular architecture.[1,32]

The giant protein titin/connectin is a highly modular protein, composed of hundreds of immunoglobulin and fibronectin-3-like domains that are arranged in patterns specific for sarcomeric subcompartments like the A-band or Z-disc.[1,33] This long and slender protein is the only single molecular entity able to span the entire half-symmetric unit of the sarcomere from Z-disc to M-band. Titin forms numerous interactions with sarcomeric proteins along its length of over 1000 nm and is thus ideally suited to define the axial position of these proteins along the half-sarcomere.[1] Titin is an elementary component of myofibril assembly. Even small deletions of the protein severely affect primary sarcomere assembly, as shown by several titin knockout models in the myogenic BHK-J cell line,[27] a myogenic heterozygous M-band deletion cell line[34] and a homozygous complete M-band knockout ES cell line.[35] Conversely, internal M-band deletions are compatible with initial sarcomere assembly[36-39] but lead to later myofibril disassembly and muscle failure.

The filamentous protein nebulin, only significantly expressed in skeletal muscle, forms a contiguous alpha-helix closely associated with the actin filament. The presence of repetitive patterns matching the repeats of actin and actin-associated proteins suggested that nebulin may be the ruler protein responsible for determining actin filament length beyond the generic roughly one micrometer observed in the absence of nebulin. The presence of interaction sites for tropomodulin,[40] desmin[41] and capZ[42] suggest that indeed nebulin may be important for coordinating thin-filament assembly and the integration of Z-disks with the intermediate filament system. However, two recent nebulin knockout models[42,43] suggest that the situation seems to be more complex, as initial sarcomere assembly proceeds without nebulin. The absence from the nebulin knockout models of the narrow length distribution of actin filaments observed normally in skeletal muscle, suggests a contribution of nebulin to length regulation at least at the pointed end. Nebulin also obviously has diverse functions that may relate to subunit exchange control[44] or be partly regulatory.[45]

Microtubules

The process of myofibril formation appears to require not only the molecular ruler functions encoded in the giant sarcomeric proteins titin, nebulin and possibly obscurin, but also proteins of temporary importance and transient sarcomeric association. The microtubule network emerges as an essential dynamic scaffold for sarcomere assembly. Morphological analysis demonstrated that microtubules form an extensive network closely associated with sarcomeres in heart muscle.[46] Induction of cardiac hypertrophy and thus the formation of new sarcomeres, is associated with a rapid increase in the number of microtubules and their reorganisation parallel to the myofibrillar axis (see also progressive alignment of microtubuli in embryonic mouse hearts depicted in Fig. 3).[47-50] At the onset of myogenic differentiation, microtubule dynamics is reduced by the formation of stable arrays of glutaminated tubulin (Fig. 2), with a parallel reduction of the dynamic pool of tyrosinated tubulin.[51] The ablation of microtubules by nocodazole results in defective myofibrillogenesis in redifferentiating adult cardiomyocytes, but does not affect neonatal cardiomyocytes, presumably because their myofibrils are preformed and thus independent of microtubules.[52] This is supported by pharmacological studies using the microtubule-depolymerising drug colcemid. Muscle cells differentiate in the presence of colcemid and form all myofibrillar components, but these do not assemble with precise lateral alignment.[53] A role of microtubules in the dynamic reorganisation of the cytoskeleton during myofibril assembly was also suggested by pharmacological experiments with the microtubule-stabilising drug taxol, which induces bizarre pseudo-sarcomeres

with interdigitating assemblies of myosin and microtubules, lacking normal actin filaments.[53-55] Furthermore, morphological analysis in cultured cardiomyocytes suggested a direct role in the transport and integration of sarcomeric myosin into nascent sarcomeres (Fig. 2),[56,57] as well as possibly for the localisation of myosin mRNA.[29] Indeed, the transport of myosin to nascent myofibrils occurs along glutaminated microtubules and is plus-end directed.[57]

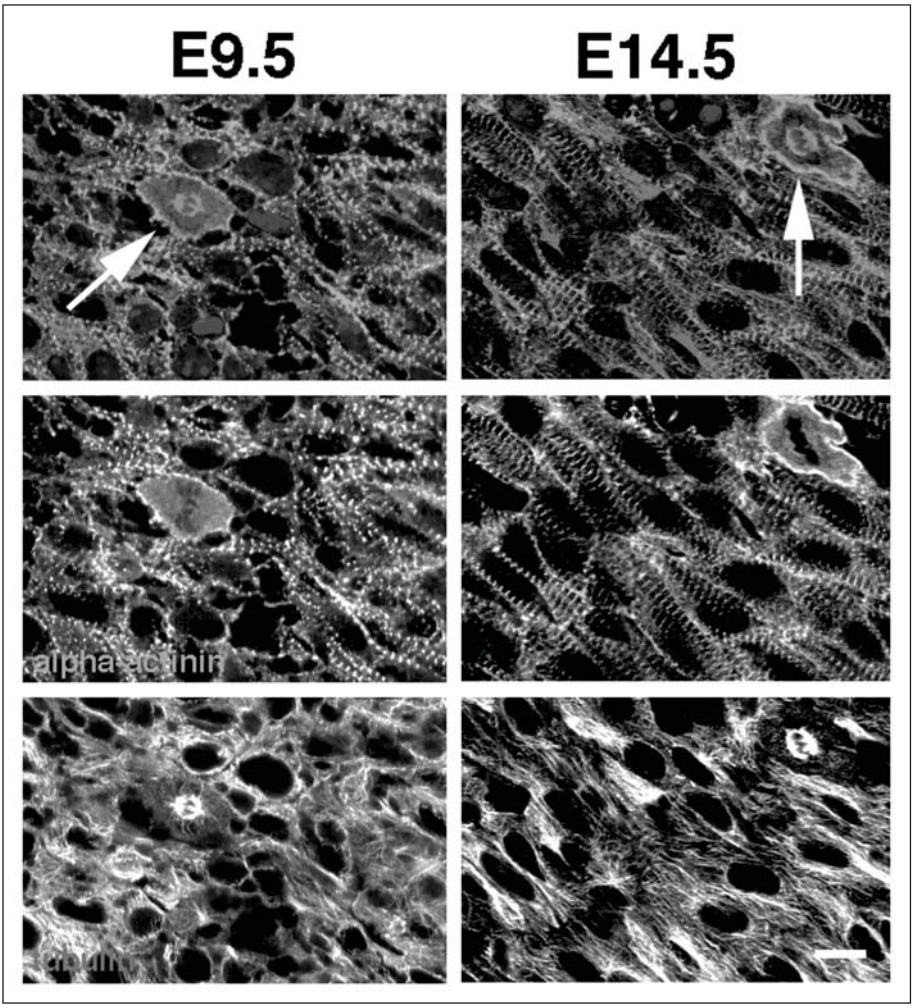

Figure 3. Single confocal sections of triple-labelled embryonic mouse heart whole mount preparations at embryonic day 9.5 (E9.5; left column) and 14.5 (E14.5; right column) stained with antibodies against sarcomeric alpha-actinin (green signal in overlay, middle panels) and against tubulin (red signal in overlay, bottom panels) and DAPI to visualise the DNA (blue signal in overlay). The increase in myofibril alignment and thus increase in a truly striated appearance of the Z-discs between these two stages is apparent. In addition, cell division occurs in differentiated cardiomyocytes (arrows; mitotic spindle shown with tubulin), which leads to a disassembly of the myofibrils (diffuse signal for alpha-actinin). The close association of the microtubule network and nascent myofibrils is apparent. Bar = 10 micrometres. A color version of this figure is available at www.eurekah.com.

A family of muscle-specific microtubule-binding proteins (MURF) was identified initially as putative interactors of the serum response factor[58] with a crucial role in myofibril assembly. The MURFs are transcribed from three genes, (chromosomes 1p31.1-p33, 8q12-13, 2q16-21). They contain an N- terminal RING, followed by a B-box zinc-finger domain and a coiled-coil sequence and thus belong to the RBCC subfamily of RING finger proteins usually acting as ubiquitin E3 ligases.[58-60] MURFs can multimerise via the coiled-coil domain, resulting in homo- or hetero-multimers.[58,59] Intriguingly, the known cellular localisations of MURFs are variable and include microtubules and Z-discs (MURF2, 3), or both Z-discs and M-bands (MURF1, 2).[58,59,61,62] Their exact functions are unclear, although roles in cytoskeletal dynamics and ubiquitin-mediated transcription regulation and cell signalling are emerging.[62] For MURF3, a stabilising effect on microtubules is established.[58] This may involve a similar ubiquitin-mediated effect on protein dynamics as that of the highly homologous MID1 protein,[63] which mediates ubiquitin-specific modification and degradation of the catalytic subunit of the translation regulator protein phosphatase 2A (PP2A).

MURF proteins are involved in early myofibrillogenesis with multiple cellular localisations: on microtubules, M-bands and in the nucleus. The ablation of MURF3 by antisense RNA impairs the organisation of expressed myosin heavy chain into sarcomeric structures, but also dramatically suppresses myogenic differentiation on the transcriptional level.[58] This suggests that the ubiquitin ligase activity of MURF proteins is not only necessary for protein degradation in sarcomere turnover and atrophy, but plays a role in the control of muscle differentiation. This agrees well with the early onset of MURF expression. Apparently, neither MURF1 nor MURF2 could complement the MURF3 defect, which might point to an important role of MURF3 in muscle differentiation. It is also conceivable, however, that in hetero-multimeric MURF complexes all three MURFs cooperate for their structural and putative transcriptional functions. Such hetero-multimers might also translocate to the nucleus and in any case may allow an impressive number of possible permutations and hence computations. A role for MURF3 in muscle gene transcription could be inferred from the possible interaction with SRF, which is likely to be posttranslationally regulated and hence difficult to characterise in vitro.[58] MURF1, also known as SMRZ,[64] was found to localise to the nucleus when transfected in C2C12 myoblasts. The significance of this observation is yet unclear, since both titin and MURF are not expressed in myoblasts. MURF1 can interact via the highly conserved RING domain with the ubiquitin-like SUMO-2/SMT3b[64] and importantly acts as a ubiquitin E3 ligase on a number of sarcomeric and nonsarcomeric target proteins (reviewed in ref. 65). These observations suggest that active transport mechanisms are essential for myofibril formation. Both microtubule and actin based transport may be involved, with both systems being possibly interdependent.

Contractility and Sarcomere Assembly

Not only pharmacological interference with cytoskeletal proteins such as tubulin affects myofibril assembly, but also inhibitors of myosin. Myosin-based contractility is required for proper myofibrillogenesis, since interference using BDM (2,3-butanedione monoxime) or BTS (N-benzyl-p-toluene sulphonamide), both inhibitors of myosin-actin based motility, suppresses myofibrillogenesis in skeletal muscle cells in culture.[66,67] Similarly, inhibition of calcium-mediated activity of myosin-light chain kinases seems to inhibit myofibrillogenesis.[68,69] Especially in cultured cardiomyocytes, attachment to the substrate is a prerequisite for sarcomere assembly. In cultured chicken cardiomyocytes, a tight correlation between myofibril assembly and the spreading of cells on the substrate was observed[70] and cardiac myocytes that are kept in suspension cultures cannot maintain a mature myofibrillar apparatus.[71] This may also explain why nonmuscle myosin 2 is essential for sarcomere assembly in cultured muscle cells, since it is required to provide the necessary tension in spreading areas of the cells, analogous to its function in growth cones.[72]

Chaperone-Assisted Sarcomere Assembly

The complex folding events required during the co-assembly of the many and diverse sarcomeric proteins are not only baffling to the observer, but seem to pose a genuine challenge for the cell. Both the transient involvement of chaperones that assist protein folding, as well as of proteases that could remove misfolded proteins from the sites of sarcomere assembly are now emerging. The heat-shock proteins Hsc70 and Hsp90 are required for correct folding and incorporation of the multimeric complexes of nascent myosin filaments. Myosin heavy chain associates transiently with Hsc70 and Hsp90 as intermediates in the folding and assembly pathway of muscle myosin (Fig. 2).[73] Initially, only the myosin rod is folded, but the motor domain is unfolded and remains so in the absence of chaperones. The correct folding of the complex motor domain therefore seems to absolutely require the presence of Hsc70 and Hsp90.[73] Hsp90 interacts further with steif/unc-45b, an evolutionary highly conserved protein containing three tetratricopeptide repeats and a CRO1/She4p homology domain[74,75] and both genes are co-expressed in skeletal muscle. Knockdown of Hsp90a leads to impaired myofibril formation, similar to reduced levels of Steif/Unc-45b activity. These observations suggest a requirement of Steif/unc-45b and Hsp90a for the assembly of the contractile apparatus in the vertebrate skeletal musculature.[76] Unc-45 is highly conserved between nematodes and vertebrates, suggesting evolutionarily ancient and crucial functions in myofibril assembly. In nematodes over-expressing UNC-45, myosin assembly is defective, with decreased myosin content and a mild paralysis phenotype. The reduced myosin accumulation is the result of degradation through the ubiquitin/proteasome system and proteasome inhibition is able to restore myosin protein and worm motility. These findings suggest that UNC-45-related proteins contribute to the degradation of myosin during myofibril assembly, possibly by removing misfolded and hence potentially toxic protein from the assembly sites.[77] Further functional interdependence of myofibril assembly and ubiquitin-mediated protein degradation is suggested by the participation of the ubiquitin-selective chaperone p97, which plays roles in substrate recruitment and ubiquitin-chain assembly.[78]

Interestingly, genetic ablation of both RBCC proteins, MURF1 and -3 in mice results also in a muscle pathology with myosin storage,[79] suggesting that the ubiquitin E3 ligases responsible for myosin ubiquitylation may be MURF-members.

Sarcomere assembly and protein degradation are thus closely interlinked (Fig. 2), as was also shown by the tight colocalisation of proteasomal complexes with nascent myofibrils in skeletal and cardiac myocytes.[80,81] Apart from the ubiquitin-proteasome system, the calcium-regulated cysteine protease calpain-3, which is specifically expressed in skeletal muscle, is also implicated in myofibril assembly, as shown by myofibrillogenesis defects in calpain-3 (p94) knockout animals. Calpain-3 knockout mice showed signs of muscle atrophy, but myogenic cells fused normally in vitro. However, they lacked well-organized sarcomeres and showed mis-aligned A-bands.[82] Interestingly, mutations in calpain-3 lead to limb-girdle muscular dystrophy 2A, suggesting that a possible disruption of myofibril turnover might be involved in the pathogenesis. A role tightly linked to the progression of myofibrillogenesis is also suggested by studies of the postdifferentiational splicing of calpain-3. These isoforms, expressed during myofibril assembly, show various localisations and change their sarcomeric location in response not only to developmental state, but also to stretch and sarcomere length.[83]

Accessory Proteins

Recently, a growing number of novel sarcomeric proteins have been discovered.[6,84] They include structural proteins like the alpha-actinin binding proteins myotilin or gamma-filamin, but also an increasing number of potentially regulatory proteins like ZASP/cipher, telethonin,[84] or obscurin (Fig. 1).[85] The role of many of these proteins in myofibril assembly is yet unclear; however, evidence from knockout animals suggests that not all of these proteins are indispensable for primary sarcomere assembly. Rather, many seem to have a function in load-adaptation and myofibril turnover. For instance, the knockout of myotilin shows no discernible phenotype,[86] despite the fact that point mutations in myotilin are frequently associated with limb girdle muscular dystrophy type 1A.[87,88]

Sarcomere Assembly in Vivo: What Is Different?

Although cell culture systems have provided invaluable insight into the basic mechanisms of sarcomere assembly, allowing high-resolution and time-resolved analysis of this process, there are important differences to the situation in vivo. As in differentiating skeletal muscle cells in vitro, sequential onset of expression of sarcomeric proteins is observed in skeletal muscle during embryonic development, usually following a time-course from IZI to M-band proteins. The intermediate filament protein desmin comes first, followed by sarcomeric alpha actinin and titin.[89,90] Thick filament proteins such as sarcomeric myosin, MyBP-C and myomesin are only detected with a delay. This is in contrast to the situation in the developing heart, where most sarcomeric components are co-expressed well before the first myofibrils are seen (e.g., in the 6 somite stage in chicken) and localised in a completely diffuse fashion throughout the cytoplasm of the cardiomyocytes.[25] This difference can be explained by the distinct times that cardiac and skeletal muscle take to mature in the mammalian embryo. The heart is the first functional organ and any delay or impairment of its function is incompatible with survival, while skeletal muscle only becomes crucial immediately after birth, when the diaphragm has to start working.

However, apart from the onset of expression of sarcomeric proteins, the basics of sarcomere assembly are remarkably conserved between skeletal and cardiac muscle and are also reminiscent of many observations made on cultured myocytes. The first assembled complexes always consist of alpha-actinin, the N-terminus of titin and actin filaments, thus representing I-Z-I complexes like those in cultured muscle cells as starting points of myofibril assembly.[89] In the developing embryonic heart, these complexes are always found in close association with the plasma membrane of the cardiomyocytes.[25] A major difference between the first organised complexes in cardiomyocytes in situ compared to premyofibrils and SFLS, respectively, in cultured cardiomyocytes appears to be their spacing, which even at the earliest stages is never as narrow as in cultured cells. Another difference is the apparent absence of nonmuscle myosin 2B from these earliest complexes in the early embryonic chick and mouse heart.[25] The next step in sarcomere assembly is again similar to observations made in cultured myocytes, namely the organisation of the C-termini of titin[25,91] and the integration of thick filaments. This is presumably achieved via the M-band protein myomesin, which appears to play a role as a crosslinker between the elastic (titin) filament system and myosin filaments, analogous to the Z-disc protein alpha-actinin as a crosslinker between titin and the actin filaments. These observations suggest the existence of a basic framework that is indispensable for sarcomere assembly and consists of alpha-actinin at the Z-disc, myomesin at the M-band and titin stretching in between. These components appear to be essential to integrate thin and thick filaments as well as the plethora of additional sarcomeric proteins that were identified over the last decade.

Once the initial sarcomeres are assembled, the next steps involve the maturation of the thin filaments, in particular the definition of their exact length. The skeletal muscle actin filament length regulator nebulin seems to play only a minor role in ventricular cardiomyocytes, judged by its lack of expression as visualised in lacZ mice[43] and given that actin filaments in embryonic cardiomyocytes show a highly variable length distribution.[92] Defined I-bands can only be detected once the pointed-end capping protein tropomodulin becomes firmly integrated into the sarcomeres.[92]

The next steps in myofibrillogenesis in situ are essentially the same as in cultured myocytes, namely the lateral extension of Z-discs and the alignment of myofibrils (compare the thin, dot-like arrangements of sarcomeric alpha-actinin in the E9.5 heart with the extended, better aligned Z-disks in the E14.5 heart in Fig. 3). In the embryonic heart, there is a continuous switch between myofibril assembly and disassembly, since individual differentiated embryonic cardiomyocytes are still able to undergo cell division (arrows in Fig. 3), but can only do so when their myofibrils are disassembled (hence the diffuse localisation of alpha-actinin in the dividing cells in Fig. 3; see also 93). At present, the regulation of this complex dynamic behaviour of the myofibrils is completely unclear; however, it is possible that this process may be an exaggerated and speeded-up version of the normal adaptive behaviour of myocytes and that it may be regulated by similar signalling pathways also involved in hypertrophy and atrophy (see chapter by Dr. Mathias Gautel).

Lessons from Knockout Animals

In the last decade, homozygous null mice were generated for a number of sarcomeric proteins with partly surprising results, since an unexpected amount of functional redundancy was found. For example, desmin, which is one of the first cytoskeletal marker proteins to be expressed in muscle cells and which was proposed to be essential for skeletal muscle development,[94] turned out to be dispensable, as desmin knockout mice are viable. These animals assemble normal sarcomeres, but have problems with their maintenance, leading to a cardiomyopathy with strong involvement of the mitochondria.[95,96] Myosin binding protein-C was also thought to be an essential scaffold for thick filament assembly.[23,97] Nevertheless, cardiac MyBP-C knock-out mice assemble normal sarcomeres in the absence of other MyBP-C family members, but again show problems later on with the development of hypertrophic cardiomyopathy.[98] Similarly, mice that are deficient for nebulin are able to assemble sarcomeres initially, albeit with shorter thin filaments and display lethality within the first two postnatal weeks, possibly due to feeding problems.[42,43]

Nonmuscle myosin 2B, which was proposed as a space holder for sarcomeric myosin in the premyofibril model, may also be more important for myoblast motility and the assembly of the contractile acto-myosin ring during cell division, than for sarcomerogenesis. Nonmuscle myosin 2B knockout mice can assemble sarcomeres, but show enlarged embryonic cardiomyocytes and cardiac defects in agreement with a defect in cell migration and die on their first postnatal day.[18] A subset of the animals show upregulation of nonmuscle myosin 2A expression; however, a recent attempted rescue by overexpressing nonmuscle myosin 2A in these knockout mice failed to rescue the cell division defect in the cardiomyocytes.[20]

In the case of titin, only partial deletions were analysed so far: A nonsense mutation in the N2B region of cardiac titin in zebrafish leads to the lack of any sarcomeres, although thick and thin filaments are initially assembled separately.[99] A mouse strain in which a subset of titin's M-band region Mex1 and Mex2 (which also encompass the titin kinase domain) was conditionally ablated, displayed different phenotypes depending on the onset of cre-mediated recombination.[36,39] Knockout of this region at the germ-line stage surprisingly still allowed for initial sarcomere assembly, as demonstrated by alternated spacing of alpha-actinin and myomesin in E9.5 hearts; however they are eventually disassembled. While it was initially thought that a lack of interaction with MURF1 was the driving mechanism for the observed unravelling of the sarcomeres, the knock-out strategy also deleted the only known binding site for the structural protein myomesin, which is thought to provide the crucial link between titin and myosin filaments. Additionally, binding sites for FHL2 and possibly other members of the highly homologous FHL protein family such as FHL1 and regulatory phosphorylation sites are deleted in this model. Antiparallel myomesin dimers may still be attached to a certain extent to the myosin filaments via their head domains, providing a weak link that eventually cannot be maintained in a working sarcomere in the absence of its binding region on titin. In addition, it cannot be excluded that titin could form homo-dimers via its extreme C-terminal regions, which are still present, or that titin's C-termini could be at least transiently connected by additional, yet unidentified proteins. Support for the latter hypothesis comes from experiments in differentiating ES-cells, which are homozygous for a deletion of the complete M-band region of titin and show a complete failure to progress during sarcomere assembly beyond initial Z-disc complexes, not even managing to complete A-band formation.[35]

While the loss of expression of the fast muscle specific isoform of alpha-actinin (ACTN3) is a naturally occurring phenomenon that leads to enhanced endurance tolerance,[100,101] no knock-out of the constitutive sarcomeric alpha-actinin (ACTN2) has been reported so far, not even at the level of homozygous ES cells. The same is the case for myomesin, although here indirect conclusions can be drawn from mouse models that affect either myomesin expression levels or destroy its binding sites. As mentioned above, the known myomesin-binding site is ablated in the partial M-band titin knock-out model by Gotthardt and colleagues[36,39] and this seems to be incompatible with the maintenance of ordered sarcomeres in contracting myofibrils. Another indirect myomesin knock-out was created by the Olson lab by deleting Mef2c in a skeletal muscle-specific fashion.[102] This transcription factor appears to directly affect myomesin transcription

and its absence from skeletal muscle leads to dramatically reduced myomesin expression levels and disorganised sarcomeres.[102]

In summary, apart from titin, which truly seems to be indispensable for sarcomere formation and the until now not definitely proven essential role of sarcomeric alpha-actinin and myomesin, there seems to be a certain extent of functional redundancy in the assembly of myofibrils. This can be partly explained by the existence of different isoforms of sarcomeric proteins and partly by the possibility that other proteins can take over at least for the initial stages of sarcomere assembly. It is only under continuous contraction when it becomes apparent that the delicate balance between the expression and localisation of different sarcomeric proteins is crucial for long-term maintenance; an observation that is supported by the data on mutated sarcomeric proteins in humans and resulting different forms of cardiomyopathy and skeletal muscle diseases.

Future Perspectives

The complex machinery involved in sarcomere formation will likely be better understood in the near future, with the identification of more of the proteins transiently involved in the intermediate stages of sarcomere assembly. The use of genetic models like zebrafish (see Chapter by Dr. John Sparrow et al) and the possibility to manipulate individual components therein by morpholino oligonucleotides offers the chance of very detailed loss of function studies, often without the limitation of embryonic lethality observed in vertebrate models. However, due to the partial duplication of the zebrafish genome, this approach may not always be necessarily straightforward. A steady stream of sarcomeric knockout mice is produced from several leading laboratories and will complement work on the cellular level (e.g., using siRNA interference experiments) and in invertebrate model organisms. Also, we expect that the interplay of sarcomere assembly and disassembly by proteolytic systems will become clearer. This will produce important information on the mechanisms of those muscle diseases where the balance of sarcomere formation and degradation, or myofibril turnover, is perturbed.

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Chapter 2

Skeletal Muscle Alpha-Actin Diseases

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Abstract

Skeletal muscle α-actin is the principal protein component of the adult skeletal muscle thin filament. The interaction between skeletal muscle α-actin and the various myosin heavy chain proteins in the different muscle fibre types generates the force of muscle contraction. Skeletal muscle α-alpha actin is thus of fundamental importance to normal muscle contraction. To date over 140 different disease-causing mutations have been identified in the skeletal muscle α-actin gene *ACTA1*. These mutations are associated with histologically distinct congenital myopathies, including nemaline myopathy, actin myopathy, intranuclear rod myopathy, congenital fibre type disproportion and myopathy with cores. Mutations in *ACTA1* are associated with a wide range of clinical severity. although the majority of patients tend to have severe congenital-onset disease. Most of the patients have de novo dominant mutations not present in either parent. However mild *ACTA1* disease may be dominantly inherited and there are also recessive mutations. The recessive mutations are either genetic or functional null mutations. Patients with no skeletal actin retain cardiac actin, the fetal isoform of actin in skeletal muscle. Information from the clinic suggests that exercise and L-tyrosine may benefit some patients and that in the future decreasing the proportion of mutant actin may ameliorate the disease in some patients.

Introduction

Skeletal muscle α-actin is one of the six actin isoforms in vertebrates.[1] The other actins are α-cardiac, α-smooth muscle, β-actin, γ-actin and γ-enteric actin.[1] β-actin and γ-actin form the actin cytoskeleton of every cell in the human body except the inner ear hair cells where only gamma actin is expressed.[2] The other actins are restricted in their expression to the various adult tissues included in their names.

Actin is an ancient protein. All actins are extremely highly conserved with skeletal muscle α-actin identical in mice and man and 87% conserved at the amino-acid level with actin in rice.[3]

Skeletal muscle α-actin is the principal protein component of the thin filament of adult striated skeletal muscle. The ATP-dependent interaction between skeletal muscle α-actin in the thin filament and the various myosin isoforms in the thick filament generates the force of muscle contraction. This crucial importance of actin in the sliding filament model of muscle contraction has been known for many decades.[4] However, mutations in skeletal muscle α-actin that cause human disease were first described only in 1999.[5] At that time, mutations were identified in three different histologically defined congenital myopathies: most commonly in nemaline myopathy (NEM), characterised by the presence of nemaline bodies in the sarcoplasm, but also in actin myopathy, characterised by accumulation of actin thin filaments in muscle fibres and in intranuclear rod myopathy.[5] We now know that mutations in skeletal muscle α-actin are also associated with

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myopathy with core-like features[6] and congenital fibre type disproportion.[7] Mutations in skeletal muscle α-actin cause around 20% of all nemaline myopathy, but a higher proportion of the severe cases.[8,9] Mutations in skeletal muscle α-actin are thus a significant cause of congenital myopathies, especially severe congenital myopathies and therefore of infant morbidity. Currently the skeletal muscle α-actin diseases are incurable. Prenatal diagnosis is possible once the family mutation has been identified, but most of the patients have de novo mutations not present in the peripheral blood lymphocyte DNA of either parent.[10]

Skeletal muscle α-actin mutations affect the fundamental basis of muscle contraction, the core of muscle activity and because of this and the sheer volume of muscle in the human body, skeletal muscle α-actin mutations represent a significant challenge to muscle researchers in developing effective treatments.

Clinical Features

In 1997, Goebel and colleagues reported three patients with congenital myopathy and excess accumulation of α-actin-containing thin filaments; two of these patients also had nemaline bodies.[11] Two of the three patients had died from respiratory insufficiency. Using a candidate gene approach, Nowak and colleagues identified mutations in *ACTA1* in these patients and went on to screen a series of 59 cases with severe neonatal, intermediate and milder forms of nemaline myopathy.[5] In total, they identified 15 mutations in 14 families. Many other *ACTA1* mutations have now been identified in patients with nemaline myopathy and other congenital myopathies, with varying clinical presentations and inheritance patterns, further confirming the heterogenous nature of the actin-related diseases.[6-8,10,12,13] The majority of patients are sporadic (have new dominant mutations). The frequency of germline mosaicism is unknown, although it has been demonstrated (Laing, unpublished observations).

Range of Severity Associated with ACTA1 Mutations

Patients with *ACTA1* mutations exhibit marked clinical variability, ranging from severe congenital-onset weakness with death from respiratory failure in the first year of life to a mild childhood-onset myopathy with survival into adulthood (Table 1). Clinically a patient with an *ACTA1* mutation cannot be distinguished from patients with other genetic forms of nemaline myopathy or the other pathological subtypes (e.g., CFTD, core myopathy). The range of phenotypes associated with mutations in *ACTA1* thus falls into the same range of diagnostic subclasses that apply to nemaline or congenital myopathy in general. The clinical features of nemaline myopathy are reviewed in detail in North et al 1997[14] and Ryan et al 2001.[13] The *severe neonatal* form presents at birth with severe hypotonia and muscle weakness, little spontaneous movement, difficulties with sucking and swallowing, gastroesophageal reflux and respiratory insufficiency. Decreased fetal movements and polyhydramnios may complicate the pregnancy and death in utero or at birth can occur. Early mortality is common and is usually due to respiratory insufficiency or aspiration pneumonia. Patients with the *intermediate congenital* form of NEM have antigravity movement and independent respiration at delivery but have weakness sufficient to prevent achievement of motor milestones, develop joint contractures early and generally require a wheelchair and/or ventilatory support by the age of 11 years. Distinction between intermediate and typical congenital NEM may therefore be possible only with increasing age. The *typical congenital* form of NEM usually presents in the neonatal period or first year of life with hypotonia, weakness and feeding difficulties. Spontaneous antigravity movements are present and respiratory involvement is less prominent. Some weakness of the respiratory musculature is usual, but this may be subclinical with insidious nocturnal hypoventilation or manifest as frequent lower respiratory tract infections. A minority of cases present after one year of age, with delay of gross motor milestones, an abnormal waddling gait, or bulbar weakness manifesting as hypernasal speech or swallowing difficulties. Cardiac involvement is rare. Weakness is usually static or very slowly progressive and most patients are able to lead an independent, active life. Later-onset forms presenting in *childhood,* or even *adulthood*, can occur occasionally.

Table 1. Clinical and histological details of patients from two large series of ACTA1 NEM

	Agrawal et al 2004[8] (%)	Wallgren-Pettersson et al 2004[9] (%)
Total NEM cases studied	109	Not stated
No. of families with *ACTA1*	28	30
No. of patients	38	34
Category of nemaline myopathy		
Severe	14 (50%)	18 (60%)
Intermediate	3	not stated
Typical	10 (36%)	8 (27%)
Mild/childhood	0	3
Adult	1	0
Other	0	5 (clinical severity not stated)
Family History		
Sporadic/Singleton	23	25
Autosomal recessive	1	2
Autosomal dominant	4	1
Mosaic parent	0	2
Deceased	10	16

Table 1 summarises the findings of two large series of patients with *ACTA1* nemaline myopathy. These data demonstrate that the severe form of nemaline myopathy is the most common clinical presentation. Indeed in the series of 109 cases of nemaline myopathy screened for *ACTA1* mutations by Agrawal et al (2004),[8] mutations in *ACTA1* accounted for 56% of the severe and usually lethal, congenital onset form. Notably their series also included the first (and only one to date) reported case of *ACTA1* mutation manifesting as adult onset disease.

The cardinal features of all forms of *ACTA1* myopathy are diffuse weakness and hypotonia. Weakness is usually most severe in the face, the neck flexors and the proximal limb muscles. In some patients there is also involvement of distal muscles. In congenital-onset forms of NEM, the face is often elongated and expressionless, the mouth tent-shaped and the palate high-arched. There may be retrognathia, with relative micrognathia due to abnormal muscle tension on the developing mandible. The extraocular muscles and cardiac muscles are usually spared. Lingual and pharyngeal muscle weakness results in dysarthria, dysphagia, excessive drooling, a hypernasal voice, wasting of the tongue and an absent gag reflex and patients with congenital-onset NEM require gavage feeding or gastrostomy during the first few years of life.

Respiratory muscle weakness is common in congenital NEM, not only in the neonatal period but also throughout life. Even patients without respiratory symptoms have a reduced vital capacity. There is an increased risk of insidious nocturnal hypoxia and regular sleep studies are recommended for all patients. Many patients have hypermobility of joints in infancy and early childhood, developing joint contractures and deformities with time. Severe kyphoscoliosis may develop in late childhood and reduce respiratory capacity.

Intelligence is usually normal in NEM.

Cardiac muscle is usually spared in *ACTA1* nemaline myopathy. This is likely due to the fact that the α-cardiac actin isoform is the predominant form of sarcomeric actin at the protein level in the human heart, with low or undetectable levels of skeletal muscle α-actin.[15] However hypertrophic cardiomyopathy has been reported in two related patients with core disease and a heterozygous missense mutation (E334A) in *ACTA1*[6] and more recently in a patient with a de novo heterozygous missense mutation (K336E) in *ACTA1*.[16] It was demonstrated that this K336E mutation resulted

in a slower crossbridge turnover rate and a 10-fold reduction in the affinity of the mutant actin for the Z-line protein α-actinin.[16] The authors suggested that this would result in lower force generation and impaired force transmission in both skeletal and cardiac muscle.[16]

Histopathology

The commonest histopathological diagnosis associated with *ACTA1* mutations is nemaline myopathy.[5,10] The diagnostic feature of nemaline myopathy is the presence of distinct rod-like inclusions, nemaline bodies, in the skeletal muscle fibres (Figs. 1A, 2A). Nemaline bodies are not visible on haematoxylin and eosin (H&E) staining, but appear as red or purple structures against the blue-green myofibrillar background with the modified Gomori trichrome stain. Nemaline bodies are considered to be derived from lateral expansion of the Z-line. Additional pathologic features seen in the muscle of patients with nemaline myopathy include type 1 fibre predominance, fibre atrophy and/or fibre hypertrophy.[17]

ACTA1 mutations are responsible for a number of other congenital myopathy subtypes including intranuclear rod myopathy (IRM);[5,10] myopathy associated with accumulation of actin (actin myopathy);[5,10] core myopathy[6] and congenital fibre type disproportion (CFTD).[7] (Figs. 1 and 2) The variety of changes in muscle histology likely result from fundamental differences in the way that *ACTA1* mutations disrupt muscle function.[10,18,19] For example, not all *ACTA1* mutations result in fibre type disproportion.

Pathologic changes that may specifically be indicative of an *ACTA1* mutation include abnormal accumulation of actin filaments[11] and abnormal localization of sarcomeric actin on immunocytochemical staining.[12] Patients with recessive *ACTA1* disease, which is caused by genetic or functional null mutations, may express high levels of α-cardiac actin in their skeletal muscles and no skeletal muscle α-actin.[8,20]

Ryan et al (2003)[17] examined muscle pathologies associated with mutations in *ACTA1* in 19 patients with pathological and genetic diagnosis of *ACTA1* nemaline myopathy (Table 2). Percentages of rod-containing fibers and rod size varied widely in patients and showed some correlation with disease severity. 84% of all patients with *ACTA1* mutations had rods in 50-100% of fibers, as compared to 43% of patients with nemaline myopathy in whom mutations in *ACTA1* were excluded ($p = 0.002$). Rods were present in both type 1 and 2 fibers in all but one biopsy. Two patients had intranuclear rods. Light microscopy in these cases commonly revealed increased fiber size variation and internal nuclei. Type 1 fiber predominance was seen in 67% of biopsies and type 1 fiber atrophy in 39%; type 2 fiber atrophy was seen in eight biopsies (42%) and was more common in patients with *ACTA1* mutations than in patients without *ACTA1* mutations ($p = 0.03$). Perimysial connective tissue was increased in four cases. Findings in single biopsies included collections of whorled thin filaments within fibers and occasional central cores, seen on EM. Immunohistochemical staining revealed co-expression of fast and slow MHC isoforms in up to 50% of fibers in three of eight patients in whom this staining was performed. Three patients with *ACTA1´* mutations underwent multiple biopsies. Little pathologic evolution was seen on repeat biopsy after as long as 22 years.

Electron microscopy increases detection of nemaline bodies, intranuclear rods, glycogen accumulation and markedly atrophied fibers that may not be seen on light microscopy. Disruption of the sarcomeric register, in association with accumulations of thin filaments was most marked in patients with severe congenital onset nemaline myopathy and those with large numbers of rods; the extent of disruption of sarcomeric structures on EM appears to correlate better with clinical severity of nemaline myopathy than do light microscopic abnormalities.[17] Increased sarcoplasmic and intermyofibrillary glycogen stores also appear to be more common in patients with nemaline myopathy and *ACTA1* mutations compared to other forms of nemaline myopathy (p value < 0.00001).[17]

Genetics

The first disease-causing mutations identified in the skeletal muscle α-actin gene (*ACTA1*) were published by Nowak et al in 1999.[5] We now know of more than 140 different mutations in actin. There are 80 mutations listed in the Human Gene Mutation Database (HGMD) (http://

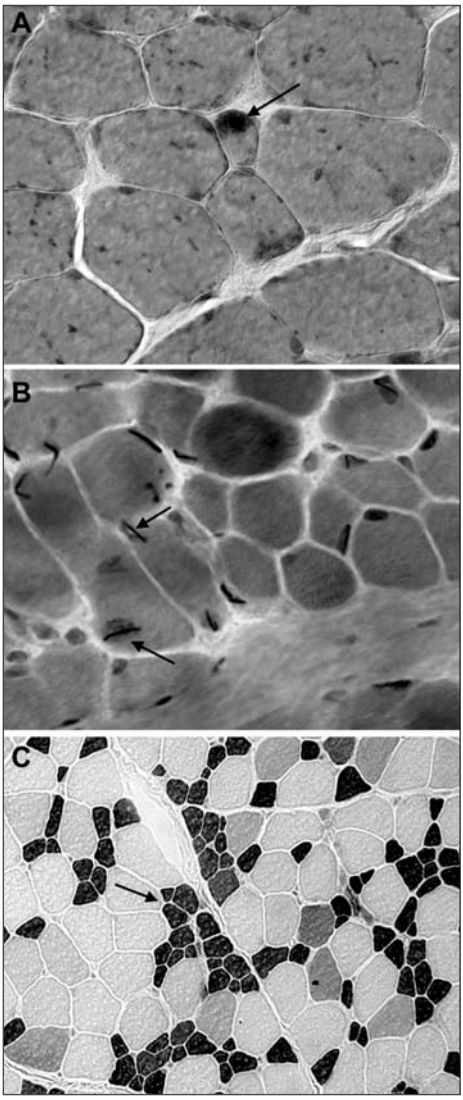

Figure 1. Light microscopic analysis of pathologies associated with skeletal muscle alpha-actin disease. Gomori trichrome staining of muscle from nemaline myopathy patients depicting (A) cytoplasmic rods (arrow) and (B) intranuclear rods (arrow). C) ATPase staining (at pH 4.6) of muscle from a patient with congenital fibre type disproportion (CFTD) illustrating the small type 1 fibres (dark stain, arrow). Panel C courtesy of Dr. Nigel Clarke.

www.hgmd.cf.ac.uk/ac/index.php), 10 further published mutations not listed in HGMD e.g.,[21] and we know of over 50 novel unpublished mutations (manuscript in preparation). A number of the mutations have been found multiple times in unrelated families, but many are private mutations restricted to individual patients.

Mutations in *ACTA1* may cause either dominant or recessive disease, but most patients have de novo dominant mutations not present in the peripheral blood lymphocyte DNA of either parent. Actin diseases are thus largely the result of new mutations.[5,10] Mutations that cause relatively

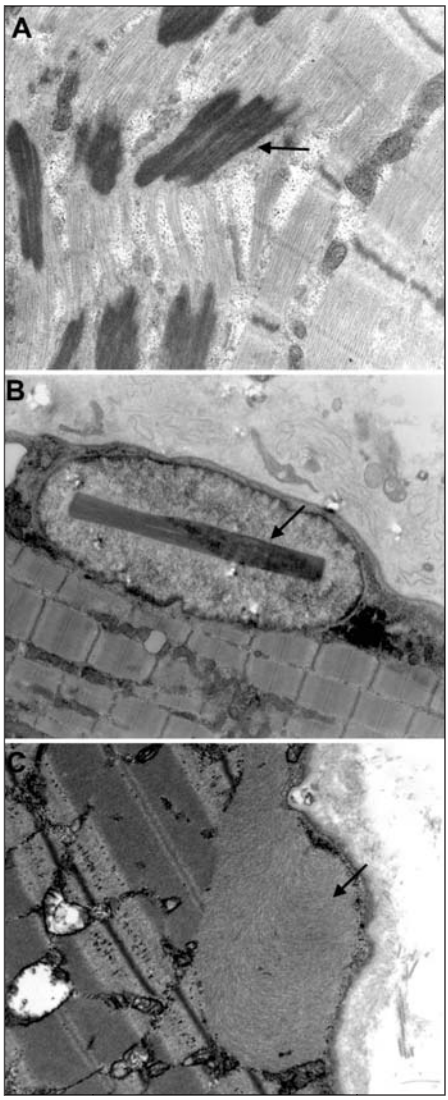

Figure 2. Electron microscopic analysis of pathologies associated with skeletal muscle alpha-actin disease. Muscle from patients with nemaline myopathy illustrating (A) cytoplasmic rods (arrow), (B) intranuclear rods (arrow) and (C) filamentous accumulations of actin (arrow). Panel B courtesy of Dr. D. Hutchinson.

mild disease may be dominantly inherited. Recessive mutations are either genetic null mutations or, where this has been determined, functional null mutations—nonsense, frameshift, splice-site and certain missense mutations.[5,18,20,22]

Somatic mosaicism for *ACTA1* mutations has also been demonstrated in mildly affected parents of severely affected patients[5,23] (unpublished observations). We have also shown definite gonadal mosaicism in one family. In this family the first affected child had a heterozygous missense mutation not present in the peripheral blood of either parent, but the same missense mutation was identified by prenatal diagnosis in a subsequent pregnancy.

Table 2. Pathological features of ACTA1 *nemaline myopathy (adapted from Ryan et al 2003[17])*

Finding (No. of *ACTA1* Patient Biopsies Examined for Each Parameter)	*ACTA1* NEM			
	Severe Congenital	Intermediate Congenital	Typical Congenital	Childhood Onset
Patients	6	3	8	2
Rods < 50% fibers (19)	-	-	2	1
Rods > 50% fibers (19)	6	3	6	1
Rods seen only on EM (16)	2	1	-	-
Rods primarily sarcomeric (19)	1	-	2	-
Rods primarily subsarcolemmal (19)	3	1	3	1
Mixed distribution rods (19)	2	2	3	1
Intranuclear rods (19)	1	1	-	-
Rods restricted to type 1 fibers (12)	1	-	-	-
Increased fiber size variation (19)	5	3	7	2
Type 1 fiber predominance (18)	3	1	7	1
Type 1 fiber hypertrophy (18)	1	-	2	-
Type 1 fiber atrophy/hypotrophy (18)	2	2	3	-
Type 2 fiber predominance (18)	-	-	-	-
Type 2 fiber hypertrophy (18)	-	-	-	-
Type 2 fiber atrophy/hypotrophy (18)	4	2	2	-
Inability to fiber type on myosin ATPase (18)	-	-	-	1
Grouped fiber atrophy (19)	-	1	-	-
Fiber type grouping (19)	-	-	-	-

The actins are one of the most highly conserved protein families known, with most of the amino-acid residues invariant back to insects and yeast.[3] The >140 mutations so far identified affect 97 of the 375 amino-acid residues in the mature actin protein. The mutations are spread throughout the structure of the actin monomer. The rate of discovery of novel mutations does not yet appear to be tailing off, raising the question of whether eventually disease-causing mutations will be identified in all the amino-acid residues in skeletal muscle α-actin. Some amino-acid residues are mutated multiple different ways. The current largest number of mutations known at one residue is for Met229 in which 5 different mutations have been identified: ATG-GTG (valine); ATG-ACG (threonine); ATG-ATA, ATG-ATC and ATG-ATT (all isoleucine), while Gly270 (GGT) is mutated to serine (AGT); arginine (CGT); cysteine (TGT) and aspartate (GAT).

There are only a few relatively mutation free regions in the protein (Fig. 3). Mutations in these regions may either (1) not cause disease, (2) cause an in utero lethal phenotype, or (3) a disease phenotype we do not yet recognise as being caused by *ACTA1* mutations.

Currently, there are no known amino-acid variants in skeletal muscle α-actin that are definitely nondisease causing polymorphic variants.

Genotype—Phenotype Correlations

ACTA1 mutations generally breed true in that the same mutation in multiple different families will cause the same histopathological phenotypes. For example a mutation that causes nemaline myopathy in one patient will cause nemaline myopathy in others, or a mutation that causes actin myopathy in one patient will cause actin myopathy in another.[5,10] Mutations also tend to breed true in tissue culture in that mutations that cause intranuclear rod myopathy in patients also tend to cause intranuclear rods in culture.[18] Similarly there is a tendency for the same mutation, or mutations with the same resulting protein defect to result in similar diseases. For example, the three different mutations of the stop codon recently described, although they change the stop codon to different amino-acids, all have the same effect of adding 46 amino-acids to the length of the actin protein translated from the 3′UTR and all three patients had severe nemaline myopathy requiring ventilatory assistance.[24] This is not always the case however. There may be variability in the severity of the disease caused by the same mutation. One mutation may be associated with interfamilial variation in disease severity and variability in disease severity has been reported between the different affected family members of one family.[25] In addition, reduced penetrance has been suggested for two mutations in two families.[8] These results suggest modifying effects of either other genes, epigenetics or environment.

The *ACTA1* mutation identified in a patient may thus be used only cautiously in prognosis.

Molecular Modelling of the Effect of *ACTA1* Mutations

ACTA1 mutations that cause the same pathology do cluster to some extent. The best examples are the mutations that cause intranuclear rod and actin accumulation which cluster around the nucleotide binding cleft or can be argued to affect the cleft.[10] Most *ACTA1* mutations however cause nemaline myopathy and the mutations that cause nemaline myopathy are well scattered through the actin monomer. It is as if nemaline myopathy was the default disease state.

What Do the Patients Tell Us about the Pathophysiology of *ACTA1* Diseases?

Most of the mutations in *ACTA1* cause dominant disease through dominant negative effects. Actin monomers polymerise into filamentous actin and therefore it is easy to see that mutation of one monomer may influence neighbouring monomers in the filament.

Actin interacts with multiple actin-binding proteins, in multiple functions, a fact that has been used to explain the highly conserved nature of actin proteins.[3] Different dominant mutations affect different actin functions.[18,22] The severity of the disease caused by a particular *ACTA1* mutation may be determined by the effect of the mutation on muscle fibre architecture, or on a particular function of actin.

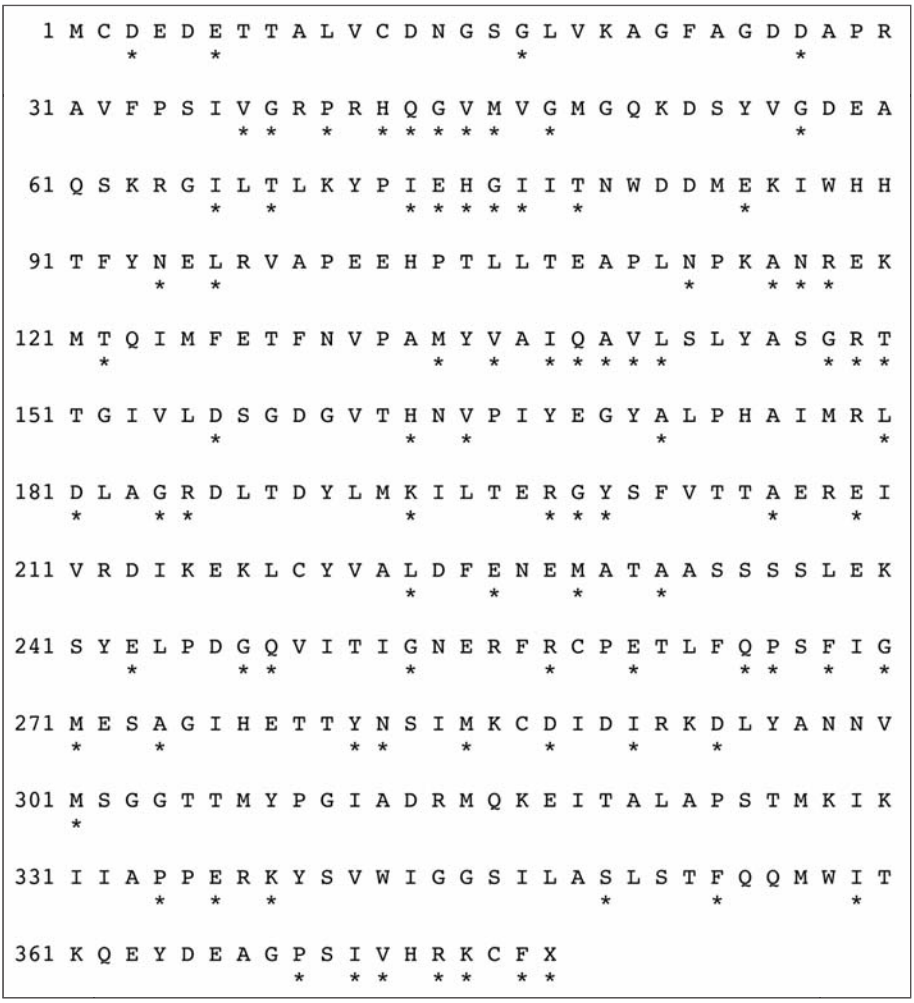

Figure 3. Distribution of currently known *ACTA1* missense mutations. Mutated amino-acid residues are denoted by an asterisk beneath the residue. The STOP codon mutations are also noted.

The effects of dominant *ACTA1* mutations are starting to be tested in various systems and correlated with disease severity.[16,26,27] This work is still in relative infancy, but, as stated above, one mutation that caused a severe skeletal myopathy and cardiomyopathy resulted in a 10-fold reduction in the affinity of actin for actinin.[16]

Recessive mutations in *ACTA1* are either genetic or functional nulls. The missense mutations that are recessive, where this has been tested, are functional nulls, in that they cause mis-folding of the actin monomer which then cannot incorporate into the thin filament.[22] The parents of patients with recessive disease have only one functioning *ACTA1*, but are clinically unaffected.[5,8,20] This indicates that one functioning normal *ACTA1* (haploinsufficiency) is sufficient for life and normal muscle function.

The autosomal recessive skeletal actin null patients have no mutant protein in their muscles, yet nemaline bodies and zebra bodies form.[20] Why is this so? Does this mean that nemaline

bodies form when there are altered ratios of the different sarcomeric proteins present during sarcomerogenesis? Nemaline bodies also form in recessive nemaline patients null for slow alpha-tropomyosin (*TPM3*)[28] and slow troponin T (*TNNT1*).[29]

What Do the Patients Tell Us about Possible Treatments?—Lessons from the Clinic in the Development of Therapies

At least one patient demonstrates that strict exercise regimes may benefit at least some *ACTA1* patients.[12](North unpublished observations) Interestingly this patient is also one of the first described with nemaline myopathy.[30] This patient, now in his 50's, presented with typical congenital onset nemaline myopathy. In his early teens, he found that repeated exercise going up and down stairs strengthened his proximal muscles, so that he could eventually arise from the floor without using a Gowers' manoeuvre. In his teens, he took up cycling—and eventually became a regular competitor in endurance cycling races. His quadriceps muscle biopsy after instituting these exercise regimes showed marked hypertrophy of most muscle fibres, over twice the mean diameter for his age. Interestingly this patient also reports that he is unable to sprint—which likely is due to the marked type 1 fibre predominance in his muscles. Subsequently, we have recommended low impact exercise such as cycling and swimming to many patients with nemaline myopathy with and without *ACTA1* mutations. Patients generally report improved strength and stamina after regular exercise: conversely periods of inactivity, particularly forced inactivity due to illness, result in loss of muscle strength. Interestingly the mouse model of *TPM3* nemaline myopathy has compensatory hypertrophy of muscle fibers compared to wild type that appears to delay onset of muscle weakness[31] and exercise following inactivity can reverse muscle weakness and pathological changes in muscle[32].

These data suggests that exercise and factors that can produce muscle fibre hypertrophy may be possible therapeutic agents in some patients with *ACTA1* nemaline myopathy.[33]

Kalita[34] reported improvement in muscle strength, appetite and weight gain, pharyngeal secretions and drooling in two individuals with nemaline myopathy after dietary tyrosine supplementation. These patients, a father and son, were subsequently found to have an *ACTA1* mutation (A. Beggs *personal communication)*. We have subsequently given dietary supplementation with L-tyrosine (250-3000 mg/day) to five patients (four infants and one adolescent) with nemaline myopathy. All four infants were reported to have an initial decrease in drooling and suctioning requirements and an increase in energy levels. The adolescent showed improved strength and exercise tolerance. No adverse effects of treatment were observed. The means by which L-tyrosine may ameliorate bulbar dysfunction in this disorder is not clear. Tyrosine conversion to L-dopa by tyrosine hydroxylase is the rate-limiting step in catecholamine synthesis. There may be an increase in peripheral catecholamine production in response to tyrosine therapy, which may cause vasoconstriction and decreased saliva production; however the basis for the effect on muscle stamina and strength is not clear.[33] There is no reason to expect this effect to be specific to nemaline myopathy. This anecdotal evidence raises the possibility that dietary tyrosine supplementation may improve bulbar function, activity levels and exercise tolerance in nemaline myopathy, but randomised blinded placebo-controlled trials are needed to confirm a true effect.

The patients with recessive mutations in *ACTA1* and absence of skeletal α-actin have increased cardiac actin in their skeletal muscles.[8,20] Cardiac actin is the fetal isoform of actin for skeletal muscle, normally present before birth, but switched off around the time of birth.[15] The level of cardiac actin retained in the recessive patients seems to some extent to determine disease severity.[20] This suggests that, at least for skeletal actin null patients, an alternative isoform of actin, such as the naturally occurring retention of cardiac actin, may ameliorate the disease severity. These results in human families with recessive *ACTA1* mutations are mirrored by skeletal actin knock out mice. The *ACTA1* knockout mice are, remarkably, indistinguishable from their normal and carrier littermate controls at birth but are all dead by day 9 postnatal despite retention of some cardiac actin.[35]

The identified somatic mosaic parents of severely affected children have mild disease, so mild that they can consider themselves unaffected[5,23] (and unpublished observations). This suggests

that even for dominant *ACTA1* mutations, the relative dose of the mutant protein determines the severity of the disease and that dilution of the mutant protein by a normal actin isoform, or allele-specific knock down of the mutant allele, might ameliorate the disease.

Similarly, most *ACTA1* mutation patients do not have heart disease. This may result from the fact that the predominant actin isoform in the adult heart is cardiac actin.[36] This may dilute out the effect of the mutant skeletal actin present in the heart to a sufficiently low percentage of the total actin that it has little effect. However, an *ACTA1* mutation that has a particular effect on actin biology may also cause cardiomyopathy. For example a mutation that produces an actin monomer that prevents further extension of the actin filament, for example an end-capping mutation,[10] might affect cardiac muscle as much as skeletal muscle. The dose of the mutant actin may not matter in this situation, since one mutant actin monomer in a filament may be sufficient to stop elongation of the filament.

The variability of disease severity that exists for the same mutation between patients and even within individual dominant families suggests that there are factors that can modify disease severity.[12] However, we currently do not know what those modifying factors are and therefore the known variability cannot currently guide possible therapies.

Future Directions

It is entirely possible that other clinical and histopathological phenotypes that we have not yet recognised as being *ACTA1* diseases are caused by *ACTA1* mutations. It is important to keep screening other phenotypes for *ACTA1* mutations, perhaps especially patients at the severe end of the congenital onset spectrum.

Antenatal screening for *ACTA1* diseases is currently available only for known families. However *ACTA1* disease largely results from de novo mutations with catastrophic consequences on actin function. This is similar to other diseases such as Duchenne muscular dystrophy and Neurofibromatosis type 1. New technologies are coming and some time in the future it may be possible to analyse all the disease genes with a known high prevalence of de novo mutations or perhaps even sequence an entire human genome for so little cost and so rapidly, that antenatal screening might be possible for these catastrophic de novo mutations.

It will be important to gain greater understanding of *ACTA1* disease mechanisms through using tissue culture and animal models, which have been and are being developed, as discussed in other chapters.

The ultimate aim must be to develop effective treatments for the *ACTA1* diseases. It may be that there is no single treatment that will overcome all the *ACTA1* mutations. For example, some *ACTA1* mutations may have such an effect on thin filament function that no amount of dilution of the mutant actin will alleviate the disease. Alternately, if we can identify genes or other factors that modify the severity of *ACTA1* diseases, these may become targets for possible future therapeutic intervention.

Acknowledgements

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Chapter 3

Nebulin—A Giant Chameleon

Katarina Pelin* and Carina Wallgren-Pettersson

Abstract

Nebulin is an enormous protein of the muscle sarcomere. It is a determinant of thin filament length, Z-disk structure and fiber contractility. The nebulin gene contains four regions of alternative splicing, providing a wealth of different isoforms of the protein. The precise function of these numerous isoforms in various types of muscle tissue remains to be elucidated, as does their role in the maintenance of normal muscle strength and activity. Understanding these basic mechanisms is a prerequisite for the development of specific therapies for the disorders caused by mutations in the nebulin gene. Such mutations are the main cause of autosomal recessive nemaline (rod) myopathy, especially of the typical form of this congenital myopathy. Further known disorders caused by nebulin mutations are several other subcategories of recessively inherited nemaline myopathy and a novel distal myopathy caused by homozygous missense mutations in the nebulin gene. Because of the giant size of the gene, molecular genetic testing methods are difficult to design for routine diagnostic use.

Introduction

Nebulin is a giant structural protein of the sarcomeric thin filaments.[1] It is expressed in vertebrate skeletal muscle where it comprises approximately 3% of the myofibrillar proteins. A lower expression of nebulin has been detected in cardiac muscle.[2] Nebulin binds to actin and regulates the assembly and the lengths of the thin filaments in the muscle sarcomere.[3] Nebulin is not required for myofibrillogenesis in the mouse, but it is crucial for the normal contractile function of its skeletal muscles.[4,5]

Mutations in the nebulin gene (*NEB*) are the most common cause of autosomal recessive nemaline myopathy.[6] Homozygous missense mutations have recently been associated with a novel autosomal recessive distal myopathy, named distal nebulin myopathy.[7]

The Nebulin Protein

Nebulin (NEB) is one of the largest known proteins. It is a component of the thin filament of the striated muscle sarcomere. For a long time nebulin was thought to be absent from cardiac muscle sarcomeres, where the homologous, but smaller protein nebulette is expressed.[8] Nebulin is, however, expressed in cardiac muscle, mainly in atrial cardiomyocytes and in a low percentage of ventricular cardiomyocytes.[2,4]

The nebulin protein has a highly repetitive modular structure with the capacity to bind and stabilize the actin filaments in the sarcomere.[9] The elastic properties of nebulin have been estimated to be two to three orders of magnitude smaller than that of an actin filament, indicating that nebulin stiffens the thin filament.[10] During muscle differentiation nebulin first appears in the Z disk and later extends into the A bands.[11] Nebulin repeat modules in the C-terminal

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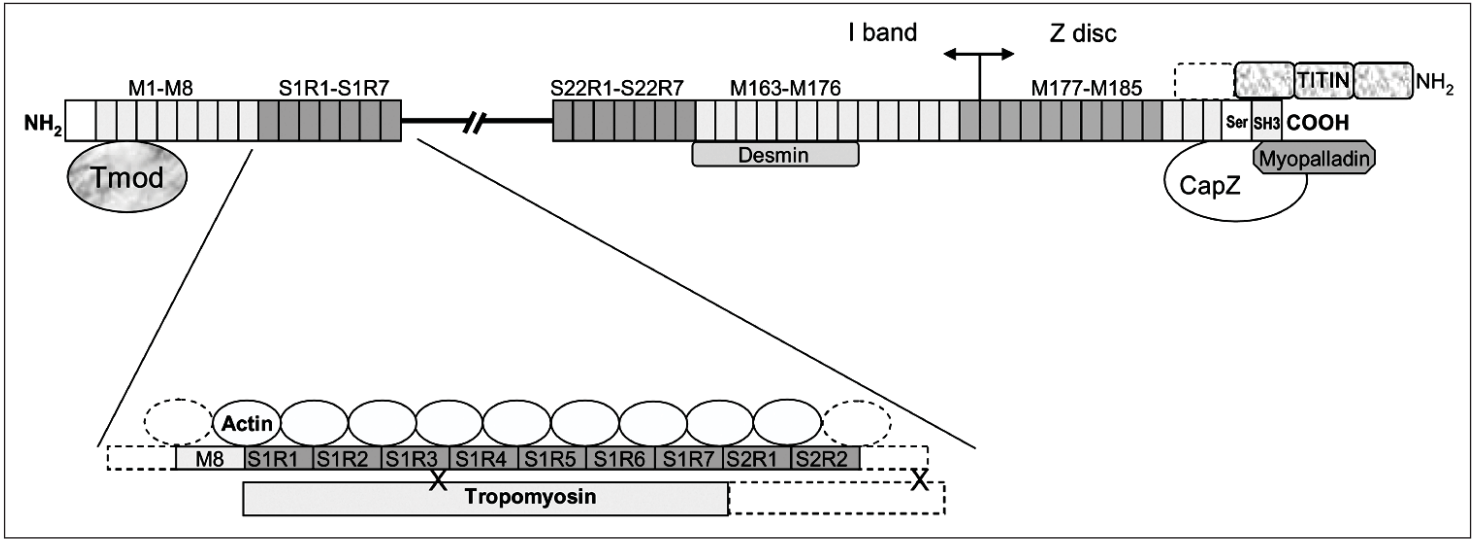

Figure 1. A schematic presentation of the nebulin protein structure adapted from Labeit and Kolmerer[14] and the known protein-binding partners as reported by McElhinny et al,[16] Bang et al,[17,19] and Witt et al.[5] The lower part of the figure shows a detailed view of one nebulin super repeat (S1) consisting of seven simple repeats (R1-R7) and the binding to actin and tropomyosin. Conserved actin-binding motifs (SDXXYK) are present in the simple repeat boundaries as defined by Labeit and Kolmerer.[14] Tropomyosin-binding motifs are present in the third simple repeat (R3) of every super repeat. The proteins are not drawn to scale. Abbreviations: Tmod tropomodulin, Ser Serine-rich domain, SH3 Src homology domain, M1-M8, M163-M176 simple repeats not organized into super repeats, S1R1-S1R7, S22R1-S22R7 super repeats of seven simple repeats.

portion of the protein have a higher tendency to form α-helices and a higher actin-binding affinity compared with N-terminal modules. This may reflect the need of directionality during thin filament assembly that is provided by a higher actin-binding affinity of the nebulin modules close to the C-terminus, which is anchored in the Z disk. Once these modules have bound to actin, the whole length of the nebulin polypeptide may then associate with the thin filament in a manner similar to the closing of a zipper.[12] Each actin subunit has three nebulin-binding sites and it has been suggested that nebulin moves on the actin filament during muscle contraction in a similar fashion as does tropomyosin.[13]

Nebulin is encoded by one gene which undergoes extensive alternative splicing resulting in isoforms ranging from 600 to 900 kilodalton.[14,15] Up to 97% of nebulin consists of ~30-35 amino acid long repetitive modules called simple repeats. Most of the simple repeats are arranged into seven-module super repeats. Based on the alternative splicing pattern of nebulin,[15] we have calculated that human nebulin isoforms can include between 179 and 239 simple repeat modules, with the potential to bind 179-239 actin monomers. Each simple repeat has a predicted α-helical secondary structure and an SDXXYK-motif, present at each 5.5 nm along the peptide. The SDXXYK-motif serves as an actin-binding site. A second motif, WLKGIGW, is present once in each super repeat at 38.5 nm intervals. This motif is probably involved in the organization of the tropomyosin and troponin periodicity of the thin filaments (Fig. 1).[14]

Both the 8 kilodalton N-terminal and the 20 kilodalton C-terminal ends of nebulin contain unique protein domains. Protein domains in the N-terminus interact with tropomodulin at the pointed end of the thin filaments.[16] The C-terminal end is anchored in the Z disk of the muscle sarcomere and contains a conserved SH3 domain. The sarcomeric protein, myopalladin, links nebulin to α-actinin in the Z disks.[17] The nebulin SH3 domain binds titin and CapZ.[5,18] The Z-disk peripheral region of nebulin has been shown to bind desmin (Fig. 1).[19] The C-terminus contains a large number of consensus phosphorylation sites and it has been demonstrated that nebulin is a phosphoprotein, but the functional significance of nebulin phosphorylation is unknown.[20]

The first evidence that nebulin may act as a thin filament protein ruler was obtained when a correlation was observed between nebulin size variants and thin filament lengths in different skeletal muscles.[21,22] Functional evidence supporting the ruler hypothesis has recently been provided by RNA interference studies in cultured rat cardiomyocytes[3] and nebulin knockout mice.[4,5] Knockdown of nebulin by RNA interference in rat cardiomyocytes resulted in dramatic elongation of the pre-existing thin filaments. Depolymerized thin filaments reassembled to unrestricted lengths in the absence of nebulin. Thus, nebulin is crucial for thin filament length regulation and assembly. Furthermore, the differentiation of primary rat cardiomyocytes into myotubes was disturbed in cells with reduced nebulin levels.[3]

However, recent results from studies on nebulin-deficient mice indicate that nebulin is not crucial for the normal assembly of sarcomeres, although these mice had substantially shorter skeletal muscle thin filaments than wild-type mice.[4,5] The diversity of thin filament lengths was also smaller. The structure of the Z disks was abnormal and nemaline bodies were reported for one of the mouse models.[5] Nebulin-deficient mice do not differ phenotypically from their wild-type littermates at birth, but growth retardation soon becomes evident and muscle contractility is impaired. The majority of the mice die within the first two weeks after birth.[4,5]

The Nebulin Gene

The nebulin gene is located on the long arm of chromosome 2 (at 2q22).[23] We have determined the genomic sequence of the entire gene; it is 249 kb in size and comprises 183 exons. The size of the exons varies from 42 to 596 bp and the size of the introns from less than 100 bp to >9 kb.[15] The central region of the gene harbors a ~8.2 kb region spanning eight exons which is repeated three times (exons 82-89, 90-97 and 98-105). The replicated segments are 99% identical. Exons 82-105 encode a total of 1458 amino acids, corresponding to 42 simple repeat domains or six super repeats.[15] Our results indicate that these exons are alternatively spliced, but the precise splicing pattern is difficult to elucidate due to the high homology of the duplicated

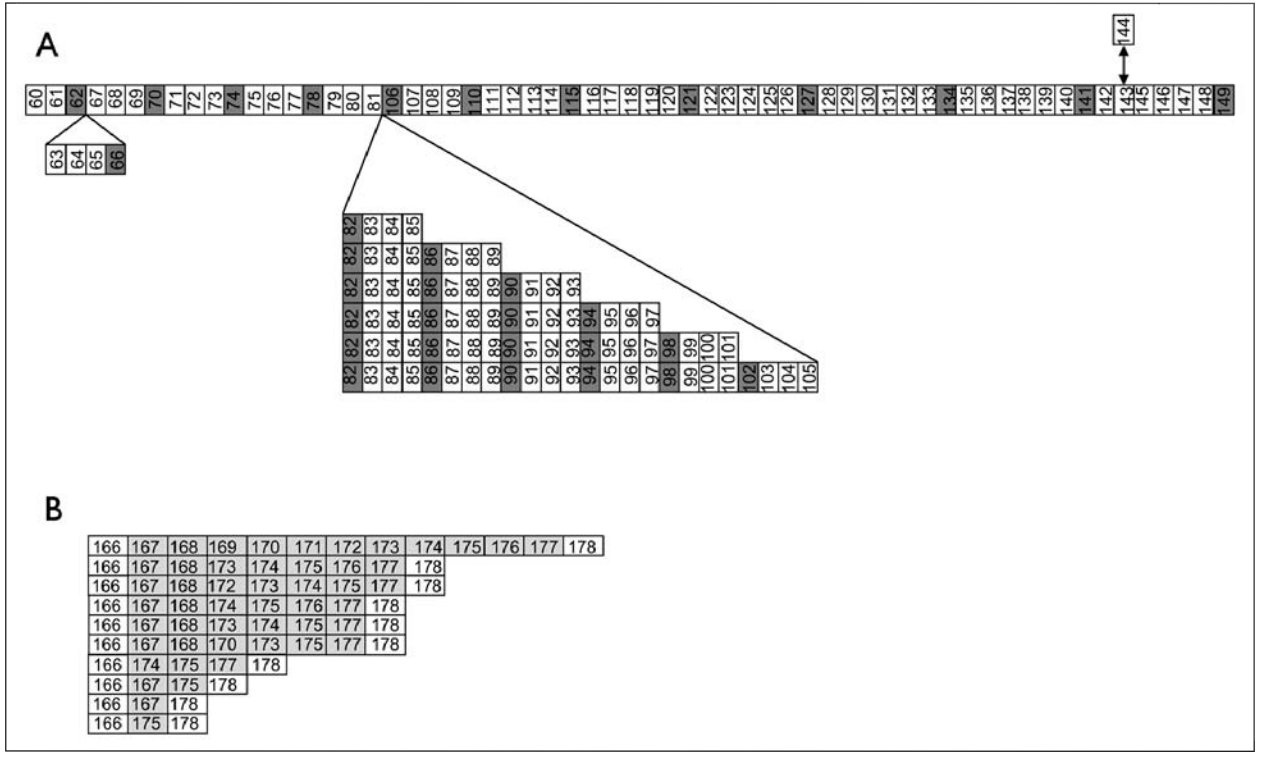

Figure 2. A) Alternative nebulin transcripts from three regions in the super repeat-encoding part of *NEB*. Only the region spanning exons 60-149 is shown. Exons encoding tropomyosin-binding domains are shown in gray. Exons 63-64 and 82-105 are spliced in groups of four exons in order to maintain the super repeat organization of *NEB*. Exons 143 and 144 are mutually exclusive. B) The fourth alternatively spliced region of *NEB*, exons 166-178 in the Z-disc simple repeat domain encoding part of the gene. Exons 167-177 are spliced independently of each other. The longest splice variant has been detected in a muscle biopsy from a nemaline myopathy patient[26] and the nine shorter variants in normal human tibialis anterior muscle.[15]

exons. However, in order to keep the super repeat organization of nebulin with evenly spaced actin- and tropomyosin-binding sites intact, these exons should not give rise to more than seven different length variants (Fig. 2A).

One region preceding the duplicated segments is also involved in alternative splicing, i.e., exons 63-66. These exons encode seven additional protein repeat modules highly homologous to super repeat 11. We have identified transcripts lacking exons 63-66, i.e., exon 62 spliced to exon 67, in cDNA reverse transcribed from adult human tibialis anterior muscle RNA. Human fetal muscle expressed only transcripts including all of the exons 63-66 (Fig. 2A).[15] The observed splicing pattern is in accordance with the super repeat organization of nebulin.

The 3′ end of *NEB* harbors two regions with alternatively spliced exons, i.e., exons 143-144 and exons 167-177. Exons 143 and 144 are mutually exclusive and give rise to two different transcripts, i.e., transcripts expressing either exon 143 or exon 144 (Fig. 2A). Fetal muscle in human seems to express only transcripts including exon 143. Both transcripts can be found in adult human tibialis anterior muscle, in adult mouse hind leg muscle and in 7-day-old mouse embryos. Adult human gastrocnemius, rectus femoris and cardiac muscles seem to express only transcripts including exon 144. Exons 143 and 144 encode 35-amino-acid-long simple repeat domains differing in both charge and hydrophobicity. The domain encoded by exon 144 is predicted to have a protein kinase C phosphorylation site. No phosphorylation sites are predicted in the domain encoded by exon 143.[15] Our recent results from a quantitative RT-PCR study of the corresponding mouse *Neb* exons, i.e., exons 127 and 128 show a developmental and muscle-type specific expression pattern of these exons. The expression pattern does not however appear to correlate with the fiber-type composition of the muscles.[24]

Exons 167-177, encoding the Z-disk region simple repeats M176-M182, represent so called cassette exons which are spliced independently of each other, with the potential to produce 121 different splice variants. Ten different splice variants from this region are shown in Figure 2B. We have identified 20 different transcripts by sequencing of RT-PCR products from this region of *NEB* in the human tibialis anterior muscle and it seems likely that several more exist.[15]

Based on the organization of the nebulin protein and our observations of the splicing pattern of *NEB*, we predict the theoretical number of different nebulin isoforms to be 3388, calculated from: 7 (exons 82-105) × 2 (exons 63-66) × 2 (exons 143-144) × 121 (exons 167-177).

Mutations in the Nebulin Gene

To date, all mutations identified in *NEB* have been recessive. Sixty-four different mutations have been published segregating with autosomal recessive nemaline myopathy in 55 families[6,25,26] and one further mutation has been identified in the North American Jewish population.[27] The majority (55%) of the mutations in *NEB* are frameshift or nonsense mutations predicted to cause mRNA instability or premature truncation of nebulin. Point mutations (25%) or small deletions (3%) affecting conserved splice signals are predicted in the majority of cases to cause in-frame exon skipping, possibly leading to impaired nebulin-tropomyosin interaction along the thin filament. Nine missense mutations (14%) affect conserved amino acids at or in the vicinity of actin or tropomyosin binding sites.

The frequency of large deletions, duplications and rearrangements in *NEB* is largely unknown, due to the mutation analysis methods used to date. However, one 2.5-kb deletion including exon 55 is associated with recessively inherited nemaline myopathy in the Ashkenazi Jewish population, where a number of patients have been found to be homozygous for the mutation.[27] The carrier frequency in the North American Ashkenazi population studied was about 1:100. The majority of nemaline myopathy patients in other populations (in 49/55 families whose mutations have been published) are, however, compound heterozygous for two different *NEB* mutations. The mutations are located in both constitutively and alternatively expressed exons throughout *NEB* and no mutational hotspots are evident.

Patients with more severe clinical pictures tend to have mutations predicted to be more disruptive than patients with milder forms. Immunohistochemical labeling shows that the nebulin protein

is present in the muscles of NM patients, despite their truncating mutations, but the expression pattern may be abnormal and some epitopes may be missing.[26,28] Given the enormous size of the gene with mutations scattered all along its length and the fact that most patients have two different mutations, more precise genotype-phenotype correlations will only be possible to define after the identification of a very great number of mutations.

DNA-Diagnostic Dilemma

The size of the gene and the wide-spread mutations in it pose a major challenge for designing a mutation analysis battery suitable for use in diagnostic service laboratories. Denaturing High Performance Liquid Chromatography (DHPLC) is a promising tool for the identification of small heterozygous alterations,[6] but it needs to be complemented by a method for the detection of large deletions. This might be either Multiplex Ligation-Dependent Probe Amplification (MLPA)[29] or a microarray.

Autosomal Recessive Nemaline (rod) Myopathy

Nemaline myopathy is a primary muscle disorder characterized by weakness of voluntary muscles and the presence in the muscle fibers of rod-like or threadlike nemaline bodies, their name being derived from the Greek word for thread (*nema*)[30-33] (Fig. 3). At light microscopy, the nemaline bodies can be seen with the modified Gomori trichrome stain as reddish bodies, usually just under the muscle sarcolemma and at electron microscopy they appear to be aberrations of the muscle Z disk. They are composed of Z-disk and thin filament proteins. α-Actinin is a main constituent, while nemaline bodies can be labeled with antibodies against myotilin and telethonin also, with actin, tropomyosin and desmin being present at the periphery.[34-40] Patients often have a deficiency of either or both subtypes of the fast, type 2 muscle fibers[41] and the exact fiber types may be difficult to define histochemically.[42]

The two most common causes of nemaline myopathy are mutations in the genes for nebulin and slow skeletal α-actin, while in rarer cases, α-tropomyosin, β-tropomyosin, troponin or cofilin may be mutated.[26,43-49] Autosomal recessive nemaline myopathy is most commonly caused by mutations in *NEB*.[6,27,50]

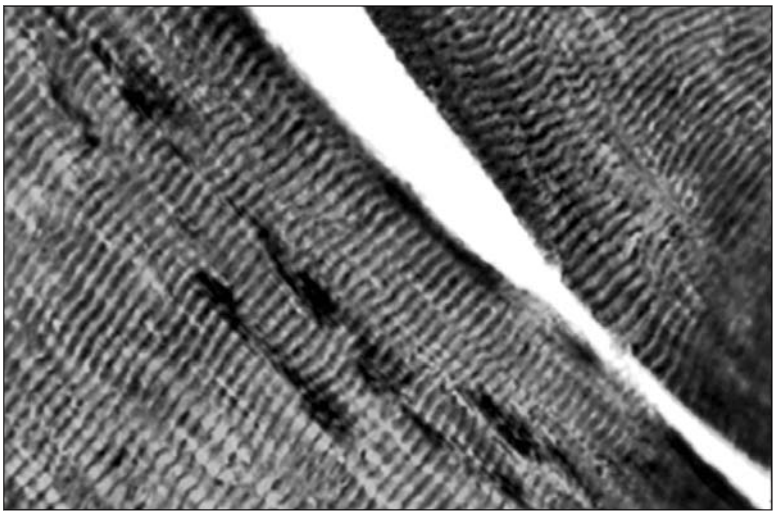

Figure 3. Longitudinal section of muscle biopsy showing the characteristic nemaline (rod) bodies in the muscle fibers. Modified Gomori trichrome stain with labeling of nemaline bodies with antibodies against alpha-actinin. Reprinted from: Wallgren-Pettersson C. J Neurol Sci; 89:1-14;[15] ©1989 with permission from Elsevier.

The European Neuromuscular Centre International Consortium on Nemaline myopathy has defined six clinical categories of nemaline myopathy, based on knowledge of the typical form and of how some groups of patients differ from those with that form. The six categories are: the severe congenital form with patients lacking spontaneous movements or respiration at birth, or with fractures or severe contractures at birth, the intermediate congenital form with patients moving and breathing at birth but who are later unable to achieve ambulation or respiratory independence, the typical congenital form with typical distribution of muscle weakness, motor milestones delayed but reached and slowly progressive or nonprogressive course, mild nemaline myopathy with childhood onset, adult-onset nemaline myopathy and other forms of nemaline myopathy with unusual associated features.[42] Among these, the typical form, in addition to being the most common form of nemaline myopathy, is also the one most commonly caused by mutations in *NEB* (Fig. 4). Mutations in this gene may rarely cause the severe congenital form,[51] the intermediate or the mild form,[6,50] but *NEB* mutations have not been identified to date in patients with verified onset of symptoms in adulthood.

Symptoms and signs are restricted to those caused by weakness of striated muscle. Although a low level of nebulin expression has been noted in cardiac muscle,[2,4] there is usually no primary involvement of the heart. In the typical form of nemaline myopathy, the patients mostly have a nasal

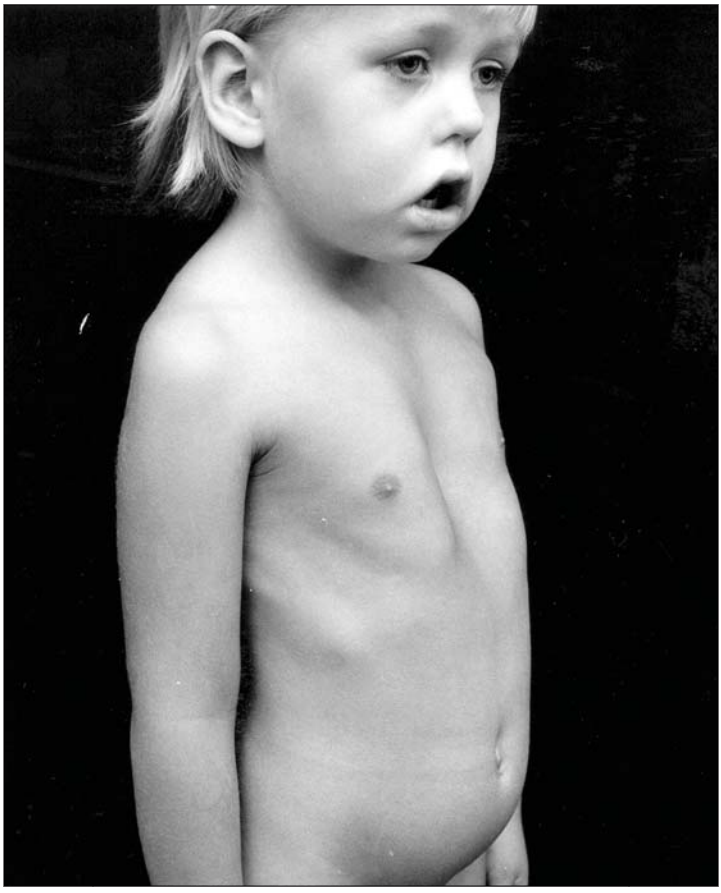

Figure 4. Patient with the typical form of nemaline myopathy. Note the myopathic facies, the hyperlordosis and the chest deformity. Reprinted from: Wallgren-Pettersson C et al. Neuromuscul Disord; 5:93-104;[38] ©1995 with permission from Elsevier.

voice or even dysarthria and the facial and bulbar muscles are weak, but the extra-ocular muscles are spared. Most patients are unable to lift their heads in the supine position.[52,53] Involvement of limb muscles is initially mainly proximal, with a later distal involvement, including foot drop. Axial muscles are usually weak and scoliosis commonly develops in the prepubertal period of rapid growth, during which some patients lose ambulation.

A study of 60 patients with mutations in the nebulin or actin genes provides an example of selective muscle involvement in sarcomeric disorders.[50] Patients with nebulin mutations showed more pronounced weakness of the knee flexors than of the extensors, while the opposite was the case in patients with actin mutations. The ankle dorsiflexors were very weak in patients with *NEB* mutations, while they were better preserved in patients with actin mutations. These differences in patterns of weakness were corroborated by comparisons of muscle MRI results of patients with mutations in the same genes.[54]

All patients with congenital nemaline myopathy are at risk of respiratory insufficiency and their respiratory capacity should be monitored throughout life.[52,55] Even if they are symptom free, most patients, including ambulant ones, will show restriction of their respiratory capacity on testing. Asymptomatic patients also run a great risk of insidious nocturnal hypoxia and several patients have experienced sudden respiratory failure. An international group of experts has agreed on strategies for ventilatory support in the congenital myopathies.[56]

Although no curative treatment is currently available for nemaline myopathy, much can be achieved for the patient through appropriate management by a multidisciplinary team familiar with the treatment of neuromuscular disorders. The goals of the treatment include prevention of respiratory complications, alleviation of swallowing and speech difficulties, prevention of spinal and joint deformities and maintenance of ambulation and independence in the activities of daily living. Genetic counseling should be offered to all families where a diagnosis of nemaline myopathy has been made.

Autosomal Recessive Distal Nebulin Myopathy

Recently a novel disease entity has been found to be caused by *NEB* mutations.[7] Two missense mutations were identified in homozygous form in seven Finnish patients from four unrelated families with childhood or adult-onset foot drop (Fig. 5). Both mutations, when combined in compound heterozygous form with more disruptive mutations, are known to cause nemaline myopathy.[6] Muscle weakness in this disorder predominantly affects the ankle dorsiflexors, the finger extensors and the neck flexors, a distribution different both from the patterns of weakness seen in nemaline myopathy caused by *NEB* mutations and those of the previously known recessively inherited distal myopathies. Laing distal myopathy (MPD1, OMIM #160500) however is clinically similar.[57,58] It is caused by heterozygous mutations in the myosin heavy chain gene *MYH7* and some cases have been caused by de novo mutations, making this disorder an important differential diagnosis in sporadic cases. This myopathy presents in childhood or young adulthood with weakness of the anterior leg muscles and neck flexors.[59] Later, the finger extensors become weak and in some there is an additional mild involvement of shoulder and hip muscles. The pattern of weakness of the ankle dorsiflexors and the finger extensors is thus very similar in the two disorders. Histologically, there are no definite, specific differences between Laing distal myopathy and distal nebulin myopathy.[7,59.]

Histologically, the novel distal nebulin myopathy differs from nemaline myopathy in that nemaline bodies were not detectable with routine light microscopy and they were inconspicuous or absent even at electron microscopy. Rimmed vacuoles were not a feature, differentiating this myopathy from most other distal myopathies. Thus, homozygous missense mutations in *NEB* cause a novel distal myopathy. It remains to be seen whether a similar condition may be caused by other missense mutations present in homozygous or compound heterozygous form.

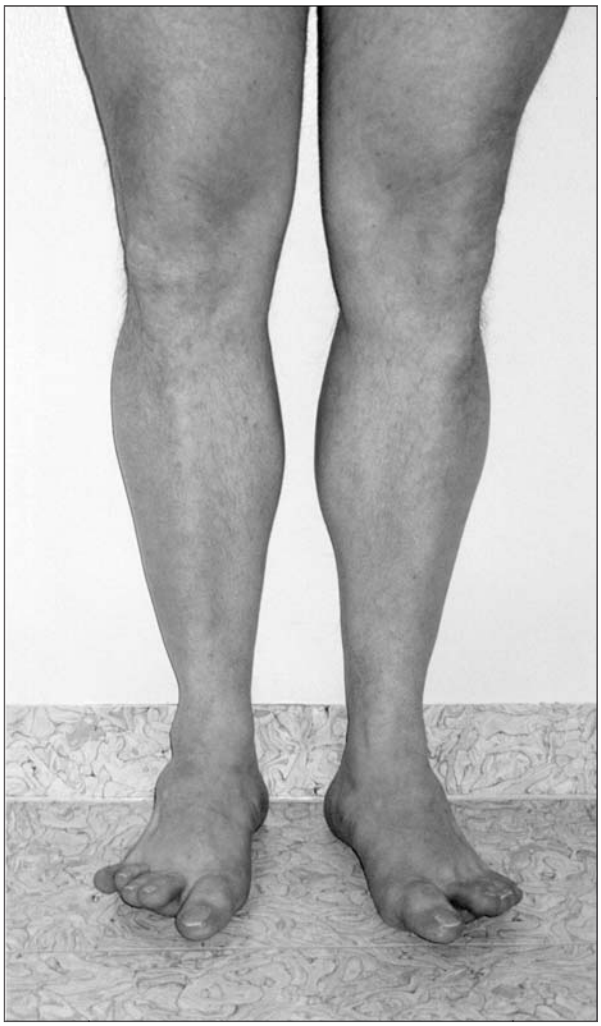

Figure 5. Patient with distal nebulin myopathy. Note the small muscle bulk in the anterior leg muscles in contrast with the otherwise well-preserved muscle mass (and a slight pseudohypertrophy) and the inability to dorsiflex the feet.

Conclusions (Future Applications, New Research, Anticipated Developments)

An ultimate goal for research into nebulin and its aberrations is finding specific therapies for the disorders caused by these aberrations. This is however not an easy task. The sarcomere develops during fetal life and forms an important component of the postmitotic and rather stable muscle tissue, constituting around 40% of the body.

Nebulin plays important roles in the sarcomere. Therefore the recent finding that nebulin-deficient mice do form sarcomeres was perhaps somewhat surprising. However, in these mice, thin filament lengths were shorter and length diversity was reduced. Neither of the mouse models is an exact replicate of nemaline myopathy in human patients as it is known today, since no patient with this disorder has been found to be a null mutant for nebulin. Nevertheless, some of the findings in

the mouse models were similar to those in patients with nemaline myopathy caused by mutations permitting the expression of nebulin. Z-disk structure and sarcomere maintenance was abnormal and muscle contractility was impaired.

A first requirement for designing specific forms of treatment for muscle disorders caused by mutations in *NEB* is sufficient understanding of the pathogenesis. The reasons for the formation of the characteristic nemaline bodies are poorly understood in nemaline myopathy caused by mutations in the nebulin gene, as well as in other forms of nemaline myopathy. The number of nemaline bodies on the other hand does not appear to correlate with muscle weakness. Also, patients with distal nebulin myopathy have weakness in the absence of nemaline bodies. Thus, a key to the pathogenesis of disorders caused by mutations in *NEB* may lie in deciphering which isoforms of the protein are necessary for the maintenance of sarcomere function in the various muscle types, rather than in elucidating nemaline body formation as such.

Genotype-phenotype correlations have been difficult to discern. This is due to the daunting size of the gene, to most patients being compound heterozygotes for two different mutations and to the mutations being spread all over the gene. More distinct genotype-phenotype patterns may emerge after the identification of a greater number of mutations.

For the same reasons, mutation analysis is not yet feasible in routine laboratory settings. A combination of DHPLC and a method for the identification of large deletions may be needed for diagnostic purposes.

A long-standing question has been why mutations in a gene as large as *NEB* cause a disorder that is rare, i.e., nemaline myopathy. Based on knowledge of average mutation frequencies, it would have been expected that any disorder caused by mutations in *NEB* would be common. A first hypothesis was that mutations in regions encoding the Z-disk part of the protein might cause nemaline myopathy, while mutations in other parts of the gene might cause other disorders. Mutational results however do not support this idea; mutations have been identified all along the length of the gene.

Another explanation might lie in the fact that isoforms of the protein are *legio*. Possibly, the existence of the numerous isoforms reflects a certain redundancy, which would cause many alterations in the gene to be harmless.

A further possibility, corroborated by the recent discovery of the novel disease entity, distal myopathy caused by mutations in the nebulin gene, is that certain types of mutations cause disorders other than nemaline myopathy. Additional entities may remain to be discovered.

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Chapter 4

Skeletal Muscle Disease Due to Mutations in Tropomyosin, Troponin and Cofilin

Nigel F. Clarke*

Abstract

Tropomyosin (Tm) and the troponins (troponin I, troponin T and troponin C) are proteins that work cooperatively to regulate muscle contraction, making actin-myosin interactions sensitive to cytosolic calcium levels. Several isoforms exist for each component in this group, each having a specific expression pattern that enables cardiac, slow skeletal (type 1) and fast skeletal (type 2) muscle fibers to have distinct contractile properties. Mutations in all components of this complex have been associated with skeletal muscle disease. The first disease associations were with nemaline myopathy, but recently other congenital myopathies ('cap disease', congenital fiber type disproportion) and other clinical entities (distal arthrogryposis, multiple pterygium syndrome) have been linked to mutations. A homozygous mutation in *CFL2*, the gene for muscle cofilin, has been associated with nemaline myopathy in one family to date. Researchers have begun to decipher the mechanisms by which these mutations result in muscle weakness and contractures using a variety of in vitro assays to assess the effects of individual mutations on protein function and on sarcomere dynamics.

Introduction

The tropomyosins are a group of highly conserved actin-binding proteins that play important roles in a wide range of actin-related intracellular processes. All tropomyosins either homo- or heterodimerise in parallel coil-coiled continuous alpha helices and then dimers assemble head to tail into long filaments that lie along actin polymers (see ref. 1 for an overview). In mammals tropomyosins are encoded by four genes that show highly homology and that are differentially spliced to create over 40 different isoforms.[2] The three so-called striated muscle tropomyosin isoforms have very similar amino acid sequences. Each is 284 amino acids in length and is transcribed from a different gene; α-tropomyosin$_{fast}$ (αTm$_{fast}$) from *TPM1*, β-tropomyosin (βTm) from *TPM2* and α-tropomyosin$_{slow}$ (αTm$_{slow}$) from *TPM3*. Through interactions with α-skeletal actin and α-cardiac actin, these tropomyosin isoforms are central to the regulation of muscle contraction in skeletal and cardiac muscle.[3]

The expression and dimerisation patterns of the three striated muscle isoforms of Tm are complex. The αTm$_{slow}$ isoform is expressed in type 1 (slow-twitch) skeletal muscle fibers while αTm$_{fast}$ is expressed in type 2 (fast-twitch) muscle fibers and is the most abundant isoform in the heart.[4-6] The βTm isoform is expressed in both type 1 and type 2 muscle fibers and accounts for a minor percent

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of tropomyosin in the human heart.[6] In skeletal muscle, the muscle isoforms exist predominantly as heterodimers;[7,8] as αTm_{slow}-βTm heterodimers in slow fibers and as αTm_{fast}-βTm heterodimers in fast fibers, although as a proportion, slow fibers contain more βTm than fast fibers.[5,9]

The troponin complex is made up of three proteins (troponin C, troponin I and troponin T) that bind as a unit to tropomyosin to make the process of muscle contraction sensitive to calcium levels. When membrane depolarisation triggers a rise in cytosolic calcium, calcium ions bind to troponin C and a conformation change in the troponin complex results in the movement of tropomyosin relative to the actin filament. This exposes myosin binding sites on actin and permits force generation through actin-myosin interactions (reviewed in ref. 10). As for tropomyosin, each component of the troponin complex has tissue-specific isoforms that are encoded by different genes. Troponin I and troponin T have separate isoforms for cardiac, type 1 and type 2 muscle fibers while two isoforms of troponin C serve type 2 and cardiac/type 1 fibers respectively. Minor differences in the amino acid composition between the troponin and tropomyosin isoforms (and variations in tropomyosin isoforms ratios) change the dynamics of calcium-mediated muscle contraction in the different fiber types to suit their different physiological roles.

Mutations in *TPM1*,[11] *TNNT2*,[11] *TNNC1*[12] and *TNNI3*[13] that respectively encode the predominant isoforms of tropomyosin, troponin T, troponin C and troponin I in the heart, are well established genetic causes of cardiomyopathy, but to date they have not been associated with skeletal muscle disease. The body of research into the range of mutations and the pathophysiological effects of those mutations on cardiac function exceeds that for skeletal muscle disease and has been an important source of inspiration and techniques for skeletal muscle researchers.

Mutations in Tropomyosin Associated with Skeletal Muscle Disease

Beta-Tropomyosin, Encoded by TPM2

The first patient identified with a *TPM2* mutation had nemaline myopathy (NM)[14] but this study suggests it is an uncommon cause of the disease, responsible for less than 5% of families. Recently, further *TPM2* mutations have been identified in association with a range of clinico-pathological presentations. Although *TPM2* mutations remain rare, with only seven disease-causing mutations identified to date (Table 1), this suggests that we may have been looking in the wrong patient groups. Some intriguing patterns are emerging, such as association of *TPM2* mutations with two disease phenotypes, congenital myopathy and distal arthrogryposis, that historically have been consider quite separate entities. Congenital myopathies typically have congenital hypotonia and weakness that remains relatively stable and congenital contractures are usually only seen in patients with severe congenital weakness and in large joints.[15] The main clinical feature in distal arthrogryposis (DA) is nonprogressive congenital contractures in distal joints usually without other evidence of neuromuscular disease (such as muscle weakness).[16] A variety of DA subtypes have been proposed based on clinical phenotype.[17] Two families have some features of both phenotypes, indicating there may be a continuous spectrum of disease that spans the two entities.[18,19]

Congenital Myopathies Associated with *TPM2* Mutations

Four mutations have been associated with typical clinical features of a congenital myopathy in individual families (Table 1).[14,20,21] Although histological patterns have varied, all patients have followed similar clinical courses, having stable generalized muscle weakness without early joint contractures. Most presented in infancy, the only exception being a patient with mild NM (p.Q147P) who presented at age 12 years with walking difficulties.[14] All affected individuals have had mild to moderate generalized weakness and all achieved independent ambulation. Typically the weakness has been more prominent in proximal limb and neck muscles (those for neck flexion especially). Facial weakness was common and ankle contractures and scoliosis developed with age in some. Ambulation was sometimes lost during adulthood and three patients required nocturnal ventilatory support due to reduced respiratory muscle function.[20,21]

Three different histological patterns have been reported in association with the four congenital myopathy mutations. Although experience is extremely limited at present, there are early signs that

Table 1. Mutations in the tropomyosin genes associated with skeletal myopathy

Family No.	Mutation	Exon	Isoforms Affected	Disease Assoc.	Inheritance Pattern	Reference
TPM2						
1	c.440A > C p.Q147P	4	m + nm	NM	de novo dominant	14
2	c.349G > A p.E117K	3	m + nm	Myopathy	AD	14
3	c.271C > G p.R91G	3	m + nm	DA1	AD	24
4	c.397C > T p.R133W	4	m + nm	DA2B + muscle weakness	AD	19
5	c.415_417delGAG p. E139del	4	m + nm	Cap	de novo dominant	20
6	c.121G > A p.E41K	2b	m + nm	NM + Cap	AD	21
7	c.628C > T (hom) p.Q210X	6b	m	NM/MTS	AR	18
TPM3						
1	c.26T > G p.M9R	1a	m	NM	AD	33
2	c.94C > T (hom) p.Q32X	1a	m	NM	AR	37
3	c.857A > C (c.het) p.X286S	9b	m	NM	AR	39
3	IVS9b-1G > A (c.het)	9b	m	NM	AR	39
4, 5, 6	c.503G > A p.R168H	5	m + nm	NM + CFTD	AD	30,34,38
7	c.298C > A p.L100M	3	m + nm	CFTD	AD	34
8	c.502C > G p.R168G	5	m + nm	CFTD	Likely de novo dominant	34
9	c.502C > T p.R168C	5	m + nm	CFTD	de novo dominant	34
10	c.505A > G p.K169E	5	m + nm	CFTD	de novo dominant	34
11	c.733A > G p.R245G	8	m + nm	CFTD	de novo dominant	34
12, 13	c.855delA (hom) p.I285fs × 360	9b	m	NM	AR	40

Mutations are listed in approximate order of discovery. All mutations associated with autosomal dominant disease occur in the heterozygous state. An exon numbering system that is consistent between tropomyosin isoforms is used.[31,32] m: muscle-specific isoform; nm: 'nonmuscle' isoform; AD: autosomal dominant; AR: autosomal recessive; NM: nemaline myopathy; DA: distal arthrogryposis; Cap: 'cap disease'; CFTD: congenital fiber type disproportion; MTS: multiple pterygium syndrome: hom: homozygous; c.het: compound heterozygous.

these may represent variable expressions of a common pathogenic process on muscle structure. *TPM2* mutations were first found in one patient with NM (p.Q147P)[14] and in a second patient who had nonspecific histological changes on muscle biopsy (p.E117K).[14] More recently, the p.E139del mutation has been associated with 'cap disease' and marked type 1 fiber hypotrophy.[20] Cap disease is characterised by abnormal protein accumulations peripherally in muscle fibers that stain negatively for myosin ATPase and positively for a wide range of thin filament proteins.[21,22] A fourth mutation (p.E41K) was found in a mother and daughter who were diagnosed with NM and cap disease, respectively, in association with complete type 1 fiber predominance.[21] The presence of both cap disease and NM in the same family suggests there is a significant overlap in the pathophysiology of these two histological patterns. In addition, several of these patients have had more than one biopsy.[20,21] In these patients, proteins accumulations (either caps or nemaline bodies) were seen in biopsies taken in adulthood but were much less obvious or absent in biopsies taken earlier in life, suggesting that age can be an important factor in the development of protein accumulations. Nemaline bodies were also found in children homozygous for a recessive *TPM2* mutation (p.Q210X) but since distal arthrogryposis and multiple pterygia were the most striking clinical features, they have been included in the next section.[18]

Distal Arthrogryposis (DA) Associated with *TPM2* Mutations

Mutations in *TPM2* have been associated with DA, or variants of that, in three families to date, each in association with a different mutation. The p.91R > G mutation tracked with distal arthrogryposis type 1 (DA1) in 14 members of a single family.[23,24] The p.R133W mutation was associated with dominant distal arthrogryposis type 2B, a more complex phenotype that is also known as variant Freeman-Sheldon syndrome or Sheldon-Hall syndrome.[19] As well as congenital ankle/foot (pes equinovarus) and hand contractures, the affected mother and daughter also had limited mouth opening, small jaw, neck webbing, kyphoscoliosis and proximal joint contractures.[19] There was also muscle weakness, more pronounced in distal muscle groups and that worsened with age, which is atypical for DA. In a third family, children homozygous for a recessive nonsense mutation in *TPM2* (p.Q210X) that abolishes βTm expression, had skin webs (pterygia) across multiple proximal joints at birth in addition to distal arthrogryposis, hypotonia, facial weakness, ptosis and ocular strabismus.[18] Remarkably, these children walked from mid-childhood and remained stable to early adulthood, without apparent cardiac or respiratory dysfunction.

Muscle histology has been studied in two of these families. In two patients with p.R133W the only abnormality noted was type 1 fiber predominance.[19] In the quadriceps muscle of a child homozygous for the p.Q210X mutation, numerous nemaline bodes were seen.[18] Therefore this family and a family with DA and progressive weakness (families 4 and 7, Table 1) have some features of a congenital myopathy, as well as DA.

Molecular Aspects of *TPM2* Mutations

All *TPM2* mutations reported to date, except one, have been associated with autosomal dominant disease, causing muscle weakness in the heterozygous state (see Table 1). Sometimes these have affected a single individual in a family, most likely arising de novo, but in four families there has been autosomal dominant transmission. The only known *TPM2* mutation associated with recessive disease (p.Q210X) is particularly interesting because there is good evidence that it completely abolished βTm expression.[18]

In βTm, the six dominant missense mutations are spread widely and cause a diverse range of amino acid substitutions. To date, the functional effects of only two associated with DA have been studied in any depth. Both the p.91R > G and p.R133W mutations alter charged arginine residues that occupy '*g*' positions of the Tm alpha-helix. In this position, amino acid side chains are directed outward from the Tm dimer for potential involvement in protein-protein interactions and salt bridges between residues in positions '*g*' and '*e*' can help to stabilise the Tm dimer.[25,26] Recent biochemical studies suggest that both mutations result in abnormal Tm-actin interactions but indicate different pathophysiological effects. Contractility studies using skinned patient muscle fibers carrying the p.R133W mutation showed a reduction in specific force generation that was

significant in type 1 fibers only. This is likely to be due to an increased inhibitory effect of tropomyosin on myosin-actin interactions and this may account for the ongoing muscle weakness in these patients.[9] In contrast, the p.91R > G mutation was associated with increased activation of myosin ATPase, a finding also associated with several troponin mutations associated with DA.[27] There is also evidence that this mutation destabilises the coiled coil structure of the Tm dimer.[27] Therefore in vitro studies on *TPM2* mutations associated with DA have not shown consistent abnormalities. This may to some extent stem from differences in the methodologies used but may also reflect differences in the clinical phenotypes.

At present, the reason that mutations in *TPM2* can be associated with a range of phenotypes is unclear but one can hypothesise that this arises because different mutations affect βTm function in different ways. The association between *TPM2* mutations and both distal arthrogryposis and pterygia also raises the possibility that βTm has a special role during fetal development, particularly in distal muscles, that may differ from its roles in postnatal life.

Alpha-Tropomyosin$_{slow}$, Encoded by *TPM3*

The *TPM3* gene encodes the α-Tm isoform that is specific for type 1 muscle fibers, as well as a number of nonmuscle isoforms. In slow muscle fibers αTm$_{slow}$ accounts for around a third of Tm and preferentially forms dimers with βTm.[4,28] Twelve families have been reported with mutations in *TPM3*, more than for *TPM2*, but the clinical and histological findings are less diverse. Two systems have been used to number amino acids in αTm$_{slow}$, which has the potential to cause confusion, depending on whether the first N-terminal methionine, which is translated but cleaved from the mature molecule, is included. The other skeletal Tm isoforms lack this extra methionine and so this issue does not arise. In this chapter the numbering of amino acids in *TPM3* includes the first cleaved Met according to standard nomenclature.[29] Therefore, for example, the missense change associated with c.503G > A is notated here as p.R168H, while in some references this is listed as p.R167H.[30] In addition, several systems for numbering tropomyosin exons have been used. Here a numbering system that is consistent between isoforms is used.[31,32]

Histological Aspects of *TPM3* Mutations

TPM3 mutations have been associated with two main histological patterns to date. Mutations were first associated with NM,[33] but as for the *TPM2* gene, *TPM3* appears an uncommon cause of this histological pattern. Recently, *TPM3* mutations were found to account for around a quarter of patients with congenital fiber type disproportion (CFTD), in which the primary abnormality is a consistent hypotrophy of type 1 fibers, relative to type 2 fibers (Fig. 1).[34] There is good evidence that for *TPM3* there is a large overlap in pathophysiology between NM and CFTD although important difference may exist for other genes.[35] Small type 1 fibers, the hallmark feature of CFTD, is a secondary feature in most *TPM3*-NM patients (Fig. 1)[33,36,37] and some mutations have been associated with both histological patterns, such as p.R168H.[30,34,38] Age at biopsy may be one variable that influences whether nemaline bodies are found since there is a trend for nemaline bodies to be more common with increasing age,[15] but it is likely that some *TPM3* mutations have a very low propensity to cause nemaline bodies. For example, no nemaline bodies were found in three patients with the p.L100M mutation, including two adult males biopsied in their 30s.[34] On the muscle biopsy, there is no single pathognomonic abnormality that reliably indicates a mutation in *TPM3*, although type 1 hypotrophy is common and when nemaline bodies are present, they are confined to type 1 fibers.

Clinical Aspects of *TPM3* Mutations

Although patient numbers are limited, clinical patterns are emerging that can aid the diagnosis of patients with *TPM3* mutations. The six children with recessive mutations have been the most severely affected. A boy homozygous for a nonsense mutation in exon 1 died at age 21 months from bronchopneumonia.[37] He survived the neonatal period without respiratory or feeding difficulties and had normal intelligence and language acquisition, but he could never sit or stand unaided and never achieved anti-gravity head control. Although there was no confirmation of protein

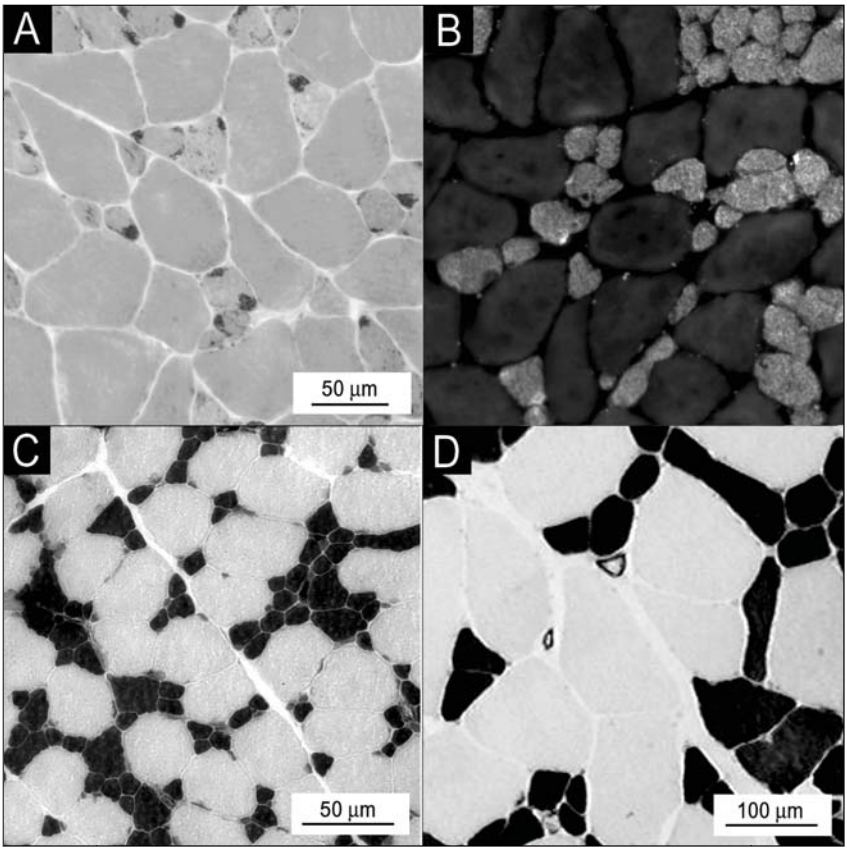

Figure 1. Histological abnormalities associated with *TPM3* mutations. A and B; Biceps muscle biopsy from an adult patient with the p.M9R *TPM3* mutation. A) Gomori trichrome. Abundant nemaline bodies are restricted to small (type 1 fibers) fibers. B) Immuno-fluorescence for slow myosin. Most small fibers are type 1 (stain positively) and most large fibers are type 2. C and D) biopsies of CFTD stained by ATPase (pH 4.3). Both biopsies show hypotrophy of most type 1 fibers (that appear dark) and hypertrophy of type 2 fibers (pale) as the only significant abnormality. C) quadriceps biopsy from a boy aged 2 years with the p.R245G mutation. D; forearm muscle biopsy from a man aged 56 years with the p.R168H mutation. Adapted with permission from Clarke NF, Kolski H, Dye DE et al. Mutations in *TPM3* are a common cause of congenital fiber type disproportion. Annals of Neurology 2008. ©2008 American Neurological Association.

levels, due to the proximal position of the nonsense mutation it is likely that this boy made no functional protein and that he displays the phenotype resulting when αTm_{slow} is lost completely from sarcomeres. The second reported patient with recessive disease was a compound heterozygote for two mutations affecting the last exon of the *TPM3* gene. This child was hypotonic at birth, walked at age 17 months and had progressive weakness from age 2, losing ambulation at age 6.[39] By age 10 he had moderate restrictive lung disease. Four children homozygous for a common mutation in the final exon of αTm_{slow} also had severe phenotypes.[40] All presented in early infancy and developed early scoliosis, marked axial weakness and chest deformities. Three of the four children walked in mid childhood, but with difficulty and two require non-invasive nocturnal ventilation in late childhood.

In comparison, to date, patients with dominant mutations have all walked and remained ambulant, although some were still young children at the time of reporting. The weakest muscle groups are typically those of the neck (flexors especially), proximal limbs, ankle dorsiflexors, scapular stabilisers, trunk and face.[34] Mild ptosis is often reported (Fig. 2) and asymmetry in strength is not uncommon.[34] This pattern is very similar to the congenital myopathy phenotype associated with *TPM2* mutations. Although there is a common pattern of muscle involvement the muscle groups most affected vary, some patients presenting with distal weakness (foot drop),[36] while others have had mainly proximal limb weakness.[34] The variability in the muscles most severely affected does not appear to be solely a mutation-specific phenomenon since the same mutation (p.Arg168His) has been associated with mainly proximal limb-girdle weakness in some patients[34] and significant distal weakness in others.[38] Similarly the overall severity of disease can vary, even within the same family.[34] Respiratory muscle weakness is a significant source of morbidity and mortality. Most adult patients require non-invasive nocturnal ventilatory support despite remaining ambulant and children as young as three years of age have presented in respiratory failure.[34]

Molecular Aspects of *TPM3* Mutations

The child homozygous for a nonsense mutation in exon 1, which is predicted to terminate protein production after 31 amino acids, most likely represents the clinical situation when αTm_{slow} is completely lost from sarcomeres in type 1 fibers, together with possible added effects of nemaline bodies.[37] Although this child had severe congenital hypotonia and weakness, this suggests that αTm_{slow} is not necessary for survival in the antenatal and neonatal periods, but that the deficiency becomes more problematic with growth. The fact that the parents of this child (carriers of one nonsense mutation) were asymptomatic also strongly suggests that haploinsufficiency for αTm_{slow} is not an important disease mechanism.

A second child with NM due to recessive mutations in *TPM3* represents a complex molecular situation.[39] This child had two different recessive mutations, the first of which changes the stop codon in the final exon of the skeletal muscle isoforms (9b also called 9sk) so that it codes for

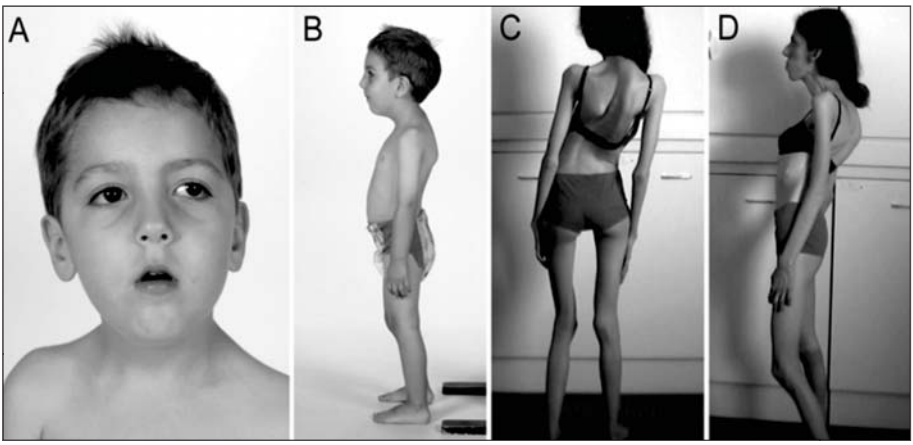

Figure 2. Patients with congenital fiber type disproportion due to mutations in *TPM3*. A and B) A boy at age 3.5 years with a heterozygous p.K169E *TPM3* mutation. Note the mild bilateral ptosis, facial weakness, increased lumbar lordosis and thoracic kyphosis. C and D) A patient at age 33 years with a heterozygous p.R168C *TPM3* mutation. There is generalized muscle wasting and thin habitus, kyphoscoliosis and neck contractures. Adapted with permission from Clarke NF, Kolski H, Dye DE et al. Mutations in *TPM3* are a common cause of congenital fiber type disproportion. Annals of Neurology 2008 (*in press*). © 2008 American Neurological Association

a serine residue. The predicted effect of this mutation is to add 57 further amino acids to the C-terminus of αTm_{slow}. An abnormally high molecular weight form of Tm was found on Western blot of patient muscle, suggesting that some mutant protein is produced and is present in skeletal muscle at low levels. The second mutation, which alters the splice acceptor site for the same final exon (9b), appeared to abolish splicing of this exon, removing the normal stop codon. Interestingly, some mRNA transcripts of αTm_{slow} were found that used an alternative final exon (9d, also called 8nm) that by chance, encodes completely normal αTm_{slow} protein. The full pattern of spliced αTm_{slow} transcripts was not fully assessed and it is unclear how much normal protein results from the aberrant splicing of exon 9d. The phenotype in this boy was less severe than that of the child with complete absence of αTm_{slow},[37] suggesting that some functional αTm_{slow} was present.

A homozygous single base pair deletion has been reported in two Turkish families with recessive disease.[40] The deletion affects the last nucleotide before the stop codon in exon 9b and induces a frame shift that removes the normal stop codon and is predicted to add 75 further amino acids to the C-terminus of αTm_{slow}. It is unknown whether this elongated αTm_{slow} is expressed and incorporated into sarcomeres or is a component of the numerous nemaline bodies that were present in type 1 fibers. It is interesting that all recessive mutations to date have involved exons only used in the αTm_{slow} isoform and that all nonmuscle isoforms transcribed from the gene are predicted to be unaffected.

Only one dominant missense *TPM3* mutation (p.M9R)[33] has been studied at the molecular level in any detail, but the potential effects of other mutations have been assessed using in-silico Tm models.[34] This modelling suggested there are two broad groups of mutations so far. Two mutations (p.M9R[33] and p.L100M [34])involve amino acids in position '*a*' of the alpha-helix, that is implicated in Tm dimerisation.[41] The second group of mutations alter charged residues with side chains that project from the dimer and these mutations may alter interactions with other proteins.[34] In fact four mutations alter two adjacent charged amino acids that are implicated in the principle actin binding site in αTm_{slow} (amino acids Arg168 and Lys169; Table 1).[34]

The effects of the p.M9R mutation have been studied in many ways. Two transgenic mouse lines have been generated[42] and mutant protein has been synthesised artificially.[43,44] A more detailed description of many of these studies is included in the chapters by Dr. Biljana Ilkovski, Dr. Mai-Anh Nguyen and Professor Edna Hardeman of this book. There is evidence that the p.M9R mutation alters αTm_{slow} function in many ways, that include a reduction in binding of F-actin[43,44] and tropomodulin[45] and result in weaker muscles at short sarcomere lengths[46] and an altered Ca^{2+} sensitivity of muscle contraction.[47] A consistent result in patient and transgenic mouse muscle was reduced levels of βTm, likely due an increased tendency of p.M9R mutant protein to form αα homodimers rather than αβ heterodimers.[28] The relative importance of these abnormalities to the pathogenesis of weakness in patients is uncertain. What is clear from these studies is that Tm biology is complex in-vivo and that the mechanism of weakness may involve alterations at many levels, including Tm dimerisation, ratios of Tm isoforms, thin filament stability and direct or indirect changes to the dynamics of the actin-myosin contractile unit.

Skeletal Muscle Disease Due to Mutations in the Troponins

Troponin T

Mutations in two isoforms of troponin T have been associated with human skeletal muscle disease; *TNNT1* that encodes the troponin T isoform for slow muscle fibers (troponin T_{slow}) and *TNNT3* that encodes the fast skeletal muscle isoform (troponin T_{fast}).

Mutation of *TNNT1* in Amish Nemaline Myopathy

Using linkage analysis, a homozygous nonsense mutation was identified as the cause of autosomal recessive NM in the Old-Order Amish population.[48] Due to extensive consanguinity and a high proportion of carriers, around 1 in 500 children are affected in this population but it has not been reported elsewhere. Affected children have tremors and mild proximal joint contractures at birth but relatively normal strength.[48] Progressive generalized weakness and progressive contractures

develop during infancy and children usually die by age 2 years from respiratory insufficiency, associated with pectus carinatum chest deformities. Histological analysis of several quadriceps muscle biopsies has shown consistent type 1 fiber hypotrophy, abundant nemaline bodies in type 1 fibers and regions of myofibrillar disarray on electron microscopy.[48] Amish NM is due to a single base change in exon 11 that introduces a premature stop codon at amino acid position 180, which is predicted to shorten the troponin T_{slow} protein by 83 amino acids. This truncated protein cannot be detected in patient muscle or in cultured muscle cells transfected with a mutant construct, even though mutant mRNA is abundant.[49,50] This provides strong evidence that the mutant protein, which lacks several key protein binding sites, cannot incorporate into the sarcomere and is rapidly degraded. Therefore the phenotype in children with Amish NM arises from a complete loss of troponin T_{slow} from slow muscle fibers. The relatively mild phenotype at birth is likely due to the presence of fetal and cardiac troponin T isoforms in skeletal muscle in fetal life, that are transcribed from the *TNNT2* and *TNNT3* genes and which most probably compensate for the absence of troponin T_{slow}.[49] During early infancy, the progressive weakness, amyotrophy and contractures likely arise due to downregulation of the developmental Troponin T isoforms with loss of their protective effects.[49]

Troponin T Fast (*TNNT3*)

Abnormalities in *TNNT3* have been linked to human disease in only one family to date. A heterozygous missense change (p.R63H) tracked with distal arthrogryposis type 2B in a mother and two children, following autosomal dominant inheritance.[51] While it is still possible that this is a chance association, several lines of evidence suggest that this change is disease causing. The missense change was not found in over 200 ethnically matched healthy individuals, the amino acid substitution affects a highly conserved arginine residue and a mutation in the homologous amino acid in the cardiac isoform of troponin T (*TNNT2*; R94L) has been linked to hypertrophic cardiomyopathy.[52] The identification of further patients with changes in *TNNT3* or studies that show that this mutation significantly alters troponin T function are awaited to confirm this association.

Troponin I Fast (*TNNI2*)

A locus for autosomal dominant distal arthrogryposis type 2B was established on chromosome 11p15.5 by linkage analysis in a large family.[53] Subsequently, two different mutations were found in the *TNNI2* gene that encodes the fast skeletal muscle isoform of troponin I in four out of 34 families with autosomal dominant distal arthrogryposis.[24] Since then, four further families with heterozygous mutations have been identified (Table 2), confirming that this is a recurrent, but relatively uncommon cause of this phenotype.[54-57] The severity of clinical features has varied, even within the same family. The most common presentation is the DA2B phenotype, in which mild facial features (such as down-slanting eyes, small mouth, prominent nasolabial folds), scoliosis and large joint contractures are seen as well as congenital contractures of the hands and feet.[24,54,57] Some family members have been more typical of the milder DA1 phenotype in which the main abnormalities are distal limb contractures.[55] Patients do not usually have muscle weakness on neurological examination.[56] Creatine kinase is often mildly elevated when measured.[56] Other established causes of the DA2B phenotype are the *TPM2* gene (see above), *TNNT3* (see above) and *MYH3*,[58] the most common of which is proving to be *MYH3*.[58]

Histological Aspects of *TNNI2* Mutations

Most patients with *TNNI2* mutations have not undergone muscle biopsy but tibialis anterior muscle biopsies were studied in detail from four adult patients from one family with the p.K175del mutation.[56] Common features were type 1 fiber predominance, type 1 fibers that were smaller than type 2 fibers, frequent internalised nuclei (mainly in type 2 fibers), scattered regenerating fibers and a mild increase in endomysial connective tissue. These changes and the mildly elevated CK suggest that this *TNNI2* mutation results in increased muscle fiber damage and repair in distal muscles. These abnormalities are most likely centred in type 2 muscle fibers where *TNNI2* is

Table 2. Mutations in the troponin genes associated with skeletal myopathy

Family No.	Mutation	Exon	Disease Assoc.	Inheritance Pattern	Refs.
TNNI2					
1, 2	c.521G > A p.R174Q	6	DA2B	AD	24
3, 4, 5	c.466C > T p.R156X	6		AD	24,54
6, 7	c.523_525delAAG p.K175del^	6	DA2B, DA1	AD	55,56
8	c.496_498delGAG p.E167del	6	DA2B	AD	57
TNNT3					
1	c.188G > A p.R63H	9	DA2B	AD	51
TNNT1					
1*	c.579G > T p.E180X	11	Nemaline	AR	48

DA: distal arthrogryposis; AD: autosomal dominant; AR: autosomal recessive.
^Identical mutation notated differently in two papers. *Many Amish families have been ascertained, all presumed to be descended from a common founder.[48]

expressed and the histological changes are reminiscent of a mild dystrophic process, even though progressive weakness is not a feature of this disorder.

Molecular Aspects of *TNNI2* Mutations

All mutations identified to date in *TNNI2* have clustered in the C-terminal region of the protein. One mutation (p.R156X) involves a premature stop codon that is predicted to remove 26 amino acids from the C-terminus. An assessment of this truncated protein in an in vitro muscle contracture model indicates that the mutant protein is most likely expressed and incorporates into sarcomeres in-vivo where it most likely has a dominant negative effect.[27] The three other mutations reported to date either result in the deletion of a single amino acid (p.K175del[55,56] and p.E167del[57]) or in the substitution of a conserved C-terminal amino acid (p.R174Q).[24] It is interesting that three of the four reported mutations have been found in two or more families, almost certainly arising de novo each time (Table 2).

Two reported mutations in *TNNI2* (p.R174Q and p.R156X) along with single mutations in *TNNT3* and *TPM2* that were also associated with distal arthrogryposis have been studied using several in vitro measures of sarcomeric dynamics.[27] A consistent pattern of abnormalities was found to account for the common DA phenotype. All four mutations increased the level of myosin ATPase activity across a wide range of Ca^{2+} concentrations even though the mutations are in three different genes. Therefore each mutation is predicted to increase force generation after membrane depolarisation and during the relaxation phase and result in a hypercontractile situation. The C-terminus of troponin I mediates the inhibitory effects of troponin I on actin-myosin interactions,[59] and so it is consistent that mutations in this region all increase myosin ATPase activity. Reduced joint movement during fetal life is considered an important factor in the development of congenital joint contractures and in DA, increased muscle contraction due to altered sarcomeric regulation has been proposed as the specific pathogenic mechanism.[27] The congenital nature of the contractures and their distal predominance suggests that distal muscle groups are particularly vulnerable to overactive muscle contraction during fetal development.[27] To date, the only result

at odds with this hypothesis is a study of the p.R133W mutation, which showed a reduction in specific force generation (see *TPM2*).[9]

Cofilin *(CFL2)* Is a Rare Cause of Nemaline Myopathy

The cofilins, together with actin depolymerisation factor (ADF), form a group of proteins that catalyse the depolymerisation of actin filaments in a pH-dependent manner.[60] The *CFL2* gene encodes the muscle isoform of cofilin and has been directly implicated in human disease in only one family to date. Due to its role in actin filament turnover in muscle, *CFL2* was consider a good candidate for nemaline myopathy and it was directly sequenced in 113 unrelated patients with nemaline myopathy of unknown genetic basis and 58 patients with other muscle pathologies.[61] A homozygous missense change was found in two sisters from a consanguineous family of Middle Eastern origin.[61] Both children had typical clinical features of a congenital myopathy that included congenital hypotonia, delayed early milestones, frequent falls and an inability to run. Walking was precarious in the older sister at age 16 years. Nemaline bodies were seen on muscle biopsy at age 2 years in one child, together with occasional minicore lesions and actin filament accumulations. A muscle biopsy of the older child at age 4 years showed nonspecific abnormalities.

Both sisters were homozygous for a c.103C > A change, which is predicted to substitute threonine in place of a highly conserved alanine 35 residue.[61] While the possibility remains that this is a chance association, there is good supportive evidence that this change is pathogenic. The associated LOD score in the family was 1.9, the change was not found in over 200 healthy individuals (almost half of whom were ethnically matched) and reduced cofilin 2 levels were found in patient muscle biopsies by Western blot and immunohistochemistry.[61] In addition the mutant protein tended to precipitate abnormally when expressed in bacterial cells, suggesting the mutation causes protein misfolding.[61] Molecular modelling has suggested the mutation may disrupt a beta sheet directly adjacent to the nuclear localisation signal.[61]

To confirm this disease association, it would be ideal to identify other patients with mutations in *CFL2*, but indications are this will be difficult. After screening a large patient cohort, Agrawal et al concluded that *CFL2* is a rare cause of nemaline myopathy, accounting for less than 1% of patients.[61]

Concluding Remarks and Future Directions

Skeletal myopathies due to mutations in tropomyosin and troponin remain rare disorders, although recent discovery of *TPM3* mutations in 20% of a CFTD cohort[34] suggests mutations may be more common in some patient groups than first anticipated. As we identify more patients with mutations in these genes, the phenotypes (for *TPM2* in particular) and the histological patterns that can be associated continue to broaden. The first mutations were identified in patients with distinct histological patterns, such as NM. Recent discoveries indicate that some mutations may only cause abnormalities in fiber sizes, such as CFTD. It may be that we will need to consider patients with 'nonspecific' histological changes to identify all patients with mutations. In addition, the recent discovery of mutation of *TPM2* in multiple pterygium syndrome[18] suggests we may not yet appreciate the full spectrum of clinical phenotypes that can arise.

This phenotypic complexity complicates the diagnosis process. There is a large clinical and histological overlap in the diseases caused by the tropomyosins and troponins and with other known causes of NM, CFTD and DA. None of these genes has a reliable clinical or histological sign, which is specific or always present. It is likely that using clues from both histological and clinical examinations will prove the best approach to identifying the causative gene. It is also important to remember that histological appearances can change with age and that rebiopsy may provide useful information.

It is also possible that other tropomyosin or troponin genes are causes of skeletal myopathies. The *TNNC1* and *TPM1* genes, which are recognized causes of cardiomyopathy are also expressed in type 1 and type 2 skeletal fibers, respectively. The β-myosin gene (*MYH7*) shows us this is possible[62] since some *MYH7* mutations are associated with just cardiac manifestations[63] while others

principally cause skeletal muscle weakness.[64] In addition, the *TNNI1* gene, which encodes the type 1 fiber isoform of troponin I, is yet to be linked to muscle disease.

Several interesting issues are raised by the patients who have homozygous recessive truncating mutations in either *TPM2* (βTm),[18] *TPM3* (αTm_{slow})[37] or *TNNT1* (troponin T_{slow}),[48] which all are likely to abolish protein production. The fact these patients survived through infancy (albeit with severe weakness) suggests that none of these proteins is essential for life and that other skeletal isoforms can compensate to maintain adequate respiratory function, for example. These patients may also represent the most severe phenotypes that arise due to mutation of these genes, unless some mutations disrupt muscle function more severely through dominant negative mechanisms. Overall it suggests that there may not be lethal congenital forms of these diseases.

Twelve out of 13 reported dominant *TPM2* and *TPM3* mutations are located in exons that are used in both muscle-specific and nonmuscle tropomyosin isoforms transcribed from these genes. Only the dominant p.M9R mutation breaks this pattern. Therefore many nonmuscle tropomyosin isoforms are also predicted to contain the missense mutations. In none of the patients were there nonmuscle pathologies that can be ascribed to the effects on nonmuscle isoforms, except perhaps deafness in the mother and daughter with DA2B associated with the p.R133W *TPM2* mutation.[19] The effects of these mutations on the function of the nonmuscle isoforms have not been studied but it is possible that differences in dimerisation patterns (nonmuscle isoforms preferentially homodimerise) or functional redundancy between Tm isoforms protects from disease.[65] In contrast, all of the recessive mutations in *TPM2* or *TPM3* are located in exons only used by the skeletal Tm isoforms (exons 1a, 6b and 9a).

In the last five years there has been enormous progress in identifying the genetic basis of various forms of congenital myopathy but even in the most well characterised subtypes, such as nemaline myopathy, many patients remain undiagnosed at a genetic level. For nemaline myopathy, the formidable size of nebulin is hampering efforts to exclude this in many families, but the finding of *CFL2* mutations in one family suggests there may be many further genetic causes to discover and that some of these may be very rare. It is likely the search for new causes of congenital myopathy will keep us occupied for many years.

The accumulation of information on histological changes and clinical phenotypes, together with basic research into the properties of different mutations is beginning to shed light on the pathophysiological processes that underlie the muscle weakness, contractures and histological abnormalities. It is vital we understand these processes well for this will enable us to devise sound therapeutic strategies to overcome the muscle dysfunction, so that patients can lead longer, stronger lives.

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Chapter 5

Investigations into the Pathobiology of Thin-Filament Myopathies

Biljana Ilkovski*

Abstract

Mutations in actin and tropomyosin, identified in patients with myopathic disease have been used in tissue culture models and functional studies with a view to understand how these mutations impact on skeletal muscle structure and function and result in muscle weakness. The likely mode of pathogenesis in these disorders is via a dominant negative effect i.e., the production of 'poison' proteins that interfere with the normal function of the native protein. Tissue culture models and in vitro binding studies highlight the defects of different actin mutants including abnormal folding, aggregation and altered polymerization which would likely impact on skeletal muscle structure and function. The most widely studied mutation in tropomyosin is the M9R substitution identified in a large Australian family with nemaline myopathy. The M9R mutant protein has a reduced affinity for actin, does not bind to tropomodulin in a model peptide and results in reduced sensitivity of isometric force to activating calcium in cardiac myocytes. The pathological consequences of mutations identified in troponin, nebulin, and cofilin are also discussed. Although mutations in α-actinin have not been associated with NM, tissue culture models using tagged constructs of different regions of the α-actinin gene suggest that this protein plays a role in nemaline body formation.

Introduction

Since the discovery of the first disease-causing mutation in *TPM3* identified in nemaline myopathy (NM) over 10 years ago[1] there have been significant advances in the identification of genes that cause NM. Mutations in nebulin are thought to be the most common cause of NM,[2] however, sequencing of this gene has proven to be difficult due to its large size and the high number of repetitive regions. The majority of mutations have been identified in the α-skeletal actin gene (*ACTA1*) and represent the second most common cause of NM, accounting for ~20% of all cases.[3-5] Since the discovery of the different mutations that cause NM, various attempts have been made to elucidate the functional roles of the mutant isoforms and the effects they have on skeletal muscle structure and function. Various tissue culture models, functional assays, recombinant proteins and synthetic peptides have been developed to aid in our understanding of how these mutant proteins impact on muscle and cause weakness.

Actin

Mutations in the muscle α-skeletal actin gene (*ACTA1*) are associated with a number of different pathological phenotypes. The majority of mutations in *ACTA1* have been identified in patients with nemaline myopathy (NM) with characteristic nemaline bodies.[4] Some NM patients display pathologi-

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cal variants within their muscle including intranuclear rods and/or filamentous actin accumulations.[4] Recently, *ACTA1* mutations have been identified in two additional histopathological phenotypes including central core myopathy[6] and congenital fibre type disproportion (CFTD).[7]

Although we do not entirely know how the various actin mutations cause muscle weakness and lead to the different pathologies observed in patient muscle, a number of recent studies have provided important insights into the pathogenesis of actin-related myopathies. Tissue culture and functional models have been developed to study a range of different actin mutations and have assisted in our understanding of the behaviour of mutant actin protein and how it leads to disease. Cell biology studies have examined the effects of mutant actins on protein folding, aggregation and polymerization and binding interactions to other proteins.

Actin is one of the most widely studied proteins and is responsible for cell movement, intracellular transport and contractile force. It is the major protein component of the thin filament and is involved in muscle contraction. The discovery of the crystal structure of actin[8] enabled the development of a molecular model, which has been useful for making predictions about the structure of actin in the presence of disease-causing mutations. Using a molecular modeling approach, Sparrow et al[4] attempted to predict the structural and functional consequences of 69 *ACTA1* mutations identified in myopathic patients, to gain insight into the molecular effects of the different *ACTA1* mutations and how they interfere with the normal function of actin. The authors predicted that actin mutations that cause NM affect the stability or conformation of actin which interferes with actin-actin contacts or interactions with other actin-binding proteins.[4] Hence, NM actin mutations are likely to differentially affect specific actin-binding interactions and they may also invoke distinct structural changes within the actin molecule or filament.[4] Actin mutations that result in accumulations of filamentous actin are all located in close proximity to the nucleotide binding cleft of the actin molecule, or are postulated to affect the cleft and are predicted to affect nucleotide binding and hinge flexibility. On the other hand, mutations that result in intranuclear rods are likely to affect nucleotide binding and exchange.[4]

Studies so far suggest a dominant negative mode of disease pathogenesis in dominant actin-related myopathies. Using 2D gel electrophoresis, Ilkovski et al[9] detected mutant actin protein in whole muscle lysates extracted from skeletal muscle biopsies from two severe NM patients harbouring R183G and E72K *ACTA1* mutations. In both cases the mutant protein was expressed at similar levels to wild-type actin, as would be expected in heterozygotes. Extraction of muscle into soluble (actin monomers and short actin filaments) and insoluble (cytoskeleton and sarcomeric apparatus) protein pools using the detergent triton–X illustrated that the mutant protein was present in appreciable quantities in the insoluble pool, suggesting that the mutant actin isoforms incorporate into the sarcomeric thin filament. Subsequent studies in a number of different patients provide further support for a dominant negative effect in actin-related myopathy. D'Amico et al[10] used the same experimental approach as Ilkovski et al[9] to determine the relative levels of mutant and wild-type actin in skeletal muscle preparations from a patient with hypertrophic cardiomyopathy and NM associated with a K336E *ACTA1* mutation. The mutant protein was detected in myofibrillar extracts at a level of ~30% of wild type actin, suggesting incorporation into the sarcomere.[10] In addition, mutant D292V actin protein was identified to comprise ~50% of the actin in skeletal muscle in a CFTD patient.[11]

Mass spectrometry has recently been used to detect the relative amounts of mutant and wild-type polypeptides in cases where the mutation does not result in an amino acid charge change. Clarke et al[11] used mass spectrometry to confirm the mutant load in the D292V patient muscle and showed in a CFTD patient with a P332S *ACTA1* mutation that the mutant protein was expressed at 25-30% of total actin protein in the filamentous pool. Wallefeld et al[12] used Western blotting to detect a mutant isoform from a severe NM patient with a heterozygous *ACTA1* stop codon mutation (X376TyrX*47) that is 47 amino acids larger and expressed at lower levels than wild type actin. Marston et al[13] isolated mutant messenger RNA from skeletal muscle from a NM patient with a M132V *ACTA1* mutation and identified that 61% of the *ACTA1* transcript is mutant by restriction enzyme analysis. The lower than expected amounts of mutant protein detected in the

patients harbouring the K336E, P332S and X376TyrX*47 actin mutations may be attributed to degradation of the mutant actin, alterations in transcription or translation, or incorporation of the mutant actin into insoluble structures.

Although there is strong evidence for a dominant negative mode of disease pathogenesis, the possibility of haploinsufficency (shortage of normal actin protein) could not be excluded, especially in the case of mutant actin isoforms detected in NM patients, which could incorporate into nemaline bodies or filamentous aggregates of actin. Recently, however 7 severe NM patients from 6 different families were found to be homozygous for 3 different actin null mutations.[14] All of the patients are consanguineous and the parents who are heterozygous for the actin null mutation are unaffected, suggesting that 50% of functional actin is sufficient for normal actin function. This is supported by the α-skeletal actin knock-out mouse. Transgenic mice that are heterozygous for *ACTA1* (+/−) do not display any muscle weakness, whilst homozygous mice (−/−) have reduced muscle strength and die within 9 days after birth, apparently from starvation.[15] These results also suggest that haploinsufficiency is not the cause of dominant actin-related myopathies.

Several investigators have used in vitro cell culture models to examine the ability of mutant actin isoforms to incorporate into the actin cytoskeleton in both muscle and nonmuscle cells. Ilkovski et al[9] generated C-terminal enhanced green fluorescent protein (EGFP) tagged constructs of 10 different actin mutations for expression in C2C12 myoblasts. Expression of WT-actin$_{EGFP}$ resulted in incorporation into stress fibres indicating normal localization of actin into the cytoskeleton. Expression of several mutant actin isoforms also localized to stress fibres but, the most striking feature was the formation of abnormal actin aggregates. V163L-actin$_{EGFP}$ and V163M-actin$_{EGFP}$, modeled on patients with intranuclear rod myopathy, produced highly fluorescent intranuclear aggregates reminiscent of those observed in the patients' muscle. I136M-actin$_{EGFP}$ produced exclusively cytoplasmic aggregates. This mutation is based on a patient with NM who had only cytoplasmic rods on muscle biopsy. The intranuclear aggregates labeled with phalloidin, indicating that they contain filamentous actin, but the cytoplasmic aggregates produced by I136M-actin$_{EGFP}$ did not label suggesting that these aggregates may not contain filamentous actin or that they are folded in such a way to prevent phalloidin binding.[9]

Costa et al[16] also used an in vitro cell model to express several mutant actin isoforms using N-terminal myc tagged constructs into fibroblast cells and observed similar results to Ilkovski et al.[9] They detected abnormal aggregates in cells transfected with V163L and H40Y that labeled with phallodin , but other constructs modeled on patients with NM resulted in cytoplasmic aggregates that did not label with phalloidin. The formation of mutant actin C-terminal EGFP tagged aggregated proteins has recently been described in two independent studies. Expression of the *ACTA1* stop codon mutation X376TyrX*47 produced abnormal aggregates in C2C12 myoblasts and differentiated myotubes.[12] The actin also localized to stress fibres and sarcomeres suggesting that it is capable of normal actin function. However, transfection of the mutant construct resulted in greater cell death compared to WT-actin$_{EGFP}$ suggesting that high levels of this mutant actin are cytotoxic and may relate to the muscle weakness in the patients.[12] Expression of C-terminal EGFP tagged D292V actin construct in C2C12 cells also resulted in abnormal cytoplasmic actin aggregates.[11] This was an unexpected finding as this mutation is modeled on a patient with CFTD who did not show rods upon muscle biopsy and has relatively normal sarcomeric structure. The majority of the D292V-actin$_{EGFP}$ induced aggregates were highly fluorescent and similar to those observed with I136M-actin$_{EGFP}$ (modeled on an NM patient with cytoplasmic aggregates) by Ilkovski et al.[9] However, some aggregates had less intense non-uniform fluorescence, which was unique to the D292V mutation. The formation of abnormal aggregates in cultured cells likely represents abnormal protein folding and may not be indicative of nemaline body formation.

A number of actin mutants expressed in cultured cells behaved like wild type actin ie incorporated into stress fibres and/or sarcomeres and did not induce abnormal actin aggregates.[9,11,16] This suggests that either the effects observed in cultured cells are mutation specific such that certain mutations have a greater propensity to aggregate compared to others, or that myoblasts are not the ideal system to use as they do not represent the human disease. Hence expression of mutant actin

isoforms in myotubes may represent a better system. Bathe et al[17] recently explored the ability of mutant actin to contribute to the cytoskeleton in differentiated C2C12 cells. They generated 7 actin-myopathy related mutations using C-terminal EGFP tagged constructs and expressed them in differentiated C2C12 myotubes. Generally, actin mutations linked to NM resulted in abnormal aggregates or diffuse staining in myoblasts but incorporated well into sarcomeres in differentiated myotubes supporting the notion of a dominant negative effect through a functional defect rather than mislocalisation. Mutations linked to intranuclear rod mopathy, V163L and H40Y resulted in intranuclear aggregates in myoblasts and upon differentiation were found less frequently in the case of V163L and not at all in H40Y. The majority of the mutant actin had incorporated into stress fibres. The authors attempted to make comparisons between rods produced in cell culture and those identified in patient muscle. Staining of rods created in cell culture did not label with sarcomeric α-actinin whilst rods identified in patients typically label with α-actinin. The authors suggest that this may be due to antibody inaccessibility or that the different kinds of rods represent a specific molecular defect and are not related to rods observed in the patient muscle. This is supported by human studies that show a lack of rods in some muscles in patients with NM,[9,18,19] suggesting that rods may not entirely contribute to muscle weakness. The authors acknowledge that the effects observed in tissue culture could be due to differential expression levels and that an endogenous actin promoter would be a better system to use for the expression of mutant actin isoforms.[17]

Actin exists as a polymer in the form of filamentous actin (F-actin) within the sarcomere. Filamentous actin is formed by a complex process of polymerisation, which involves the addition of actin monomers to the filament. The actin monomer is a globular protein (G-actin) and it's addition to the filament occurs through a highly ATP-dependent process involving a number of key regulatory proteins such as cofilin and profilin. Alterations in actin polymerisation have been explored in patients with mutations in *ACTA1* using in vitro studies.

Ilkovski et al[9] expressed C-terminal FLAG tagged actin mutant constructs in C2C12 myoblasts and differentiated myotubes and examined the ability of the mutant actin isoforms to incorporate into the actin cytoskeleton and the ability to polymerise with endogenous actin. Two mutant actin isoforms showed significant differences in their capacity to polymerise with endogenous actin isoforms compared to wild type actin. The V163L-actin$_{FLAG}$ mutant isoform, based on a patient with intranuclear rod myopathy, had a greater propensity to incorporate into filaments in myoblasts but not myotubes compared to WT-actin$_{FLAG}$, suggesting enhanced polymerisation with cytoplasmic isoforms present in myoblasts. Whereas R183G-actin$_{FLAG}$, modeled on a patient with severe NM, had a reduced ability to incorporate into filaments in both myoblasts and myotubes compared to wild type-actin$_{FLAG}$. Impaired polymerisation of actin mutants was also explored by Marston et al[13] who showed increased levels of short actin filaments following in vitro polymerisation of skeletal actin purified from muscle from a NM patient with a M132V *ACTA1* mutation. Costa et al[16] used a panel of different actin mutations to explore alterations in polymerisation. Using an in vitro translation assay the authors showed that several actin mutants had a reduced capacity to copolymerise with wild type actin when compared to the native protein.

Costa et al[16] also examined the ability of a range of different mutants to fold correctly using a reticulocyte assay which endogenously contains cyclase-associate protein (CAP) and the actin folding machinery: cytosolic chaperonin (CCT) and prefoldin. Proteins that remain bound to CCT and prefoldin indicate impaired folding. Two recessive *ACTA1* mutations, L94P and E259V, did not fold properly or release from CCT or prefoldin, suggesting that these patients produce nonfunctional actin. A number or mutants showed increased binding to CAP. CAP has a high affinity for the nucleotide free state of actin and hence this result suggests that these mutant proteins have unstable folding. These mutations are located near the nucleotide-binding cleft of actin and are predicted to affect nucleotide binding. The majority of the actin mutants examined folded properly and bound normally to the actin monomer binding proteins DNaseI, Vitamin D binding protein (DBP), thymosin-β4 and profilin. Ilkovski et al[9] also showed that the expressed mutant I357L actin isoform had an altered mobility on native PAGE gels compared to wild type actin, suggesting altered conformation and a less compact tertiary structure.

In vitro motility assays have also been used to examine the effects of mutant actin isoforms on skeletal muscle function. Marston et al,[13] examined the movement over heavy meromyosin of thin filaments reconstituted from actin extracted from a patient with a M132V *ACTA1* mutation. Copolymerisation of M132V with wild type actin resulted in altered regulatory properties when thin filaments were reconstituted with tropomyosin and troponin. Notably, the mutant isoform resulted in faster sliding of thin filaments at pCa5 compared to wild type actin. The authors suggest this effect may be due to a structural change or a change in the dynamics of actin. In contrast, the K336E mutant actin isoform identified in a patient with hypertrophic cardiomyopathy and NM resulted in a slower sliding speed compared to wild type actin for pure actin filaments and calcium regulated thin filaments reconstituted with skeletal muscle troponin and tropomyosin.[10] Imposition of an internal load by the addition of α-actinin resulted in an apparent increase in relative force of M132V mutant actin compared to wild type actin.[13] This may reflect a lower affinity of mutant actin for α-actinin rather than a difference in motor properties and may explain the formation of nemaline bodies observed in the patient.[13] α-Actinin binding affinity was explored in an assay with the mutant K336E actin isoform. This mutant actin isoform showed a 10-fold reduction in binding affinity for α-actinin compared to wild type actin.[10] The authors hypothesized that this may result in lower force generation which may contribute to the muscle weakness observed in the patients.

Recently, Clarke et al[11] examined the structural and functional effects of *ACTA1* mutations identified in three patients with CFTD and compared these mutations with mutations associated with NM. There was no clear difference between *ACTA1* mutations associated with CFTD and NM in the ability of mutant actin to aggregate or polymerize in vitro. However, in vitro motility studies showed a significant reduction in the motility of D292V F-actin containing filaments compared to wild type actin in the presence of tropomyosin. Upon addition of the troponin complex the loss of motility persisted indicating that it cannot restore normal tropomyosin function. These results indicate that the position of tropomyosin is altered relative to the actin polymer in the presence of mutant D292V actin which blocks normal actin-myosin interactions.[11]

Most recently, Domazetovska et al[20] examined the mechanisms underlying muscle weakness in a three generation family with intranuclear rod myopathy harbouring a V163M mutation in *ACTA1*. Using mass spectroscopy, the mutant V163M protein was found at lower levels (~3-20%) than wild-type α-skeletal actin and detailed examination of muscle from the patients showed normal sarcomeric structure in >90% of muscle fibres. These results may help to explain the mild phenotype observed in the patients. Introduction of the V163M mutation in a drosopohila model showed gross sarcomeric disorganisation. In the indirect flight muscle of drosophila, the protein turnover is low, there are no satellite cells and only one actin isoform is expressed. However, in the patient human muscle features of regeneration were observed and the presence of other actin isoforms, which may have a compensatory role to ameliorate the pathological phenotype in the patients.[20] Domazetovska et al, also co-expressed the V163M mutant with wild-type α−skeletal actin in C2C12 myobasts and showed that wild type α−skeletal actin accumulates within intranuclear aggregates and its localisation within cytoplasmic microfilaments is reduced. This suggests that the V163M mutation can disrupt the actin microfilament system of cells.

The mechanisms leading to intranuclear aggregate formation were examined in a tissue culture model using the V163M mutation. Domazetovska et al,[21] used live cell imaging of muscle and nonmuscle cells transfected with a V163M-$_{EGFP}$ tagged construct and showed that nuclear aggregates of actin form within the nuclear compartment and are highly motile and dynamic structures. α-Actinin was found to label 'star-like' intranuclear aggregates but not needle-shaped aggregates suggesting that α-actinin cross-links intranuclear aggregates and dictates their morphology. Domazetovska et al,[21] also showed that under conditions of cell stress (using ATP depletion and microfilament disruptors latrunculin and cytochalasin D), wild-type α-skeletal actin formed intranuclear aggregates similar to those observed with V163M, suggesting a common mechanism of intranuclear rod formation. Cells containing intranuclear aggregates have a significantly reduced

mitotic index, suggesting that they impact on cell function and may contribute to the muscle weakness in patients with intranuclear rod myopathy.[21]

In summary, mutations in *ACTA1* are likely to result in a dominant negative mode of disease pathogenesis rather than haploinsufficiency. A number of functional studies using tissue culture models and in vitro binding studies highlight the defects of different actin mutants including abnormal folding, aggregation and altered polymerization which would likely impact on skeletal muscle structure and function. Tissue culture models have greatly assisted us in understanding the behaviour of different actin mutants. However, there are limitations to these studies and animal models will help us to better define the impact of these mutations on sarcomeric structure and muscle weakness.

Tropomyosin

The first disease-causing mutation in NM was identified over a decade ago in a large autosomal dominant Australian family.[1] The mutation substitutes an arginine for a methionine at position 9 in the α-tropomyosin$_{slow}$ gene (*TPM3)*. Since it's discovery there have been a wide range of investigations on this specific mutation including functional studies using tissue culture models and bacterially expressed proteins to determine the effect of the M9R mutation on skeletal muscle structure and function.

Moraczewska et al.[22] introduced the M9R mutation into rat striated fast α-tropomyosin cDNA and expressed it in *E. coli* cells. In vitro expression of mutant M9R protein resulted in a 30-100 fold reduction in the affinity for actin compared with wild-type tropomyosin. This could alter end-to-end interactions of mutant tropomyosin along the length of the thin filament, making assembly more difficult and resulting in less stable actin filaments. The weaker actin affinity could also be due to the lack of acetylation of the N-terminus in E. *coli*, since the N-terminus in eukaryotic cells is normally acetylated. The N-terminus of tropomyosin needs to be acetylated to bind effectively to actin in human muscle. To test this hypothesis, Moracazewska et al[22] introduced the mutation in a model polypeptide, TM Zip- a chemically synthesised tropomyosin protein containing the first 14 residues of rat α-tropomyosin$_{fast}$. Using this model peptide, the authors showed that the M9R mutation destabilised the tropomyosin coiled-coil which would be expected to impair end-to-end association between tropomyosins on the thin filament. It is hypothesised that the mutation may alter thin filament assembly in muscle due to decreased tropomyosin-actin binding and altered binding of the N-terminus of tropomyosin to tropomodulin at the pointed end of the filament.[22]

Moraczewska et al[22] also examined the effect of the M9R mutation on calcium sensitivity and showed that it was normal, although the activation of actomyosin S1ATPase was reduced even at much higher concentrations of tropomyosin. The lower activation in the presence of calcium suggests impairment in switching the thin filament to an open force producing state, which may reflect the reduced affinity for actin or relate to dissociation of tropomyosin and may explain the muscle weakness in the NM patients with the M9R mutation.[22]

Akkari and colleagues[23] also explored the binding affinity of tropomyosin to actin and showed similar results to Moracewska et al.[22] They used a baculovirus expression vector system (BEVS) to introduce human α-tropomyosin$_{slow}$ into insect cells as it contains the necessary machinery to permit posttranslational modifications of proteins such as acetylation. Tropomyosin requires an acetylated N-terminus for proper function. The mutant tropomyosin protein produced using BEVS was as biologically active as wild-type tropomyosin. Standard Western blotting illustrated that the protein was full-length and isoelectric focusing confirmed the presence of the mutant isoform. Using in vitro binding methods, the authors showed that the acetylated form of M9R mutant tropomyosin was at least 100 times less effective at binding actin than wild-type tropomyosin.[23]

Greenfield and colleagues[24] employed circular dichroism techniques to study the interaction of two α-tropomyosin$_{fast}$ chimeric proteins AcTM 1a ZIP (lacking the acetyl group at the N terminus) and AcTM1bZip (chemically synthesized with an acetylated N terminal) with the actin pointed end capping protein tropomodulin. The authors showed that tropomyosin requires an

intact N-terminus to interact with tropmodulin.[24] Using this in vitro system, the M9R mutation located in the N-terminal region was found to destroy the binding to tropomodulin, suggesting that the mutation could affect filament length and sarcomere assembly.

The M9R mutation has also been introduced into the human fast skeletal α-tropomyosin gene using a C-terminal FLAG tag adenoviral vector. Introduction of this construct into differentiated rat adult cardiac myocytes resulted in expression of the mutant protein and correct assembly into sarcomeres, suggesting that it exerts a dominant negative effect rather than haploinsufficiency.[25] There was no formation of rods in this model, suggesting that nemaline bodies are a secondary phenomena in the pathogenesis of NM. This is supported by human studies in NM patients in whom rods were not identified in some muscles.[9,18,19,26] Cardiac myocytes possessing the mutant tropomyosin contract normally and contract even at reduced calcium concentrations.[25] The primary defect detected is a decrease in the sensitivity of contraction to activating Ca^{2+} suggesting that the M9R mutation disrupts the signalling pathway from the Ca^{2+} binding troponin subunit and alters Ca^{2+}-activated force production. On the basis of these findings it was hypothesised that the observed decrease in myofilament sensitivity to activating Ca^{2+} would directly contribute to reduced force production and the muscle weakness seen in NM.

Further studies using the transgenic mouse model of the M9R mutation in $\alpha\text{-tropomyosin}_{slow}$ have examined the ratio of tropomyosin isoforms and the impact on tropomyosin dimer formation.[27] This study demonstrates that the onset of the disease coincided with a drop in β-tropomyosin levels and an increase in the expression of $\alpha\text{-tropomyosin}_{slow}$ in transgenic mice. The degree of reduction of β-tropomyosin did not correlate with the number of fibres containing rods. This observation is consistent with observations in a patient with the M9R mutation where the levels of $\alpha\text{-tropomyosin}_{slow}$ were markedly increased relatively to β-tropomyosin.[27] In all transgenic mouse muscles examined, the contribution of β-tropomyosin to the total tropomyosin protein pool was reduced relative to wild-type mice even though the expression of endogenous skeletal tropomyosin mRNA was not affected by the M9R mutation. Hence, it was hypothesised that the M9R mutation could interfere with the association of tropomyosin dimers. The authors examined tropomyosin dimer formation using either recombinant proteins or muscle extracts and found that the M9R mutant protein had a reduced preference for β-tropomyosin. The M9R mutation altered the preference of $\alpha\text{-tropomyosin}_{slow}$ from the formation of the preferred α/β heterodimers to α/α dimers. The change from α/β heterodimers to α/α or β/β homodimers would likely change the affinity of tropomyosin for other thin filament regulatory proteins and may contribute to the reduction in force production and muscle weakness observed in NM.[27]

In summary, cell biology studies illustrate that the introduction of the M9R mutation in $\alpha\text{-tropomyosin}_{slow}$ results in a number of functional defects that may contribute to the sarcomeric disruption and muscle weakness observed in the patients. The M9R mutant protein has reduced affinity for F-actin by up to 100-fold[22,23] and reduces the formation of preferred α/β heterodimers in favour of α/α homodimers.[27] Using a synthesized polypeptide, the M9R mutation can cause local destabilization of the coiled coil[25] and circular dichroisim studies have shown that the binding of an N-terminal tropomyosin fragment containing the M9R to tropomodulin is abolished.[24] Whilst substitution of M9R within the human $\alpha\text{-tropomyosin}_{fast}$ gene resulted in reduced sensitivity of isometric force production to activating calcium, in adenovirally transduced rat cardiac myocytes.[25]

Nebulin

Mutations in nebulin have been identified in patients with NM[28-31] and more recently in patients with a distal myopathy.[32] Nebulin mutations are thought to be the most common cause of NM, but routine sequencing of this gene has been difficult because of its large size and repetitive regions.[2] Protein analysis using a range of different nebulin antibodies detected abnormal or absent staining in a subset of patients with mutations in nebulin.[33,34] Western blotting using protein muscle lysates from patients with nebulin mutations has detected truncated nebulin protein.[34] In some cases, there is absent nebulin protein suggesting degradation of the mutant protein. Abnormal nebulin

expression was also observed in some NM patients with proven *ACTA1* mutations,[9] suggesting that protein analysis may not be a reliable diagnostic tool for detecting primary abnormalities in nebulin. The reason for the abnormal nebulin expression in *ACTA1* NM patients is unclear but may reflect instability and increased turnover of the thin filament, whereby synthesis of the extremely large nebulin protein is unable to keep up with turnover.[9]A nebulin deficient mouse model has been generated by two independent research groups.[35,36] Both groups showed that the mice displayed features observed in patients with NM, namely muscle weakness and the presence of rods within muscle fibres. The mice had shorter thin filaments and altered muscle contractility.[35,36] Further analysis of the animal models and tissue culture studies would be useful in providing further insights into the functional consequences of the different nebulin mutations.

Troponin

The troponin complex consists of troponin C (calcium binding), troponin T (tropomyosin binding) and troponin I (inhibitory) and is responsible for regulating calcium mediated muscle contraction. A homozygous nonsense mutation (E189X) has been identified in the slow skeletal muscle troponin T gene (*TNNT1*) in NM patients of Amish descent.[37] The mutation results in a stop codon and protein analysis from patient muscle showed a complete absence of troponin.[38] The absence of mutant protein is also a feature of a subset of NM patients with homozygous mutations in *ACTA1*[14] and *TPM3*.[39] All of these patients have the severe-lethal form of NM and rods within their muscle fibres. Although the pathobiology is not certain, rod formation in these patients may be due to altered stoichiometry of the other sarcomeric proteins. The absent protein may be partially replaced by another similar isoform, for example, cardiac actin in the case of skeletal actin null patients.[14] Wang et al[40] further examined the expression of mutant troponin T in Amish NM patients. Troponin T mRNA was detected in patient muscle, suggesting normal transcription and RNA splicing. Introduction of a mutant troponin T cDNA resulted in expression of the truncated protein in *E. coli* but not in C2C12 myoblasts or myotubes. Troponin T mRNA was produced in C2C12 cells indicating muscle cell-specific degradation of troponin T when it is not integrated into myofilaments.[40] This result suggests that rapid degradation of the truncated protein rather than instability of the nonsense mRNA is responsible for the absence of truncated slow troponin T protein in patient muscle.[40] Using a solid phase protein binding assay Wang et al[40] showed that truncated troponin T did not bind to tropomyosin, consistent with the loss of one or two tropomyosin binding sites.

α-Actinin 2

Although nemaline myopthy is generally considered to be a disease of the thin filament, the Z-line associated protein α-actinin 2, has been suggested as a possible candidate for nemaline myopathy, because the rods are thought to be derived from extensions of the sarcomeric Z-disk of muscle, appear to be in structural continuity with the Z-disk and are of similar electron density. α-Actinin 2 cross-links actin filaments at the Z-line and α-actinin 2 antibodies positively stain the rods in the majority of NM patients. The Z-line anchors the thin filaments and maintains structural support and tension during muscle contraction. Hence, rod formation may be due to an inability of actin to be cross-linked by α-actinin 2 at the Z-line which may affect interactions with other Z-line associated proteins. Considering α-actinin 2's fundamental role in the maintenance of muscle structure integrity and possible role in rod formation, the *ACTN2* gene was screened for mutations in ~40 patients with NM but no disease-causing mutations were identified (Kathryn North, Alan Beggs, unpublished data). Nevertheless, a number of different tissue culture models of α-actinin 2 have been developed which provide some insight into the pathogenesis of rod formation.

In 1992, Schultheiss et al[41] showed that chick myotubes transfected with a truncated α-actinin construct resulted in the formation nemaline-like bodies and hypertrophied Z bands. Subsequent studies were performed to identify the region in α-actinin 2 responsible for these pathological phenotypes. In 1998, Lin et al[42] expressed four different tagged sarcomeric α-actinin peptides in embryonic day 11 chick skeletal myoblast cells. The constructs included (1) full-length sarcomeric

α-actinin, (2) an N-terminal deletion that removed the actin-binding site only, (3) a peptide that consisted of the actin-binding site only and (4) an N-terminal deletion that removed the EF-hands and titin binding domains. All peptides incorporated into normal sarcomeric Z bands in myotubes. The peptide lacking the EF-hands region and the titin-binding domains resulted in the formation of nemaline-like bodies and disruption of thick and thin filaments. The relative proportion of thin filaments compared to thick filaments was reduced. It was hypothesised that the EF-hands or the C-terminal titin-binding domain inhibits the longitudinal distribution of sarcomeric α-actinin along the thin filaments. However, subsequent studies using a peptide lacking the titin binding domain alone did not result in nemaline bodies, suggesting that it is the absence of the EF-hands region and not the titin-binding domain that induces the nemaline-like bodies (unpublished observations of Hijikata et al, 1998).

Cofilin

Cofilin is a key regulator of actin dynamics. It is directly involved in actin polymerization by binding to actin monomers and is activated by dephosphorylation. Recently, Agrawal et al[43] identified an A35T homozygous mutation in the muscle specific isoform cofilin-2 in two siblings with NM and minicores. This is the first gene identified that causes NM and is involved in the regulation of actin dynamics, rather than encoding a protein component of the thin filament. Reduced levels of cofilin were detected in the patient muscle by immunohistochemistry, hence representing a useful diagnostic tool. Using 2D gel electrophoresis and Western blotting analysis, the authors detected reduced levels of cofilin 2 and an absence of phosphorylated cofilin isoforms in the patient muscle compared to control. The authors suggest that the reduced levels of cofilin 2 in the patient muscle is likely due to reduced protein stability and/or some other post-transcriptional mechanisms based on quantitative rt-PCR of cofilin mRNA.[43]

A number of different cell biology approaches were used to examine the functional effect of the mutant A35T cofilin protein. There was no difference in localization of WT or mutant cofilin in C2C12 myoblasts transfected with either an EGFP or V5-tagged construct. However, after several days of differentiation both nuclear and cytoplasmic rod structures were apparent. Expression of A35T His/V5 tagged mutant cofilin in *E. coli* cells resulted in a less stable and less soluble protein compared to wild type. The mutant protein is likely to be misfolded or its tertiary structure be destabilised, leading to degradation of the protein in vivo, which may explain the reduced levels of cofilin observed in the patient muscle. This in turn may result in reduced depolymerization of actin filaments which may accumulate in rods.[43]

Conclusions and Future Directions

The characterization of various mutant thin filament proteins that cause myopathy has provided us with insights into how the different mutations affect muscle structure and function and ultimately result in muscle weakness. The molecular and cellular effects observed for *ACTA1* mutations, namely altered folding, impaired polymeristaion and aggregate formation is likely to be mutation specific, suggesting that they affect different functional domains of the actin molecule. This may account for the variability in severity observed in patients with actin-related disorders. The M9R mutation in *TPM3* likely affects binding to actin and tropomodulin and force production to activating calcium. These factors likely contribute to the muscle weakness observed in the patients. The mechanisms of rod formation are still largely unknown and studies performed in tissue culture will hopefully shed light on this in the future. A combination of functional models, tissue culture studies and animal models will be needed to further determine the causes of muscle weakness due to the various mutations and to devise future therapies for thin filament-related disorders.

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Chapter 6

Mouse Models for Thin Filament Disease

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Abstract

Thin filament integrity is important for the ordered structure and function of skeletal muscles. Mutations within genes that encode thin filament and thin filament-associated proteins can cause muscle disruption, fiber atrophy and alter fiber type composition, leading to muscle weakness. Analyses of patient biopsy samples and tissue culture systems provide rapid methods for studying disease-causing mutations. However, there are limitations to these techniques. Although time consuming, many laboratories are generating and utilizing animal models, in particular the mouse, to study the disease process of various myopathies. This chapter reviews the use of mouse models for thin filament diseases of skeletal muscle and in particular, concentrates on what has been achieved through the generation and characterization of transgenic and knock-in mouse models for the congenital thin filament disease nemaline myopathy. We will review potential therapies that have been trialled on the nemaline models, providing indications for future directions for the treatment of nemaline myopathy patients and muscle weakness in general.

Introduction

As described in the preceding chapters, mutations within genes that encode for the components of the thin filament or thin filament-interacting proteins, actin, tropomyosin, troponin, nebulin and cofilin, result in human skeletal muscle diseases. These include nemaline myopathy,[1-11] actin aggregate myopathy,[1] "cap" disease,[12] congenital core myopathy,[13] congenital fiber type disproportion,[14,15] distal arthrogryposis[16-18] and distal myopathy[19] as well as cardiomyopathies[3,20-28] when mutant protein isoforms are expressed in the heart (Table 1).

Analyses of patient biopsy samples and tissue culture systems provide valuable insights into myopathy-causing mutations. However, these approaches have limitations. Firstly, muscle biopsies only provide a snapshot of the state of the muscle at the time of sampling. It is difficult to extend these findings to investigate the aetiology of the disease process or the association between the development of muscle pathologies and muscle weakness. In addition, different muscles have distinct contractile and enzymatic characteristics and as well, biopsies from muscles such as the heart or diaphragm are difficult to obtain. Because of this, it is difficult to correlate muscle pathologies and disease severity. Tissue culture systems can be utilized to examine the expression of disease-causing mutant proteins and the potential development of a subset of pathologies. However, muscle cells in culture do not progress to mature muscle fibers and do not experience the influence of innervation and biomechanical load. Therefore, extrapolating findings in muscle culture to muscle in the body is limited. Another limitation that researchers are faced with is that many disease-causing mutations are rare, many patients carry sporadic mutations and thus lack other affected family members, making it difficult to reconfirm findings.

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The Sarcomere and Skeletal Muscle Disease, edited by Nigel G. Laing.

Table 1. Human skeletal muscle and cardiac myopathies caused by mutations in thin filament proteins and current mouse models

Gene Affected	Human Myopathy	Mouse Model
α-Skeletal actin	Actin myopathy[1]	
	Nemaline myopathy[1,2]	*ACTA1*(H40Y) transgenic and knock-in mouse lines. (Unpublished, Hardeman laboratory)
	Congenital fiber type disproportion[14,15]	
	Congenital core myopathy[13]	
	Nemaline myopathy with hypertrophic cardiomyopathy[3]	
	Skeletal actin null myopathy[4]	Skeletal actin knock-out mouse[56]
α-Cardiac actin	Familial hypertrophic cardiomyopathy[20]	
α-Tropomyosin slow	Nemaline myopathy[5,6]	*TPM3*(M9R) transgenic mouse[48]
β-Tropomyosin	Nemaline myopathy[7]	
	Distal arthrogryposis[16]	
	Cap disease[12]	
α-Tropomyosin fast	Familial hypertrophic cardiomyopathy[2]	FHX α-TM175[57] and FHC α-TM180[58] transgenic mouse lines.
	Dilated cardiomyopathy[21]	DCM α-TM54 transgenic mouse[59]
Skeletal troponin T fast	Nemaline myopathy[8]	
	Distal arthrogryposis[17]	
Skeletal troponin I fast	Distal arthrogryposis[18]	
Cardiac troponin I	Familial hypertrophic cardiomyopathy[22,23]	cTnI-G203S[60] and TnI(146Gly)[61] transgenic mouse lines
	Restricted cardiomyopathy[24]	
	Dilated cardiomyopathy[25]	
Cardiac troponin T	Familial hypertrophic cardiomyopathy[26]	
	Dilated cardiomyopathy[25,27]	
Cardiac troponin C	Familial hypertrophic cardiomyopathy[28]	
	Dilated cardiomyopathy[25]	
Nebulin	Nemaline myopathy[9,10]	Nebulin knock-out mouse lines[53,54]
	Distal myopathy[19]	
Cofilin	Nemaline myopathy[11]	

To address these limitations, animal models in particular mouse models have increasingly been developed to study the disease processes of various myopathies. These animal models can be utilized to investigate the effectiveness of potential therapies and therefore, address the lack of treatment that is currently available. Finding potential treatments is of interest to many since a large number of mutations in thin filament-coding genes result in severe, debilitating muscle weakness that affects the patient's quality of life and may lead to premature death. This chapter will concentrate on mouse models that are currently available for thin filament diseases of skeletal muscle.

Nemaline Myopathy

Nemaline myopathy (NM) is a clinically highly heterogeneous congenital myopathy caused by mutations in thin filament or thin filament-associated proteins: α-skeletal actin,[1,3] α-tropomyosin$_{slow}$,[5,6] nebulin,[9,10] troponin T,[8] β-tropomyosin[7] and recently, cofilin.[11] The severity and age of onset of skeletal muscle weakness in NM varies from severe, fetal[29] or neonatal[30] onset to mild, adult onset.[31] Patients are classified into subcategories based on the age of onset and the severity of motor and respiratory muscle involvement. Skeletal muscle weakness can be static or slowly progressive and inactivity may exacerbate muscle weakness in nemaline patients. The facial and bulbar muscles are usually affected. Although cardiac involvement is rare, nemaline rods have been detected in cardiac fibers.[32] The majority of nemaline patients with cardiac involvement present with dilated cardiomyopathy.[33,34]

Although a large number of mutations in six different genes result in NM, the skeletal muscles of nemaline patients are characterized by a common feature—accumulation of nemaline rods in the cytoplasm and/or nucleus of muscle fibers. Nemaline rods appear to be extensions of the Z-line and are composed of Z-line and Z-line associated proteins. These include α-actinin,[35] α-actin,[36] myotilin,[37] desmin and vinculin.[34] At the electron microscopic level, nemaline rods appear as electron dense, crystalline lattice structures.[38] Additional pathologies of nemaline muscles include an increase in or a predominance of slow/oxidative fibers,[39] decreased fiber size (atrophy)[40-42] and accumulation of glycogen and actin filaments in patients carrying certain α-skeletal actin mutations.[43] Nemaline muscles may also contain increased amounts of connective and adipose tissue.[4] Analyses of human biopsy samples have suggested two possible mechanisms for the predominance of slow fibers in nemaline muscles: conversion from type 2 to type 1 fibers[44] and/or a disruption of normal muscle fiber development.[39]

Skeletal Muscle Alpha Actin (*ACTA1*) Diseases

Mutations in the skeletal muscle alpha actin gene *ACTA1* are associated with multiple histopathological phenotypes including NM,[1,3] actin aggregate myopathy, intranuclear rod myopathy,[1] congenital core myopathy[13] and congenital fibre type disproportion.[14,15]

Mutations in *ACTA1* most often cause NM, accounting for approximately 20% of NM patients (See Chapter by North and Laing). The majority of nemaline-causing *ACTA1* mutations are single base changes that result in the production of a mutant thin filament protein. However, recently, seven nemaline patients were described having homozygous null mutations for the α-skeletal actin protein.[7] This discovery, along with previously described NM patients that lack α-tropomyosin$_{slow}$[6] and troponin T,[8] raises the possibility that NM may also arise from an imbalance in the stoichiometry of thin filament proteins.

In addition to the pathologies characteristic of NM, the skeletal muscles of *ACTA1* NM patients may also contain aggregations of actin filaments. This pathology can occur in the absence of nemaline rods and as such, individuals are classified as actin myopathy patients.[1] Mutations within the α-skeletal actin gene can also result in congenital fiber type disproportion or CFTD[14,15] and congenital core myopathy.[13] The skeletal muscles of CFTD patients contain characteristic small, atrophic slow/type 1 fibers. Unlike actin myopathy, NM and congenital core patients, CFTD patients lack any obvious protein aggregations.

Nebulin (*NEB*) Diseases

Mutations in nebulin cause NM[9] and most recently, homozygosity for missense mutations in nebulin have been associated with an early-onset distal myopathy.[19] Early-onset distal myopathy patients caused by mutations in the nebulin gene are phenotypically similar to those caused by mutations in the slow skeletal/beta cardiac myosin gene (*MYH7*)[45] (see Chapter by Pelin and Wallgren-Pettersson). However, the skeletal muscles of nebulin distal myopathy patients do not display distinctive pathologies, including those typical of distal myopathies such as rimmed vacuoles.[19]

Tropomyosin Diseases

Mutations in both the α-tropomyosin$_{slow}$ gene (*TPM3*) and the β-tropomyosin gene (*TPM2*) appear to cause diseases of skeletal muscle, while mutations in α-tropomyosin$_{fast}$ (*TPM1*) cause cardiomyopathy (Table 1). So far, mutations in *TPM3* cause NM[5,6] while mutations in *TPM2* have been shown to cause NM, distal arthrogryposis and most recently "cap" disease.[12] In "cap" disease, the muscle fibers have "caps" of aggregated protein. The majority of the proteins present in the "cap" aggregates are thin filament and Z-line proteins, similar to, but more extensive than those found in nemaline rods.[46] Nemaline rods may also be found in the skeletal muscles of "cap" disease patients[47] (see Chapter by Clarke). The muscle pathologies in distal arthrogryposis are not well-characterized, but may be limited to only increased slow/oxidative muscle fibers.[47]

Troponin and Cofilin Diseases

Mutations in skeletal muscle troponin T fast and troponin I fast are associated with distal arthrogryposis,[17,18] while mutations in muscle-specific cofilin (CLF2) have recently been associated with NM with minicores.[11]

Tropomyosin Mouse Model for Nemaline Myopathy

The first mouse model for nemaline myopathy was reported by Corbett et al (2001).[48] This model was generated by expressing a dominant negative mutation of α-tropomyosin$_{slow}$, α-tropomyosin$_{slow}$(Met9Arg) or *TPM3*(M9R), specifically in the skeletal muscles of transgenic mice using the human skeletal actin promoter. *TPM3*(M9R) was the first mutation identified as being disease-causing in nemaline patients and results in a childhood onset form of the disease.[5] All pathologies identified in nemaline patients carrying the *TPM3*(M9R) mutation were detected in the skeletal muscles of transgenic mice.[48] These pathologies included the presence of electron dense nemaline rods (Fig. 1), an increase in the percentage of slow/oxidative fibers and a late onset of muscle weakness at 5-6 months of age in the mouse. In addition, this mouse model assisted in the detection of a novel feature of this disease: a mild, chronic repair mechanism as characterized by increased satellite cell activity and centralized myofiber nuclei.[49,50] This demonstrates a potential advantage to using mouse models because they may assist in the identification of novel pathologies which can facilitate patient diagnosis and impact on potential therapies.

Although at two to three months of age the *TPM3*(M9R) mouse does not display whole body weakness, the isometric force of muscles in these mice was lower than littermate controls.[51] This finding indicates that alteration in muscle function is present in the *TPM3*(M9R) muscle prior to detection of whole body weakness.

A careful examination of various muscles of this nemaline mouse revealed variable percentages of rod-containing fibers between muscles. Indeed, some muscles did not have rods. This demonstrated that the mutant protein does not elicit the same pathologies in all muscles. Since nemaline patients are generally diagnosed by the presence of nemaline rods in a muscle biopsy, this finding highlights the importance of selecting a 'correct' muscle for the diagnosis of nemaline patients or performing more than one biopsy in a patient. It also suggests that NM might be under-reported.

By comparing the expression level of the *TPM3*(M9R) mutant in various transgenic lines with the presence of nemaline rods, Corbett and colleagues[48] determined the expression threshold that is required for rod formation. For this particular mutation and potentially for tropomyosin mutants in general, a comparable expression level of mutant to endogenous protein is required. In addition, comparing the transgene expression in various muscles and the corresponding level of muscle disruption indicated that the amount of mutant tropomyosin protein above threshold level did not define the degree of muscle disruption.

As previously mentioned, transgenic muscle displayed an increased percentage of slow/oxidative fibers. Comparing the myosin heavy chain (MyHC) composition of muscles obtained from transgenic and control littermates at various ages allowed researchers to identify a possible mechanism for the increase in slow/oxidative fibers. The fiber type content was established in transgenic hindlimb muscles by 2 months and did not alter in composition with age. This suggests that the

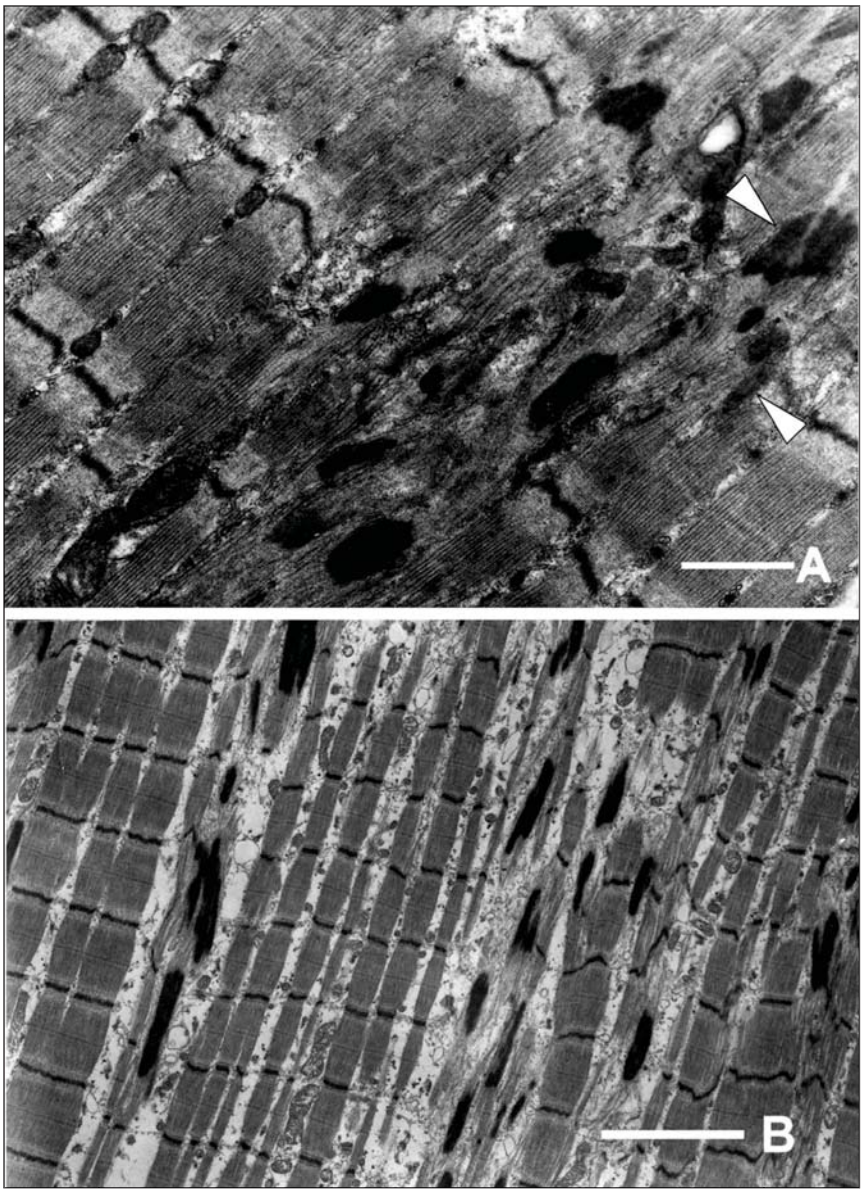

Figure 1. Electron micrographs showing electron dense nemaline rods in (A) *TPM3*(M9R) transgenic muscle (arrowheads) (20100x; scale bar = 1μm) and (B) human *TPM3*(M9R) nemaline patient muscle (4000x; scale bar = 5μm) (from Corbett et al., 2001).[48]

mechanism for the increase in slow/oxidative fibers observed in the *TPM3*(M9R) model was due to a disruption of the fiber maturation process. In addition, transgenic muscles with similar MyHC composition displayed different percentages of rod containing fibers, suggesting that fiber type composition is not associated with rod content.

Analysis of skeletal muscle of the *TPM3*(M9R) mouse further revealed a possible mechanism for the muscle disruption and weakness observed in patients carrying this mutation.[52] Corbett and colleagues determined that expression of the *TPM3*(M9R) protein perturbed the composition of tropomyosin isoforms in muscles from both patients and transgenic mice. In particular, a reduction in the β-tropomyosin isoform and a reduction in the preferred α/β tropomyosin heterodimer were detected in mutant muscles. By assessing a postnatal timecourse of expression of tropomyosin isoforms in both *TPM3*(M9R) and control muscles, the authors determined that the onset of *TPM3*(M9R) expression coincides with the decline in β-tropomyosin expression. This suggests that the expression of the *TPM3*(M9R) protein perturbs tropomyosin isoform levels and dimer preference. This is the first report of a mechanism that could underpin muscle disruption in NM. Potentially, this mechanism could be extrapolated to include nemaline patients carrying any tropomyosin mutation.

Nebulin Null Mouse Models

The thin filament template protein nebulin is 800kDa. Due to its large size, it is difficult to examine the role that nebulin plays in muscle fibers using current in vitro and in vivo expression systems. Because of this, two groups have generated nebulin null mice by disrupting the first exon of the nebulin gene.[53,54] Homozygous null mice die between postnatal day 8 to 20 demonstrating that nebulin is important for muscle function and survival. To determine the expression pattern of nebulin in striated muscles, the Chen laboratory mated heterozygous knock-out mice with Rosa26 lineage reporter mice. This revealed that nebulin is expressed in the heart, which was originally thought to only express another family member, nebulette. The skeletal muscles of nebulin deficient mice showed reduced thin filament lengths and expanded Z-lines. This suggests a role for nebulin in the maintenance of myofibrillar integrity, in particular, thin filament length. The absence of correct thin filament length resulted in decreased isometric contraction force in knock-out muscles. Gene expression profiling of cardiac and skeletal muscles of nebulin null mice suggest that absence of nebulin may disrupt calcium homeostasis and glycogen homeostasis.[53] This provides an insight into additional roles that nebulin plays in striated muscles and therefore, potentially, the detrimental effects caused by the absence of nebulin. Mutations in the nebulin protein in NM patients cause expanded Z-lines, or Z-line streaming, as well as nemaline rods. However, unlike the nebulin null mice, nemaline patients with frameshift, nonsense or splice site mutations in the nebulin gene may still express a portion of the nebulin protein in skeletal muscles.[55]

α-Skeletal Actin Mouse Models for Nemaline and Other Myopathies

Due to the major role that α-skeletal actin plays in the thin filaments of the sarcomere, it was originally assumed that any α-skeletal null patients would not survive until birth. Recently, however, seven α-skeletal actin null nemaline patients were identified.[4] All skeletal muscles examined showed nemaline rods and three of these muscles displayed zebra bodies. The absence of α-skeletal actin protein caused severe muscle weakness and six of these nemaline patients died by 22 months of age.

A homozygous null α-skeletal actin mouse model was generated originally to investigate the developmental and functional effects that the absence of α-skeletal actin protein has on skeletal muscles.[56] In the mouse, a complete lack of α-skeletal protein causes severe muscle weakness, resulting in death by post natal day 10. Surprisingly, heterozygous null mice survive into adulthood without any overt muscle phenotypes. An increase in α-cardiac actin was detected in the skeletal muscles of both human and mouse homozygous null individuals. The skeletal muscles of heterozygous null mice also showed increased expression of all three muscle actin isoforms. These observations suggest that increasing the production of other actin isoforms may partially compensate for the absence of α-skeletal actin and therefore, potentially provide a therapeutic route for skeletal actin null patients.

However, it must be noted that nemaline rods were not reported by Crawford et al (2002). Because of this, it is not known whether the absence of α-skeletal actin protein results in formation

of nemaline rods in the mouse. Therefore, it is not known whether the α-skeletal actin null mouse is a robust mouse model for *ACTA1* null NM.

To generate a mouse model for NM that displays severe, early onset of muscle weakness, members of the Hardeman laboratory utilized both transgenic and knock-in technology to express an α-skeletal actin mutant protein, *ACTA1*(H40Y), in skeletal muscles of mice (unpublished data). The *ACTA1*(H40Y) mutation leads to formation of nemaline rods in both the cytoplasm and nuclei of muscle fibers of patients and mice allowing researchers to study the development of both pathologies (Fig. 2). In preliminary studies, skeletal muscles from both transgenic and knock-in *ACTA1*(H40Y) mouse models display pathologies observed in patients carrying the mutation. An increase in percentage of slow/oxidative fibers, accumulation of glycogen and accumulation of abnormally large subsarcolemmal mitochondria were also detected. These findings suggest that metabolism is altered in nemaline muscles. Preliminary data also demonstrate severe muscle weakness in the *ACTA1*(H40Y) mice as evidenced by decreased forearm strength and a shortened lifespan.

Comparing pathologies present in the *ACTA1*(H40Y) and *TPM3*(M9R) models could potentially identify factors that contribute to severity of muscle weakness. This will also allow researchers to determine whether certain pathologies are specific to the disease-causing gene. Indeed, there are indications that the mechanism of increased slow fiber type composition differs between the two nemaline mouse models. In addition, analyses of transgene expression in the skeletal muscles of the *ACTA1*(H40Y) mouse revealed the potency of this dominant negative mutation. The presence of mutant *ACTA1*(H40Y) protein at less than 4% of total sarcomeric actin protein is sufficient for nemaline rods to form. This finding has implications for actin isoform substitution therapy. Up-regulating another actin isoform, for example α-cardiac, may be beneficial for α-skeletal actin null nemaline patients, but sufficient expression may not be elicited to counter the effects of a potent dominant negative *ACTA1* mutation.

Mouse Models for Cardiomyopathies

Several transgenic mouse models have been generated for various cardiomyopathies[57-61] (Table 1). These mouse models all express mutant forms of thin filament proteins present in cardiomyocytes. These mutations resulting in hypertrophic cardiomyopathy or dilated cardiomyopathy affect the function of transgenic hearts. It would be interesting to compare pathologies and gene expression profiles from cardiomyopathy mouse models and existing skeletal muscle thin filament disease models to help tease out the difference between the function of mutant thin filament proteins in the heart and skeletal muscle.

Therapies for NM—Insights from Mouse Models

Currently, there is a lack of treatments available to address the debilitating muscle weakness that thin filament myopathy patients experience. It is not feasible to target individual disease-causing mutations specifically since so many different mutations cause NM. Therefore, current strategies aim to identify therapies that alleviate general pathologies and clinical conditions such as muscle weakness. Various forms of exercise have previously been utilized to improve the strength of aged and diseased muscles.[62-64] It is not certain, however, whether exercise is beneficial to nemaline patients. In particular, the skeletal muscles of nemaline patients are structurally disrupted so there is a possibility that exercise may exacerbate this condition. In order to investigate the effects that exercise has on NM, adult *TPM3*(M9R) transgenic mice were exercised on a treadmill over four weeks.[65] This study demonstrated that although *TPM3*(M9R) mice ran with an altered gait, they were capable of completing the exercise regime. Importantly, the study found that endurance exercise did not have a detrimental effect on nemaline muscles. In particular, nemaline muscles responded to endurance exercise in a similar manner to control muscles.

The *TPM3*(M9R) mouse was modelled on a mild form of nemaline myopathy and does not display severe muscle weakness. To induce severe muscle weakness in this model and in particular to mimic the prolonged period of inactivity that some nemaline patients experience, the hindlimbs of

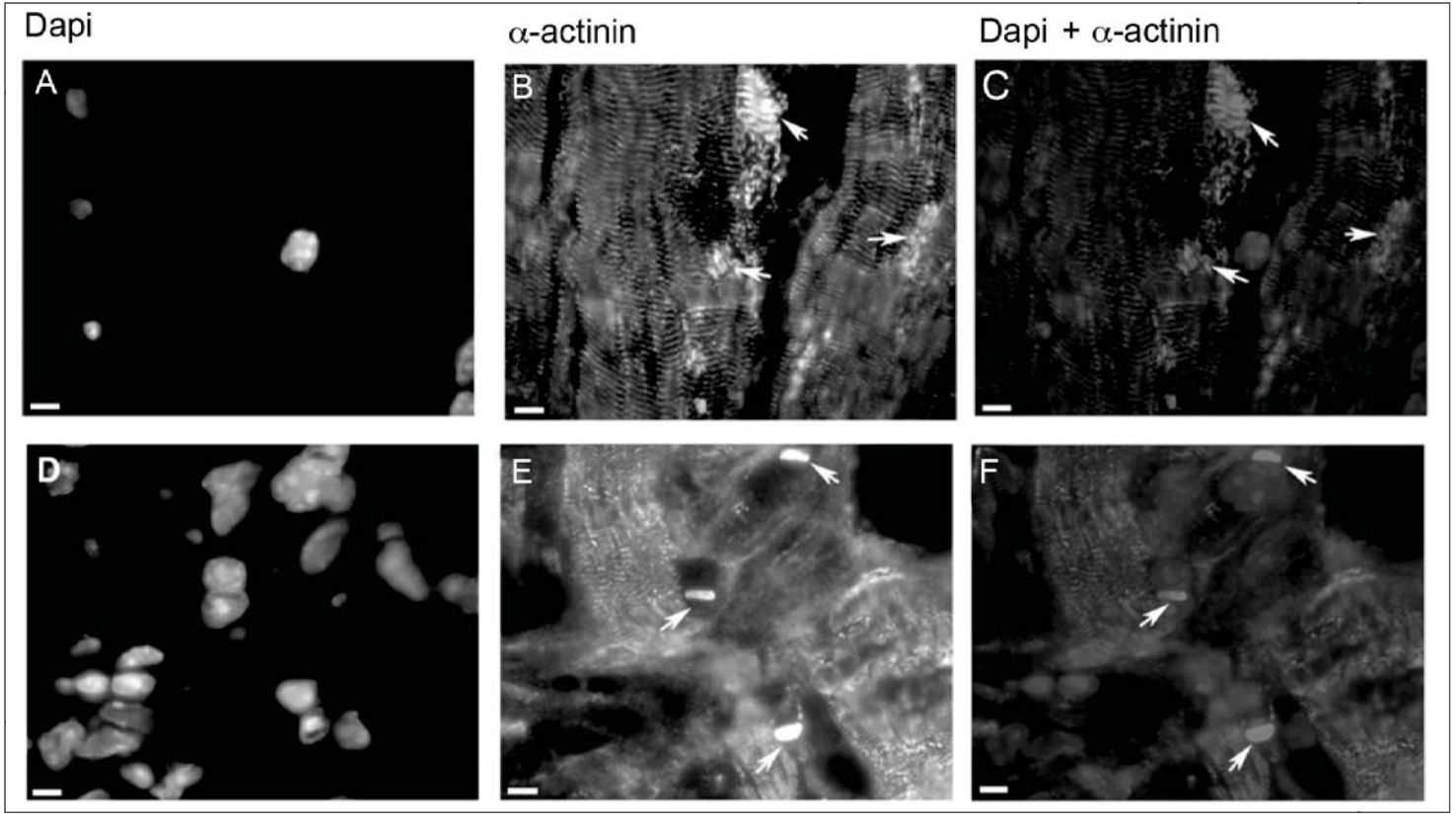

Figure 2. Detection of cytoplasmic and intranuclear rods in the *ACTA1*(H40T) knock-in mouse model. Muscle nuclei are visualized using DAPI (A & D) and nemaline rods are detected using an α-actinin antibody (B & E) in sections of the diaphragm muscle from a 6 week-old *ACTA1*(H40Y) male mouse. White arrows indicate cytoplasmic rods (B & C) and intra-nuclear rods (E & F). (100x; scale bar = 10μm).

TPM3(M9R) mice were immobilized for four weeks.[49] To assess whether exercise could help to alleviate the pronounced muscle atrophy and muscle weakness that results from immobilization/inactivity, post-immobilized nemaline and control mice were exercised on a treadmill or given a free-wheel for 4 weeks. Inactivity impacted on the strength of the *TPM3*(M9R) mice more severely than littermate controls. Immunohistochemical analysis of immobilized nemaline muscles detected increased muscle disruption and fiber atrophy, suggesting two mechanisms underpinning the detrimental effects that inactivity has on nemaline patients. Two major findings were reported. Firstly, it was determined that either voluntary or endurance exercise was sufficient to restore fiber size, resolve nemaline rod content to some extent and importantly, improve the strength of nemaline mice. Secondly, this paper identified a chronic repair process present in nemaline muscles.

Increasing the size of muscle fibers, hypertrophy, has been shown to improve muscle strength in the elderly[66] and patients with neuromuscular diseases.[67] There is some circumstantial evidence suggesting that fiber hypertrophy may be of some benefit to nemaline patients. This is mainly through the observation that nemaline patients with mild muscle weakness have large fast/glycolytic fibers.[42] The *TPM3*(M9R) mouse model which displays a late onset, mild muscle weakness also has larger fast/glycolytic fibers.[48] In order to examine the therapeutic potential of hypertrophy on nemaline muscle function, members of the Hardeman laboratory tested hypertrophy-promoting agents on the *ACTA1*(H40Y) mouse model. Not all agents proved beneficial, but preliminary data suggest that insulin like growth factor 1 (IGF-1) and four and a half LIM domain protein 1 (FHL1) improved mobility and could alleviate some fore-arm weakness.

Recently, supplementation of the amino acid L-tyrosine has been implicated in improving the muscle strength of nemaline patients.[68] To investigate and confirm these beneficial effects, members of the Hardeman laboratory assessed the effects that daily oral L-tyrosine administration has on the *ACTA1*(H40Y) mouse model. Preliminary data suggest that a brief period of treatment is sufficient to increase the mobility of nemaline mice. Importantly, tyrosine treatment did not appear to cause any detrimental effects in either normal or nemaline mice.

Future Directions

Several mouse models for thin filament diseases are currently available and these models cover three disease-causing genes as well as two categories of nemaline patients. Early results from the current models for NM suggest exercise, muscle hypertrophy and L-tyrosine supplementation as potential therapies for nemaline patients and potentially, congenital myopathy patients in general. The potential for future applications of animal models of thin filament diseases are substantial.

The methods for induction of muscle hypertrophy or the dosage of L-tyrosine will need to be investigated to maximize the potential impact of these therapies on patients. This includes methods for the effective delivery of therapies into the most severely affected muscles. The diaphragm needs to be targeted since patients with severe muscle weakness require ventilatory support. In addition, although preliminary results indicate that muscle hypertrophy has a beneficial effect in the nemaline mouse model, hypertrophy could only moderately alleviate fore-arm weakness. It needs be determined whether this is sufficient to improve the quality of life of patients in general. L-tyrosine supplementation can increase the mobility of the nemaline mice; however, it may not improve muscle strength of nemaline mice. It may be that combinations of treatments are required for significant improvement of muscle function in NM. These treatments could be L-tyrosine plus resistance exercise that would promote muscle hypertrophy or a ventilation exercise that would improve the function of the diaphragm.

There are indications that modifier loci exist in NM. Firstly, patients that carry the same nemaline-causing mutation may display different degrees of muscle disruption, time of onset and severity of muscle weakness. Skeletal muscles from both the *TPM3*(M9R) and *ACTA1*(H40Y) mouse models display variable degrees of muscle disruption. In addition, the effect of the *ACTA1*(H40Y) mutation is more potent in male knock-in mice causing increased muscle weakness (with age), increased mobility deficits, leading to deaths of male mice (unpublished data). Mouse models are ideally suited to aid in the identification of modifier loci in nemaline myopathy.

The elevated level of cardiac actin in both α-skeletal actin null patients and the α-skeletal actin null mouse model suggests that an increase in cardiac actin expression could potentially provide compensation for the deleterious effects of mutant α-skeletal actin. The *ACTA1*(H40Y) transgenic mice indicates that only a small amount of mutant actin protein is sufficient for nemaline rod formation in muscles. However, it is tempting to contemplate whether extensive up-regulation of normal α-skeletal actin or α-cardiac actin expression could improve strength in nemaline patients.

Characterization of the two mouse models for NM suggests that some nemaline pathologies develop via different mechanisms. In particular, the increase in slow/oxidative fibers present in *TPM3*(M9R) muscles is most likely due to a disruption of the fiber maturation process. However, analayses of MyHC content in *ACTA1*(H40Y) muscles at various ages suggest that the increase in percentage of slow/oxidative fibers in this model is due to an active conversion from a faster to slower fiber type. This suggests that different NM-causing mutations or mutations in different NM-causing genes may elicit the same muscle pathologies via different mechanisms. What impact this has on the effectiveness of potential therapies is not known and would need to be assessed.

There are several thin filament diseases of the skeletal muscle for which mouse models have not yet been generated. These include congenital fiber type disproportion, actin aggregate myopathy, cap disease, core myopathy and nebulin distal myopathy. In addition, when multiple different pathologies are caused by mutations in one gene, for example *ACTA1*, it is unlikely that a single transgenic or knock-in mouse model will reproduce all of these different pathologies. Some mouse models might display more than one pathology, but many individual mouse models might have to be generated in order to reproduce all of the pathologies. It must be noted that although rewarding, the generation and characterization of a mouse model is a time consuming process. Therefore, the cost benefit ratio of what may be achieved by utilizing the mouse model must be considered carefully before generating additional models.

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Chapter 7

Thick Filament Diseases

Anders Oldfors and Phillipa J. Lamont*

Abstract

Hereditary myosin myopathies are a newly emerged group of diseases caused by mutations in skeletal muscle myosin heavy chain (MyHC) genes. The phenotypes of these diseases are varied, ranging from prenatal nonprogressive arthrogrypotic syndromes to adult-onset progressive muscle weakness. They are caused by mutations in skeletal muscle myosin heavy chain (MyHC) genes. Mutations have been reported in two of three MyHC isoforms expressed in adult limb skeletal muscle: type I (slow/β-cardiac MyHC; *MYH7*) and type IIa (*MYH2*). Most of the mutations described in *MYH7* are associated with hypertrophic/dilated cardiomyopathy, with no skeletal muscle involvement. However, some mutations are associated with two distinct skeletal myopathies, namely Laing distal myopathy and myosin storage myopathy. Although initially thought not to have associated cardiac involvement, recent reports have indicated co-existent cardiac and skeletal muscle disease can occur in both. A myopathy associated with a specific mutation in *MYH2* is associated with congenital joint contractures and external ophthalmoplegia. Mutations in embryonic MyHC (*MYH3*) and perinatal MyHC (*MYH8*) are associated with distal arthrogryposis syndromes with no or minor muscle weakness. This may be expected in myosin isoforms expressed predominantly during muscle development. Clinical findings, muscle morphology and molecular genetics in hereditary myosin myopathies are summarized in this chapter.

Introduction

Myosin heavy chains (MyHC) are motor proteins that convert the chemical energy derived from hydrolysis of ATP into mechanical force. This drives diverse motile processes in eukaryotic cells including vesicular transport, cytokinesis and cellular locomotion. The MyHCs have been divided into classes and Class II includes the sarcomeric MyHCs that self associate to form filaments and function enzymatically to effect contraction in striated muscle. Muscle myosin is a heterohexamer consisting of two myosin heavy chains and two nonidentical pairs of myosin light chains. The seven MyHC isoforms identified in mammalian skeletal muscle include two developmental isoforms, three adult muscle isoforms, the extraocular isoform and MyHCβ/slow which is also expressed in cardiac muscle. The developmental isoforms are MyHC-embryonic (*MYH3*) and MyHC-perinatal (*MYH8*). The adult isoforms are MyHC IIa(*MYH2*), MyHCIIb (*MYH4*) and MyHCIIx/d (*MYH1*). MyHC-extra ocular (*MYH13*) is expressed primarily in extrinsic eye muscles.[1,2]

Six MyHC genes are found in a tightly linked cluster on chromosome 17 (*MYH1, 2, 3, 4, 8 and 13*), with *MYH6* and *MYH7* on chromosome 14.[1] A mutation in *MYH6*, which encodes cardiac myosin has been associated with atrial septal defect.[3] *MYH7* encodes one myosin which is both β-cardiac myosin in the heart and MyHC I expressed in type 1 muscle fibres. Mutations in *MYH7* can cause either cardiomyopathy or skeletal muscle disease or both. *MYH11* encodes smooth muscle myosin and mutations are associated with thoracic aorta aneurysm, dissection and patent ductus arteriosus.[4] There are other nonmuscle myosins which will not be discussed further.

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The Sarcomere and Skeletal Muscle Disease, edited by Nigel G. Laing.

The skeletal muscle diseases discussed in the following chapter are associated with mutations in *MYH 2*, *MYH3*, *MYH7* and *MYH8* and are summarized in Table 1.

The two pairs of light chains of muscle myosins stabilize the long alpha helical neck of the myosin head. In addition, myosin light chain-2 (*MYL2*) is an important protein in the regulation of myosin ATPase activity in smooth muscle. Familial hypertrophic cardiomyopathy (HCM) has been associated with mutations in the *MYL2* gene.[5] Three of these HCM patients had microscopic changes in soleus or deltoid muscle biopsies, although no clinical weakness in the skeletal muscles was reported. Thus, no unequivocal skeletal myopathies have so far been associated with mutation of the myosin light chains.

Myopathies Associated with *MYH2* Mutations

Autosomal Dominant Myopathy with Congenital Joint Contractures, Ophthalmoplegia and Rimmed Vacuoles

This myopathy was identified as an autosomal dominant disease in a large Swedish family with many affected individuals. The index case was a four-year-old girl who was investigated because of suspected congenital myopathy. She had hip dislocation and joint contractures at birth. She had proximal muscle weakness affecting pelvic and shoulder girdle muscles and also slight external ophthalmoplegia but no ptosis. Her mother and maternal grand-father also had had congenital joint contractures, they showed proximal muscle weakness and external ophthalmoplegia, which was nearly total in the grandfather. A thorough survey of the family disclosed 19 affected individuals.[6] Several of them were unknown to each other and they had at different hospitals obtained various diagnoses. Since muscle biopsy revealed rimmed vacuoles and inclusions of 15-20 nm tubulofilaments, the disease was considered a variant of hereditary inclusion body myopathy (hIBM) and has been assigned hIBM3. By positional cloning strategy it was later demonstrated that the disease was caused by a mutation in the gene encoding MyHC IIa (*MYH2*).[7,8]

Clinical Features

The majority of the affected individuals had had joint contractures and/or hip dislocation at birth. The contractures frequently involved fingers and hips but were generalized in some cases. The contractures resolved spontaneously during early childhood. The affected individuals were not hypotonic at birth and onset of walking was delayed only in rare cases. During childhood the condition appeared nonprogressive. Most patients experienced deterioration from 30 to 50 years of age (Fig. 1). They then developed difficulties in running and walking up stairs. The muscle weakness was predominantly proximal with atrophy of the quadriceps femoris muscle, which was severe in some cases affecting ambulation. Some individuals developed kyphosis or scoliosis. EMG showed clear myopathic changes with low amplitude and polyphasic potentials in severely affected individuals. There were no signs of motor or sensory nerve involvement. s-CK levels showed 2- to

Table 1. Diseases associated with mutations in skeletal muscle myosin heavy chains

Protein	Gene	Disease
MyHC IIa	MYH2	• Autosomal dominant myopathy with congenital joint contractures, ophthalmoplegia and rimmed vacuoles
Embryonic MyHC	MYH3	• Freeman-Sheldon syndrome • Sheldon-Hall syndrome
MyHC I (β-cardiac MyHC)	MYH7	• Familial hypertrophic/dilated cardiomyopathy • Laing distal myopathy • Myosin storage myopathy
Perinatal MyHC	MYH8	• Trismus-pseudocampylodactyly syndrome

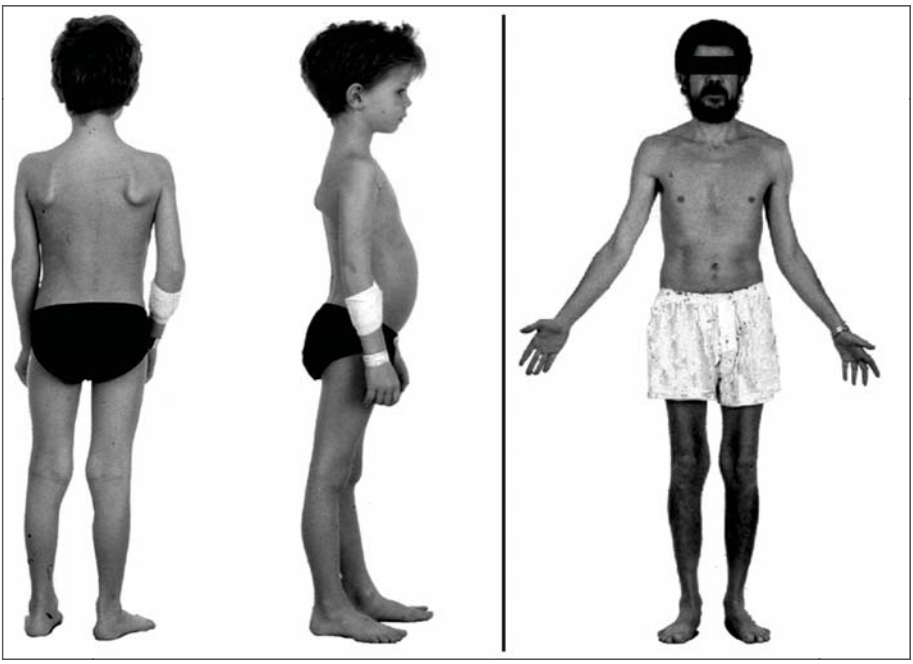

Figure 1. A 6 year old boy and his father with "Autosomal dominant myopathy with congenital joint contractures, ophthalmoplegia and rimmed vacuoles" caused by a *MYH2* mutation. The boy has winging of the scapulae, hyperlordosis, extended knees and thin thighs. The father has severe atrophy of the thigh muscles at age 37 years, Reproduced from *Ann Neurol* 1998; 44:242-248, with permission.

10-fold increase but only in those individuals who experienced deterioration. Physical exercise by an endurance training program on a stationary exercise bicycle increased the obtained peak watt but no change in muscle strength.[9,10]

Muscle Morphology

In young and mildly affected individuals there were minor changes. Increased variability of fiber size and mini-cores in some muscle fibers were the most obvious changes. The type 2A fibers were more or less selectively affected. They were either absent, very few and small or showed disorganization of the myofilaments (Fig. 2).[8,11] Interestingly there was an apparent increase in the expression of the MyHC IIa isoform with increasing age and severity of muscle disease.[11] Adult individuals who experienced a progressive course exhibited in their muscle biopsies a dystrophic pattern with marked fiber size variability, internalized nuclei and increase of interstitial fat and connective tissue.[6,11] The muscle biopsies from patients with progressive course showed muscle fibers with rimmed vacuoles and ubiquitinated inclusions similar to those in sporadic inclusion body myositis (s-IBM) (Fig. 3a). As in s-IBM there were also cytoplasmic and occasional intranuclear collections of 15-21 nm tubulofilaments (Fig. 3b). Pathological changes were present in all fiber types, but rimmed vacuoles were seen only in fibers expressing type IIa MyHC.[11] Cytochrome c oxidase deficient muscle fibers as can be seen in s-IBM and to some extent in normal aging were present in muscle of adult individuals and associated with clonal expansions of mtDNA with deletions as in aging and in s-IBM.[12]

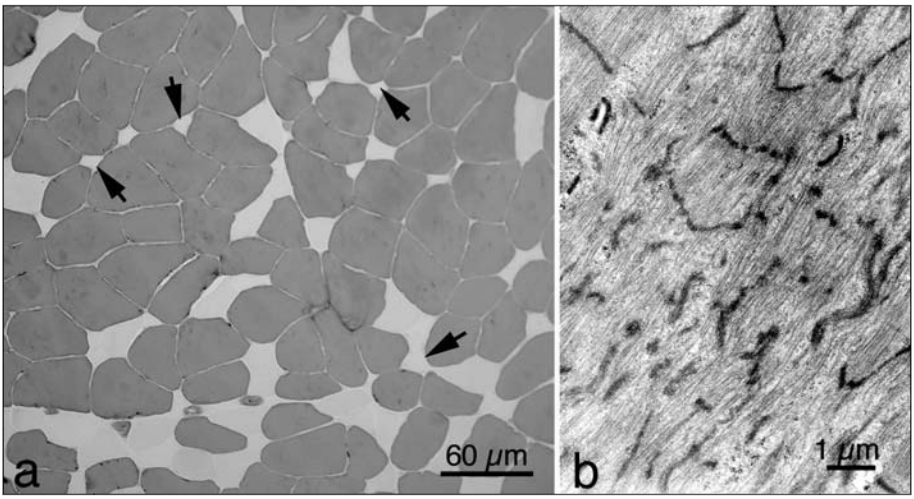

Figure 2. Muscle biopsy from a clinically mildly affected hIBM3 patient showing hypoplastic type 2A muscle fibers (a) (arrows) and focal disorganization of myofilaments (b).

Molecular Genetics and Pathogenesis

The disease in this family is caused by a mutation, Glu706Lys, in the gene encoding MyHC IIa (*MYH2*). This MyHC isoform is expressed in type 2A muscle fibers, which may explain why the pathological alterations affect mainly type 2A fibers. The mutation is located in a region that is extremely conserved, namely the SH1 helix in the motor domain of the MyHC. Experimental studies applying an in vitro motility assay on myosin from isolated muscle fibers from the patients demonstrated reduced motility of myosin from type 2A muscle fibers.[13] This result was in accordance with studies on the equivalent mutation in *Dictyostelium*[14] and *Caenorhabditis elegans*.[15]

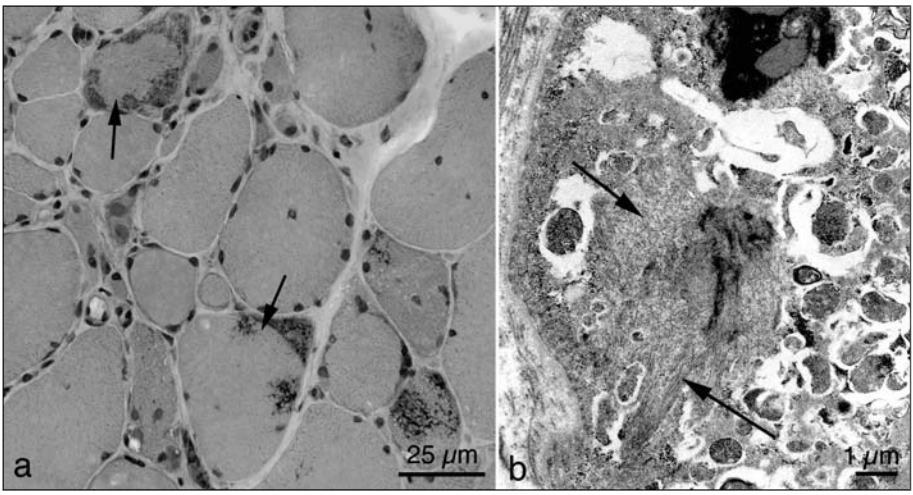

Figure 3. Muscle biopsy from the quadriceps femoris muscle of the father in Figure 1 showing a dystrophic pattern and multiple muscle fibers with rimmed vacuoles (a) (arrows). Tubulofilamentous inclusions are seen by electron microscopy (b) (arrows).

In a study on sequence variability and search for other familial myopathies caused by *MYH2* mutations six polymorphic sites in *MYH2* were identified.[16] Two families with myopathy and missense mutations in *MYH2* were identified, but definite proof for a causal relationship between the respective mutation and muscle disease was not obtained.

Myopathies Associated with *MYH3* Mutations

It has recently been demonstrated that syndromes with congenital contractures that preferentially affect hands and feet and with no or minor muscle weakness, so-called distal arthrogryposis (DA) syndromes are sometimes caused by mutations in sarcomeric proteins that are expressed during fetal development.[17-23] Therefore it has been postulated that the pathogenesis of such syndromes involves a developmental myopathy. Freeman-Sheldon syndrome (Distal arthrogryposis 2A; DA2A) and Sheldon-Hall syndrome (Distal arthrogryposis 2B; DA2B) (Fig. 4) are such syndromes, which have been associated with mutations in the embryonic isoform of MyHC, encoded from *MYH3*.

Clinical Features

Freeman-Sheldon syndrome (DA2A) is a rare disease with contractures of hands and feet, usually camptodactyly and clubfoot in addition to contractures of facial muscles.[24-26] The facial muscle contractures cause the mouth to be very small, resulting in feeding difficulties. The facial dysmorphism has characteristic features. In addition to the small, pursed mouth there is also downslanting palpebral fissures, prominent nasolabial folds and a "H"-shaped dimpling of the chin. The majority of the patients develop scoliosis. Cryptorchidism and strabismus are common additional complications and thirty percent of the DA2A patients have hearing loss.

Sheldon-Hall syndrome (DA2B) is a milder variant with less prominent facial dysmorphism compared to DA2A.

Muscle Morphology

No systematic reports on muscle pathology in DA2 are reported.

Molecular Genetics

In a systematic study of Freeman-Sheldon and Sheldon-Hall syndromes, 23 of 28 DA2A patients and 12 of 38 DA2B patients had a *MYH3* mutation. Missense mutations affecting Arg672 were present in 72% of the DA2A patients but were never observed in DA2B. Most other mutations

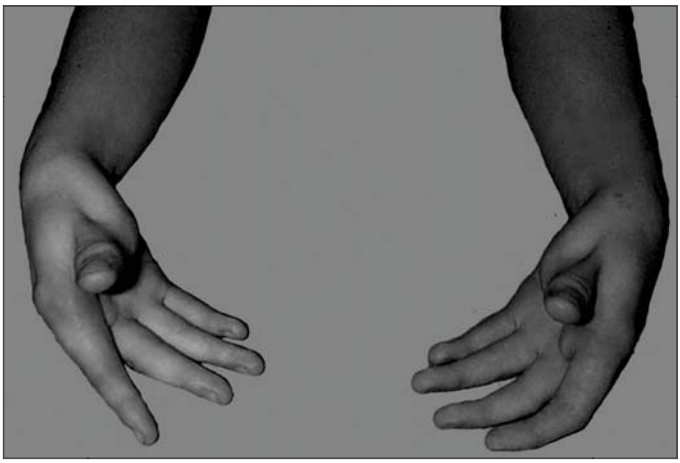

Figure 4. Hands of a woman with Sheldon-Hall syndrome (DA2B) associated with a *MYH3* mutation. There is palmar malposition of the thumbs. Photograph courtesy of Eva Kimber.

were also missense mutations located in the globular head of the MyHC. One single amino acid deletion mutation was identified in the neck region. Two missense mutations were located in the rod region.

Skeletal Myopathies Associated with *MYH7* Mutations

The majority of the more than 200 dominant missense mutations in *MYH7* are associated with hypertrophic/dilated cardiomyopathy without signs or symptoms of skeletal myopathy (http://genetics.med.harvard.edu/~seidman/cg3/). These will not be discussed further.

Laing Distal Myopathy

This form of distal myopathy, with autosomal dominant inheritance, was linked to the *MYH7* gene on chromosome 14q11 in 1995, based on a study of an Australian family with 9 affected

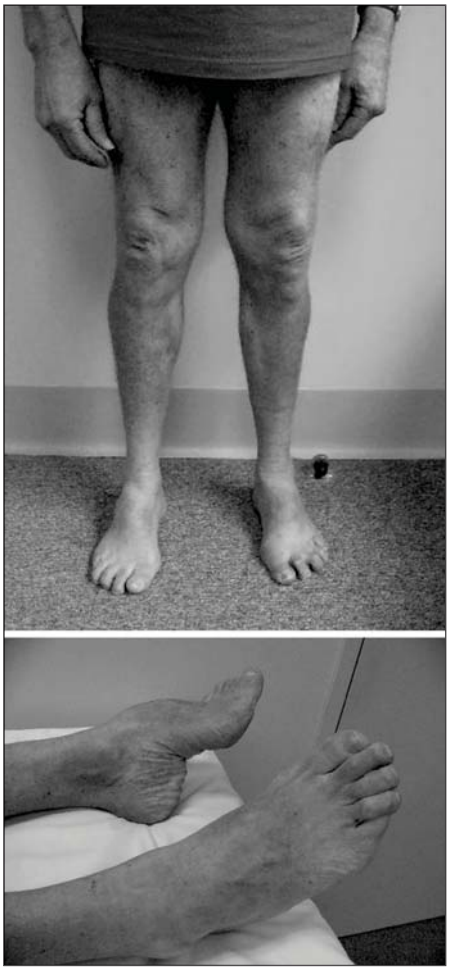

Figure 5. 65 year old man with Laing DM. Top: In his legs there is wasting of the muscles below the knees, anterior more than posterior. Bottom: The patient is unable to dorsiflex the ankles because of a combination of tight tendo achilles and muscle weakness; weakness of toe flexion leads to a "hanging great toe".

individuals.[27] Several additional familial and sporadic cases have subsequently been reported from various parts of the world and mutations in *MYH7* have been identified in most of these cases.

Clinical Phenotype

Laing distal myopathy (Laing DM) is often referred to as "early onset". However, a review of five previously published familial cases and three unpublished sporadic cases showed that onset could be as late as 34 years.[28] Most commonly, however, weakness is noted in the first four years of life. Initial site of weakness is usually in the distal leg, with wasting of the anterior compartment below the knee and weakness of toe and ankle dorsiflexion (Fig. 5). This has been called the "hanging toe" sign.[29] It leads to a high stepping gait and tightness of the tendoachilles. Within several years, most patients also notice weakness of finger extension (Fig. 6). Weakness of neck flexion develops early in all cases except one family where it did not occur until the fifth decade.[30] Over the ten years following onset there is proximal spread of weakness to the upper and lower limb girdles, trunk muscles and the face, particularly orbicularis oris and oculi. However, the progression of weakness remains slow, with patients remaining ambulant into their sixth decade of life.

In the past, it was not clear whether cardiac disease in Laing DM was incidental, as most patients with this form of distal myopathy do not have cardiac disease. There had been only a single early report of a father and son with Laing DM and echocardiographic evidence of a dilated cardiomyopathy.[30] Similarly, patients with a primary cardiomyopathy caused by *MYH7* gene mutations had not had skeletal muscle weakness reported although histological abnormalities such as central cores, type I fibre atrophy and mitochondrial abnormalities had been seen in skeletal muscle.[31,32] However, there are two recent reports where patients have both skeletal muscle weakness as well as a cardiomyopathy, due to a *MYH7* mutation.[33,34] Thus it seems that *MYH7* mutations can cause both cardiac and skeletal muscle disease in the same patient. The two recently described mutations occur in the region of the *MYH7* gene usually thought to cause cardiomyopathy, rather than the light meromyosin domain hitherto associated with skeletal muscle disease.

One patient in the original family described in 1995.[27] developed a pronounced kyphoscoliosis during adolescence which has been stable over the past 40 years. This is presumed to be incidental. One patient was demonstrated to have hypomyelinated neuropathy on sural nerve biopsy,[29] but again the significance of this is unclear.

Figure 6. This 65 year old man with Laing DM is unable to fully extend his fingers, because of weakness of the long finger extensors.

Laboratory Findings and Muscle Biopsy

The serum creatine kinase level can be elevated to three times normal in the first two decades, but is often normal. Electromyography is often reported as normal, although nonspecific features such as occasional fibrillation potentials may occur. A recent case was reported to have clear myopathic changes, with small polyphasic units in distal muscles.[34]

In contrast to myosin storage myopathy, no pathognomonic histological features in muscle have been described to aid in diagnosis. Excessive variation in fibre size, particularly type 1 fibre hypotrophy, is common. However, the variation can be confined to type 1 fibres, or seen in both type 1 and 2 fibres and both hypertrophy and atrophy are reported.[28] Similarly, either type 1 or 2 predominance is described. Recently the muscle biopsy from a seven year old boy was reported as showing numerous hypotrophic type 1 fibres, but the apparent absence of type 2B fibres (Fig. 7).[33] Necrotic and regenerating fibres are seen but never prominent. Rimmed vacuoles are seen uncommonly and in low numbers. Excessive central nucleation is common and Z-disc streaming and nuclear clumps occur. Biopsies of severely affected muscles were described as "end-stage".[28] Four of the more recently described cases have shown abnormal immunostaining for fast and slow myosins. They demonstrated either co-expression of both fast and slow myosin or nearly complete conversion to fast myosin expression.[28,34] It may be postulated that this conversion to fast myosin expression is consistent with a mutation in a gene responsible for slow myosin protein production.

Molecular Genetics and Pathogenesis

Until recently, the *MYH7* mutations causing Laing DM were confined to exons 32, 34, 35 and 36, all within the light meromyosin region of tail of the gene.[35] However, two recent mutations

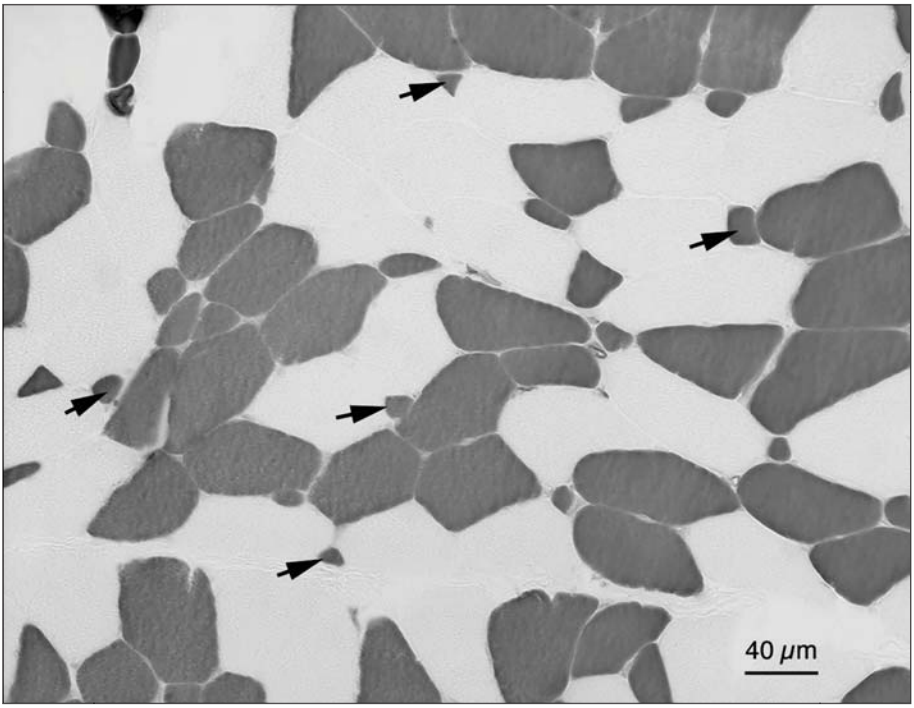

Figure 7. Hypoplastic type 1 muscle fibers (arrows) and lack of type 2B fibers in a 7 year-old boy with distal myopathy and cardiomyopathy associated with a *MYH7* mutation. ATPase pH 4.6.

fall outside of this region, in exons 16[34] and 14,[33] in the globular head of *MYH7*. Initially it was postulated that mutations within the tail of the myosin molecule disrupted the formation of the normal coiled coil, thus disrupting its function.[30] However, with the more recent discovery of mutations in the globular head also causing distal limb weakness, it may be that disruption of binding of myosin to titin, myomesin, or M-protein is playing more of a role.

Myosin Storage Myopathy

Myosin storage myopathy (MSM) is a rare myopathy with subsarcolemmal accumulation of eosinophilic, unstructured, hyaline material in type 1 muscle fibers. It was first described in 1971 as a "Familial myopathy with probable lysis of myofibrils in type I fibers".[36] In that report two siblings were described, a two year old boy and his five year old sister, with congenital muscle weakness and hypotonia. The weakness was general but mainly proximal with waddling gate and positive Gower sign. They had slightly increased levels of serum creatine kinase (s-CK). The parents and four other siblings were apparently unaffected and therefore an autosomal recessive pattern of inheritance was suspected. Muscle biopsy revealed type 1 fiber predominance and peripheral zones in type 1 fibers, which stained intensely for myofibrillar ATPase. With other enzyme-histochemical methods the peripheral zones were generally unstained. Electron microscopy revealed abrupt transition from regular sarcomeres to the peripheral zones that were composed of finely granular material. The authors suggested that there was accumulation of myofibrillar fragments in the abnormal areas of the muscle fibers which could be due to breakdown of myofibrils or a storage disorder.[36] In 1977 "A new congenital myopathy" characterized by subsarcolemmal bodies, which showed ATPase activity, was reported.[37] The patient was a 50-year-old woman with difficulties in walking and climbing stairs since childhood. She developed foot drop already at one year of age and from age 6 she had difficulties in combing her hair. She had a scapulo-peroneal distribution of muscle weakness, but she was also weak in pelvic muscles. S-CK levels were normal. The muscle biopsy demonstrated subsarcolemmal ATPase positive bodies in type 1 muscle fibers. By electron microscopy these bodies consisted of granular and filamentous material measuring 20-60 Å. Nuclei with prominent nucleoli were frequently present within the abnormal areas. It was concluded that the bodies had actomyosin-like properties. The authors suggested that the pathogenesis involved a maturational or developmental arrest rather than a degenerative process.[37] The subsarcolemmal bodies described in these two reports were later called hyaline bodies[38] and in some subsequent reports the disease was described as congenital myopathy with hyaline bodies[39] or hyaline body myopathy.[40-43] When it had been clarified that the accumulated material consisted of myosin and that the gene defect was in the slow/ß cardiac myosin heavy chain gene (*MYH7*), the disease was named myosin storage myopathy,[44] a term subsequently used in the literature.[1,45-49]

Clinical Features

The clinical manifestations have been described in some 40 patients with MSM.[36-37,39-49] Most of these patients were demonstrated to be heterozygous for a missense mutation in *MYH7*. There are no distinct clinical features common to all patients with MSM but some features appear frequently. High variability of the clinical expression may occur also within the same family.[42,49] Age at onset of symptoms is also highly variable. Affected individuals can occasionally present as floppy infants[36] but onset later in childhood is more common with signs and symptoms such as frequent falls, difficulties in running or climbing stairs, delayed motor milestones or a waddling gate.[37,39,41,43-44,48-49] Onset in adulthood has been reported in many cases and early symptoms have then been difficulties in running and problems lifting the arms, especially above shoulder level.[41,43] These are common symptoms in many patients and the distribution of muscle weakness is frequently scapuloperoneal with winging of scapulae and drop foot[37,40-43,49.] Other patients may show a mainly limb girdle muscle weakness.[36,39,45] Calf muscle pseudohypertrophy is a frequent finding. Kyphosis or scoliosis and respiratory insufficiency may supervene. The course is usually slowly progressive over many years but the course can also be nonprogressive or severely progressive.[42] There are seldom dysmorphic features or facial muscle involvement.

Cardiac involvement is typically not present, but a few patients have been described with cardiac hypertrophy that could possibly be attributed to other causes.[39,45,48] However, three siblings with MSM associated with hypertrophic cardiomyopathy and heart failure were described.[47] The onset of muscle weakness and cardiac disease were in late adolescence or adulthood and two of the siblings died in cardiorespiratory failure and hyaline bodies were identified in the cardiomyocytes. In this family the parents were second cousins and a homozygous mutation in *MYH7* was identified, indicating recessive mode of inheritance.

Serum creatine kinase (s-CK) levels are usually slightly elevated, but may be normal or moderately increased. The single case reported to have very high s-CK levels was one of the siblings who was homozygous for a *MYH7* mutation and also had cardiomyopathy.[47]

Muscle Morphology

The morphological hallmark of MSM is large subsarcolemmal accumulation of material that looks unstructured by light microscopy and hence has a hyaline appearance (Fig. 8a). In hematoxylin-eosin staining it appears light red and in Gomori-trichrome staining it turns light green. Enzyme-histochemical and immuno-histochemical stainings have indicated that the material consists of myosin, since it shows myosin ATPase activity and immunoreactivity to myosin antibodies (Fig. 8b). There is frequently increased staining of NADH-TR (Fig. 8c), as well as increased

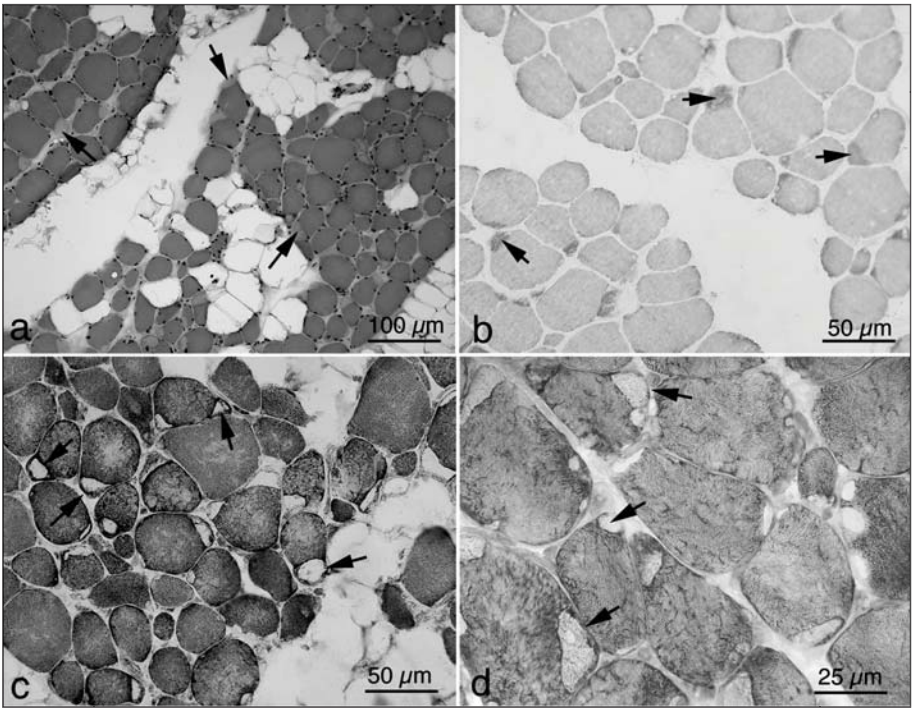

Figure 8. Muscle biopsy findings in a patient with myosin storage myopathy: a) Hematoxylin-eosin staining demonstrating muscle tissue partly replaced by fat tissue and subsarcolemmal hyaline structures in many muscle fibers (arrows). b) Immunostaining with an antibody against slow/β-cardiac myosin heavy chain demonstrating intense immuno-reactivity in subsarcolemmal inclusions (arrows) of type 1 muscle fibers. c) NADH-tetrazolium reductase staining demonstrating unstained subsarcolemmal inclusions surrounded by a rim of intense staining (arrows). d) Periodic acid and Schiff reagent (PAS) staining showing that the storage material is only faintly PAS-positive (arrows) and that the intermyofibrillar network is irregular in several fibers.

immunoreactivity of desmin and other sarcomeric proteins at the border of the inclusions. The accumulated material seems to be negative for most proteins except myosin and also other compounds such as ubiquitin, α-B-crystallin and glycogen (Fig. 8d).[42] There is usually type 1 fiber predominance but not type 1 fiber uniformity. The inclusions are restricted to type 1 fibers that are frequently smaller than the type 2 fibers.[36,37,42] The proportion of type 1 fibers showing inclusions may vary considerably from >95% to zero %.[42,49] There may be marked atrophy with replacement of muscle fibers by fat and connective tissue. In addition to the accumulation of myosin the intermyofibrillar network can be disorganized as revealed by staining of NADH-TR and periodic acid and Schiff reagent (PAS) (Fig. 8c and d) and also by electron microscopy. The accumulated material in MSM appears at the ultrastructural level as a granular substance. Electron-micrographs may also give the impression that there are abortive filaments present (Fig. 9).

Molecular Genetics

The so far identified mutations associated with myosin storage myopathy are located in the distal rod region of the MyHC, in or close to the so-called assembly competence domain.[50] MyHC dimers will not assemble into thick filaments in vitro unless this part of the molecule is present. Four mutations have been identified and they all cause the change of a conserved amino acid. The most common mutation appears to be Arg1845Trp, which has been identified in several unrelated patients from different parts of the world. In most instances the mutations associated with MSM are dominant, since they are present as single heterozygous mutations and several families show an apparent dominant mode of inheritance. In one family, in which the MSM was associated with lethal cardiomyopathy, affected siblings were homozygous for the mutation E1883K and the parents who were second cousins had no symptoms of muscle disease or cardiomyopathy.[47]

Myopathies Associated with *MYH8* Mutations

MYH8 encodes a perinatal form of myosin heavy chain found in immature muscle fibres as well as regenerating type 2c fibres.

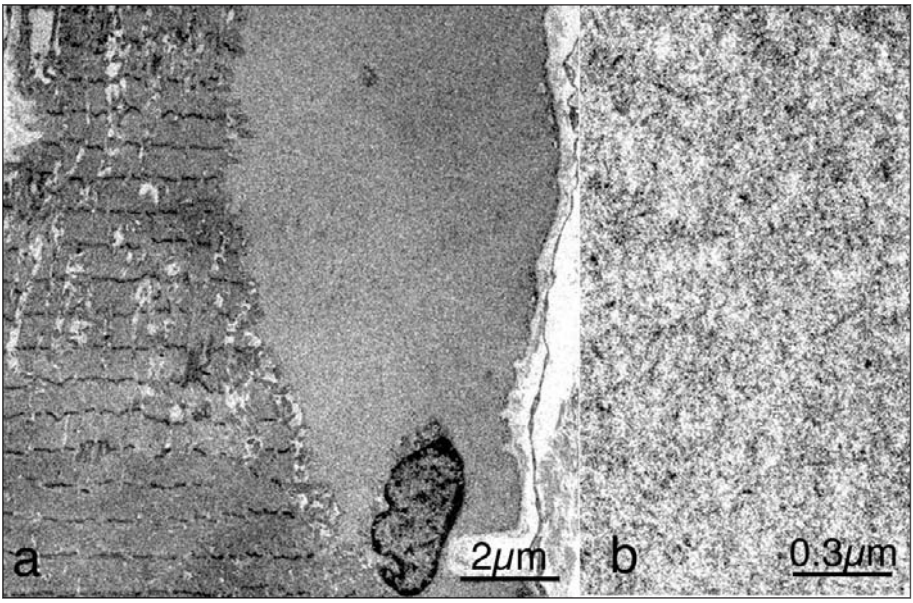

Figure 9. Electron-micrograph demonstrating the granular structure of the accumulated material in the subsarcolemmal region of a muscle fiber.

Trismus-Pseudocamptodactyly Syndrome

Clinical Phenotype

A *MYH8* mutation has been shown to cause a rare autosomal dominant form of distal arthrogryposis (DA7) called trismus-pseudocamptodactyly syndrome (TPS).[21,23] It is characterised by inability to fully open the mouth (trismus) and shortening of the long finger flexor tendons, so that there is involuntary flexion of the fingers when the wrists are extended (pseudocamptodactyly) (Fig. 10). Other reported features include short stature, small mouth, shortened hamstring muscles and equinovarus of the foot. However, there is significant phenotypic variation and all features are not usually present in every patient. Over 20 families with TPS have been described. In the family where the *MYH8* mutation was initially described, 3 of 19 members had the additional feature of cardiac myxomas. This was reported as a variant of the Carney complex, a syndrome with skin pigment abnormalities, endocrine tumours/overactivity, myxomas and schwannomas. However, further genetic studies of four large TPS kindreds, as well as 49 independent cases of Carney complex casts doubt on a shared pathogenesis between the two conditions.[21]

Molecular Genetics and Pathogenesis

To date only a single *MYH8* mutation, R674Q, has been reported to cause TPS. Haplotype analysis has confirmed that this mutation arose independently in North American and European TPS pedigrees.[21] The arginine residue affected by this mutation is conserved in all known human genes that encode myosin heavy chains and homologs of *MYH8* in a variety of species, suggesting that this arginine plays a crucial role in the normal function of myosin heavy chain 8. It has been suggested that it may disrupt the catalytic activity of myosin.[21] As *MYH8* is only expressed in the perinatal period, this disruption leads to nonprogressive contractures which appear prenatally.

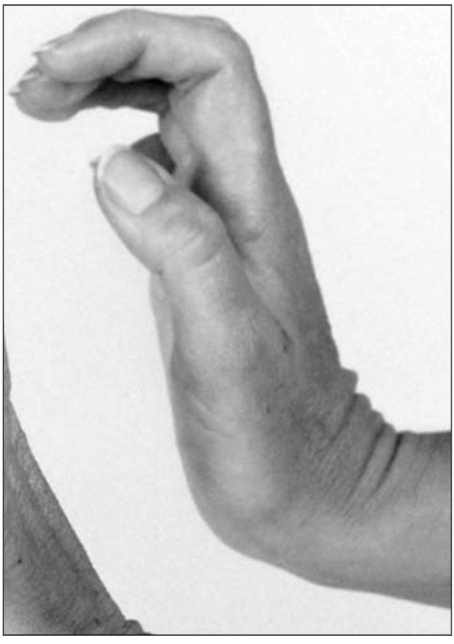

Figure 10. Pseudocamptodactyly: shortness of the long finger flexors leads to involuntary flexion of the fingers when the wrist is extended; this flexion is not present when the wrist is flexed or in the neutral position.

Future Perspectives, New Applications and Anticipated Developments

Considering the abundance (15-25% of total body protein) and importance of myosin for muscle function, it is likely that myosin mutations will be found to cause other muscle disorders that so far do not have a molecular diagnosis. Increasing the number of "myosinopathies" and also discovering further mutations within already identified genes, will guide investigation of the pathogenesis of these gene defects. It is fair to say that our understanding of the molecular pathophysiology of these disorders is just beginning. Clues to the etiology of myosin myopathies may be obtained by studying morphological changes in muscle biopsies, although sometimes these morphological changes are not present despite changes in the motor function of myosin.[32] Functional studies are useful, although many sarcomeric proteins are very large with multiple functions, making interpretation of such studies sometimes difficult. Ultimately, it is the molecular pathophysiology that will govern the type of therapeutic intervention likely to be of benefit.

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Chapter 8

Acute Quadriplegic Myopathy:
An Acquired "Myosinopathy"

Lars Larsson*

Abstract

Acquired neuromuscular disorders have been shown to be very common in critically ill patients receiving prolonged mechanical ventilation in the intensive care unit (ICU). Acute Quadriplegic Myopathy (AQM) is a specific acquired myopathy in ICU patients. Patients with AQM are characterized by severe muscle weakness and atrophy of spinal nerve innervated limb and trunk muscles, while cranial nerve innervated craniofacial muscles, sensory and cognitive functions are spared or less affected. The muscle weakness is associated with altered muscle membrane properties and a preferential loss of the motor protein myosin and myosin-associated thick filament proteins. Prolonged mechanical ventilation, muscle unloading, postsynaptic block of neuromuscular transmission, sepsis and systemic corticosteroid hormone treatment have been suggested as important triggering factors in AQM. However, the exact mechanisms underlying the loss of thick filament proteins are not known, though enhanced myofibrillar protein degradation in combination with a downregulation of protein synthesis at the transcriptional level play important roles.

Introduction

Acute Quadriplegic Myopathy (AQM) is considered a consequence of modern treatment in anesthesiology and intensive care. The first AQM case report was published three decades ago by MacFarlane and Rosenthal,[1] who described a case of acute quadriplegia affecting spinal nerve innervated muscles and with intact sensory, cognitive and craniofacial muscle function in a 24-year-old woman during treatment of status asthmaticus with mechanical ventilation, neuromuscular blocking agents and high systemic corticosteroid hormone doses. Besides AQM, this disease has been given a number of different descriptive titles, such as critical illness myopathy, thick filament myosin myopathy, acute myopathy in severe asthma and myopathy of intensive care.[2] AQM was initially regarded as a rare condition of limited clinical significance and the AQM diagnosis was for many years lumped together with muscle paralysis of neurogenic origin, such as critical illness polyneuropathy and Guillain-Barré syndrome. However, the muscle paralysis and severe muscle wasting in AQM has a primary myogenic origin. The inclusion of AQM among neuropathies is, in part, due to misinterpretations of electroneurographic and electromyographic recordings which may mimic neurogenic lesions due to altered muscle membrane excitability in AQM.[3-7] While acute quadriplegia in the ICU was initially thought to be a rare event, we now know that neuromuscular dysfunction is found in up to 30% of the general ICU population and 70-80% of certain sub-groups.[2,8-10] This potentially lethal condition prolongs the recovery of critical care patients, thereby, increasing the median ICU treatment costs three-fold per patient.[11,12] Additional

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substantial costs are associated with the subsequent extended rehabilitation requirements and drastically impaired quality of life for long durations after hospital discharge.[13,14]

Patients with AQM are typically diagnosed relatively late during ICU treatment, i.e., 2-3 weeks after ICU admission and exposure to causative agents. This is, at least in part, due to the frequent use of sedatives that may disguise the muscle paralysis. The slow weaning from the ventilator is frequently the first sign of AQM. In general, the prognosis of AQM is good, if the patients survive the primary disease, but the overall mortality (36-55%) is relatively high in this group of critically ill patients.[15] Complete recovery from the generalized muscle paralysis may occur within weeks, but most patients require intense rehabilitation for several months and a small number of patients with AQM do not become ambulatory. There are very few long-term follow up studies of AQM, but, recent studies on patients that have survived critical illness in the ICU have demonstrated persistent impairment in neuromuscular function 1-2 years after hospital discharge.[13,14]

A preferential loss of myosin and myosin associated proteins has been repeatedly documented in patients with AQM using electron microscopy, electrophoretic separation of myofibrillar proteins, enzyme- and immuno-cytochemistry.[16-19] Thin filament proteins are, on the other hand, spared or less affected than the thick filament proteins (Fig. 1). The preferential loss of myosin causes decreased force production at the muscle fibre level, as well as generalized muscle wasting and weakness by reducing the number of motor proteins interacting with the thick filament.[18] This results in decreased specific tension (force normalized to muscle fiber cross-sectional area) and altered cross-bridge cooperativity at low Ca^{2+} concentrations.[20] However, it is important to emphasize that the acute paralysis in AQM is primarily the consequence of an altered muscle membrane excitability.[3,4,6,7] Following the initial acute phase of AQM and during recovery, the myosin loss has a significant impact on muscle structure and function.

Widespread myosin loss has been considered to be essentially pathognomonic of AQM. Electrophoretic separation of myofibrillar proteins and measurement of myosin:actin ratios has therefore been used as a sensitive diagnostic method to detect ICU patients with AQM.[18] However, focal myosin loss has been reported in other disorders, such as dermatomyositis[2] and a dramatic preferential loss of myosin was recently observed in a patient with small cell lung cancer and cachexia.[21] Thus, a decreased myosin:actin ratio is not a pathognomonic finding in AQM, but distinguishing the muscle weakness/paralysis in patients with cancer cachexia from ICU patients with AQM should not impose a diagnostic problem due to differences in clinical history, phenotype, electroneurography and electromyography.

Underlying Mechanisms

With appreciation of the frequency of acquired myopathies in ICU patients, there has been growing interest in their risk factors and underlying pathophysiological mechanisms.[4,5,10,18,22-25] Mechanical ventilation, neuromuscular blockers, muscle unloading, sepsis, circulating active factors and/or corticosteroids have been proposed as triggering factors. The severe and rapid muscle wasting in patients with AQM has been suggested to be associated with activation of different proteolytic pathways, i.e., the ubiquitin-proteasome, cytoplasmic (calpain) and lysosomal (cathepsin) pathways.[2,10,22,25-27] Di Giovanni and coworkers[27] demonstrated that the muscle atrophy in AQM distinguished itself from neurogenic atrophy by showing a stronger upregulation of ubiquitin-dependent proteolysis, including the ubiquitin ligase, atrogin-1. In AQM, the proapoptotic transforming growth factor β (TGFβ), MAPK pathway, RAS cascades and cell cycle inhibitors were activated.[27] A model for muscle cell atrophy in AQM was proposed where TGFβ receptor activation, by stress response corticosteroid/RAS pathways, initiates constitutive intracellular signaling of the MAPK cascade in muscle, resulting in proteolysis and muscle wasting.[27] This pathway is not activated in neurogenic muscle atrophy or in inflammatory myopathies, such as dermatomyositis, polymyositis or inclusion body myositis.[27]

In contrast to the relatively large number of studies on muscle wasting and myofibrillar protein loss due to enhanced protein degradation in patients with AQM there are very few studies to date focusing on the impact of an altered transcriptional regulation of protein synthesis in patients

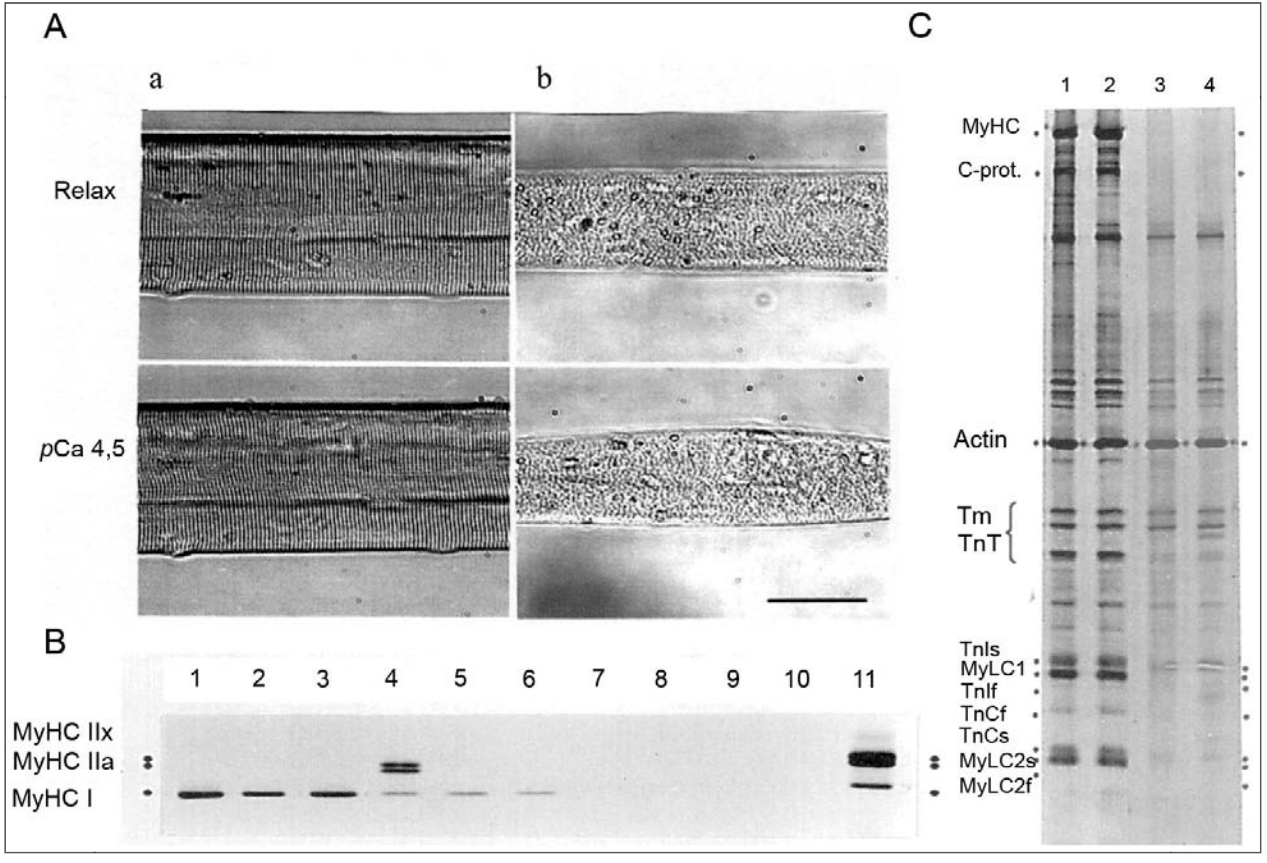

Figure 1. Legend viewed on following page.

Figure 1, viewed on previous page. Myofibrillar protein isoform composition. A) Chemically skinned single muscle cells from the tibialis anterior muscle from a normal control subject (a) and a quadriplegic patient (b) in relaxing solution (Relax.) and during maximum activation (*p*Ca 4.5). Scale bar, 50 μm. B) Electrophoretic separation of MyHC isoforms by 6% SDS-PAGE. MyHCs were separated from single tibialis anterior fiber segments (1-3, 5-8), bundles of 10 tibial anterior fibers (9, 10) and from single 10 μm cross-section from a vastus lateralis muscle biopsy (4, 11) expressing three MyHCs bands (types I, IIa and IIx). Lanes 7-10 are from the quadriplegic patient (lane 7 corresponds to fiber b in A) and the other lanes are from normal control subjects. C) Electrophoretic separation of thick- and thin-filament protein isoforms with 12% SDS-PAGE. Fibers 1-2 are from the tibialis anterior muscle of a patient with hemiparesis due to an upper motoneurone lesion. Lane 1 from the paretic side and lane 2 from the nonparetic normal side. Lanes 3 and 4 correspond to the fiber bundles from the quadriplegic patient, i.e., the same bundles as lanes 9 and 10 on the 6% SDS-PAGE (B).

with AQM.[18,28] This is surprising since mixed muscle proteins and MyHC synthesis rate decrease within hours in the slow-twitch soleus[29,30] and the diaphragm[31] in response to unloading caused by hindlimb suspension and mechanical ventilation, respectively. Protein synthesis is the result of multiple highly regulated events, including transcriptional and posttranscriptional regulation of protein synthesis. Results from independent studies indicate that myosin synthesis is regulated by posttranscriptional events during the first hours of reduced activity/unloading, while myosin synthesis regulated by transcription dominates at longer durations, i.e., several days-months.[31-33] In patients with AQM, approximately 2-3 weeks after ICU admission, a dramatic downregulation, or complete block, of all adult skeletal myosin heavy chain (MyHC) isoforms was observed at the mRNA level.[18,28] The transcriptional regulation of the thin filament protein actin was, on the other hand, normal or less affected according to in situ hybridization or realtime PCR experiments.[18,28] Months later, in parallel with recovery of muscle mass and function, transcriptional regulation of myosin synthesis returned to normal.[18,28]

Experimental Animal Models

The incomplete understanding of basic mechanisms underlying AQM in the clinical setting is in part due to the fact that basic distinctions between myopathy and neuropathy have often not been clearly made and the complex clinical, electrophysiological and histological abnormalities are often incompletely reported.[34] Diagnosis and classification has frequently been based on clinical observations and electrophysiological measurements, but both are weak diagnostic indicators.[18,35] The study of generalized muscle weakness is further complicated by the co-existence of more than one factor underlying muscle paralysis in the ICU patients. Different primary diseases, large variability in pharmacological treatment and collection of muscle samples several weeks after admission to the ICU and exposure to causative agents are other factors complicating mechanistic studies of AQM in the clinical setting. There is, accordingly, compelling need for an experimental animal model mimicking the ICU conditions, including long-term exposure to neuromuscular blockers, high-dose systemic corticosteroids, mechanical ventilation and muscle unloading.[36]

The prevailing AQM model used to date, is a rodent model where peripheral denervation of distal hindlimb muscles is combined with corticosteroids.[5] This model has proved useful in studies on the mechanisms underlying the altered muscle membrane properties in patients with AQM.[37,38] However, this model lacks several of the key features that occur in the ICU, such as muscle unloading, mechanical ventilation and exposure to neuromuscular blockers. Further, gene expression analysis between patients with AQM have demonstrated significant differences in the activation of proteolytic pathways in patients with neuropathies and AQM.[27] This clinical finding is supported by significant differences in myofibrillar protein and gene expression in response to peripheral denervation or long-term neuromuscular blockade in rats[39] and in the expression of transcription factors involved in myofibrillar protein synthesis between rats exposed to peripheral denervation and long-term neuromuscular block of distal hindlimb muscles.[40]

In an attempt to mimic the ICU condition, a large mammal porcine AQM model has been introduced.[41] The initial experiments using this model have been very encouraging and after five

days exposure to sepsis, neuromuscular blockers, corticosteroids and mechanical ventilation, pigs have demonstrated a clinical picture resembling the disease in ICU patients with AQM, i.e., a down-regulation of thick filament protein synthesis at the transcriptional level, intact sensory nerve conduction velocities and amplitudes and decreased compound muscle action potentials upon supramaximal stimulation of the motor nerve.[41] Encouraged by these initial results, a new series of pig experiments have been performed where pigs have been exposed to corticosteroids, neuromuscular blockers, or sepsis separately or in combination. A control group with mechanically ventilated animals receiving no other treatment was included. Preliminary results from these experiments demonstrate significant differences in gene expression and contractile properties with the most dramatic changes taking place in animals exposed to sepsis, neuromuscular blockers, corticosteroids, muscle unloading and mechanical ventilation for 5 days, i.e., a dramatic decrease in specific tension in spite of no signs of muscle fiber atrophy.[42]

The major advantage with the porcine model is the similar metabolism and protein turnover rate to humans, allowing drugs to be administered in similar concentrations as in patients and there is also reference material available from research on sepsis and mechanical ventilation where pigs have been used extensively over a number of years. However, the porcine AQM model also suffers from some major disadvantages such as very high costs, logistic problems and intrinsic limitations that have restricted the experiments to a maximum of five days. Myofibrillar proteins have a very slow turnover rate and changes in protein expression due to altered protein synthesis rates cannot be evaluated satisfactorily during a five day experimental period. Dworkin and colleagues have over a number of years developed an experimental rat model to study regulation of blood pressure.[43-46] In this model, rats are pharmacologically paralyzed postsynaptically and mechanically ventilated for long durations (several weeks), allowing detailed studies of both myofibrillar gene and protein expression. This model effectively simulates the ICU condition and it has recently been used in pilot studies on regulation of skeletal muscle myofibrillar protein synthesis and degradation.[47,48] In these studies, the different effects on spinal- and cranial nerve-innervated muscles were confirmed. In addition, significant differences were observed in myofibrillar gene and protein expression between the diaphragm and other spinal nerve innervated muscles, including other respiratory muscles as well as fast- and slow-twitch hindlimb muscles.[47,48] However, in order to improve our understanding of the mechanisms underlying the muscle wasting and paralysis in ICU patients, these studies will need to be expanded to include both detailed short- and long-term effects of mechanical ventilation, muscle unloading and exposure to neuromuscular blockers and corticosteroids, in combination and separately, on regulation of muscle contraction and myofibrillar protein synthesis and degradation.

Future Perspectives

The clinical awareness of the neuromuscular dysfunction and the presence of a specific myopathy in ICU patients, resulting in a partial or complete depletion of myosin and myosin-associated proteins, has increased significantly in the past years. However, our knowledge of mechanisms underlying AQM is still incomplete and interventions are limited to reduction of triggering factors (neuromuscular blockers and corticosteroids) and nutritional compensation for the negative protein balance.[49-52] The unraveling of the intracellular signaling pathways controlling myofibrillar protein synthesis and degradation in patients with AQM is an important future challenge. A detailed understanding of these complex pathways will allow for targeted pharmacological intervention strategies. In this context, the mechanical loading experiments performed by Griffiths and coworkers[53] are of specific interest, demonstrating positive effects on average muscle fiber cross-sectional area and protein loss in response to unilateral continuous passive movement for 3 hours per day during 7 days in mechanically ventilated and neuromuscular blocked critically ill ICU patients. The interpretative value of these observations are limited, as cautioned by the authors, by the relatively small number of subjects (n = 5) and variability in the severity of the critical illness between patients.[53] The experimental animal AQM models will allow detailed evaluation of both short- and long-term effects of pharmacological and mechanical loading intervention strategies on skeletal muscle structure and function as well as on myofibrillar protein synthesis and degradation.

Acknowledgements

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Chapter 9

Third Filament Diseases

Bjarne Udd*

Abstract

The backbone of the third filament system of the sarcomere is the huge titin molecule, spanning from the sarcomeric Z-disc to the M-line. Proteins in direct interaction and functionally integrated with titin, such as calpain 3 and telethonin, are part of the third filament system. The third filament system provides support to the contractile filament systems during development and mature states including mechanical properties and regulatory signaling functions. The first mutations in the third filament system causing human muscle disease were identified in calpain 3 in 1995, followed by telethonin and titin. In spite of some early ideas on what is going wrong in the muscle cells based on the defective proteins, the exact molecular pathomechanisms leading to muscle atrophy in patients with these disorders are still unknown. However, preparations for direct trials of gene therapy have already been launched, at least for calpainopathy.

Introduction

The term 'third filament system' was introduced by the researchers working on titin in the 1980's. It was clear that the highly ordered actin-myosin structure and the return of these filaments to their original positions after rounds of contractions of the sarcomere needed an elastic scaffolding ruler for the assembly, maintenance and positioning of thick filaments. Titin with its repetitive modular structure and unchallenged length over the half sarcomere was the ideal molecule to account for these properties (Fig. 1A).[1-4]

Titin

Studies with antibodies to different epitopes along the giant protein revealed that the A-band portion of titin remained in place during contraction whereas the I-band portion was moving, providing the functional properties of an elastic spring.[5] About 100 kDa of titin's N-terminus is fixed in the Z-disk of the sarcomere. The next 1,500-kDa region constitutes the elastic portion in the I-band of skeletal muscle and another 1,750 kDa part is used for the multiple interactions of titin with myosin through myosin binding protein C (MYBP-C) in the A-band. Finally, the C-terminal 250 kDa of titin is located in the M-line of the sarcomere, with less modular and more complex sequence properties, including a kinase domain, interaction domains for myomesin and unique sequence regions is1-is7 with unknown functions (Fig. 1A,B).[6] These features, together with later discoveries of titin interacting regulatory proteins in the M-line, N2A-line and Z-disk regions, indicated essential signaling functions for titin.[7,8] Recently, the kinase domain in M-line titin was identified as a sensor of mechanical contraction activity in the myofiber with a physically interacting complex of signaling proteins (Fig. 1).[9] This signaling complex includes MURF2, which in the normal state of muscle activity stays in the complex in the M-line. However, if muscle activity is blocked chemically or by denervation, MURF2 dissociates from the complex and shuttles to

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The Sarcomere and Skeletal Muscle Disease, edited by Nigel G. Laing.

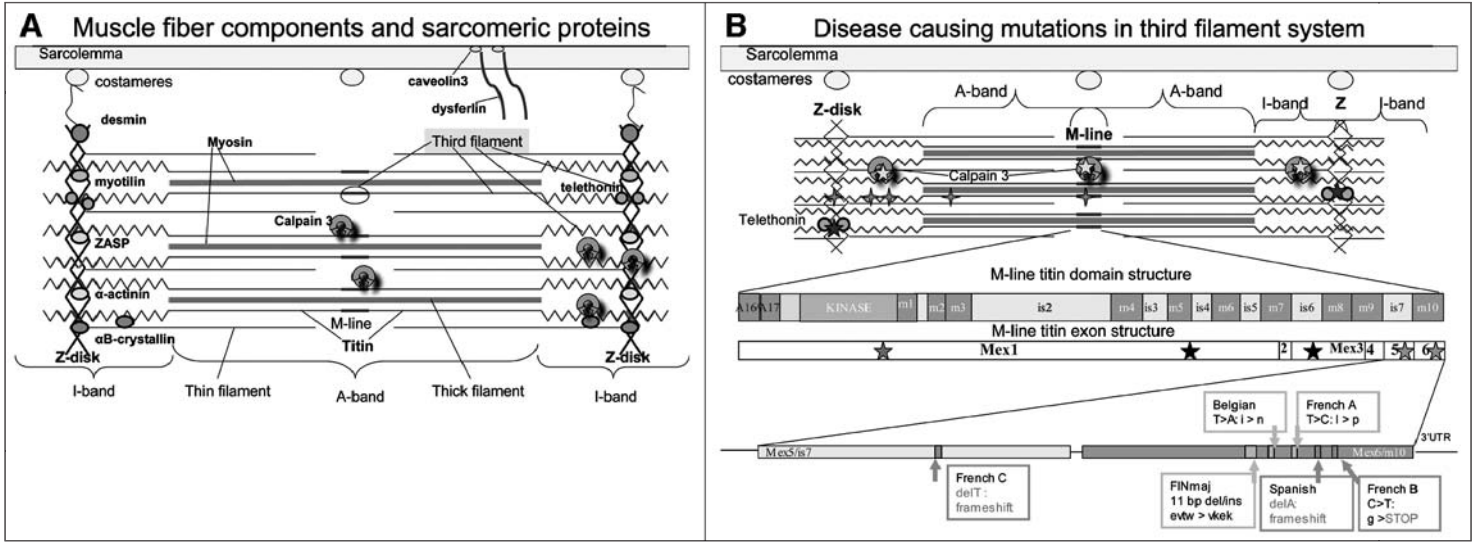

Figure 1. Schematic drawing of sarcomere structure with emphasis on proteins involved in the third filament system (A). In (B) the same schematic sarcomere structure detailed for mutations in the third filament system known to cause human disease. With magenta simple stars: mutations in titin causing cardiomyopathies. Yellow star: mutations in calpain 3. Blue star: mutations in telethonin. Red star: mutation in the kinase domain of C-terminal titin associated with HMERF disease. Black stars: mutations in C-terminal titin exons Mex1 and Mex3 causing severe muscular dystrophy and lethal cardiomyopathy. Green stars: mutations in C-terminal titin causing TMD/LGMD2J titinopathy (B). A color version of this figure is available online at www.eurekah.com.

the nucleus where it inhibits the activity of the transcription factor SRF.[9] M-line titin apparently has important signaling functions for regulation of transcription and turnover in relation to the highly variable demands in skeletal muscle.

Titins from both halves of the sarcomere meet and overlap in the M-line center and titins from adjacent sarcomeres also overlap in the Z-disk (Fig. 1A). Serially coupled titin molecules thus form a continuous filament system from sarcomere to sarcomere over the whole length of the muscle fiber (Fig. 1A). Titin is the largest single polypeptide in nature. During myofibrillogenesis titin is expressed before the other filament systems of the sarcomere. However, titin is subjected to extensive alternative splicing particularly in the I–band region but also in the M-line.[10] Longer I-band isoforms (up to 3700 kDa) are present in skeletal muscle compared to cardiac muscle (2970–3300 kDa). In the Z-disk, cardiac titins contain more Z-repeat motifs than skeletal titins.[11,12] Close to the PEVK region in the I-band, titin contains an N2-A element in skeletal muscle, while all cardiac titins express an N2-B and some also an N2-A element.[13] Of these, N2-B isoforms are stiffer.[14] A C-terminally truncated 700 kDa isoform of titin is also expressed in striated muscle.[10] The C-terminal M-line region of titin is expressed in at least two different splice isoforms: with or without the second last exon, Mex5 encoding the is7 region and the proportions of the Mex5 + and Mex5—isoforms vary in different skeletal muscles.[2,6,12] The exact role and the different properties of these isoforms are not well known, but shifts in expression of isoforms have been related to pathological conditions, i.e., heart failure.[15] More recent studies suggest a role in protein metabolism, compartmentalization of metabolic enzymes, binding of chaperones and positioning of the membrane systems of the T-tubules and sarcoplasmic reticulum.[16]

Titin Binding Proteins

Telethonin (or T-cap) is a ligand of titin in the Z-disk and caps the N-terminus of titin (Fig. 1A). Calpain 3 has multiple interaction sites with titin, the main portion being tied up with titin in the N2A region of I-band and another portion located in the M-line interacting with the C-terminus of titin. Recently, interaction of N-terminal calpain 3 with central parts of alpha-actinin was confirmed (Fig. 1A).[17] Other examples of well established ligands of titin are alpha-actinin, CARPs, MYBP-C, MuRF1, FHL-2/DRAL and myomesin, but no primary genetic defects are currently known in these proteins in the context of human skeletal muscle disease.

Third Filament Diseases

The first human disease defects in this third filament system were published in 1995, when mutations in calpain 3 were shown to cause a recessive limb-girdle muscular dystrophy (LGMD2A).[18] A similar phenotype, recessive limb-girdle muscular dystrophy LGMD2G, was the result of mutated telethonin.[19] In 2002, a number of mutations in titin were published. Mutations in the I-band and A-band regions of titin were associated with dominant cardiomyopathy.[20,21] In the same year, mutations in the last domain of the titin C-terminus were reported in complex genotype-phenotype relationships; heterozygote mutation carriers developed a mild late onset distal myopathy, whereas rare homozygotes for one of the mutations developed a severe early onset limb-girdle muscular dystrophy, LGMD2J.[22] Recently, new mutations in the kinase domain and other parts of C-terminal titin have been associated with complex muscle disease phenotypes.[9,23]

Titinopathies

Cardiomyopathies

Genetic titin defects underlying human muscle disease were first reported with dilated cardiomyopathy (DCM). A frameshift mutation in A-band titin was identified in a German family.[20] In another family, a tryptophan > arginine missense mutation was detected in the Z-disc/I-band transition zone.[20] The mutation was predicted to disrupt a highly conserved protein sequence in this region. Two different Z-disc titin mutations were reported to affect binding affinities of titin to telethonin and alpha-actinin.[21] Two other mutations were located in the cardiac specific N2-B region of titin, one of them, a nonsense mutation, presumably leading to a non-functional

truncated titin.[21] Hypertrophic cardiomyopathy in single patients has been reported to be possibly associated with mutations in Z-disc titin.[24,25]

The causal relationship between observed titin mutations and a disease is not easy to determine. Recently a frameshift mutation in A-band titin was reported to cause dominant DCM.[26] In this case the mutant transcript was present in skeletal muscle but no mutant protein was detected. Since the mutation was not fully penetrant, the causal relationship is not definite. Nonsense mediated decay (NMD) of the mutant transcript would result in most of the titin protein being normal and a minor amount of mutant transcript may not have produced sufficient amounts of harmful mutant protein to cause a phenotype. Some of the cardiomyopathy mutations are located in the cardiac titin isoform specific N2B region and were suggested to alter binding to the signaling protein FHL-2/DRAL (Fig. 1B).[27]

On the whole, patients with mutations causing truncated titins in the Z-disk, I- and A-band regions of titin seem to develop autosomal dominant cardiomyopathy and heart failure with variable age of onset, usually without clinically overt skeletal muscle disease.

Skeletal Muscle Titinopathies—TMD and LGMD2J

Mutations in C-terminal titin (Fig. 1B) were identified to be responsible both for a milder distal myopathy, tibial muscular dystrophy (TMD, OMIM #600334, Udd myopathy) and for a more severe recessive limb-girdle phenotype (LGMD2J).[22,28]

TMD and LGMD2J Clinical Phenotype

In TMD, ankle dorsiflexion weakness due to atrophy of the anterior tibial muscle typically starts after age 35, progressing during the next 10-20 years to the long toe extensor muscles with moderate foot drop (Fig. 2).[28] At onset, symptoms may be asymmetric. After age 70, patients may have mild to moderate proximal leg muscle weakness, but they rarely become wheelchair-bound even after age 85. Sparing of the extensor digitorum brevis muscles is an important clinical clue. Unlike Welander distal myopathy (WDM), hand muscles are rarely affected in TMD.[29] A recent follow-up study of 207 mutation confirmed patients showed variants of this phenotype in 9% of the patients.[30]

Epidemiology

The currently known number of patients with TMD in Finland correlates to a prevalence of 10/100 000,[30] and the calculated total number of patients indicates a true prevalence of 20/100 000. TMD in descendants of Finnish immigrants has been diagnosed in Sweden, Norway, Germany and Canada. TMD families in other populations, without any genealogical connections to Finland, have so far been identified in France, Belgium and Spain,[31,32 manuscript submitted] and there is no reason to believe that titin mutations should be restricted to certain populations.

Laboratory Findings and Muscle Pathology

Patients with TMD have normal or mildly elevated CK.[29] In affected muscles EMG shows low-amplitude, short-duration motor unit potentials on moderate activity.[29] Increased insertional activity, frequent fibrillation potentials and occasional high-frequency and complex repetitive discharges at rest may be recorded. Clinically unaffected muscles are normal or show some polyphasic potentials.[29]

Muscle imaging provides exact information on the highly selective involvement of individual muscles in TMD, i.e., anterior compartment muscles. Fatty degeneration on imaging appears in parallel with clinical weakness.[33] By imaging, lesions in soleus, medial gastrocnemius, hamstrings, gluteus minimus and tensor fasciae latae muscles are easily detected, following the later stages of disease evolution (Fig. 3).[33]

Muscle biopsy in TMD shows variation of fiber size, thin atrophic fibers, central nuclei, structural changes within the fibers, endomysial fibrosis and fatty replacement in the end stage muscle,[34] whereas necrotic fibers are rare. Both major fiber types are involved in the pathological process (Fig. 4). In the target muscle tibialis anterior the pathology is rimmed vacuolar, however,

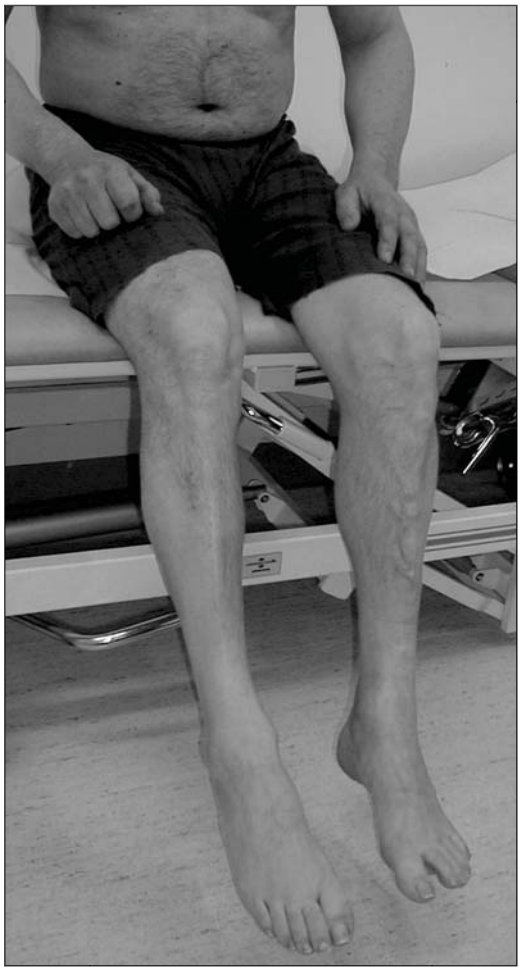

Figure 2. Legs of a 45 year old male patient with the FINmaj mutation in C-terminal titin causing TMD titinopathy (Udd myopathy), showing clear atrophy of the anterior compartment muscles, with a subsequent prominent edge of the tibia. Note also mild foot drop and normal upper limbs.

without congophilia or amyloid present by immunohistochemistry. Ultrastructural studies in TMD showed well preserved sarcomere structure, even in the homozygote LGMD2J muscle.[29] Rarely tubulofilamentous inclusions were encountered in the vacuolated fibers.[29] Rimmed vacuoles in TMD are usually not membrane bound.

Molecular Genetics

TMD/LGMD2J was linked to chromosome 2q31,[35,36] and mutations causing the disease have been identified in the last two domains of titin, the last Ig-like M10 domain and the second last serine rich unique sequence region is7.[22] These domains are encoded by the last (Mex6) and second last (Mex5) exons of the gene, respectively (Fig. 1B). The founder mutation in Finnish patients, FINmaj, is a complex in-frame del/ins of 11 consecutive base pairs exchanging four amino acids

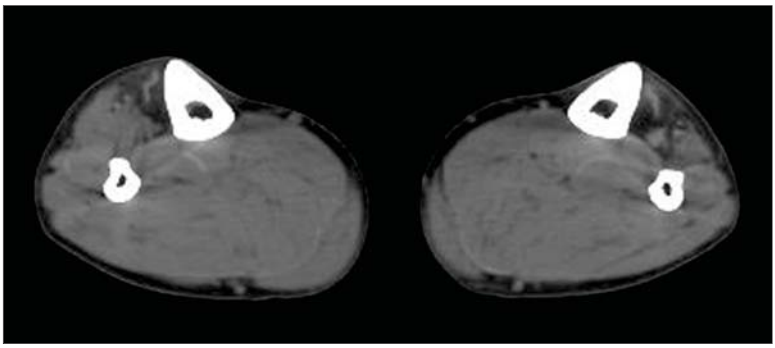

Figure 3. CT scans of lower leg muscles in a 50 year old male patient with FINmaj TMD mutation showing selective total fatty replacement of tibialis anterior muscles on both sides, severe change in extensors hallucis longus and digitorum longus on the left side and early changes only in these muscles on the right side. Normal appearance of the other lower leg muscles.

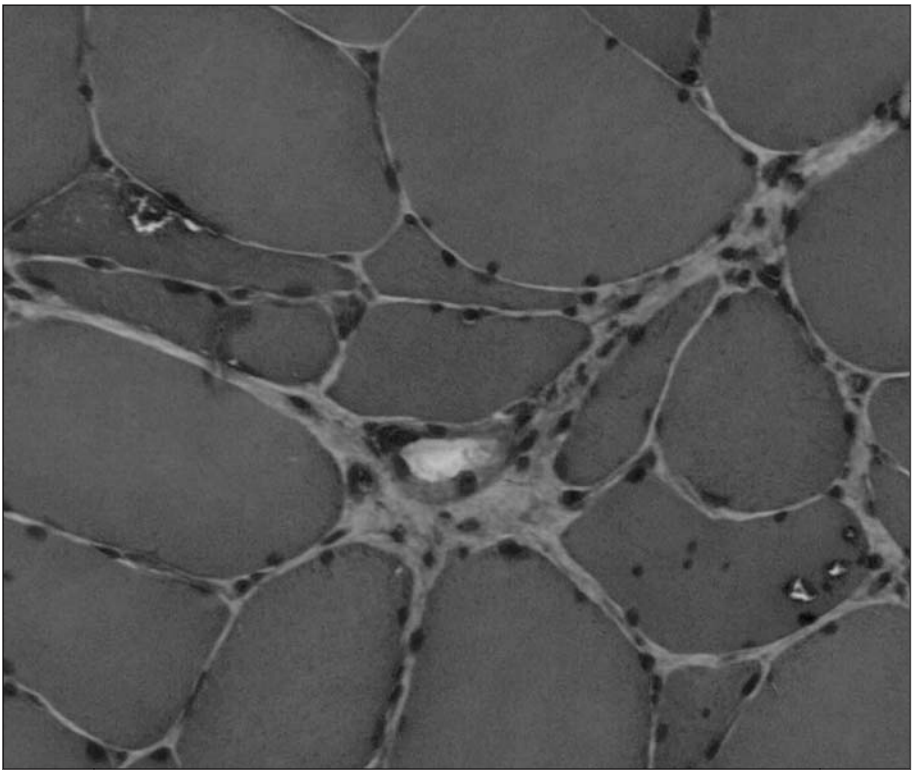

Figure 4. Hematoxylin and eosin stained tibial anterior muscle biopsy from early stages of the disease in a 40 year old TMD patient with FINmaj mutation showing large variation of fiber size, some increased internal nuclei, connective tissue and atrophic fibers with rimmed vacuolar change.

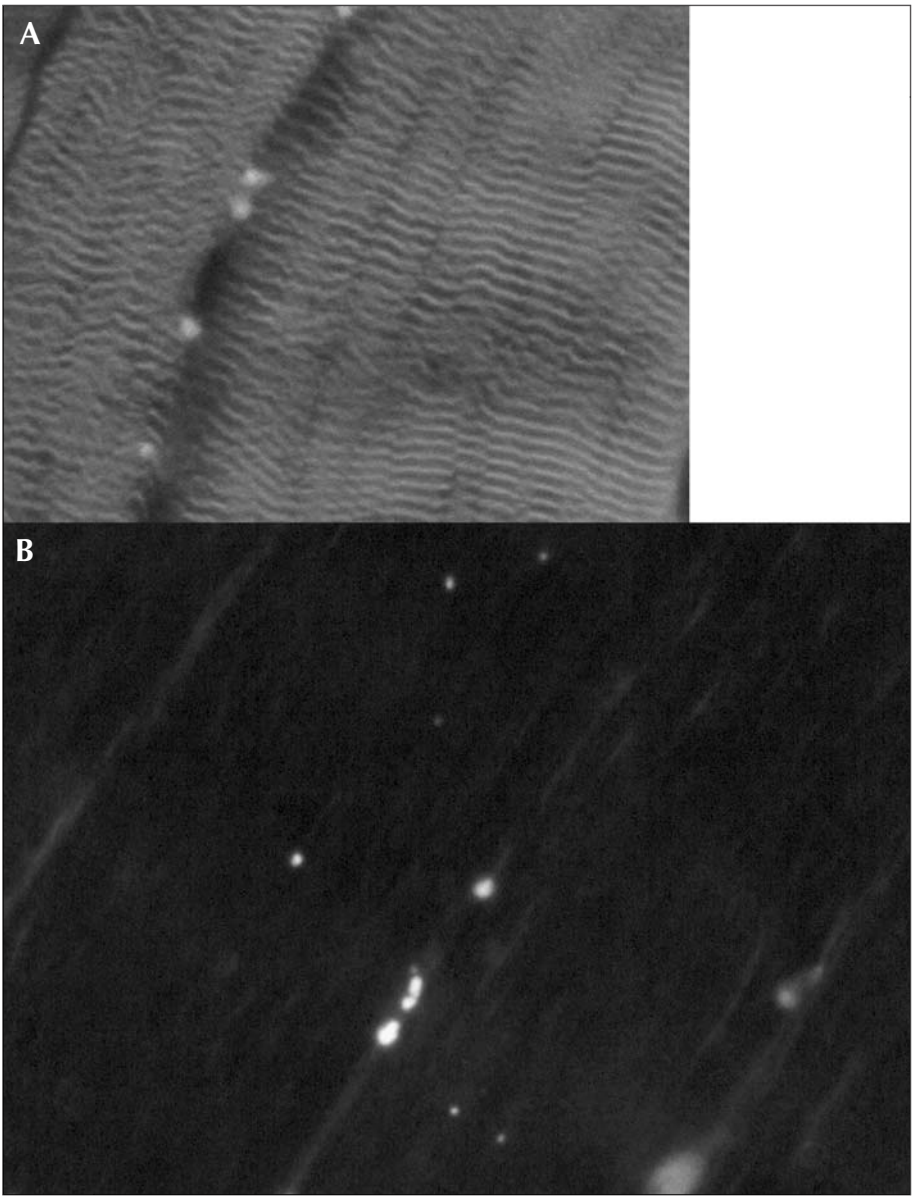

Figure 5. Titin epitope in domain M9 specific monoclonal antibody T51 immunofluorescence imaging in cortrol muscle showing regular specific M-line titin striation (A). The same T51 titin antibody applied to homozygote FINmaj mutant LGMD2J muscle shows complete absence of epitope recognition (B).

in M10.[22] The protein, however, is altered far beyond M10 since epitopes in the third last domain M9 are not recognized by specific antibodies in the homozygote muscle.[22] (Fig 5A,B).

In two unrelated French families, an identical missense point mutation changing a lysine to proline was found in the same Mex6 exon.[22,31] The third mutation in the last exon, also a missense

mutation, was identified in a Belgian TMD family.[32] Yet unpublished mutations, also in the last Mex6 exon, have been identified in Spanish and other French families.[manuscript submitted] These mutations are available for diagnostic testing. In new unrelated TMD patients searching for mutations by sequencing the last exons may be productive.

Molecular Pathomechanism of C-Terminal Titinopathy—TMD

As stated above, even in homozygous mutant muscle the overall sarcomere structure is intact on electron microscopy, suggesting no major mechanical disruption caused by the mutations.[29] The downstream chain of molecular events is, however, not yet defined. It is likely to be complex, because in the few rare patients homozygous for the FINmaj mutation the phenotype is completely different: early childhood onset proximal limb-girdle muscular dystrophy with loss of ambulation around age 25 (LGMD2J).[28,30] In TMD, the muscle involvement is focal, with initial selective targeting of the tibial anterior muscles, whereas in LGMD2J the dystrophy is generalized with fatty replacement in all large muscles already at the age when clinical symptoms with weakness of ankle dorsiflexion start to emerge in the heterozygous TMD.[30] This complete difference may suggest that the altered downstream molecular pathways are partly different in TMD vs. LGMD2J.

The only well established ligand of C-terminal titin is muscle specific calpain 3, responsible for LGMD2A when mutated.[18,37] In homozygous LGMD2J, but not in heterozygote TMD muscle, calpain 3 is severely reduced,[38] indicating that some other disrupted protein interaction may mediate pathology later in life in the heterozygotes. The myosin heavy chain tail region has been shown to interact with titin,[39] which is of mechanistic interest because mutations in the slow-skeletal/beta cardiac myosin heavy chain gene (*MYH7*), close to the region indicated to interact with titin, cause Laing distal myopathy with similar distribution of affected muscles, although with much earlier onset.[40] However, attempts to identify an *MYH7* interacting region on titin have so far failed. A direct interaction between the KY protein, deficient in the spontaneous kyphoscoliosis mouse mutant and the last M10 domain of titin was suggested based on yeast two-hybrid data. However, recent studies indicate there is no true interaction.[41] Nuclear envelope lamin A/C has also been reported to interact with the M10 domain of titin C-terminus, but this interaction has not been confirmed by others.[42]

Hereditary Myopathy with Early Respiratory Failure (HMERF)

Clinical Phenotype

HMERF is an autosomal dominant muscle disease first described in 1990 by Edstrom.[43] Patients have proximal weakness of the upper and lower extremities, early affection of neck flexors and respiratory muscles, especially the diaphragm. Ankle dorsiflexor weakness may occur and the mean age of onset is around 35 years. The disorder is progressive and patients may be wheelchair-bound 10-20 years after onset. Respiratory deficiency may be the presenting sign and patients require assisted ventilation. CK levels are normal or slightly elevated. Muscle biopsy findings revealed myofibrillar changes which, at the light microscopic level, included plaques that stained strongly with rhodamine-conjugated phalloidin, a marker for F-actin.[43] At the ultrastructural level, these bodies were seen to be composed of thin filaments and dense material mostly related to the Z-disks. Electron microscopy showed radical overall myofibrillar derangement with gradual loss of the normal sarcomere structure in late stages.[43] Early changes were abnormal Z-disks and actin aggregates, with dissolving Z-disks, I-bands and M-line structures, whereas myosin thick filaments remained preserved until very late (Fig. 6A). Later all components of the sarcomere were lost into unstructured homogeneous material before complete dissolution (Fig. 6B).

Molecular Genetics

A genome-wide screen on two large unrelated families originating from mid-Sweden mapped the disease locus to chromosome 2q24-31.[44] One French family, also with similar clinical phenotype and muscle biopsy features, was mapped to a close but different region, on 2q21.[45] Among candidate genes, titin was the strongest positional and functional candidate. A heterozygote CGG>TGG

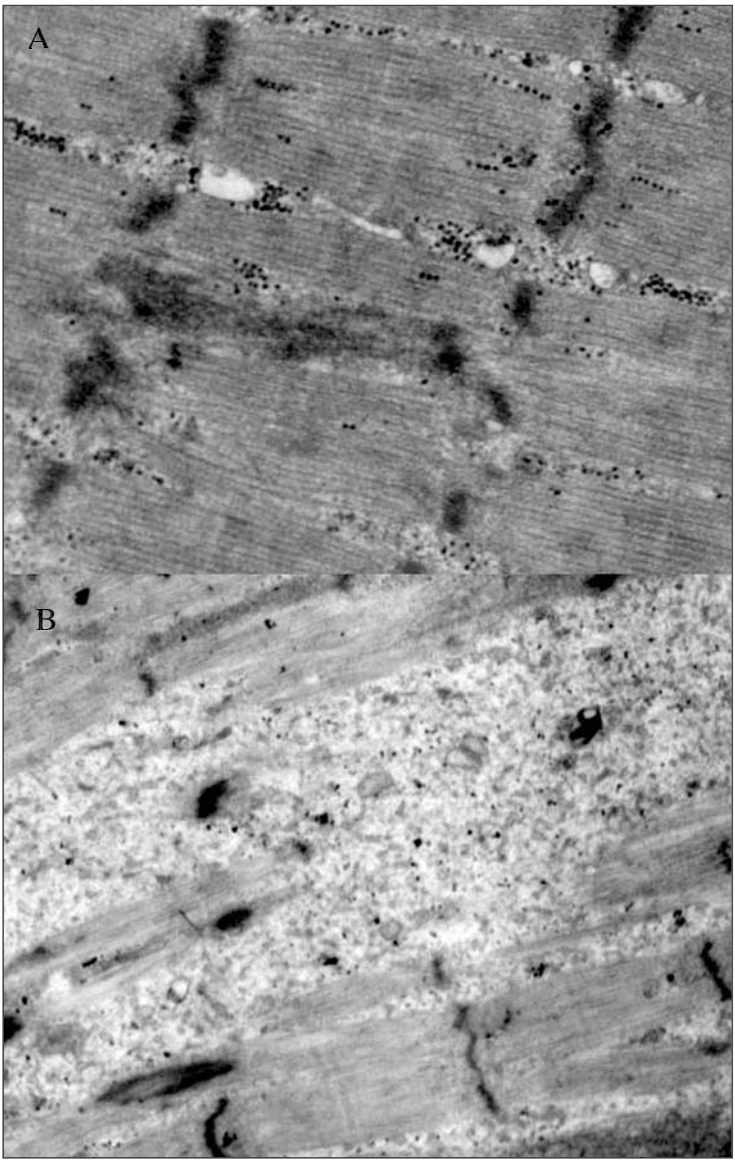

Figure 6. Electron micrographs of a patient with HMERF disease caused by mutation in the regulatory tail of the kinase domain in C-terminal titin showing early sarcomeric disorganization in a small part of the myofilament (A). At a later stage of the disease process all sarcomeric structures are in complete dissolution (B).

change at position 286133 in exon Mex1, leading to a Arg279Trp change (numbered according to kinase domain residues) of a conserved residue of the alpha-R1 helix in the regulatory tail of the kinase domain of C-terminal titin (Fig. 7), showed complete co-segregation with the disease and with a likewise segregating core haplotype, in the two families.[9] Another Swedish patient with identical phenotype but without known genealogical relation to either of the two original families

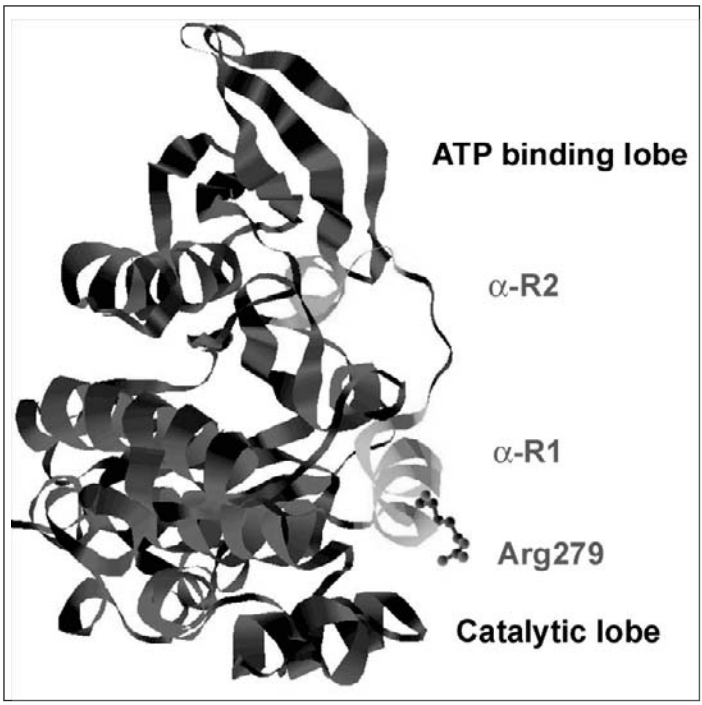

Figure 7. 3-D structure of the kinase domain with the first alpha-helix of the regulatory tail of the enzyme in light blue and the Arginine (R) in domain position 279 indicated in yellow which is changed to bulky tryptophane (W) in HMERF disease. Courtesy of Dr. Gautel.

was found to have the same mutation on the same haplotype indicating a common ancestry with the other two families.[9]

Molecular Pathomechanism

The alpha-R1 helix in the regulatory tail of the kinase contains a calmodulin-binding site in which P7, a peptide corresponding to most of the autoinhibitory domain, displays the highest affinity for CaM.[46,47] The interaction of calmodulin with P7 is dependent on specifically spaced charged and hydrophobic residues in the kinase, contributing to the intrasteric regulation of its activity.[46,47] The results of a band-shift assay showed increased calmodulin affinity with the mutant peptide (unpublished data). However, the increase in calmodulin affinity did not translate into any significant change in the activity of the mutant kinase enzyme.[9] Besides calmodulin, interaction of nbr1 with titin occurs at exactly the same alpha-helix R1. The HMERF-mutation totally abolished the interaction between nbr1 and the kinase,[9] shown also in transfection assays in non-muscle cells where wild-type kinase co-localised with nbr1, whereas the RW-mutant kinase did not.[9] The protein complex of nbr1 with p60 and MURF2 is disrupted by the HMERF mutation, leading to accumulation of p60 in many diseased muscle fibres of the HMERF patients and nuclear localisation of the transcriptional regulator MURF2 in centralised nuclei.[9] Actin showed bright aggregates and longitudinal accumulations (Fig. 8), whereas telethonin, alpha-actinin, obscurin and myomesin did not accumulate with actin. However, in fibres with Z-disk pathology, telethonin showed corresponding severe irregularities of Z-disk labelling. A titin antibody directed against the kinase domain showed normal sarcomeric labelling in non-disrupted sarcomeres.

In an experimental model of atrophy associated myofibrillar protein degradation, MURF1 and another ubiquitin ligase, atrogin-1, were shown to be highly upregulated.[48] Multiple links to serum

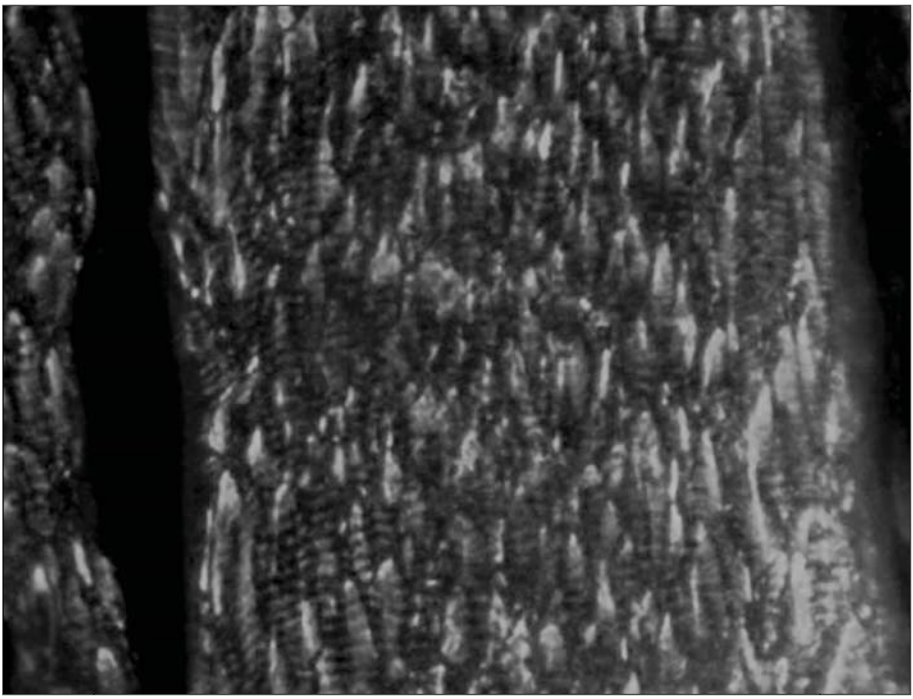

Figure 8. Anti-actin antibody immunofluorescence of muscle biopsy in HMERF disease showing increased longitudinal aggregation of actin over several sarcomeres as a result of sarcomere disintegration.

response factor (SRF) have been implied in regulating muscle growth and adaptation as well as in actin dynamics.[49-51] Downregulation of muscle gene expression can be caused by nuclear location of MURF2 in denervation models, through repression of SRF-driven gene expression.[9]

In HMERF, primary myofibril assembly seems not to be affected. The severe pathology starts in adulthood, indicating an activity-related defect in turnover or maintenance. The importance of tight control of kinase function for accurate turnover is suggested by the fact that in HMERF a heterozygous point mutation is pathogenic, despite an otherwise catalytically active enzyme and intact M-line titin. It is intriguing that most reported cardiomyopathy patients with titin mutations lack skeletal muscle weakness and that TMD/LGMD2J and HMERF patients with titin mutations suffer from muscle weakness without manifestation of heart failure. How this is regulated is not clear, but one possibility is the expression of different isoforms of MURF and titin or other components of the M-line in cardiac and skeletal muscle.

Autosomal Recessive Severe Myopathy and Lethal Cardiomyopathy

Clinical Phenotype

In two families originating from Sudan[52] and Marocco altogether five children showed delayed motor milestones during the first year of life with walking skills developing only between 2 and 4 years.[23] In early childhood they had generalized muscle weakness predominantly in lower limb muscles. Psoas, gluteus maximus, tibialis anterior and peroneus muscles were particularly weak, whereas quadriceps was less affected. Upper limbs, neck and facial muscles were weak including ptosis. Despite moderate joint and neck contractures, muscle function remained relatively stable with difficulties for climbing stairs, running and rising up from the sitting position. Meanwhile,

a progressive DCM with arrhythmia developed in all the patients at the ages of 5 to 12 years. Echocardiography showed reduced left ventricle (LV) ejection fraction, dilatation and global hypokinesia, without wall hypertrophy.[23] Heart failure associated with ventricular or supraventricular arrhythmias caused sudden death of four patients between 8 and 19.5 years. One developed scoliosis after age 11. Serum CK levels were marginally to moderately increased (1.5 to 7 times the upper normal values) and respiratory function showed restrictive pattern (FVC 63% to 76%) without clinical symptoms.[23] The parents examined at age 45 to 58 years were healthy and show no symptom or sign of heart or skeletal muscle dysfunction. Muscle MRI of two parents at 44 and 55 years showed normal muscle.[23]

Muscle biopsy of the patients showed some irregularities of oxidative enzymes and central nucleation but no overt necrosis. Endomysial connective tissue was increased and regenerating fibers were few.

Ultrastructural studies showed some sarcomere disruption devoid of mitochondria with wide M-lines and less Z-disk streaming. Left ventricle cryosections showed marked disruption of myocardial architecture, with severe endomysial fibrosis.[23]

MolecularGenetics

One family had a homozygous deletion of one base in exon 360 (Mex 3; g.291297delA) changing 21 amino acids followed by a stop codon, truncating 447 amino acids (Fig. 1B). The other family had an 8-bp deletion in exon 358 (Mex 1; g.289385_289392delACCAAGTG) with a premature stop codon, 8 exchanged amino acids and loss of the last 808 C-terminal residues downstream of the kinase domain (Fig. 1B).[23]

Molecular Pathomechanism

On the protein level, as expected because of the truncation of the CAPN3-interacting C-terminal domain is7, there was total absence of calpain 3.[23] This emphasizes the importance of calpain 3 binding in the M-line domain is7 (encoded by Mex 5) for the overall regulation of calpain 3 in the muscle cell and corroborates previous data that titin stabilizes calpain 3 from autolytic degradation. Loss of calpain 3 in these patients could be part of the skeletal myopathy but is unlikely to account for the severe cardiomyopathy, since calpain 3 is not expressed in mature heart muscle.

Interestingly, the heterozygous parents have no TMD phenotype, in contrast to the heterozygous mutations in Mex5 and Mex6. The most likely explanation for this is that the frameshift generated stop codons in both mutations induce nonsense mediated decay (NMD) of the mutant transcripts. In the heterozyogotes this would be favorable as almost all translated titin would be normal titin and the heterozygotes would escape harmful effects of the mutation. In the homozygote patients mutant titin protein is present, implying that NMD is not complete. A highly reduced supply of titin could mediate the rapidly evolving cardiomyopathy.

Another possibility would be loss of cardiac specific functions and interactions of titin domains is6 and M8, because in TMD/LGMD2J the protein is disrupted over domains M9-is7-M10 without causing a dilated cardiomyopathy phenotype.

Calpainopathy

Calpainopathy or LGMD2A (OMIM #253600) is caused by mutations in the *CAPN3* gene,[18] coding for calpain 3, a calcium-dependent cysteine protease. Calpain 3 belongs to the family of calpain proteases and shares with the other calpains a structure with an N-terminal domain I, proteolytic domains IIa and IIb, a C2-like domain III and a calcium binding domain IV. It differs from the other calpains by three specific sequences: NS, IS1 and IS2. Calpain 3 is almost exclusively expressed in skeletal muscle where it exists in a complex with titin in an inactive state. Calpain 3 is activated by intramolecular autolysis at a specific site in the IS1 region and requires further intermolecular proteolysis at other sites in IS1.[53,54] Activated calpain 3 probably has a large number of substrates, some of which have been identified in vitro*:* titin, filamin C, talin, ezrin and vinexin.[53] Binding of calpain 3 to titin at the I-band N2A element and in the M-line prevents

the autolytic processes to take place. Calpain 3 has many phosphorylation sites and some of the protein is phosphorylated in vivo, but so far no kinase and no mechanistic consequences have been determined.

Clinical Phenotype and Diagnosis

Calpainopathy is an autosomal recessive disease and considered a frequent form of LGMD,[54] but the proportion of calpainopathy compared to other recessive LGMDs may vary in different populations. Calpainopathy shows symmetric selective atrophy of the pelvic, scapular and trunk muscles especially in the gluteus maximus and thigh adductors, elevated serum creatine kinase and dystrophic features on muscle biopsies (Fig. 9).[56] The age at onset ranges from 2 to 40 years and severe disability with loss of ambulance usually occurs at 20 to 30 years after onset of symptoms. Contractures and facial weakness occur in some patients in late stages. Heart muscle is not affected in calpainopathy. On muscle biopsy, general dystrophic features characterized by the presence of

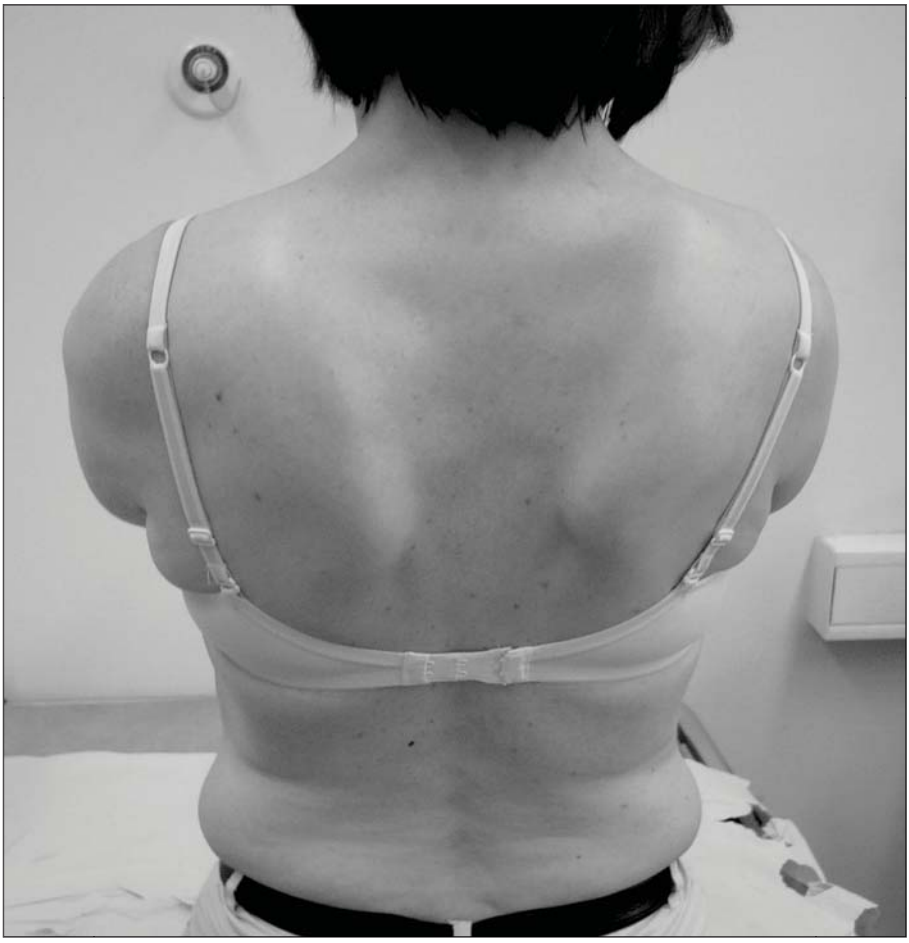

Figure 9. Clinical aspect of calpainopathy LGMD2A showing scapular winging and muscle atrophies in the shoulder girdle region in a 50 year old woman with disease onset at age 25 and barely preserved walking ability.

active necrosis and regeneration are observed and in the more chronic state, increased presence of lobulated fibers.[56]

Because the clinical phenotype is variable, the diagnosis of calpainopathy is based on protein analysis and mutation detection. Western blotting of muscle samples has remained the basic screening strategy, in spite of the fact that some pathogenic mutations do not reduce calpain 3 expression. At least eight missense mutations are known to go with normal protein amounts: T184M, G222R, G496R,[57,58] R489Q, R490Q and R490W,[59] S606L,[60] R110X/G222R.[61] Another complexity with protein analysis in calpainopathy is the relatively frequent situation of secondary reduction of calpain 3. This is well established for LGMD2B (dysferlinopathy),[57] LGMD2J (titinopathy)[38] and LGMD2I (FKRP deficiency),[62] but may also occur in other yet undetermined conditions.

Molecular Genetics and Pathomechanisms

Almost 300 mutations of all types have been described worldwide, distributed along the entire length of the gene.[63] Mutation analysis is the gold standard for definitive diagnosis of calpainopathy, however, sequencing of all the 24 exons in *CAPN3* gene for mutations is time consuming and expensive. Moreover mutations in the promoter region or in introns, as well as large deletions will not be identified by exon sequencing.[64] Many mutations are unique and only some represent founder mutations accounting for larger numbers of patients.[62] The mechanism of molecular pathology has been related to loss of function and also missense mutations may lead to loss of calpain 3 proteolytic activity.[65] However, later studies have shown that about 30% of the mutations do not decrease the proteolytic activity.[63]

Binding of calpain 3 to titin in different locations in the sarcomere and its autolytic activation without this binding suggests a tightly controlled regulation of its activity through the interaction. The phenotypic similarity between primary calpainopathy LGMD2A and secondary calpain deficiency in homozygosity for C-terminal titin mutations in LGMD2J also underline the importance of the complex for the integrity of the muscle fiber. Titin was shown to be one substrate in vitro and the key function of calpain 3 is supposed to mediate constant and controlled remodeling of the sarcomere protein machinery. Absence of calpain 3 mediated regulated cleavage of sarcomeric substrates would then lead to abnormal sarcomeres, impairment contractile capacity and muscle fiber damage.[66]

A proteomics approach comparing transgenic calpain 3 overexpressing and normal mice identified both metabolic and myofibrillar constituents as possible substrates of calpain 3. In vitro studies implied myosin light chain 1 (MLC1) as one substrate. These studies support the role of calpain 3 in sarcomere remodeling, but may also suggest a role in mitochondrial protein turnover.[67] Whether these results are connected to the early findings of clustered apoptotic nuclei in both LGMD2A and LGMD2J suggesting involvement of apoptosis in the progressive loss of muscle fibers remains to be clarified.[38,68] Another unresolved aspect of calpain 3 is the lack of its expression in mature heart muscle prompting the question what functions are essential for skeletal muscle but dispensable in heart muscle.

Telethoninopathy

The other name of the telethonin protein, T-cap, indicates its function as capping titin molecules in the periphery of Z-disks. Telethonin may thus be connected to the third filament functions rather than characterized by its location to the Z-disk region (Fig. 1A). Telethonin is shown to be a substrate of the serine-threonine kinase domain in C-terminal titin.[46] In mature muscle these locations are physically distant, suggesting either shuttling of telethonin or phosphorylation regulation only temporarily during myofibrillogenesis, when C-terminal titin and the kinase are located in emerging Z-disk regions. No further molecular pathomechanisms are detailed on the effects of mutations in telethonin reported to cause a proximal LGMD2G phenotype. Some of the patients have more distal atrophies and weakness at onset.[69] The myopathology of LGMD2G is rimmed vacuolar, but in the myofibrillar myopathies caused by mutated Z-disk proteins, the protein aggregations of desmin, myotilin etc. do not contain telethonin, suggesting that telethonin is more integrated in third filament functions.

After identification of the LGMD2G mutation, other telethonin mutations have been associated with cardiomyopathy without skeletal myopathy.[70-72]

Future Developments

Our current understanding of the molecular pathophysiology of these disorders is still highly incomplete and only emerging. Many sarcomere proteins are very large, with multiple functions and hypotheses on how defects in very large proteins, such as titin, could be overcome by therapeutic interventions are limited. In disorders with dominant gain of function and accumulations of protein as the pathological hallmark, silencing of the mutant gene with small interfering RNA molecules might be one option. In TMD this might also work since the heterozygotes with truncating mutations in Mex1 and Mex3 have no disease. On the other hand TMD, in most cases, is mild with normal lifespan, suggesting that complicated therapies may not be the first choice. In calpainopathy the situation is different with loss of function as the main alteration. Even though there is no mechanistic explanation for which functions in the muscle cell are misregulated because of the calpain 3 defect, the first steps for human gene therapy trials are already on their way. These trials will be of high value irrespective of the immediate outcome.

Beyond the exciting future for therapeutic interventions, the third filament system is far from completely understood and many more disorders will be directly linked to defects in this system.

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Chapter 10

The Z-Disk Diseases

Duygu Selcen* and Olli Carpén

Abstract

Recent studies have identified disease-causing mutations in four genes that encode Z-disk proteins. Mutations in myotilin (*MYOT*), ZASP and filamin C (*FLNC*) encoding genes cause autosomal dominant myopathy that manifests in adulthood. The clinical features and morphological changes in myopathies caused by mutations in all three genes are highly similar. The disease typically manifests as distal myopathy, but may also affect proximal muscles and the heart. The morphological findings are typical of myofibrillar myopathy (MFM) and include Z-disk alterations and aggregation of dense filamentous material visible in trichrome staining. The disease mechanism is still unclear, but may involve structural alterations of the Z-disk caused by dysfunctional proteins or their abnormal accumulation due to defective degradation. Although the fourth gene product, telethonin, is also involved in the Z-disk organization, its mutations cause a different phenotype. Telethonin mutations result in recessive muscular dystrophy, which manifests in childhood as proximal weakness. The morphologic alterations caused by telethonin mutations are not well characterized, but may share common features of MFM. Future work aims at understanding the pathophysiology of Z-disk related disorders and identification of novel genetic defects in patients with morphological features of MFM.

Introduction

The Z-disk is a structural unit, which aligns the thin filaments and links the sarcomere to the sarcolemma. In addition to structural functions, it is also involved in stretch sensing and signal transduction. The Z-disk contains a large variety of proteins, which may either be restricted to this structure or may span a larger region of the sarcomere (e.g., titin and nebulin). Z-disk abnormalities are detected in a variety of muscle disorders. These changes may be secondary or may result from alterations in core Z-disk proteins. This chapter describes diseases associated with four Z-disk components, myotilin, ZASP, filamin C and telethonin.

Clinical Findings

Myotilinopathy

In 2000, a Thr57Ile mutation in myotilin was detected in a large kinship that had previously been linked to the myotilin locus at 5q31.[1] In this kinship, proximal leg and arm muscle weakness appeared in the third decade of life, distal muscle weakness became apparent later in the course of the disease. Tight heel cords and a nasal dysarthric speech were frequently observed. The serum CK level was normal to 15-fold elevated. Some patients had hypoactive tendon reflexes. Two years later, a second kinship with a similar phenotype, at this point defined as limb girdle muscular dystrophy 1A (LGMD1A), was found to have a Ser55Phe mutation in myotilin.[2]

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The Sarcomere and Skeletal Muscle Disease, edited by Nigel G. Laing.

Since, myofibrillar myopathies (MFM) are characterized by distinct morphologic alterations that typically begin at the Z-disk and because myotilin is a Z-disk component, myotilin was screened for mutations in the Mayo Clinic cohort of patients whose muscle specimens showed MFM pathology. In 2004, this resulted in the identification of four mutations in six unrelated patients,[3] followed by detection of myotilin mutations in two other patients. The mean age of onset was 60 years. In three patients the weakness was more prominent distally than proximally. Cardiac involvement without signs of coronary artery disease was evident in three patients. Peripheral nerve involvement, reflected by clinical, EMG and histologic criteria, or a combination of these, was apparent in all patients. Subsequent studies by other investigators identified additional patients with mutations in myotilin. These patients also had progressive weakness of proximal and/or distal limb muscles, dysarthric, nasal speech, hypoactive tendon reflexes, and respiratory failure. Dominant inheritance, cardiomyopathy and intrafamily phenotypic variability were present in some kinships.[4-7]

All myotilin mutations reported to date are heterozygous missense amino acid changes and all but one mutation fall in *MYOT* exon 2.[1-6,8]

Zaspopathy

Zaspopathy involving skeletal muscle was first described in 2005 by Selcen and Engel[9] in 11 MFM patients who carried heterozygous missense mutations in *ZASP*. The mean age of onset was in 6th decade. Most patients presented with muscle weakness but one patient whose father had muscle weakness presented with palpitations and mild hyperCKemia. Seven of 11 patients had family histories consistent with autosomal dominant inheritance. The weakness was more prominent distally than proximally in 5 and distal only in 1. Two patients had only proximal muscle weakness. The three remaining patients had both proximal and distal muscle weakness.

Cardiac involvement without signs of coronary artery disease was present in three patients. In one of these, cardiac symptoms antedated the muscle weakness by 10 years. Peripheral nerve involvement by clinical, EMG, or histologic criteria was detected in 5 patients.

The mutations detected by Selcen and Engel[9] were Ala147Thr and Ala165Val in exon 6 and Arg268Cys in exon 9. The Ala165Val mutation is within and the A147T mutation is immediately before, the ZM motif needed for interaction with α-actinin.[10] Each mutated residue is conserved in the mouse.

Subsequently, a large kinship, originally described by Markesbery et al[11] as well as five other kinships with distal myopathy and MFM pathology were shown to carry the same Ala165Val mutation in *ZASP*. These six kinships and three of the zaspopathy kinships observed at the Mayo Clinic may have a common founder.[12]

Filaminopathy

In 2005, a dominant Trp2710X mutation in the last exon of filamin C was detected in 17 affected individuals of a large German kinship.[13] Eight examined patients presented between 37 and 57 years of age with slowly progressive distal anterior or posterior or both anterior and posterior leg muscle weakness. The serum CK level was elevated up to 8-fold. Four patients had signs of respiratory insufficiency, one had an incomplete right bundle branch block and three also had signs of a peripheral neuropathy. Subsequently, three MFM kinships carrying the same mutation were identified in the Mayo MFM cohort (Selcen, unpublished observation). The age of onset and the clinical presentation were similar to those reported by Vorgerd and coworkers.

Telethoninopathy

In 1997, Moreira et al described a kinship under the rubric of autosomal recessive limb girdle dystrophy, with linkage to chromosome 17q11-12.[14] Two recessive telethonin mutations in this kinship were discovered in 2000 and the disorder was defined as LGMD2G.[15] The mean age of onset was 12.5 years. The patients presented with difficulty walking, running and climbing stairs and could not dorsiflex their feet. They had both proximal and distal muscle weakness with sparing of the extraocular and facial muscles. Hypertrophy of the calf muscles but atrophy of other muscles, foot drop and hypoactive tendon reflexes were common features. Four of the six observed

patients became wheelchair-bound between the ages of 27 and 39 years. The serum CK level was elevated three to 30 fold. Cardiac involvement was observed in three of the six patients. Recently, telethoninopathy was also identified in three Chinese patients.[16] They also presented in the first or second decade of life, had proximal and distal weakness, foot drop and calf hypertrophy.

Morphologic Findings

Myotilinopathy, zaspopathy and filaminopathy display the classical MFM features.[3-5,9,12,13,17-19] In trichrome stained sections, the abnormal fibers harbor an admixture of amorphous, granular, or hyaline deposits that vary in shape and size and are dark blue or blue red in color (Fig. 1A, D and G). Many abnormal fiber regions and especially the hyaline structures, are devoid of oxidative enzyme activity (Fig. 1H). Some hyaline structures are intensely congophilic (Fig. 1K). Some muscle fibers harbor small vacuoles containing membranous material.

Signs of denervation, consisting of groups of atrophic fibers composed of fibers of either histochemical type and increased reactivity of atrophic fibers for nonspecific esterase (Fig. 1L) are observed in some patients. Abnormal accumulation of multiple proteins, some at the ectopic sites, including myotilin (Fig. 1B), αB-crystallin (Fig. 1F), dystrophin (Fig. 1J), sarcoglycans, neural cell adhesion molecule (NCAM), desmin (Fig 1E), plectin, gelsolin (Fig. 1I), ubiquitin (Fig. 1C) and filamin C are present.[3,4,9,13,19] In addition, abnormal and/or ectopic accumulation of other proteins were recently documented in myotilinopathy muscle specimens. These include: phospho-tau, actin, amyloid βA4, clusterin;[4] a mutant nonfunctional ubiquitin and p62, a multimeric signal protein;[20] and glycoxidation and lipoxidation markers, neuronal inducible and endothelial nitric oxide synthases and superoxide dismutase.[21]

Electron microscopy reveals that the earliest pathologic alterations in MFM characteristically occur at the Z-disk (Fig. 2). These changes consist of streaks of dense material (arrows in Fig. 3B) or of less dense material dappled with spots of denser material (asterisks in Fig. 3A,B) emanating from the Z-disks. In fiber regions in which the Z-disks have disintegrated, the sarcomeres fall apart and myofibrils are no longer recognizable. The dislocated membranous organelles and glycogen accumulate in clusters in spaces vacated by disintegrating myofibrils (Fig. 4). At a still more advanced stage, large fiber regions harbor fragmented filaments and Z-disk remnants that aggregate into pleomorphic inclusions. In other fiber regions, the degraded and fragmented filaments accumulate in hyaline structures of variable electron density that entrain glycogen granules (Fig. 5A,B). The dislocated membranous organelles are trapped and degraded in autophagic vacuoles (Fig. 6) that can undergo exocytosis.

In telethoninopathy, a muscle specimen of one patient showed abnormal variation in fiber size, increased internal nuclei and scattered necrotic and regenerating fibers. Rimmed vacuoles were detected in numerous fibers. There was no fiber type predominance or fiber grouping. Telethonin was absent from the muscle specimens by immunohistochemical and Western blot criteria.[15,16] Extensive immunocytochemical and ultrastructural studies were not performed. A published frozen section image, however, suggests multiple abnormal cytoplasmic areas that could be compatible with MFM pathology (Fig. 1A, ref. 14).

Z-Disk Disease Genes and Pathophysiology of Z-Disk Diseases

Myotilin

All published disease-causing mutations are missense mutations, which result in a single residue substitution.

Gene and Protein

The *myotilin* gene is located in chromosome 5q31[22] and is composed of 10 exons. There is no evidence for alternative splicing. The gene encodes a 498 amino acid protein with molecular size of 57 kDa. Myotilin is composed of a unique, serine-rich amino-terminus and a carboxy-terminus that contains two Ig-like domains.[22] All published mutations reside towards the amino-terminus, between residues 36 and 95, a sequence of unknown structure and function. The mutations (K36E,

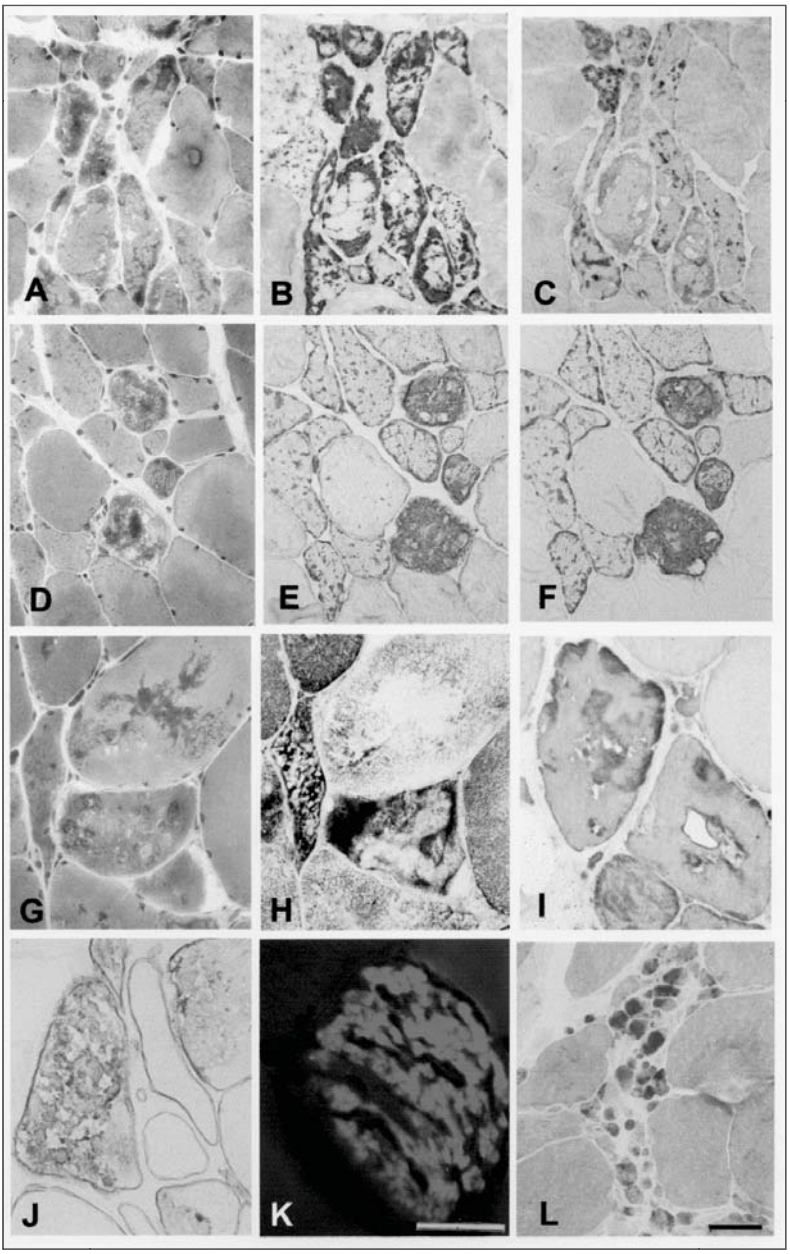

Figure 1. Histochemical and immunocytochemical features of a myotilinopathy specimen. Note accumulation of myotilin (B), ubiquitin (C), desmin (E), αB-crystallin (F), gelsolin (I) and dystrophin (J) and sharply circumscribed decreases of NADH dehydrogenase enzyme activity (H) in fiber regions that appear abnormal in serial trichrome sections (A, D and G). Serial sections for gelsolin (I) and dystrophin (J) are not shown. Intensely fluorescent congophilic deposits are associated with many hyaline deposits (K). Grouped atrophic fibers overreact for nonspecific esterase (L). Bar in L (also applies to A through J) and in K = 50 μm. (From ref. 3, with permission).

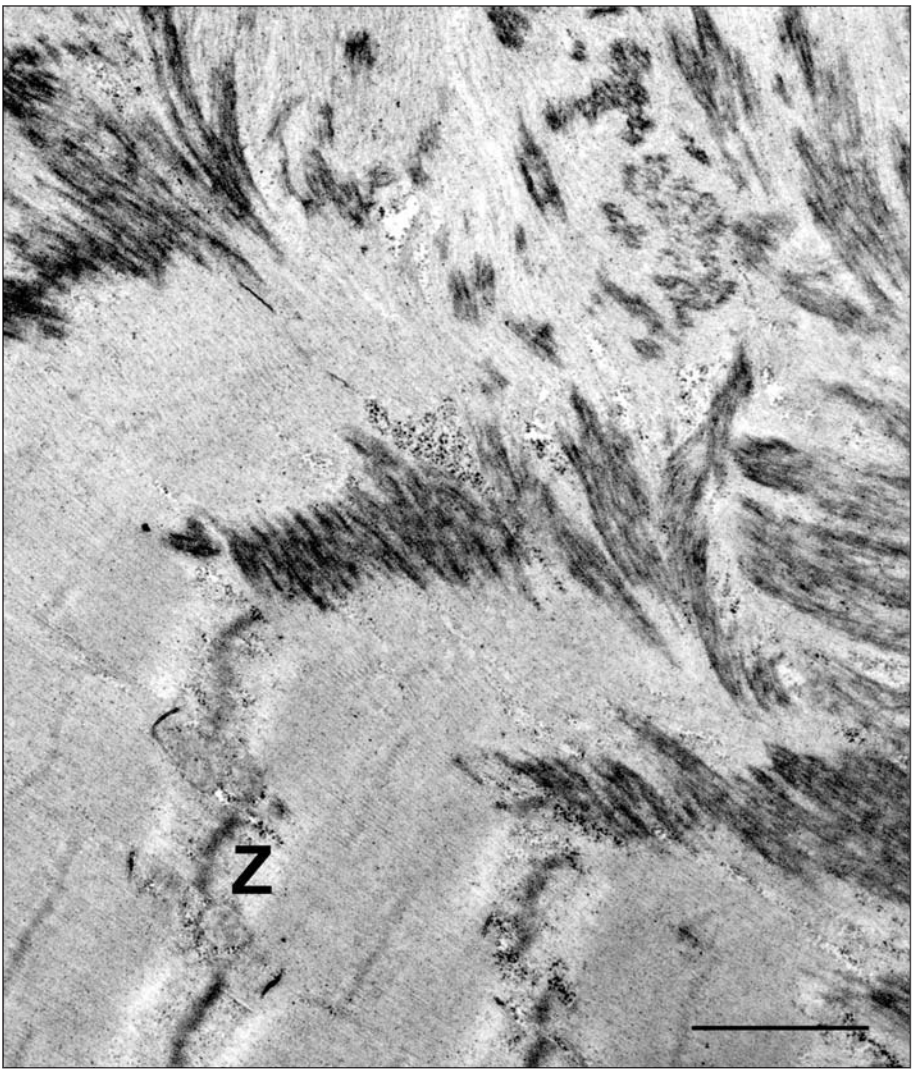

Figure 2. Normal Z-disks are replaced by stripes of dense material. The myofibrils are out of register. Z = Z-disk. Bar = 1 μm. (From ref. 19, with permission).

S39F, S55F, T57I, S60C, S60F, Q74K, S95I) either change a potential serine/threonine kinase substrate or affect the charge of the polypeptide. Expression of myotilin is restricted, with highest expression in skeletal muscle, moderate expression in heart and peripheral nerves and little or no expression in other tissues.[22] However, during mouse embryonic development, a much wider expression pattern is seen.[23] Animal studies have suggested that loss of myotilin by targeted deletion does not lead to obvious abnormalities and the morphology and strength of skeletal muscle is unaffected.[24] The only marked change is upregulation of telethonin at both the transcriptional and protein expression level. On the other hand, transgenic mice expressing a disease-causing mutant myotilin, T57I, reproduce the morphological (light microscopy, EM, immunohistochemistry) and functional features of human myotilinopathy.[25] Morphological changes are evident after 6-8

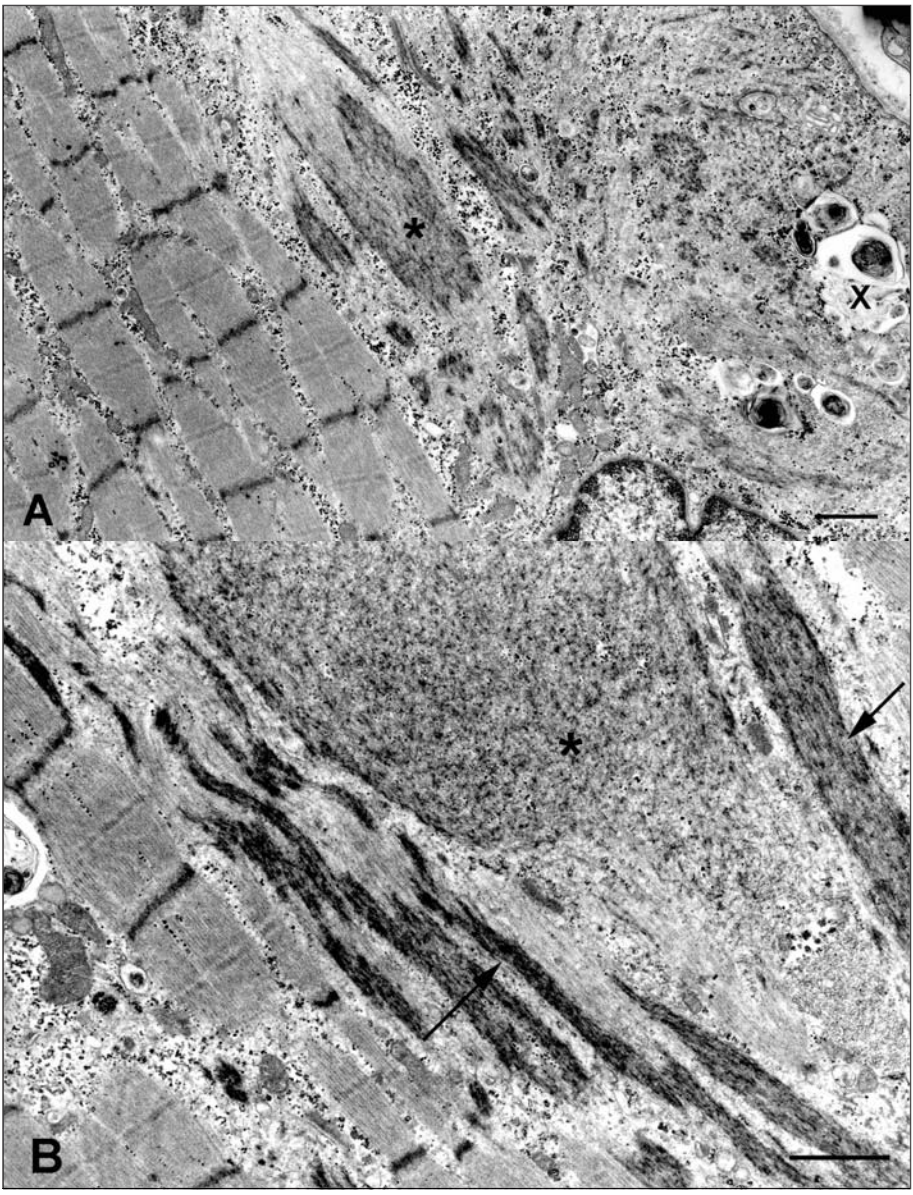

Figure 3. Streaks of dense material (arrows in B) and less-dense material are interspersed with dappled small spots of dense material (asterisks in A and B) of Z-disk origin. Degraded material is accumulating in small autophagic vacuoles (X in A). Bars = 1 μm. (From ref. 3, with permission).

months. Studies with genetically modified mice thus indicate that mutant myotilin is more harmful than loss of myotilin, whose functions may be compensated by related members of the protein family, palladin and/or myopalladin.

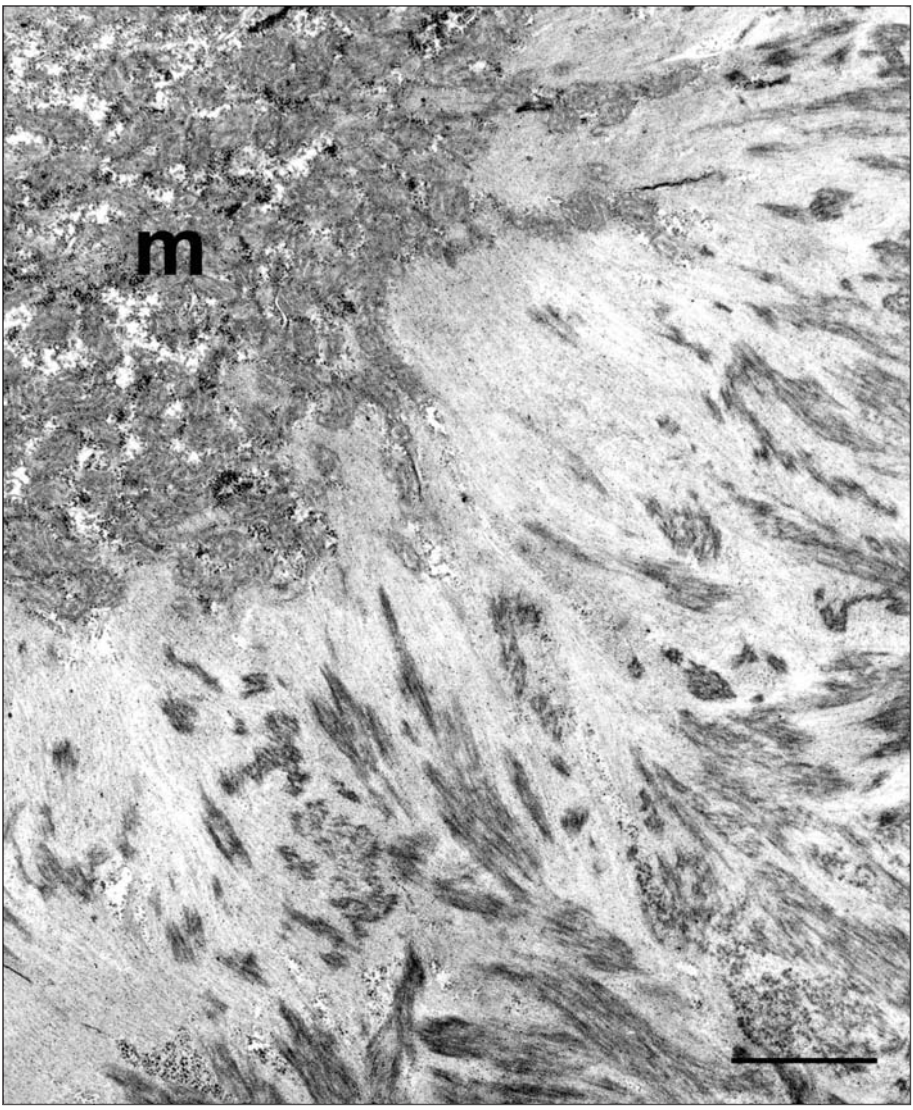

Figure 4. An abnormal fiber region harbors material that has emanated from Z-disks and a cluster of dislocated mitochondria (m). Bar = 1 μm. From: Selcen D et al. Mutations in ZASP define a novel form of muscular dystrophy in humans, Vol 57, 57:269-276. Copyright 2005. Reprinted with permission of John Wiley & Sons, Inc.

Interactions and Functions

So far, five interaction partners, all of which are Z-disk components, have been described for myotilin. These interactions link myotilin to both structural and signaling components (Fig. 7). Myotilin binds to α-actinin, an actin cross-linking protein and the backbone of the Z-disk structure.[22] α-Actinin has also several other binding partners in the Z-disk, including ZASP. A second binding partner is filamin C, the muscle specific filamin isoform,[26] whose mutation can also result in MFM. Myotilin also directly binds monomeric (G) and filamentous (F) actin and very efficiently

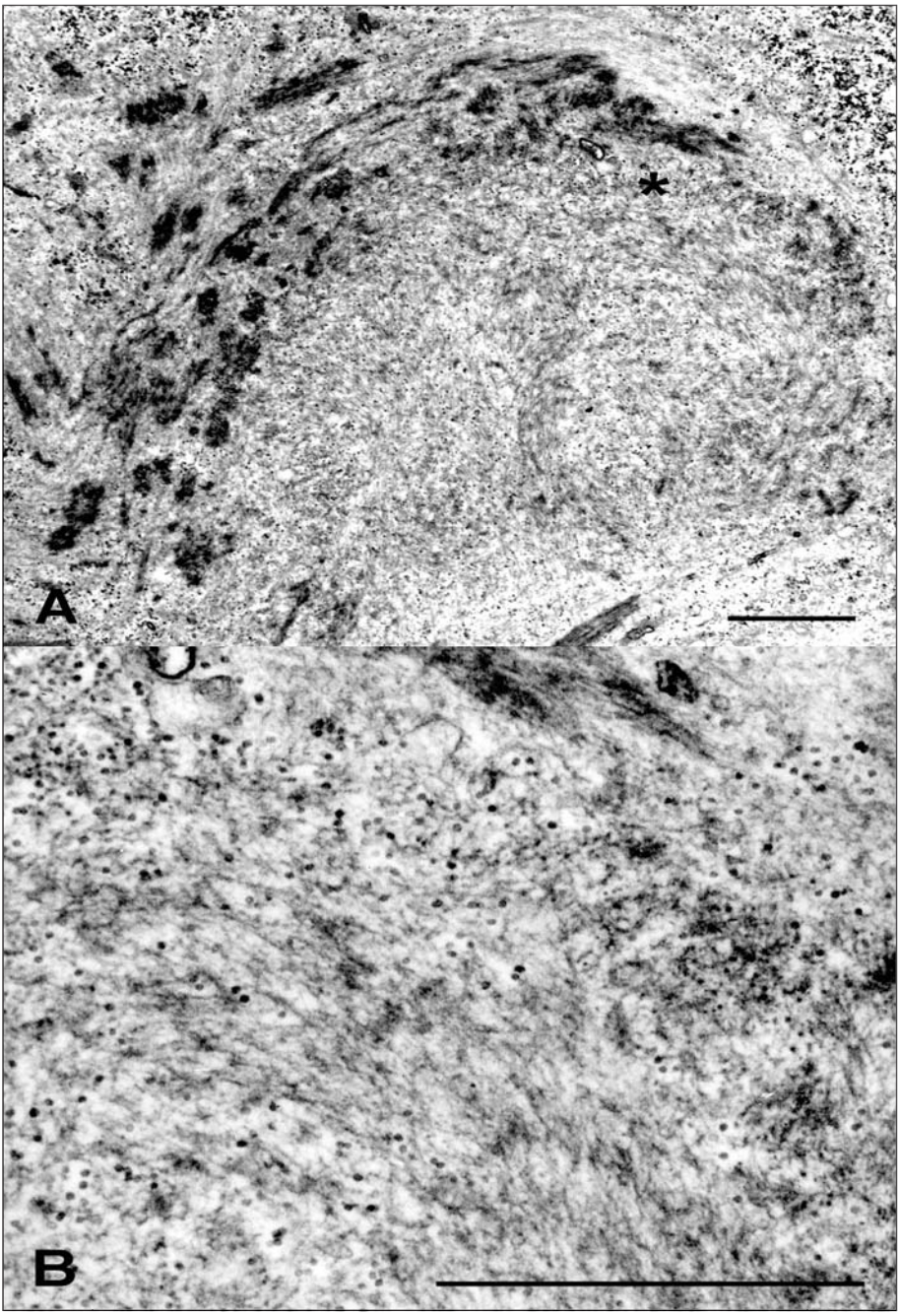

Figure 5. Large hyaline structures observed by light microscopy consist of compacted and fragmented filaments of variable electron density (A). (B) A higher magnification of region marked by asterisk in (A) resolves filamentous profiles. Bar = 1 μm. (From ref. 3, with permission).

bundles and stabilizes actin filaments.[27] The bundling effect is enhanced, when myotilin acts together with α-actinin. The homologous palladin has also significant actin bundling activity.[28]

The FATZ (also termed calsarcins or myozenin) family consists of three homologous proteins. Myotilin has been shown to interact directly with FATZ-1 and FATZ-2.[29] FATZ proteins are localized in the Z-disc, binding not only to myotilin, but also to filamins A, B and C,[29] telethonin (T-cap), α-actinin, ZASP (cypher/oracle) and calcineurin.[30-33] The latter interaction connects these structural proteins to a signaling molecule involved in cardiac hypertrophy and skeletal muscle differentiation. It has been suggested that the FATZ family may play a role in contributing to the formation and maintenance of the Z-disc[32] as well as in cell signalling, since the members bind to calcineurin. FATZ-1 and FATZ-3 are highly expressed in skeletal muscle fast-twitch fibers, while FATZ-2 is highly expressed in cardiac muscle and skeletal muscle slow-twitch fibers. Mice lacking FATZ-2 showed an increase in calcineurin activity and a concurrent increase in the percentage of slow-twitch fibers.[34] Recently, mutations in FATZ-2 were shown to be associated with hypertrophic cardiomyopathy.[35]

Myotilin binds to Muscle-specific RING-finger (MuRF) -1 and -2 E3 ubiquitin ligases.[36] Together with MuRF-3, the proteins form a subfamily of the RING-finger E3 ubiquitin ligases that are expressed specifically in cardiac and skeletal muscle. Ubiquitin conjugation allows degradation of misfolded proteins and long-lived proteins, including components of the contractile apparatus of striated muscles, via the ubiquitin proteasome system (UPS). Of the disease associated Z-disk proteins, telethonin also binds to Murf-1 and -2, whereas filamin C binds to Murf-3.[37] Interestingly, analysis of myotilinopathy specimens has indicated the presence of abnormal ubiquitin in the aggregates. It has been proposed that accumulation of protein aggregates in myotilinopathies may be related to defectively ubiquitinated abnormal protein complexes which are resistant to proteasome degradation.[20]

Myotilin forms dimers, which require its carboxy-terminal half and dimerization is apparently necessary for the actin-bundling activity. The disease mutations do not affect any of the known interactions. Due to its strong actin-bundling activity, myotilin is thought to serve as a stabilizer of the Z-disk.

Pathogenetic Mechanism

The disease results from the presence of dysfunctional myotilin instead of loss of myotilin protein. As no functions have been attributed to the region of myotilin that harbors the mutations and the structural/functional differences between wild-type and mutant myotilin have not been characterized, the pathogenetic mechanism is still unclear.

Zasp

All published disease-causing mutations are missense mutations, which result in a single residue substitution of the ZASP protein.

Gene and Protein

The gene encoding for Z-band alternatively spliced PDZ-motif containing protein (ZASP) (also named LIM domain-binding factor 3, Cypher, or Oracle) is located in chromosome 10q22.2-q23.3.[38] ZASP is a sarcomeric protein expressed in human cardiac and skeletal muscle at the Z-disk.[38] It belongs to the ten member PDZ-LIM protein family that is composed of ZASP, ALP, CLP-36 (Elfin), RIL, Mystique, Enigma (LMP-1), Enigma homologue (ENH), LMO7 and two LIM domain kinases (LIMK1 and LIMK2). Of these proteins, CLP-36, ALP and ZASP are exclusively expressed in striated muscle. ZASP is expressed as several isoforms, some of which are cardiac specific whereas others are expressed in skeletal muscle. All isoforms contain an amino-terminal PDZ domain.

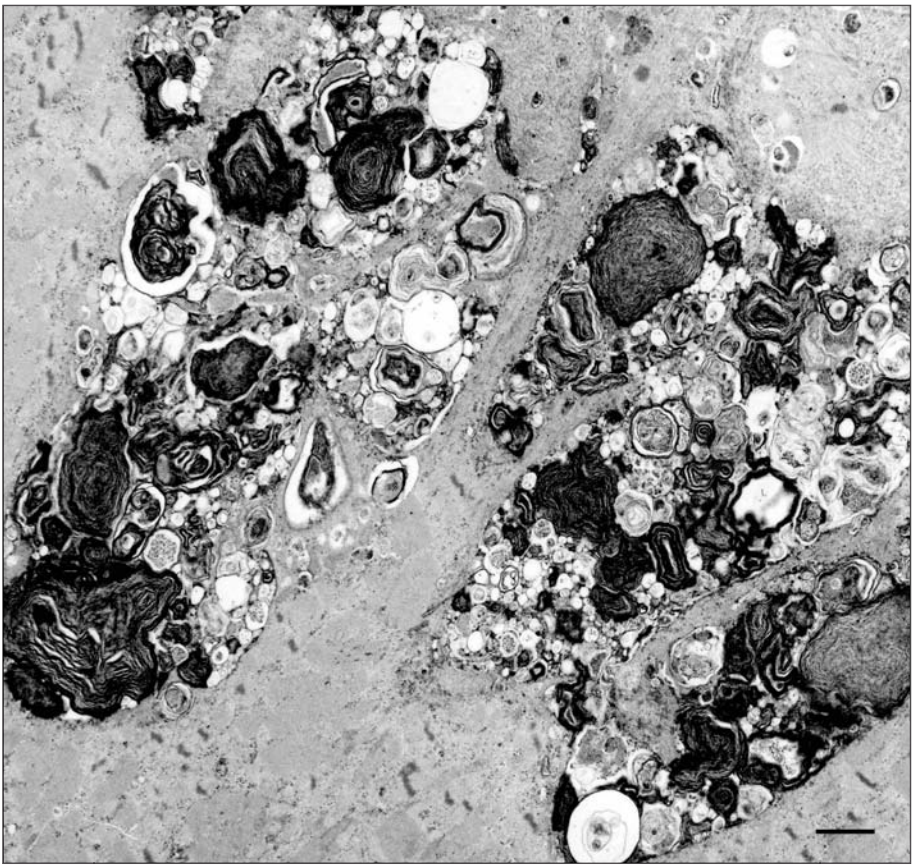

Figure 6. Large autophagic vacuoles harbor myeloid structures and debris. Bar = 1 μm. (From ref. 19, with permission).

Interactions and Functions

The ZASP PDZ domain interacts with α-actinin at micromole affinity,[38] which indicates the participation of ZASP in the mechanical anchoring and stabilization of Z-disk attached proteins. In addition, ZASP is able to bind FATZ family members.[33]

The knock-out mouse (ZASP orthologue cypher) shows severe congenital myopathy and cardiomyopathy and the mice die postnatally from functional failure in multiple striated muscles.[39] Analysis of the knock-out mice has indicated that ZASP is not required for sarcomerogenesis or Z-line assembly, but it has an indispensable function in maintenance of the Z-line during muscle contraction.

Pathogenetic Mechanism

Although loss of ZASP (cypher) is detrimental to skeletal and cardiac muscle function, there is no knowledge of the effect of disease causing mutations on ZASP. In cellular studies, the mutant and wild-type polypeptides are targeted similarly and mutations do not affect the stability of the ZASP protein.[40]

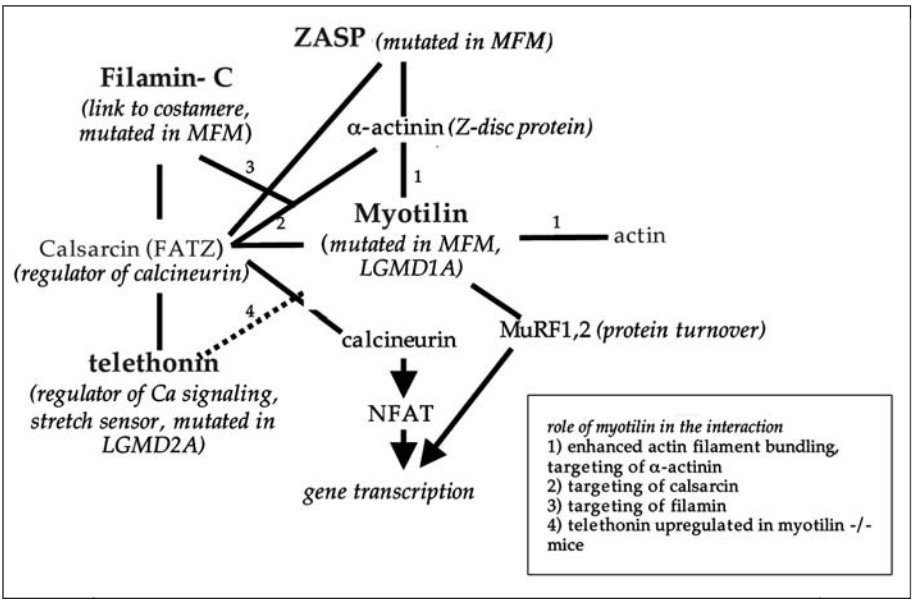

Figure 7. Interactions of myotilin within the Z-disk. Mutations in genes encoding for the proteins marked in red are known to cause (cardio)myopathies, those in bold are described in this chapter.

Filamin C

Only a single disease-causing missense mutation, which results in a single residue substitution, has been published.

Gene and Protein

The filamin family is composed of three isogenes that encode filamin A, filamin B and the muscle-specific filamin C.[41] The N-terminal portion of each of these ~560 kDa dimeric proteins contains an actin-binding domain followed by 24 Ig-like repeats. While the expression of filamins A and B is ubiquitous, filamin-C is largely restricted to skeletal and cardiac muscle cells.[42,43] The repeat 20 of filamin C contains a unique sequence thought to be responsible for its recruitment into the Z-discs. The filamins are involved in multiple processes, such as the organization of actin filaments, membrane stabilization and they serve as a scaffold for signaling proteins.[41,44] The filamin C encoding gene (*FLNC*) maps to chromosome 7q32.

Interactions and Functions

In addition to actin, filamin C interacts with several Z-disc proteins. The insert bearing Ig repeat 20 interacts with the C-terminal half of myotilin and thus, myotilin can form a link between filamin-C and α-actinin.[26] Filamin C also binds Xin, a protein implicated in actin modulation and with CAP, an adaptor protein binding to both a structural and signaling molecules.[45,46] Additional binding partners are FATZ family members that regulate calcineurin signaling[29] and Murf-3, which links filamin C to the proteasome degradation pathway.[37] At the sarcolemma, filamin C interacts with the transmembrane proteins γ- and δ-sarcoglycan.[47]

Pathogenetic Mechanism

The only known mutation, W2710X, in filamin C leads to a partial disturbance of the secondary structure of the dimerization domain of filamin C. Experimental studies have shown that the

mutant dimerization domain is less stable and more susceptible to proteolysis. As a consequence, it does not dimerize properly and forms aggregates in vitro and in cultured cells.[48]

Telethonin (T-Cap)

All identified mutations in telethonin are either frameshifting or nonsense and produce premature stop codons.[15,16]

Gene and Protein

The human *telethonin* gene consists of 2 exons and encodes for a 167 amino acid protein with molecular size of 19 kDa. There is no homology to other proteins and no structural domains have been identified in the sequence. The expression is strictly restricted to skeletal and cardiac muscle, where telethonin protein is located in sarcomeric Z disks.[49] Telethonin mRNA is one of the most abundant transcripts in skeletal muscle,[49] however the transcript and protein expression are downregulated in neurogenic atrophy.[50,51] On the other hand upregulated telethonin mRNA and protein levels are seen in mice lacking myotilin.[24]

Interactions and Functions

Telethonin binds to the Z-disk associated Ig-domains Z1Z2 of titin.[52,53] The interaction may allow telethonin to cross-link titin molecules at their N-termini and thereby provide stability to the Z-disk.[54]

Myostatin is a growth factor, which negatively regulates myoblast proliferation. Telethonin interacts with myostatin and blocks myostatin secretion in myoblasts.[55] In this way, telethonin may promote muscle cell growth and regeneration.

All three calsarcins directly interact with telethonin[56-58] (see above).

Telethonin serves also as a link between sarcomeric Z-disks and the T-tubule system, which couples excitation and contraction by regulating the intracellular ion flow. The link is formed by an association between telethonin and MinK, a component of the K^+ channel, which regulates repolarization of myocytes.[59] The interaction between telethonin and MinK is regulated by phosphorylation of telethonin at S157 (the substrate of titin-kinase), which results in loss of binding.[59]

In summary, telethonin appears to be an important Z-disk adapter protein, which links together and regulates Z-disc structural components, ion channels and signaling pathways involved in myocyte growth and differentiation.

Pathogenetic Mechanism

The disease results from loss of telethonin expression. The pathogenic mechanism is still under investigation. However, since the diseased muscles appear to have preserved sarcomere integrity, it is possible that telethonin's association with ion channel functions and/or signaling pathways, rather than its structural functions, is the underlying cause.

Conclusions and Future Directions

Recent years have seen the identification of mutations in several sarcomeric proteins as the cause of MFM and LGMD. Many open questions, however, still exist. For instance, most of the morphologically diagnosed MFM patients do not have mutations in known MFM genes. Will mutations in other Z-disk components also cause MFM. Is there a common pathway in MFMs caused by myotilin, ZASP and filamin mutations? What are the crucial steps in the molecular pathogenesis of each disease form? What is the relationship between these disorders and other MFM disorders caused by desmin and αB-crystallin mutations? The limited number of identified patients and mutations in each MFM form does not yet allow us to develop a complete picture of the phenotypic variability of the disease spectrum. Finally, further understanding of the molecular complexity of the Z-disc and clarification of the signaling pathways involved in Z-disc assembly, turnover and maintenance are needed, before the molecular pathways that result in MFM and LGMD can be defined.

Acknowledgements

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Chapter 11

Intermediate Filament Diseases:
Desminopathy

Lev G. Goldfarb,* Montse Olivé, Patrick Vicart and Hans H. Goebel

Abstract

Desminopathy is one of the most common intermediate filament human disorders associated with mutations in closely interacting proteins, desmin and alphaB-crystallin. The inheritance pattern in familial desminopathy is characterized as autosomal dominant or autosomal recessive, but many cases have no family history. At least some and likely most sporadic desminopathy cases are associated with de novo *DES* mutations. The age of disease onset and rate of progression may vary depending on the type of inheritance and location of the causative mutation. Typically, the illness presents with lower and later upper limb muscle weakness slowly spreading to involve truncal, neck-flexor, facial and bulbar muscles. Skeletal myopathy is often combined with cardiomyopathy manifested by conduction blocks, arrhythmias and chronic heart failure resulting in premature sudden death. Respiratory muscle weakness is a major complication in some patients. Sections of the affected skeletal and cardiac muscles show abnormal fibre areas containing chimeric aggregates consisting of desmin and other cytoskeletal proteins. Various *DES* gene mutations: point mutations, an insertion, small in-frame deletions and a larger exon-skipping deletion, have been identified in desminopathy patients. The majority of these mutations are located in conserved alpha-helical segments, but additional mutations have recently been identified in the tail domain. Filament and network assembly studies indicate that most but not all disease-causing mutations make desmin assembly-incompetent and able to disrupt a pre-existing filamentous network in dominant-negative fashion. AlphaB-crystallin serves as a chaperone for desmin preventing its aggregation under various forms of stress; mutant *CRYAB* causes cardiac and skeletal myopathies identical to those resulting from *DES* mutations.

Introduction

Desminopathy is associated with mutations in desmin, alphaB-crystallin and possibly other proteins that interact with desmin. Identification of pathogenic mutations in *DES* and *CRYAB* genes, analysis of the underlying human disease phenotypes and successful modeling of these conditions in cell cultures and transgenic mice helped to understand the critical events involved in the pathogenesis of desminopathy. Widespread abundant desmin-immunoreactive deposits in the cardiac and skeletal muscles and aggregation of granulofilamentous material seen at the ultrastructural level are the morphological hallmarks of desminopathy.[1,2] Transfected into cell cultures, many but not all mutant desmins are incapable of generating an intracellular filamentous network[3-5] and disrupt pre-existing endogenous intermediate filament structures.[6] Disease-associated *DES* mutations expressed in transgenic mice cause an accumulation of chimeric intracellular aggregates containing desmin and other cytoskeletal proteins. Misfolded desmin resists turnover by the cellular enzymatic machinery.[7]

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Identification of multiple causative mutations in the DES gene[6,9-11] helped to establish desminopathy as a distinct disease. The second genetically independent subset of desminopathy is myopathy associated with mutations in *CRYAB*, a chaperone that normally stabilizes proteins including desmin and prevents their irreversible aggregation.[8,12] Genetic linkage of clinically and pathologically confirmed desminopathy to other loci has also been demonstrated.[13,14] A more inclusive group of disorders named myofibrillar myopathy includes conditions associated with mutations in SEPN1, myotilin, ZASP and Filamin C.[15]

Genetic mechanisms influence desminopathy phenotype in several ways: (a) dominant, recessive and de novo mutations cause somewhat distinct syndromes; (b) desmin is expressed in skeletal, cardiac and smooth muscles, hence, combinations of damage in various tissues result in diverse phenotypes; and (c) the type and location of the mutation in *DES* or *CRYAB* may produce additional phenotypic modifications.

During the several years since previously published reviews on desminopathy, many new patients and affected families have been identified and studied. The range of clinical and myopathological manifestations has widened. As a result, the diagnostic criteria of desminopathy and interrelations with other disorders require a reevaluation.

Intermediate Filaments of the Muscle

Intermediate filament (IF) proteins are expressed in a cell-type specific manner and form 10-nm diameter filaments, which are intermediate in thickness between the 6–8-nm diameter microfilaments and the 25-nm diameter microtubules.[16] At least 65 different IFs have been identified in humans.[17] IFs are known to play a structural role in eukaryotic cells by participating in formation of the cell cytoskeleton and providing mechanical stability to the cells.[18]

The main muscle IF is desmin, a 53-kDa protein expressed in cardiac, skeletal and smooth muscles. In mature skeletal muscle, Desmin links adjacent myofibrils at the level of the Z disc and binds myofibrils to the sarcolemma at the level of the costameres, possibly through plectin.[19] This allows the formation of a continuous cytoskeletal network that maintains a spatial relationship between the contractile apparatus and other structural elements of the cell,[18] providing maintenance of cellular integrity, force transmission and mechanochemical signaling. In the heart, desmin is increased at intercalated discs and is the major component of the Purkinje fibers.[20] In smooth muscle, desmin is located in the cytoskeletal region at the dense bodies and dense plaques.

Ablation of desmin expression in mice by gene targeting[21] has demonstrated that desmin expression is crucial for maintaining the architectural and functional integrity of striated muscle. Mice lacking desmin develop numerous muscle architectural and ultrastructural defects, especially in extensively used muscles such as the heart, soleus and diaphragm. Structural abnormalities include loss of the lateral alignment of myofibrils, perturbation of myofibril anchorage to the sarcolemma and loss of nuclear shape and positioning.[22,23]

AlphaB-crystallin has its own influence on the cytoskeleton as a molecular chaperone for desmin and other proteins. AlphaB-crystallin directlty binds titin/connectin at the I-band region, more specifically at the N2B and I26/I27 domains, as demonstrated by both copurification and colocalization of alphaB-crystallin with titin/connectin.[24-26]

Molecular Genetics

Desmin and the Desmin Gene

Human desmin is encoded by a single copy gene (*DES*) located in chromosome band 2q35;[27] it encompasses nine exons within an 8.4 kb region and codes for 476 amino acids.[28] The gene is highly conserved among vertebrate species. In accordance with its function, the desmin molecule is organized into three domains: a highly conserved alpha-helical core of 308 amino acid residues flanked by globular N- and C-terminal ("head" and "tail") structures.[29] The alpha-helical core maintains a seven-residue (heptad) repeat pattern with a typical sequence of hydrophobic and hydrophilic amino acids. This heptad repeat structure guides two polypeptides into formation of

a homopolymeric coiled-coil dimer, the elementary unit of the filament. The heptad periodicity within the helical rod is interrupted in several places resulting in four consecutive helical segments 1A, 1B, 2A and 2B connected by short nonhelical linkers.

Structural analysis of the 2B helix attracted maximum attention because more than 50% of the known *DES* mutations have occurred in this region. The 2B segment located at the C-terminal part of the desmin core domain contains a discontinuity in the heptad repeat pattern, a "stutter", which is equivalent to an insertion of four extra residues at the end of the 2B eighth heptad.[30] The "stutter" is an obligatory feature of all IF proteins and its position is absolutely conserved.[31] Experimental "straightening out" of the stutter by inserting three "missing" amino acids to restore a continuous heptad repeat leads to inability of this "stutterless" molecule to anneal into longer filaments.[32] As a compensation for the stutter, the coiled coil slightly unwinds in the stutter vicinity. The local unwinding caused by the stutter modifies the assembly of the protein and its interaction properties.

Another thoroughly examined structure is the 405-YRKLLEGEESRI-416 motif at the C-terminal end of the 2B helix. Starting with several amino acids preceding the YRKLLEGEESRI motif and through the YRKLL peptide, the coiled-coil structure loosens and the alpha-helices gradually separate eventually bending away from each other at the EGEE level.[33] In vitro data demonstrate that this motif directs the proper formation of tetramers and controls the number of subunits per filament cross section.

The desmin "tail" domain contains ~30% beta-sheet with the remainder of the domain having predominantly random structure and lacking the heptad repeat pattern. The desmin tail is involved in the longitudinal head-to-tail tetramer assembly[34] and control of lateral packing, stabilization and elongation of the higher order filament structures.[35] However, the tail's major function seems to be interacting with other cytoskeletal proteins to establish a cytoplasmic intermediate filament network.[36]

Desmin normally interacts with many other structural proteins (Fig. 1), including intermediate filament-associated proteins (IFAPs) which cross-link desmin filaments into a network that anchor the cytoskeleton.[16] The inability of desmin to interact with these proteins may trigger disease development.

Nucleotide sequencing of 163 control individuals in the NIH laboratory (Goldfarb, unpublished) detected 17 DNA polymorphisms (Table 1) and several reported by others have been included in the NCBI database. c.914T>C, c.1100C>G and c.1190A>G are the most frequent. The p.Ala213Val and p.Val459Ile substitutions have been seen in unaffected control individuals, but both are thought to influence the disease phenotype (see below).

DES Mutations

The number of disease-causing *DES* mutations has reached 42 (Table 2 and Fig. 2): 37 missense mutations; 3 small in-frame deletions, deletion of exon 3 caused by mutations in splice donor or acceptor sites flanking exon 3 and an insertion of a single nucleotide resulting in premature translation termination. Thirteen mutations are recurrent and the others private. Five mutations have occurred in the "head" domain, five in the 1B segment, the most—22 mutations—are located in the 2B alpha-helix and 10 in the tail domain. No mutations have so far been identified in the 1A and 2A segments.

Mutations in the 1B Segment of Desmin

A homozygous deletion of 21 nucleotides predicting an in-frame loss of seven amino acids from Arg173 through Glu179 (Arg173_Glu179del) in the 1B helix caused a severe clinical syndrome and compromised the ability of desmin to assemble into intermediate filaments in cell culture.[10] Although the p.Ala213Val substitution was seen in four control individuals of 199 tested[40] and 2 of 86 analysed for another study,[38] the information generated so far supports the idea that this may be a modifying functional polymorphism. Although p.Ala213Val desmin created a filamentous network in SW13 cells and preserved the existing network in the C2C12 cells,[40] filament assembly experiments showed it aggregated in the viscometer; in the BMGE+H cells, the p.Ala213Val filaments

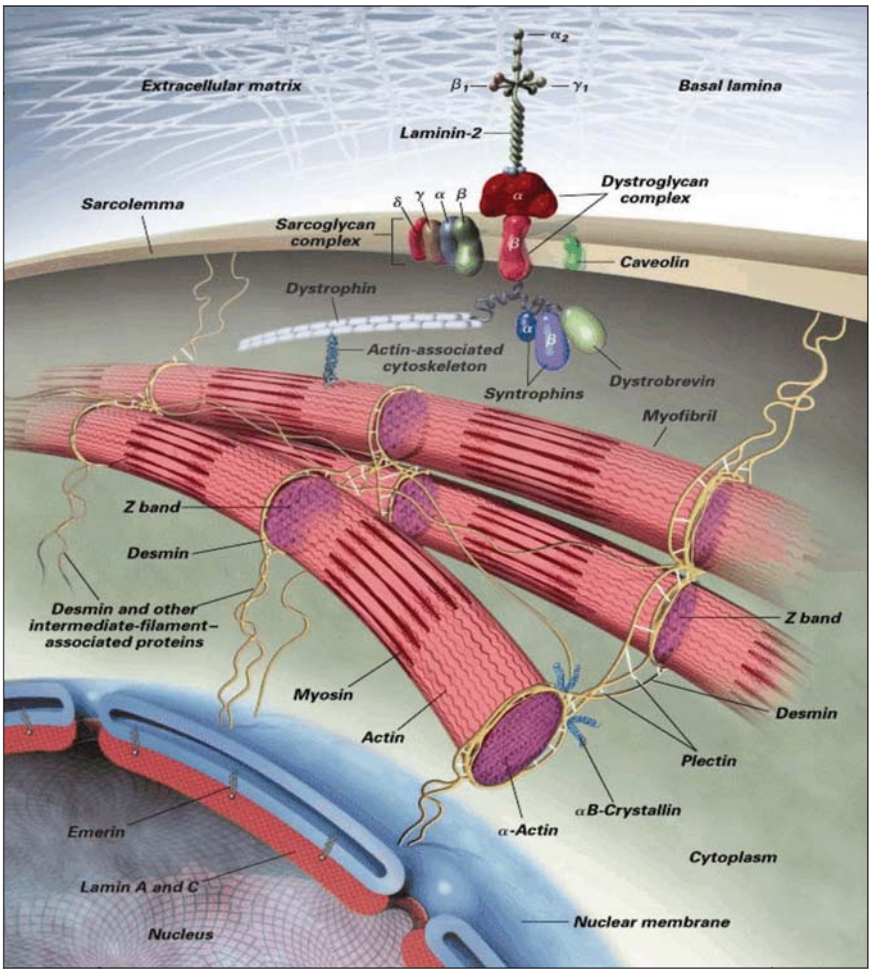

Figure 1. Cytoskeletal proteins. Reproduced from *The New England Journal of Medicine,* 2000; 342:778 with permission.

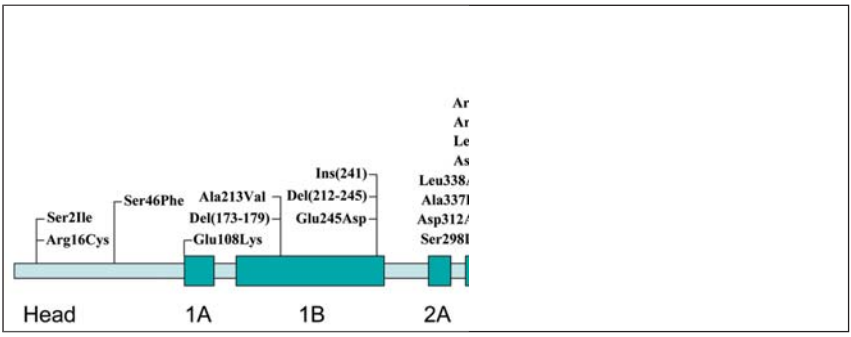

Figure 2. Schematic representation of the structural organization of desmin protein domains and predicted positions of disease-causing mutations.

Table 1. Single nucleotide polymorphisms in* DES *coding region

mRNA Position (NCBI Numbering)	Nucleotide Change	Codon Position	Amino Acid Position	Amino Acid Change	Population Frequency	Reference
c.116	C > T	3	10	Arg	0.0061	NIH Lab
c.146	G > C	3	20	Gly	0.0061	NIH Lab
c.154	G > T	2	23	Gly > Val	?	NCBI
c.161	G > A	3	25	Pro	0.0246	NIH Lab
c.179	C > T	3	31	Ser	0.0246	NIH Lab
c.269	C > A	3	61	Gly	?	NCBI
c.332	C > T	3	82	Ser	0.0061	NIH Lab
c.338	C > G	3	84	Gly	0.0061	NIH Lab
c.344	C > G	3	86	Gly	0.0061	NIH Lab
c.455	C > G	3	123	Ile > Met	0.0061	NIH Lab
c.458	G > A	3	124	Glu	0.0061	NIH Lab
c.724	C > T	2	213	Ala > Val	0.0201	NIH Lab
c.806	G > A	3	240	Lys	0.0061	NIH Lab
c.914	T > C	3	276	Asp	0.3493	NIH Lab
c.1100	C > G	3	338	Leu	0.4024	NIH Lab
c.1112	C > T	3	342	Asn	0.0122	NIH Lab
c.1157	C > A	3	357	Ala	0.0061	NIH Lab
c.1190	A > G	3	368	Ala	0.3611	NIH Lab
c.1302	C > G	3	405	Tyr	0.0324	NIH Lab
c.1367	C > T	3	427	Asn	?	NCBI
c.1461	G > A	1	459	Val > Ile	0.01*	[38]
c.1472	C > G	3	462	Ala	?	NCBI

* in African Americans

were bundle-like, suggesting that the pathomechanisms of this mutation probably involve subtle but critical interactions with non-IF components in muscle cells.[61]

A heterozygous single-nucleotide (adenine) insertion mutation occurring at the third position of codon 241 causes a frameshift leading to serial amino acid replacements: Val242Glu, His243Ser, Glu244Ala and eventually a premature termination signal at codon 245 (numbering according to the updated sequence, GenBank submission # AF167579). This mutation is predicted to create a truncated desmin molecule with molecular weight of 27 kDa.[42] Transfection studies confirmed that this mutation induces collapse of the preexisting desmin cytoskeleton. It also alters the subcellular distribution of mitochondria and affects biochemical properties of mitochondria in affected skeletal muscles.

A series of mutations has been identified in the highly conserved donor and acceptor splice sites flanking exon 3 (Table 3). The 96-bp exon 3 sequence encodes 32 complete codons, therefore total deletion of exon 3 would not interrupt the reading frame and is predicted to result in synthesis of a desmin polypeptide that is lacking 32 residues from Asp214 through Glu245 (p.Asp214_Glu245del).[3] This deletion disrupts the heptad repeat pattern and interferes with the coiled-coil structure. The presence of the deletion was confirmed on the mRNA level. Functional analysis indicates that desmin lacking the 32 amino acids was incapable of forming a filamentous network in SW13 (vim-) cells.[3] Binding sites to nebulin[62] and perhaps other interacting proteins are located within this segment.

Table 2. Phenotypic features of myopathy-causing mutations in desmin

Predicted Amino Acid Change	Domain	Type of Inheritance	No. of Fam/ Pts Studied*	Age at Onset (Diagnosis)	Initial Symptoms	Distribution of Weakness	Respiratory Weakness	Bulbar Symptoms	Cardiac Disease	Associated Mutations	Reference
p.Ser2Ile	Head	*?*	1/1	?	?	L	no?	no?	?		2
p.Arg16Cys	Head	*AR*	1/1	30	Skel	L?	?	?	R,Co		37
p.Ser46Phe	Head	*?*	1/1	?	?	L	?	?	?		2
p.Ser46Tyr			1/1	?	?	L	?	?	?		2
p.Glu108Lys	Head	*?*	1/1	60	Card	-	-	-	D, Co		38
p.Arg173_ Glu179del	1B	*AR*	1/1	15	Skel + Card	L,U,F	+	+	D,Co		10
p.Ala213Val	1B	*?*	1/1	?	Card	-	-	-	R		39
		AD	1/1	33	Skel	L,T	-	-	-	GAA**	40
		Spor	1/1	65	Card	-	-	-	D		40
		AD	1/1	31	Skel	L	+	-	-		41
p.Lys240fsX243	1B	*Spor*	1/1	18	Skel	L,U	-	-	Co		42
p.Glu245Asp	1B	*AD?*	1/1	46	Skel	L,U	-	-	R		43
			/1	20	Card	-	-	-	R		43
p.Asp214_ Glu245del	1B	*AD*	1/2	40	Card	L,U,T,F	-	-	Co		3
		AD	1/2	29-41	Card	L,U,F	+	-	Co		44
		AD	1/3	24-48	Card	-	-	-	R,Co		37
p.Ser298Leu	2B	*?*	1/1	45	Card	-	-	-	D,Co		38
p.Asp312Asn	2B	*?*	1/1	35	Card	-	-	-	D		38
		Spor	1/1	45	Skel	L,T,F	+	-	D		41

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Table 2. ***Continued***

Predicted Amino Acid Change	Domain	Type of Inheritance	No. of Fam/ Pts Studied*	Age at Onset (Diagnosis)	Initial Symptoms	Distribution of Weakness	Respiratory Weakness	Bulbar Symptoms	Cardiac Disease	Associated Mutations	Reference
p.Ala337Pro	2B	*AD*	1/2	20-38	Skel	L,U,T	-	+	Co		[11]
		?	1/1	45	Skel	L,U	+	-	Co		45
p.Leu338Arg	2B	*AD*	1/2	43-46	Skel	L,U,T,F	+	-	-		40
		AD	1/1	35	Skel	L,U	-	+	Co		41
p.Asn342Asp	2B	*AD*	1/2	23-30	Skel	L,U,T	-	-	-		11
p.Leu345Pro	2B	*AD*	1/5	24-46	Skel	L,T,F	+	+	Co		6
p.Arg350Pro	2B	*AD*	1/3	48	Skel	L,U	+	-	D,Co		46
p.Arg350Trp	2B	*?*	1/1	55	Card	-	-	-	D		38
p.Arg355Pro	2B	*AD*	1/1	36	Skel + Card	L,U	-	-	R,Co		47
p.Ala357Pro	2B	*AD*	1/3	35-45	Skel	L,U,T	+	-	-		48
		AD	1/1	Teens	Skel	L	-	-	-		41
p.Glu359_ Ser361del	2B	*AD*	2/8	31-46	Skel	L,U	-	-	-		49
p.Ala360Pro	2B	*AR*	1/3	2-10	Card	L,U,T	+	+	D,Co	*DES* As-n393Ile	11
p.Asn366del	2B	*AD*	1/1	36	Skel	L,U	+	-	Co		49
p.Ile367Phe	2B	*AD*	1/1	25	Skel	L	-	+	H,Co		50
p.Leu370Pro	2B	*AD*	1/1	28	Skel	L,U	+	-	-		48
		AD	1/2	35-40	Skel	L,U,T	+	-	D		51

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Table 2. ***Continued***

Predicted Amino Acid Change	Domain	Type of Inheritance	No. of Fam/ Pts Studied*	Age at Onset (Diagnosis)	Initial Symptoms	Distribution of Weakness	Respiratory Weakness	Bulbar Symptoms	Cardiac Disease	Associated Mutations	Reference
p.Leu385Pro	2B	*denovo*	1/1	21	Skel	L,U,T,F	+	-	D,Co		52
p.Gln389Pro	2B	*Spor*	1/1	42	Skel	L,U	-	-	D,Co		53
p.Leu392Pro	2B	*AD*	1/1	25	Skel	L,U,T,F	+	+	H,Co		50
p.Asp399Tyr	2B	*AD*	1/1	34	Skel	L	+	-	D,Co		40
p.Glu401Lys	2B	*Spor*	1/1	20	Skel+ Card	L,U	-	+	Co		40
p.Arg406Trp	2B	*denovo*	3/3	15-23	Card	L,U,T,F	+	+	D,Co		54
		denovo	1/1	24	Skel	L,U,T	+	-	Co		54
		AD	1/2	40-50	Card+ Skel	L?	?	?	R,Co		37
p.Glu413Lys	2B	*AD*	1/1	30	Card	-	-	-	R,Co		55
			/2	30-63	Card	L	-	-	R,Co		55
p.Arg415Trp	Tail	*?*	1/1	30	Skel	L	-	-	-	ZASP Ly-s251Asp	41
p.Pro419Ser	Tail	*AD*	1/2	22-25	Skel	L,U	-	+	H,Co		50
p.Thr442Ile	Tail	*AD*	1/2	27-35	Skel	L,U,T	+	-	Co		56
		denovo	1/1	38	Skel	L,U	-	-	H,Co		56
p.Lys449Thr	Tail	*?*	1/1	?	?	L	?	?	?		2
		Spor	1/1	14	Skel	U	-	-	?		41

continued on next page

Table 2. ***Continued***

Predicted Amino Acid Change	Domain	Type of Inheritance	No. of Fam/ Pts Studied*	Age at Onset (Diagnosis)	Initial Symptoms	Distribution of Weakness	Respiratory Weakness	Bulbar Symptoms	Cardiac Disease	Associated Mutations	Reference
p.Ile451Met	Tail	*AD*	1/2	15-37	Card	-	-	-	D		57
		AD	1/3	25-35	Skel	L,U,T	+	-	-		58
		Spor	3/3	38-55	Card	-	-	-	D		59
p.Thr453Ile	Tail	*Spor*	1/1	17	Card	-	-	-	R,Co		37
		AD	1/1	20	Skel	L,U,T	-	-	?		41
p.Arg454Trp	Tail	*denovo*	1/1	15	Card	L,U,T,F	-	+	H	MYOT-74	56
p.Val459Ile	Tail	*?*	1/1	34	Card	-	-	-	D,Co		38
		?	1/1	44	Card	-	-	-	D		38
p.Ser460Ile	Tail	*AD?*	1/1	29	Card	L,U	-	-	Co		56
p.Val469Met	Tail	*AD*	1/1	14	Card	L,T	-	-	H,Co	Lamin A p.R644C	60

AD: autosomal dominant pattern of inheritance; AR: autosomal recessive; Spor: sporadic; Skel: skeletal myopathy; Car: cardiomyopathy; Codon numbering according to updated sequence in GenBank submission # AF167579.
Muscle weakness predominant topography: L—lower limbs; U—upper limbs; T—trunk; F—face.
Type of cardiomyopathy: D—dilated; R—restricted; H—hypertrophic. Co—conduction abnormalities.
* Number of families and number of patients studied for the report.
** Compound heterozygosity for alpha-glucosidase (GAA) p.Asn91Asp and p.Ala261Thr mutations.

***Table 3. Splice site mutations resulting in deletion of 32 amino acids encoded by exon 3 of* DES**

Sequence	Acceptor Site (End of Intron 2)	Exon Three	Donor Site (Start of Intron 3)
Wild type	… tcccag	GAC…GAG	gtatac…
IVS2–1G→A	… tccca**a**	GAC…GAG	gtatac…
IVS2–2A→T	… tccc**t**g	GAC…GAG	gtatac…
IVS3+1G→A	… tcccag	GAC…GAG	**a**tatac…
IVS3+3A→G	… tcccag	GAC…GAG	gt**g**tac…

Nucleotide replacements are bolded.

Mutations in the 2B *DES* Segment

Current data show significant clustering of mutations and polymorphisms in exons 5 and 6 corresponding to the 2B helix. The cluster includes 22 mutations (Fig. 2, Table 2), or 52% of all known *DES* mutations within only 25% of the coding region. Ten missense mutations introduce proline. Proline is not normally present in the desmin helical rod and is known as a potent helix breaker; its dihedral angle is fixed at –65° and creates a kink in the protein structure.[63] In addition, proline destabilizes alpha-helix by its inability to form hydrogen bonds. In mutagenesis experiments, the introduction of proline residues resulted in production of short, thick and kinked abnormally assembled filaments.[64]

The p.Arg350Pro mutant is incapable of forming a desmin IF network in BMGE+H, MCF7, or SW13 cells and disrupts the endogenous vimentin cytoskeleton in 3T3 fibroblast cells.[46] The filament assembly process of the p.Arg350Pro mutant is disturbed at the unit length filament level and the lateral packing taking place in the first phase of assembly ultimately leading to abnormal protein aggregation.[46] Focal disturbances in the assembly may inhibit the proper interaction of desmin with other cellular binding partners.

A comparative study of pathogenic potentials of *DES* mutations based on their effects in cell lines indicated that the p.Ala213Val, p.Asn393Ile and even proline-inserting mutations p.Ala360Pro and p.Glu389Pro allowed *bona fide* filament formation when transfected separately.[40,61] However, when the p.Ala360Pro and p.Asn393Ile mutations were cotransfected, this caused devastating effects in each cell line used in the experiment.[40] Indeed, in a family segregating both p.Ala360Pro and p.Asn393Ile mutations, a highly aggressive early onset cardioskeletal myopathy affected only those having both mutations in a compound heterozygous fashion but spared the carriers of either mutation.[9] The p.Ala337Pro, p.Leu338Arg, p.Asp399Tyr, p.Glu401Lys and p.Arg406Trp mutations alone make desmin filaments dysfunctional and cause increasingly more severe disease that starts earlier and leads to the development of life-threatening dysphagia, cardiomyopathy, or respiratory weakness.[40]

The severity of illness caused by the mutations located in the C-terminal part of the 2B alpha-helical domain can be explained by structural relationships these mutations potentially disrupt.[40] The p.Leu338Arg mutation replaces an apolar amino acid, thus disrupting the highly conserved (*abcdefg*)n repeat pattern. The p.Asp399Tyr, p.Glu401Lys and p.Arg406Trp mutations disrupt potentially critical intrahelical and interhelical salt-bridge interactions.[40] In addition, the p.Arg406Trp mutation identified in four unrelated West European patients disrupts the fine tuned arrangements within and around the highly conserved YRKLLEGEESRI motif of the 2B helix.[54] A molecular image of a short peptide from this motif containing the p.Arg406Trp mutation shows that replacement of arginine with a larger tryptophan residue makes the peptide at least a half-turn longer and therefore changes the peptide structure and symmetry.[54] Filament assembly studies indicate that the p.Arg406Trp mutant has a reduced ability to support longitudinal annealing as well as radial compaction.[5]

The p.Glu413Lys mutation is positioned near the p.Arg406Trp mutation within the YRKLLEGEESRI segment. Structural analysis indicates that desmin residue Glu413 is still part of the coiled-coil segment.[55] Apparently, any change in this highly conserved region can influence filament assembly by disrupting interactions within the dimer and/or above the dimeric level. The results of molecular modeling indicate that the p.Glu413Lys mutation alters electrostatic interactions which are important for the proper dimer-dimer interactions during the assembly process. In addition, the intrahelical salt bridge projected to appear as a result of the p.Glu413Lys mutation would increase stiffness of the coiled-coil structure thus multiplying the destructive effects of the mutation.

Small in-frame deletions in the 2B alpha-helix have been identified in three families.[49] Molecular modeling indicate that the disease mechanism in these cases is associated with the disturbance of the dimer coiled-coil structure. The p.Glu359_Ser361del mutation creates a second stutter adjacent to the normally existing stutter (Fig. 3). This is very likely to cause additional local unwinding of the coiled coil. The p.Asn366del mutation also occurring in the vicinity of the wild-type stutter converts the latter into a heptad repeat stammer, which is expected to cause overwinding of the coiled coil.[49] Both mutations are thus expected to result in an altered coiled-coil geometry within segment 2B. As a consequence, the angular position of the tail domain with respect to the dimer axis is changed and dimer–dimer interactions and ultimately the filament assembly are very likely to be compromised. Functional studies conducted by expression of full length mutant cDNAs in SW13 and BHK21 cells confirmed the pathogenicity of these mutations.

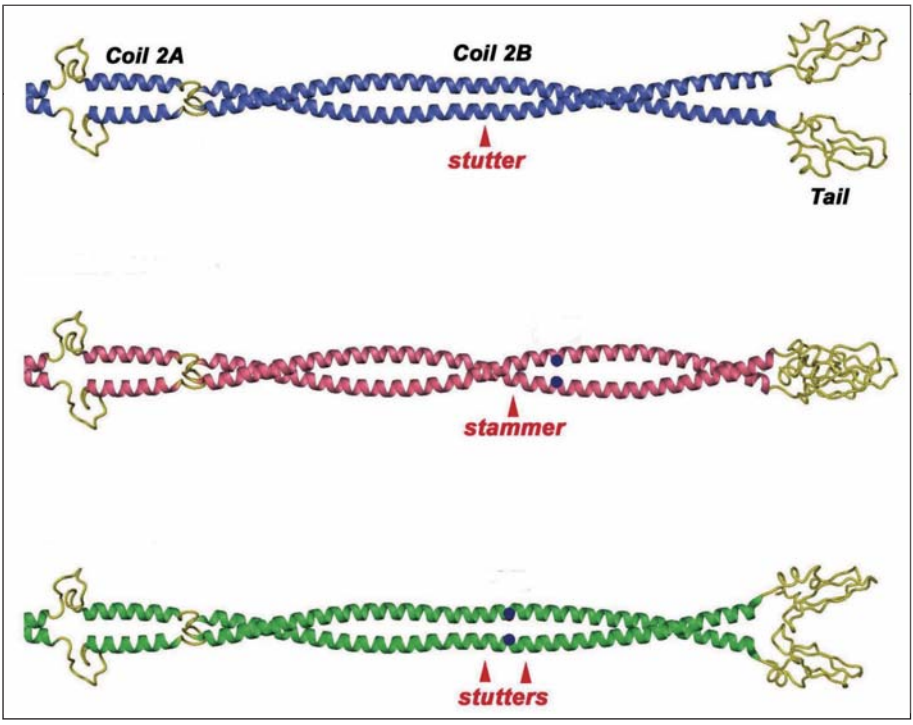

Figure 3. Molecular modeling of desmin coiled-coil segment 2B. Arrows: (1) The location of the wild-type stutter; (2) the position of the abnormal stammer occurring in the p.Asn366del mutant; (3) the positions of two stutters in the p.Glu359_Ser361del mutant. Dots indicate the mutation sites. Reproduced from Human Genetics 2004; 114:311, with permission.

The p.Ala357Pro mutation also destroys the stutter by introducing a proline residue and results in accumulation of desmin-positive aggregates in human muscle and evident perturbation of the intermediate filament network in transfected cells.[48]

Tail Domain Mutations

The disease mechanism in patients with the "tail" domain mutation is distinct from the alpha-helical rod mutations. The nonhelical tail domain lacks the heptad repeat pattern and is involved mainly in interacting with other cytoskeletal proteins to establish a cytoplasmic intermediate filament network. The inability to interact with these proteins may trigger disease development. Expression of tail mutants in SW13 cells led to formation of an apparently normal filament network, indicating that at least some of the tail mutations did not prevent normal filament assembly and network formation.[56,58] Furthermore, the p.Thr442Ile, p.Lys449Thr, p.Ile451Met and p.Val469Met mutants (but not p.Arg454Trp and p.Ser460Ile) left intact the pre-existing intermediate filament network when transfected into C2C12 cells,[56,58] suggesting that the tail mutations do not have the dominant negative effects shown for almost every alpha-helical mutation. Nevertheless, desmin tail domain mutations cause severe cardiac and skeletal myopathy similar to syndromes associated with desmin alpha-helical domain mutations. Further studies are needed to fully understand disease mechanisms associated with tail mutations.

Incomplete Penetrance

As some *DES* mutations are less pathogenic than others,[40] members of families carrying the least pathogenic mutations never develop the disease. The phenomenon of incomplete penetrance was first established in the DCM 20-032 family in which some but not all p.Ile451Met mutation carriers expressed the cardiomyopathy phenotype.[57] In "family B" with progressive skeletal myopathy and no evidence of cardiac involvement three members carrying the p.Ile451Met mutation were also clinically asymptomatic in their 50s and 60s.[58]

Modifier Genes

Five patients showed a combination of mutations in *DES* with mutations or functional polymorphisms in *DES* or other "neuromuscular" genes, *alpha-glucosidase*, *ZASP*, *MYOT*, or Lamin A/C. A compound heterozygous carrier of two substitutions in the *alpha-glucosidase* gene: a c.271A>G (p.Asn91Asp) substitution inherited from his mother and a c.781G>A (p.Ala261Thr) change inherited from the father had also a heterozygous p.Ala213Val *DES* mutation inherited from the mother.[40] None of his parents or other relatives was affected with any neuromuscular disorder. There is no indication that alpha-glucosidase interacts in any way with desmin, but some kind of synergetic influence can be expected since both Pompe disease and desminopathy cause muscle pathology.

A combination of *lamin A/C* p.Arg644Cys and *DES* p.Val469Met mutations was observed in another family.[60] The patients developed muscle weakness and complete heart block. Immunohistochemistry of the explanted heart and biopsied skeletal muscle showed desmin aggregates and granulofilamentous electron dense material on EM. Desmin and *lamin* A/C proteins are believed to be indirectly connected via other intermediate filament proteins.[11]

CRYAB *and AlphaB-Crystallin*

CRYAB is mapped to chromosome 11q22.3-q23.1 and is composed of three exons highly conserved in a variety of species.[65] AlphaB-crystallin belongs to the "small heat shock protein" family, which includes hsp20, hsp22, hspB2, B3, hsp25, hsp27 and the myotonic dystrophy kinase binding protein. Although it was originally discovered and classified as a lens protein, alphaB-crystallin is found in nonlenticular tissues and is abundant in cardiac and skeletal muscle.[66]

AlphaB-crystallin is a chaperone that responds to stressful conditions by binding to unfolded proteins and preventing their denaturation and aggregation;[67] it binds to desmin and cytoplasmic actin and helps to maintain cytoskeletal integrity.[68] When a cell is subjected to stress, alphaB-crystallin protects muscle fibers from the effects of ischemia.[69] AlphaB-crystallin participates in a number of other cellular processes, helping to modulate correct protein-folding, compartment-targeting, degradation and signaling.

***Table 4. Single nucleotide polymorphisms in* CRYAB *gene*[70]**

SNP Location	Change	Amino Acid Change	Frequency
c.-652	G > A	-	0.35
c.-650	G > C	-	0.05
c.-249	C > G	-	0.26
c.1120	C > A	p.Ser41Tyr	0.02
c.1150	C > T	p.Pro51Leu	0.02

CRYAB sequence variants not associated with any disease and their frequencies studied in Caucasian population[70] are shown in Table 4. No studies regarding functional consequences of the two amino acid altering variants have been carried out, but it is reasonable to assume that polymorphisms in the promoter region may influence gene expression.

CRYAB Mutations

Most of the currently known *CRYAB* mutations occurred in highly conserved regions; they generate adverse reactions when expressed in cell cultures[8] and a mouse model revealed a collapse of the desmin network.[12] Disease phenotypes associated with *CRYAB* mutations are clinically heterogeneous, including four mutations causing cardioskeletal syndrome with cataract present or absent and two mutations associated with cataracts alone.

A heterozygous A-to-G transition at *CRYAB* codon 120 resulting in p.Arg120Gly mutation[8] was identified in the original multigenerational French family with autosomal dominant desminopathy.[71] The Arg120 residue is located in the most conserved region shared by other small heat-shock proteins. Structural and functional studies indicate that the mutant alphaB-crystallin had reduced or completely lost chaperone function.[72,73] AlphaB-crystallin lost the ability to interact with alphaA-crystallin, but strongly interacted with wild type alphaB-crystallin,[74] suggesting a mechanism for dominant negative effect. In transiently transfected cells, mutant alphaB-crystallin accumulated in inclusion bodies (aggresomes) that may be due to misfolding of the mutant protein.[73]

Muscle cell lines transfected with p.Arg120Gly cDNA also show the presence of intracellular aggregates that contain desmin and alphaB-crystallin.[8] Transgenic mice expressing mutant alphaB-crystallin have abnormal desmin and alphaB-crystallin immuno-positive aggregates in cardiomyocytes.[12] This compromises cardiac muscle function and results in cardiac hypertrophy.

A 2-bp deletion (c.464_465CTdel) in the C terminus of alphaB-crystallin resulting in a truncated protein of 162 amino acids, instead of the normal 175, was present in a patient with myofibrillar myopathy.[75] The mutation was predicted to impair the ability of alphaB-crystallin to inhibit heat-induced protein aggregation of unfolded and denatured proteins, resulting in aberrant accumulation of proteins in muscle fibers. A c.451C>T transition resulting in a p.Gln151X substitution was also identified in a patient with myofibrillar myopathy.[75] The mutation results in a truncated protein of 150 amino acids and is predicted to be functionally deficient. Immunoblots under nondenaturing conditions showed that the mutant protein forms lower than normal molecular mass multimeric complexes with the wild type protein and exerts a dominant-negative effect.

The missense *CRYAB* p.Arg157His mutation occurring in an evolutionary conserved amino acid residue is associated with a late onset dilated cardiomyopathy. This mutation reduced the binding of alphaB-crystallin to the N2B domain of titin/connectin.[26]

Other *CRYAB* mutations have been associated with nonsyndromic autosomal dominant congenital cataracts (ADCC). A single nucleotide deletion in *CRYAB* (c.450Adel) causing termination at codon 150 was identified in a 4-generation English family with a posterior polar ADCC.[76] A p.Pro20Ser mutation of *CRYAB* cosegregated with ADCC in a large Chinese family.[77]

Pathogenesis

DES mutations are responsible for inadequate supply of normal functional desmin and toxic effects of aggregates containing mutant misfolded desmin and debris of other myofibrillar and ectopically expressed proteins that accumulate in the myofibers and eventually destroy them.[7,78] Additionally, mutant desmin hampers normal interactions with other cytoskeletal proteins. The amount of desmin in affected muscle fibres of desminopathy patients is increased as demonstrated by immunoblotting.[46,83] The pathological process in the myofibrils starts with the disintegration of the Z-disk, the functionally important site of tension transmission between the sarcomeres.[15]

Filament and Network Assembly Studies

Interference of *DES* mutations in filament-formation was tested in in-vitro filament formation experiments, generation of ordered cytoskeletal arrays and assays for the ability to integrate into an existing cytoskeletal network in various cell lines.[61] Analysis of filament assembly behavior demonstrates that some desmin mutants do not prevent filament formation (p.Ala213Val, p.Glu245Asp, p.Ala360Pro, p.Glu389Pro, p.Asn393Ile, p.Asp399Tyr). Some others interfere with the assembly process at distinct stages and these are classified into three groups: (1) mutations compromising longitudinal annealing properties (p.Leu385Pro, p.Arg406Trp); (2) mutants with enhanced adhesiveness leading to filament aggregation (p.Ala337Pro, p.Asn342Pro, p.Ala357Pro); and (3) mutants showing rapid disintegration of assembly precursors (p.Leu345Pro, p.Arg350Pro, p.Leu370Pro).[83]

It has been conclusively shown that pathogenic mutant desmin disrupts a preexisting filam-entous network in a dominant-negative fashion.[6,53,84] But some mutations located in the non-alpha-helical tail domain of the desmin molecule do not interfere with the initial IF assembly steps. Thus, the p.Thr442Ile, p.Lys449Thr, p.Ile451Met and p.Val469Met mutants, but not p.Arg454Trp and p.Ser460Ile, formed a filamentous network in SW13 cells. Furthermore, all tested tail domain mutants, including p.Arg454Trp and p.Ser460Ile, were incorporated into IF arrays of transfected C2C12 cells, suggesting that cultured myoblasts are able to neutralize the "poisonous" effect of the mutated protein.[56] Most likely, *DES* tail mutations affect multiple interactions with other cellular proteins resulting in distinct cellular malfunctions.

In cultured satellite cells taken from a patient carrying the p.Leu345Pro mutation, desmin created a fully normal network in early cell passages, however, in further passages an increasing number of cells showed abnormal accumulation of desmin-positive material with one of three patterns: perinuclear, spot-like or subsarcolemmal.[85] Nestin colocalised with the abnormal desmin deposits; alphaB-crystallin was only present in cells with a disrupted desmin network.

Animal Models

Although desmin knockout mice (*Des* $^{-/-}$) develop normally and are fertile, lack of a desmin filament network prevents organizing the cellular components spatially.[21,23,81] Some cell architecture defects such as misaligned muscle fibers, abnormal sarcomeres, swollen mitochondria and unusual distribution of myosins are seen in earlier stages of development.[86] After birth, irregularities in the myofibrillar organization are predominantly observed in the extensively used skeletal muscles such as the tongue, diaphragm and the soleus muscle.[21,23,81] Mitochondria exhibit an increase in size and number, a loss of correct positioning and finally degeneration, which was especially pronounced after exercise overload.[23]

Cardiac muscle is most susceptible to the lack of desmin. Mice develop cardiomyopathy early in postnatal life manifested by lysis of individual cardiomyocytes, invasion of macrophages, varying degree of calcification and finally fibrosis.[81] Older animals show fully characteristic morphology of muscle dystrophy.[23] Disorganized, distended and non-aligned fibers are observed in the diaphragm. Muscle fibres are gradually lost and replaced by fibrosis. The lack of desmin in growing and adult knock-out mice results in a multi-organ disorder involving severe disruption of skeletal and cardiac muscle architecture. These experiments show that desmin is essential for the structural integrity of skeletal muscle, but not for myogenic commitment, differentiation, or fusion of myoblasts.[87]

Analysis of transgenic mice has provided insights into the mechanisms of intracellular protein aggregation. In transgenic mice expressing a human mutation p.Arg173_Glu179del, examination of the myocardium reveals an accumulation of chimeric intracellular aggregates containing desmin and other cytoskeletal proteins.[7] Such inclusions are not seen in knockout mice, supporting the hypothesis that mutant misfolded proteins may act as seeds in the formation of these protein aggregates in desminopathies and likely in other myofibrillar myopathies. These aggregates clearly disrupt the continuity and overall organization of the desmin network throughout the cell.[7] The protein aggregates appear as electron-dense granulofilamentous structures proximal to the nucleus and in the inter-myofibrillar space. Misfolded desmin protein escapes proteolytic breakdown and attracts other cytoskeletal proteins into high molecular weight insoluble chimeric aggregates[82] that grow and become toxic.[88] Toxic effect of the aggregates may depend on sequestering of essential cellular proteins. Numerous fragmented filaments were found in the immediate area surrounding the aggregates.

Role of AlphaB-Crystallin

Chaperones assist normal protein folding and, if necessary, enhance ubiquitination and proteasomal degradation of abnormally constructed proteins. They help to restore proteins to their native conformation after these proteins have been misfolded by heat, ischemia, chemotoxicity, or other cellular stresses.[72] An in vitro chaperone assay demonstrated that the mutant p.Arg120Gly alphaB-crystallin becomes functionally deficient.[89] Expression of the mutant alphaB-crystallin in SW13 and BHK21 cells leads to formation of abnormal aggregates that contain both desmin and alphaB-crystallin.[8] Transgenic mice expressing mutant alphaB crystallin show the presence of abnormal desmin and alphaB-crystallin aggregates in the cardiomyocytes; formation of aggregates is the result of the loss of protection from alphaB-crystallin.[12,90]

Aggregation of amyloid-like material was recently demonstrated to be a typical feature of many human cardiomyopathies, especially those caused by mutations in *CRYAB*.[91] Physiologically, the inclusion of misfolded proteins into aggregates was proposed to be a protective mechanism in alphaB-crystallinopathy,[92] linking desminopathy to a broad class of conformational neurodegenerative diseases. Remarkably, desmin knock-out and transgenic mice show less severe pathology as compared to the *CRYAB* transgenic mice.[12]

Evidence that kinases are involved in desminopathies has come from observations of CDC2 and CDK2 overexpression in the abnormal intracytoplasmic aggregates,[93,94] but the specific role kinases may play in desminopathy has not yet been determined.

Clinical Manifestations

Definitions of Desminopathy

Desminopathy was originally described as skeletal and cardiac myopathy characterized by bilateral weakness in distal leg muscles spreading proximally, or in proximal muscles spreading distally and leading eventually to wheelchair-dependence.[95] Weakness may or may not involve upper extremities, trunk, neck flexors and facial muscles. In disease variants marked with early onset cardiomyopathy, patients experienced dizziness, syncopal and fainting episodes associated with conduction blocks requiring a permanent pacemaker. Respiratory muscle weakness is a frequent component; bulbar signs appear in the later stages of illness.

Many new affected families were identified and reported within the 10-year period since desminopathy has been classified as an independent entity; this review represents 65 published families with 98 patients. New knowledge allows defining more precisely the clinical manifestations and diagnostic criteria of desminopathy and relationships between desminopathy and other disorders.

The pattern of inheritance was reliably established in 52 desminopathy families. It was autosomal dominant in 32 families and autosomal recessive in three. Seventeen patients had sporadic disease, in seven of them both parents were available for genetic testing and evidence was obtained that the

mutations have occurred de novo. The age of disease onset in patients with autosomal dominant disease is between 14 and 48 years, while patients with recessive mutations develop the disease in their childhood or adolescence. Analysis of clinical/pathological characteristics of desminopathy outlined several sometimes overlapping clinical forms.[96]

Progressive Skeletal Myopathy

Uncomplicated progressive skeletal myopathy was observed in 13 patients from 6 families (Table 2). The disease onset in members of the p.Asn342Asp family, mother and son, was at age 30 and 23 years, respectively.[11] Initial symptoms were bilateral distal muscle weakness in the lower extremities that later spread to proximal leg muscles, upper extremities and neck flexors. ECG was normal as was the serum creatine kinase level (CK). Muscle biopsy showed abnormal accumulation of desmin-immunoreactive deposits in muscle fibers and the presence of red-rimmed vacuolated fibers. The p.Glu359_Ser361del mutation was identified in two Polish families[49] sharing a disease-associated haplotype. Eight patients were fully characterized. The age of disease onset was between 31 and 46 years. Initial symptoms were bilateral muscle weakness first in the lower and later in the upper extremities. Electrocardiogram (ECG) and echocardiogram (EchoCG) showed no abnormalities. Sections of skeletal muscle demonstrated the presence of cytoplasmic inclusions that were immunoreactive for desmin and contained electron-dense coarse granular and filamentous aggregates on EM.

Muscle CT scan studies performed in desminopathy patients carrying various *DES* mutations have identified a recognizable pattern of muscle involvement. At the thigh level, early involvement of the semitendinosus and sartorius is followed by the gracilis muscle and then the hip adductors. In advanced illness, quadriceps femoris and other muscles of the posterior thigh compartment also become affected. At the mid-calf level, initial changes occur in the peroneal group, followed by involvement of the anterior tibialis and later the posterior group[50] (Fig. 4).

Respiratory Dysfunction

Respiratory insufficiency can be a major cause of disability and death. Respiratory dysfunction causes nocturnal hypoventilation with oxygen desaturation and, eventually, daytime respiratory failure. The diaphragm, unlike other skeletal muscles, is functioning in an environment in which forces can be transmitted both in longitudinal and transverse directions during each respiratory cycle; desmin is the only known molecule having dual orientation and therefore serving as a viscoelastic element that dissipates mechanical energy in both planes. Reflecting on this critical significance of desmin in respiratory function, its content in the diaphragm is 38% higher than in the biceps femoris muscle.[98]

Progressive skeletal myopathy with early respiratory muscle involvement, but no cardiac disease, was observed in 10 patients from 5 families. In a family carrying the missense p.Ala357Pro mutation, the father and his son and daughter developed at age 35 to 45 years slowly progressive muscle weakness in the lower and upper extremities but no signs of cardiac involvement.[48] Respiratory function tests revealed progressive reduction of respiratory muscle strength that became clinically detectable between approximately the 3rd and the 8th years of illness. A gradient between vital capacity in the upright and supine position of more than 30% is suggestive of preferential diaphragmatic involvement. The patients had modest (4-times normal) elevation of serum CK and normal ECG and EchoCG studies. Muscle biopsy showed variation in fibre size, intracytoplasmic eosinophilic patches immunocytochemically identified as desmin deposits and deposits of dense granular material between myofibrils and in the subsarcolemmal space on EM.

Cardiomyopathy

Primary cardiomyopathies are classified pathophysiologically as dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC).[99] DCM is characterized by an increased ventricular chamber size and reduced systolic output.[57] RCM results from processes that stiffen the myocardium by infiltration or fibrosis leading to impaired ventricular filling and reduced diastolic

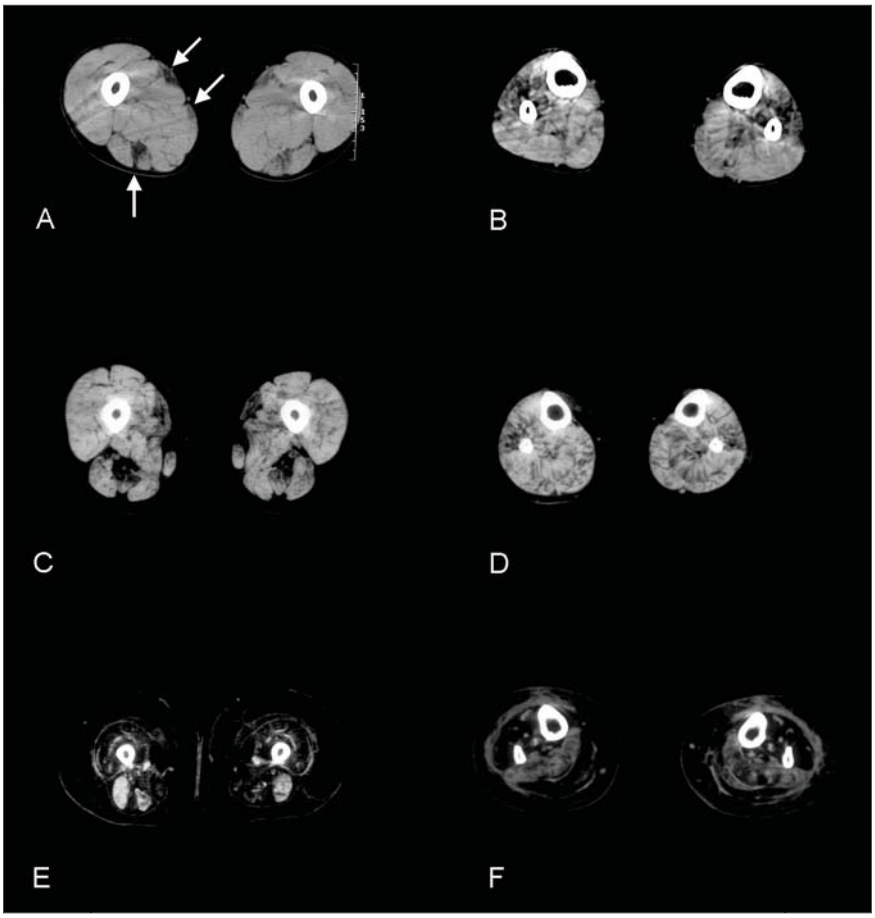

Figure 4. Muscle CT scans performed in three desminopathy patients at different stages of illness at mid-thigh (A, C and E) and mid-calf (B, D and F) levels. Two years after the initial symptoms muscle CT scan in a patient carrying a *DES* Ile367Phe mutation showed initial involvement of the semitendinosus, sartorius and gracilis muscles (arrows in A), whereas at the mid-calf level a decreased attenuation was seen in the peroneal group and anterior tibialis (B). More advanced changes are present in these muscles in a patient carrying a DES p.Arg406Trp mutation ten years after the disease onset (C and D). Almost all muscles are replaced by fatty tissue in a patient with a *DES* p.Asn366del mutation 20 years after the disease onset (E and F). A to D images are reproduced from Neuromusc Disorders 2007; 17:448, with permission.

volume of either or both ventricles with the cavity size, wall thickness of the ventricles and ejection remaining near normal.[100] Of 34 patients with cardiomyopathy in which EchoCG was performed, DCM was diagnosed in 18, RCM in 10 and HCM in 6.

Atrioventricular conduction abnormalities requiring urgent implantation of a permanent pacemaker is a frequent feature in desminopathy patients attributed to the fact that the heart conduction system is rich in desmin. Desminopathy-associated atrioventricular conduction blocks may be associated with RCM, DCM and HCM.[37,46,55]

Skeletal muscle weakness followed by cardiomyopathy was observed in 31 patients from 20 families (Table 2). A p.Leu345Pro mutation within the 2B helix[6] was detected in a family that

included 16 members suffering from gait disturbances caused by bilateral weakness in distal leg muscles that progressed to all limbs and bulbar, respiratory and facial muscles.[101] Many of the surviving patients were confined to a wheelchair or using a walker 7 to 20 years after disease onset. Six of 8 studied patients were diagnosed with cardiac arrhythmias and conduction blocks about 12 years after the appearance of myopathic symptoms and developed congestive heart failure. Histopathologically, some skeletal muscle fibers were atrophic and contained abundant desmin-positive granulofilamentous deposits in the form of a reticular meshwork.

A phenotype characterized by cardiomyopathy with subsequently developing skeletal myopathy was seen in 15 patients from nine families. This group includes two brothers with a p.Asp214_Glu245del mutation.[3] One patient developed DCM with recurrent left-sided cardiac failure, complete AV block and pulmonary hypertension.[1] Gait disturbance and weakness in the legs appeared 10 years after the onset of cardiac illness and progressed to involve both hands. The patient died of cardiac failure at age 52 years. Skeletal muscle fibers showed accumulation of cytoplasmic bodies and patch-like lesions immunoreactive for desmin, alphaB-crystallin and dystrophin and granulofilamentous material in subsarcolemmal areas. His brother developed an AV block that required a pacemaker at age 41 years, but had no skeletal muscle weakness when last examined at age 50 years. He also died from cardiac complications.

Three patients from a family with p.Glu413Lys mutation experienced recurrent episodes of syncope requiring urgent cardiac pacemaker implantation at the ages of 30, 31 and 63 years.[55] Routine ECG showed atrioventricular block. On EchoCG, there was normal morphology and robust systolic function; atria were significantly enlarged and inferior vena cava distended. Doppler examination indicated restrictive pattern of left ventricular filling with deceleration time of E wave of mitral inflow shortened and annular velocity Em reduced, consistent with RCM. Two of the three patients developed muscle weakness and atrophy of the lower limb muscles starting 5 years after the cardiac disease onset in the index case and 16 years in the second case. Skeletal muscle biopsy showed multiple intrasarcoplasmatic deposits reacting with anti-desmin antibodies and on EM granulofilamentous electron-dense material at the level of Z-lines.

The primary syndrome in seven patients from four families was RCM with atrio-ventricular block;[37] only two patients showed clinical evidence of skeletal myopathy. Desmin accumulation was demonstrated in the myocardial and skeletal muscle samples. The p.Ala213Val *DES* variant was detected in 12 unrelated desminopathy patients developing dilated[40] or restrictive[39] cardiomyopathy.

Cardiomyopathy alone with no signs of skeletal myopathy was seen in 19 patients from 15 families. Six members of an AD family bearing the p.Ile451Met mutation in the desmin tail domain developed cardiac failure between the ages of 15 to 37 years.[57] Two living patients, father and son, showed cardiomegaly and diminished left ventricular ejection fraction consistent with DCM. No signs of skeletal myopathy were observed. A cohort of six DCM patients with no skeletal muscle involvement caused by various *DES* mutations, including two patients with mutations in the tail domain, was recently reported.[38]

Correlation between Genotype and Phenotype

Soon after routine genetic screening became available for diagnostic use, convincing evidence has emerged suggesting that different mutations result in somewhat distinct clinical phenotypes.[96] Cardiomyopathy, smooth muscle myopathy, neuropathy, respiratory dysfunction, facial paralysis or cataracts may be present in some cases and absent in others. With few exceptions, phenotypic manifestations in members of the same family were concordant, making it likely that the type and location of the mutation within the desmin molecule influences the phenotype. Analysis presented in Table 5 confirms that patients with mutations in the 2B segment tend to show primarily skeletal muscle pathology, while those carrying mutations in 1B and tail domains develop predominantly a more ominous cardiac disease. The difference between the frequency of cardiomyopathy in patients with 1B mutations vs. those with mutations in the 2B domain estimated by the Mantel-Haenszel Chi-Square criteria is at the level of $p = 0.0031$. The frequency of cardiomyopathy in patients with tail vs. 2B mutations is $p = 0.049$. We conclude therefore that the location of *DES* mutation exerts

Table 5. Desminopathy clinical phenotypes caused by mutations located in different functional domains of desmin

	Desmin Domain		
Phenotypes	**1B**	**2B**	**Tail**
Isolated Progressive Skeletal Myopathy	1	11	3
Skeletal Myopathy with Respiratory Insufficiency	1	6	3
Skeletal Myopathy Followed by Cardiomyopathy	3	24	5
Cardiomyopathy Followed by Skeletal Myopathy	4	10	3
Isolated Cardiomyopathy	6	4	8
Total number of patients	15	55	22

a significant influence on phenotypic characteristics. There is also clear tendency for the age of onset to be 4 to 10 years earlier and the disease progression faster in patients with cardiomyopathy as an early feature.

A discrepancy between *CRYAB* p.Arg120Gly mutation causing cardioskeletal myopathy and p.Arg157His mutation causing isolated cardiac muscle dysfunction was also investigated and found to be dependent on reduced binding of the p.Arg157His mutant to the heart-specific N2B domain, but not the I26/I27domain of titin/connectin, while the p.Arg120Gly mutation decreased binding to both N2B and the striated muscle-specific I26/27 domains.[26] These observations suggest that the disease-causing mechanisms are different for these two *CRYAB* mutations.

Disease Severity in Patients with Autosomal Recessive Inheritance

Three autosomal recessive (AR) *DES* mutations are currently known. These patients had the earliest age of onset and the fastest progression of illness. A patient homozygous for deletion of seven amino acids in the 1B helix (p.Arg173_Glu179del),[10] developed generalized muscular weakness and atrophy, predominantly in distal muscles of the upper extremities, atrioventricular (AV) block requiring implantation of a permanent pacemaker and intestinal malabsorption.[102] EchoCG showed dilatation of the right cardiac chambers. Disease progression led to cardiac and respiratory failure and intestinal pseudo-obstruction. The patient died suddenly at age 28 years. Abundant subsarcolemmal crescent-shaped strongly eosinophilic masses in skeletal myofibers and centrally located eosinophilic bodies in the cardiomyocytes were immunoreactive for desmin and ubiquitin. Ultrastructural studies revealed electron-dense coarse granular and filamentous aggregates continuous with the Z lines.

In another AR family, three siblings were compound heterozygous for the p.Ala360Pro and p.Asn393Ile *DES* mutations.[9] They presented with syncopal episodes and complete heart block requiring insertion of a permanent pacemaker at the age of 2, 9 and 10 years. EchoCG showed moderate to severe biatrial dilatation but normal ventricle size. Cardiac catheterization revealed left ventricle diastolic dysfunction.[11] Between ages 20 and 24 years, all three developed progressive muscle weakness and wasting in the extremities and trunk, weakness in the neck and facial muscles, swallowing and breathing difficulties. All three died from congestive heart failure at 28, 30 and 32 years of age. Histopathologic findings consisted of intramyofibre accumulation of amorphous desmin-immunoreactive material with a characteristic subsarcolemmal distribution. Several older family members carrying either the p.Ala360Pro or p.Asn 393Ile heterozygous mutation had no signs of muscle or heart disease.

De Novo Mutations

Desminopathy patients associated with de novo *DES* mutations represent a complex group with even wider margins of phenotypic variability. In several so-called sporadic cases with no family history, microsatellite markers and *DES* polymorphisms were used in patients and both parents

to exclude false paternity and trace the mutant allele. If neither of the parents was affected and none showed *DES* mutations, but shared a haplotype with the affected offspring, this mutation was considered to be generated de novo.[3]

Four West European patients with de novo p.Arg406Trp mutation presented at ages 15, 18, 23 and 24 years with cardiac arrhythmia and conduction block followed in quick succession by muscle weakness.[54] The causative mutation was not present in the parents; the mutation has occurred de novo on a paternal allele transmitted to the affected offspring. All four became severely incapacitated in their twenties-early thirties and one of the patients died from decompensated congestive heart failure at the age of 28 years. Sections of skeletal muscle showed a significant accumulation of aggregates strongly positive for desmin and EM evaluation showed abnormal granulofilamentous aggregates among the myofibrils and beneath the sarcolemma.

p.Asn342Asp *DES* mutation was identified in affected mother and son, but not in the unaffected maternal grandparents.[58] The results of the haplotype analysis demonstrated that the causative mutation has occurred de novo on an allele the affected mother inherited from the grandmother and then transmitted to her son, suggesting that the grandmother was germ line mosaic.

Phenotypes Associated with CRYAB *Mutations*

The p.Arg120Gly *CRYAB* mutation was identified in a large family with autosomal dominant myopathy involving proximal and distal limb muscles often associated with neck, trunk and velopharynx muscle weakness, hypertrophic cardiomyopathy, respiratory disturbances and discrete lens opacities.[8,95,103] The age of disease onset was in the mid-30s and rate of progression was moderate. Muscle biopsy results were characteristic of desminopathy.[8,103]

Two further patients carrying c.464_465CTdel and c.451C>T (p.Q151X) *CRYA*B mutations had adult-onset cervical, limb girdle and respiratory muscle weakness in patient 1 and proximal and distal leg muscle weakness in patient 2.[75] Both had myopathic electromyogram with abnormal electrical irritability. Muscle biopsy findings were characteristic of myofibrillar myopathy and mild denervation. Disintegration begins at the Z-disk and results in abnormal local expression of desmin, alphaB-crystallin, dystrophin, neural cell adhesion molecule (NCAM) and CDC2 kinase. Neither patient had cardiomyopathy or cataracts.

The missense p.Arg157His CRYAB mutation was associated with dilated cardiomyopathy presenting after the fourth decade of life; no skeletal muscle involvement was observed.[26]

Myopathology

Desmin-reactive deposits in the cardiac and skeletal muscles and granulofilamentous material at the ultrastructural level are considered morphological hallmarks of desminopathy.[104] Although many of the myopathological features are not specific, the overall pattern is recognizable and is being used for diagnostic purposes.

Skeletal Muscle Pathology

In normal myofibres, desmin is located beneath the sarcolemma and at the Z-disk, whereas the intracellular desmin intermediate filament network is less clearly visualized.[105,106] At the electron microscopic (EM) level, desmin intermediate filaments are visible as straight 8-10 nm nonhollow filaments. Desmin and nestin are normally present at the sub-neural apparatus of the neuromuscular junctions and at myotendinous junctions.[107,108]

In patients with desminopathy, examination of skeletal muscles by light microscopy typically show irregularly shaped abnormal regions containing amorphous eosinophilic inclusions best identified with modified trichrome stain as dark green or bluish material. They are located in the subsarcolemma or within the cytoplasm. The size and shape of the inclusions varies: they may consist of "plaque-like" patches or appear as small rounded structures.[42,47,55] Frequently, both types of inclusions coexist in the same specimen (Fig. 5). In addition, hyaline structures, cytoplasmic bodies, rods and spheroid-like bodies are reported in other cases.[3,11,47]

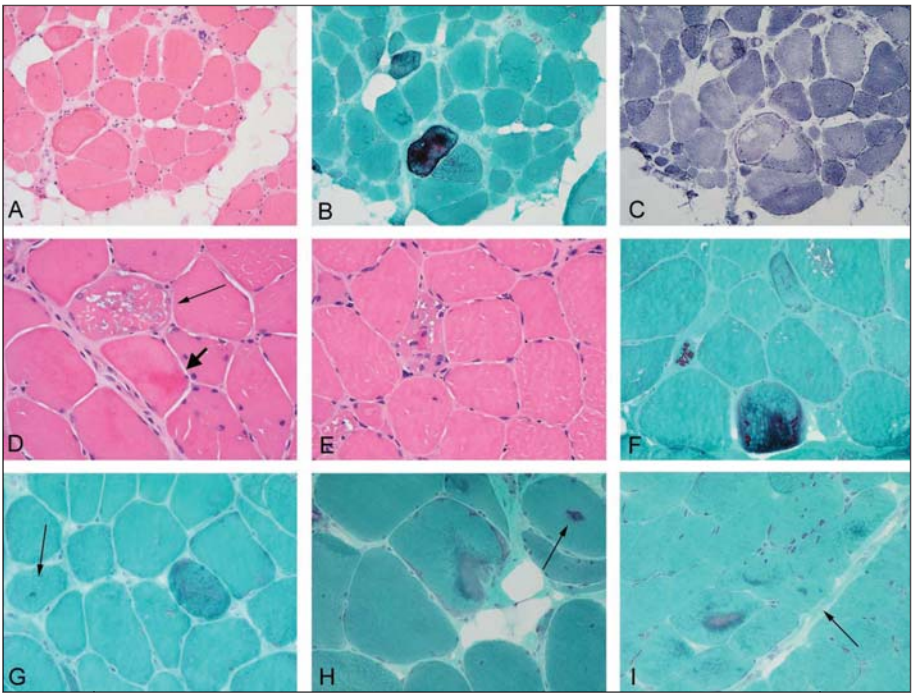

Figure 5. Types of lesions observed in patients with desmin mutations. A, B and C: muscle pathology in a patient with p.Leu392Pro *DES* mutation at advanced stage of illness showing variability in the size of fibers, an increased number of internal nuclei and areas of fibro-fatty tissue infiltration. Several fibers contain cytoplasmic inclusions that are eosinophilic on HE stain (A), blue-dark on the modified trichrome stain (B) and devoid of oxidative activity (C). D, E and F: muscle pathology in a patient with p. Ile367Phe *DES* mutation. The following features are observed: a large fibre containing rimmed vacuoles (thin arrow in D); a plaque-like inclusion in an adjacent fiber (thick arrow in D), an eosinophilic body in a fiber containing rimmed vacuoles (E); collections of small cytoplasmic red bodies in two muscle fibers, sometimes in association with a plaque-like inclusion (F). G, H and I: muscle pathology in patients with p.Pro419Ser, p.Asn366del and p. Arg406Trp *DES* mutations. Single small green inclusions (arrows) are seen in some fibers and plaque-like lesions in others. Cryostat sections stained with H&E (A, D and E), modified trichrome stain (B, F, G, H and I), NADH (C). Original magnification × 200 before reduction, except for H which is × 400.

Oxidative enzyme and ATPase activity is typically absent in the inclusions leading to rubbed-out lesions. Impairment of the mitochondrial respiratory chain complex has been reported,[42] but ragged-red fibers have not been observed. With only a few exceptions, congophilia is not reported in desminopathies.

Although not specific of desminopathy, rimmed vacuoles have been observed in a majority of cases. Other nonspecific myopathic features such as variation in fibre size, scattered atrophic fibres and an increased number of internal nuclei are also frequently observed. Muscle fibre necrosis, inflammation or regeneration have been reported in a few cases.[40,42,46] Fibrosis and fatty replacement have been found occasionally.[1,3,43,46,85] Under electron microscopy, all desminopathy muscle specimens showed granulofilamentous material representing a unifying feature.[104] The granular component of the granulofilamentous material is often more prominent than the filamentous, accumulating beneath the sarcolemma and between the myofibrils. In addition, Z-band streaming and more compact bodies of spheroid or cytoplasmic type were noted in some cases (Table 6).

Table 6. Genotype-morphotype correlations in patients with various* DES *mutations

DES Mutation	Biopsied Muscle	Myopathology	Electron Microscopy	Reference
p.Arg173_Glu179del	deltoid	subsarcolemmal crescent-shaped eosinophilic masses, greenish autofluorescence, desmin-immunoreactive atrophic fibers, increase in internal nuclei, eosinophilic desmin-positive masses	GFM, abnormal sarcomeres	10,102
p.Ala213Val	not mentioned	necrosis, desmin & alphaB-crystallin aggregates	not mentioned	40
p.Lys240fsX243	vastus lateralis	variation in fiber diameters, mild endomysial fibrosis, regenerating fibers, rubbed-out lesions, inclusions positive for desmin, alphaB-crystallin, synemin, plectin, ubiquitin	GFM	42
p.Glu245Asp	deltoid, gastrocnemius (same patient)	fiber size variation, internal nuclei, mild fibrosis, rimmed vacuoles, patches of desmin aggregates	GFM, tubulo-filamentous aggregates	43
Ip.Asp214_Glu245del	gastrocnemius	fiber size variation, internal nuclei, rimmed vacuoles, fibrosis, cytoplasmic bodies, subsarcolemmal and cytoplasmic desmin aggregates	GFM	1,3
p.Ala337Pro	not mentioned	intracytoplasmic desmin aggregates	GFM, Z band streaming	11,40
p.Leu338Arg	not mentioned	desmin aggregates	not mentioned	40
p.Asn342Asp	not mentioned	rimmed vacuoles, desmin deposits	GFM, Z band streaming	11
p.Leu345Pro	vastus & deltoid (same patient)	variation in fiber size, fibrosis, fat cell replacement, increase in internal nuclei, subsarcolemmal and cytoplasmic desmin aggregates	not mentioned	85

continued on next page

Table 6. Continued

DES Mutation	Biopsied Muscle	Myopathology	Electron Microscopy	Reference
p.Arg350Trp	not mentioned	fiber size variation (8-175μm), necrotic & regenerating fibers, increase in internal nuclei, increased endomysial fibrosis, cytoplasmic and subsarcolemmal desmin aggregates	GFM	46
p.Arg355Pro	biceps	variation in fiber size, round eosinophilic inclusions in type II fibers, single and diffuse desmin aggregates	GFM, vacuolar mitochondria, spheroid-like ellipsoid bodies	47
p.Ala357Pro	not mentioned	atrophic fibers, variation in fiber size, vacuoles, subsarcolemmal and cytoplasmic desmin aggregates	GFM	48
p.Glu359_Ser361del	not mentioned	atrophic fibers, granular desmin aggregates	GFM	49
p.Ala360Pro p.Asn393Ile	not mentioned	subsarcolemmal desmin aggregates	GFM	11,40
p.Asn366del	deltoid	marked variation in fibre size, many internal nuclei, rimmed vacuoles, ring fibres, subsarcolemmal aggregates of desmin and synemin	GFM	49, 97
p.Ile367Phe	gastrocnemius	variation in fiber size, large numbers of rimmed vacuoles, subsarcolemmal and cytoplasmic, desmin, alphaB-crystallin, dystrophin, gamma-filamin aggregates	GFM	50
p.Leu370Pro	deltoid, gastrocnemius (two patients)	fiber size variation, internal nuclei, rimmed vacuoles, subsarcolemmal and cytoplasmic desmin aggregates	GFM	51
p.Leu385Pro	anterior tibialis	variation in fiber size, fibrosis, vacuoles, desmin aggregates,	GFM	52

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Table 6. Continued

DES Mutation	Biopsied Muscle	Myopathology	Electron Microscopy	Reference
p.Leu392Pro	Biceps brachii	variation in fiber size, few rimmed vacuoles, fibrofatty tissue replacement, subsarcolemmal and cytoplasmic desmin, alphaB-crystallin, dystrophin, gamma-filamin aggregates	GFM	50
p.Asp399Tyr	not mentioned	atrophic fibers, desmin aggregates	not mentioned	40
p.Glu401Lys	deltoid	atrophic fibers, rimmed vacuoles, myophagocytosis, desmin aggregates	not mentioned	40
p.Arg406Trp	deltoid	mild variation in fibre size, few internal nuclei, ring fibers, cytoplasmic and subsarcolemmal aggregates of desmin	GFM, Z band streaming	11,97
p.Pro419Ser	gastrocnemius	variation in fiber size, rimmed vacuoles, subsarcolemmal and cytoplasmic desmin, alphaB-crystallin, dystrophin, gamma-filamin aggregates	GFM	50
p.Ile451Met	not mentioned	desmin deposits	GFM, Z band streaming	11
CRYAB p.Arg120Gly	not mentioned	cytoplasmic inclusions with reduced or absent oxidative enzyme histochemical activities, desmin, ubiquitin, alphaB-crystallin and dystrophin deposits	GMF	71,103

GFM: granulofilamentous material.

Autophagic vacuoles containing myelin-like lamellae and debris[43] and focal groupings of mitochondria[42,47,55,56] have been observed in some specimens.

Immunohistochemical studies have consistently demonstrated the presence of inclusions or deposits reacting with antibodies against desmin and other proteins such as alphaB-crystallin and, although rarely investigated, synemin,[42,85,97] syncoilin,[109] plectin,[42,43] nestin,[6,85] dystrophin,[2,43,50] merosin, alpha- and beta-dystroglycan, alpha-, beta-, gamma- and delta-sarcoglycans, utrophin, collagen VI, NOS, caveolin, dysferlin, beta- and gamma-laminin, actin, actinin, N-CAM, heat shock protein 72/73,[43] myotilin,[2,43,50] gamma-filamin,[50] vimentin, beta-spectrin[11] and ubiquitin..[43,50,102] The size, shape and localization of protein aggregates differ from one case to another. These may be restricted to the subsarcolemmal regions or within the cytoplasm; they may be diffuse or well demarcated, and in some cases both diffuse and well demarcated small deposits are found in the same specimen (Fig. 6).

Of the large number of mutations already documented in the *DES* gene, most have been published as case reports and only a few mutations described comparatively.[11,40,56] Thus, it has been difficult to establish genotype-morphotype correlations of individual mutations. Distinctive muscle pathology was reported in a patient carrying a homozygous p.Arg173_Glu179del mutation: prominent eosinophilic crescent-shape masses located under the sarcolemma in virtually each muscle fiber. These lesions stained dark green with modified trichrome, displayed autofluorescence and showed strong desmin immunoreactivity (Table 6).[10,102] Identical abnormalities have been observed in an unpublished patient carrying the same *DES* deletion (Fig. 7).

In the well studied family with *CRYAB* p.Arg120Gly mutation, muscle biopsy showed disorganization of filamentous network and characteristic regions in which the intermyofibrillar network completely disappeared (rubbed-out fibers). Affected areas contained instead abnormal aggregates immuno-positive for desmin, alphaB-crystallin, dystrophin and ubiquitin. A subsarcolemmal and intermyofibrillar accumulation of dense granulofilamentous material with various degenerative changes was observed on EM.[8,103]

Cardiac and Smooth Muscle Pathology

While cardiomyopathies are a frequent component of desminopathies, cardiac pathology has not been fully characterized. Postmortem examination of the heart from a patient carrying an p.Arg173_Glu179del mutation showed dilatation of the right chambers; cardiac cells displayed abundant centrally located globular eosinophilic bodies strongly reacting against desmin and ubiquitin.[10,102] The right ventricle was the more severely involved area. Additional cardiopathological findings have been reported based on examination of cardiac biopsies or explanted hearts.[37,43,52,56] In all described cases, microscopic examination revealed protein aggregates in the center of cardiac muscle fibre, rather than subsarcolemmally, hypertrophy of cardiomyocytes, prominent nuclei and considerable interstitial fibrosis (Fig. 8). Cardiac myocytes contain aggregates composed of desmin (Fig. 8) and other proteins such as alpha-B crystallin, ubiquitin, heat shock protein 72/73 and others.[43] At the ultrastructural level the deposits consist of granulofilamentous material, often clustered at the intercalated disks. Overall, the cardiac pathology as described is very similar to skeletal muscle pathology. Desmin-positive aggregates and other phenomena characteristic of skeletal muscle pathology were also encountered in intestinal smooth muscle cells of a desminopathy patient.[102]

Diagnosis

Recognition of desminopathy can be difficult because of the heterogeneity of clinical features and nonspecificity of the histopathology. The pattern of inheritance is also variable; most of the known mutations are dominant, but others are recessive and a significant number of mutations are generated de novo.

Diagnostic Criteria

The diagnosis of desminopathy should be consistent with the following basic criteria.[2,11,15,78,110] History of slowly progressive muscle weakness, dyspnea, dysphonia, dysphagia and cardiac symptoms.

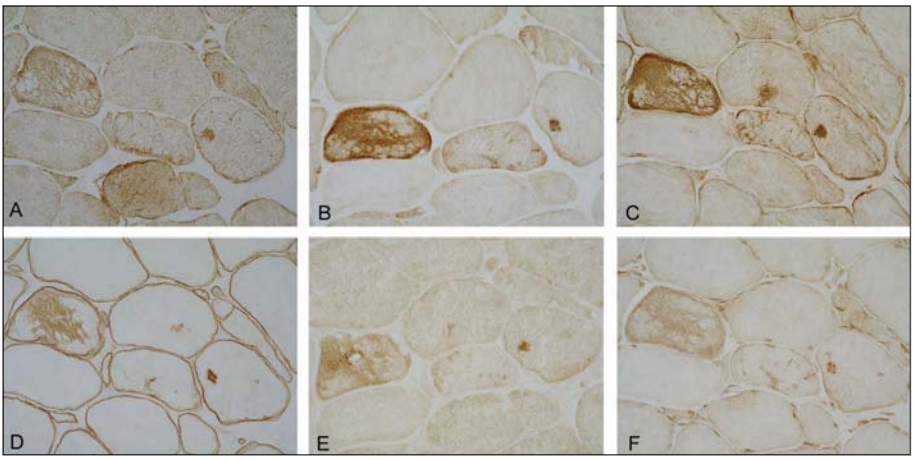

Figure 6. Diffuse or single inclusions containing desmin (A), alphaB-crystallin (B), gamma filamin (C), dystrophin (D), myotilin (E), or ubiquitin (F) in a patient with p.Pro419Ser mutation. Cryostat sections, original magnification × 400.

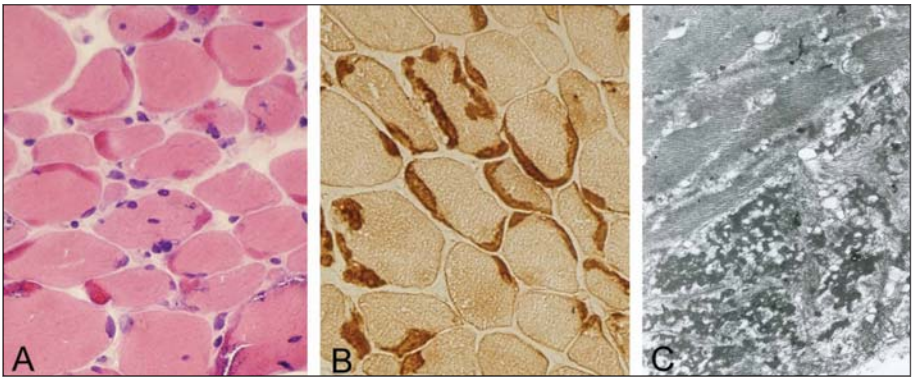

Figure 7. Muscle pathology in a patient with homozygosity for the p.Arg173_Glu179del *DES* mutation. Prominent eosinophilic masses located under the sarcolemma and sometimes associated with basophilic granular material (A), displaying strong desmin immunoreactivity (B). On electronmicroscopy, the masses are composed of a matrix of dense granulofilamentous material (C). Cryostat sections, original magnification in A and B × 200; C × 5000. Figure kindly provided by Dr. Ana Cabello.

Physical examination reveals distal and proximal weakness; trunk, neck-flexor and facial muscles are involved in some patients. Tendon reflexes are diminished or normally active. Joint retractions at ankles may be present. A restrictive ventilatory defect may result from respiratory muscle weakness. ECG shows conduction blocks in a high proportion of cases. EMG reveals abnormal electrical irritability (fibrillation potentials, positive sharp waves, complex repetitive discharges and occasional myotonic discharges) in most patients. The motor unit potentials show myopathic features or a combination of myopathic and neurogenic changes. Serum creatine kinase concentration can be normal or elevated to no greater than seven-fold above the upper normal limit.

Muscle histology reveals: (1) characteristic alterations in trichromatically stained frozen sections consisting of amorphous, or granular material in a variable proportion of the muscle fibers, (2) sharply circumscribed decreases of oxidative enzyme activity in many abnormal fiber regions,

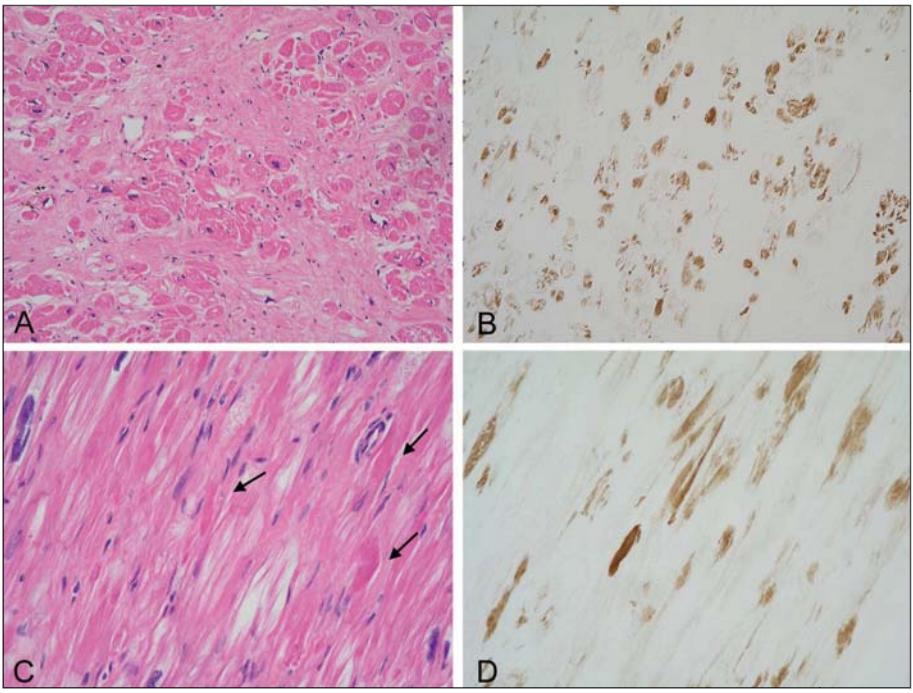

Figure 8. Formalin-fixed, paraffin embedded cardiac tissue of the explanted heart from a patient with p.Arg406Trp *DES* mutation showing severe cardiomyocyte loss and extensive fibrosis. Variability in the size of cardiac cells and prominent nuclei are observed (A). On longitudinal sections (C) masses of eosinophilic material are seen within the cardiac cells (arrows). Strong desmin immunoreactivity is present in transverse and longitudinal sections (B and D). Original magnification × 200 (A and B) and × 400 (C and D).

(3) small vacuoles in a variable number of fibers; (4) abnormal ectopic expression of desmin, alphaB-crystallin and dystrophin in immunocytochemical studies. Electron microscopy shows granulofilamentous material under the sarcolemma or within the myofibrils. Autophagic vacuoles are observed in some cases.

Recommended Investigations

- Electrophysiological investigations including nerve conduction studies and EMG examination to exclude neurogenic causes of weakness, motor neuron disease and peripheral neuropathy.
- ECG used routinely to identify arrhythmias and cardiac conduction defects. Holter monitoring is indicated if symptoms suggest an intermittent arrhythmia.
- EchoCG to detect and diagnose the type of cardiomyopathy, it should be performed even in patients with no cardiac symptoms.
- Respiratory function tests even if respiratory symptoms are not present.
- Muscle imaging to differentiate desminopathies from other MFMs.

Molecular Testing

Genetic testing has become essential to establish an accurate diagnosis of desminopathy. Clinical genetic testing for desminopathy patients is now available at a Clinical Laboratory of Baylor College of Medicine, Jeffrey A Towbin, MD, Director (http://www.genetests.org/, go to "Laboratory Directory" and type in "desminopathy"). Clinical and myopathological diagnosis must precede

genetic testing. It is expected that more patients with the clinical diagnosis of desminopathy will in fact show mutations in other interacting genes, or a combination of genes. Routine genetic testing is necessary for providing appropriate genetic counseling. The true prevalence of desminopathy may be established only when most or all patients clinically and pathologically resembling desminopathy are tested genetically. The era of gene-specific and mutation-specific treatments for inherited diseases is quickly approaching.

Relationships between Desminopathy and Other Myofibrillar Myopathies

Pathologically, desminopathies belong to a genetically heterogeneous group named myofibrillar myopathies (MFM).[78,110] The common pathological pattern is myofibrillar dissolution, accumulation of myofibrillar degradation products and ectopic expression of multiple proteins. Mutations in six genes have been identified as causing MFM: desmin, alphaB-crystallin, selenoprotein 1, myotilin, ZASP and gamma-filamin. Further genetic heterogeneity is suspected (see ref. 15 for a review).

Differences and Similarities

Although no defined canon of prescribed myopathological characteristics for individual forms of MFM exist, subtle possible differences have already emerged (Table 7). Light and EM examination of skeletal muscles in patients with *CRYAB* mutations (crystallinopathy) reveal very similar if not identical features to those described in patients with mutations in desmin.[8,71,75,103] However, immunocytochemical studies have shown lack of ubiquitin, gelsolin, or alpha1-antichymotrypsin accumulation in abnormal fibres as a distinguishing feature in some cases of crystallinopathy,[75] but not the others.[8,71,103]

Some pathologic features of desminopathy make it distinct from typical images seen in myotilinopathy. First, the inclusions found in desminopathy are usually smaller and less prominent than those observed in myotilinopathy; second, nonrimmed vacuoles have been repeatedly reported in myotilinopathy but not desminopathy; third, congophilia is an important feature associated with the hyaline lesions observed in myotilinopathy, but it is usually absent or faint in desminopathy; four, typically spheroid-bodies are part of the picture in myotilinopathy but not desminopathy. Finally, ubiquitin, gelsolin and particularly myotilin expression is much more intense in myotilinopathy than desminopathy patients.[2,50,111-114]

Histological and immunohistochemical analysis of skeletal muscles in zaspopathy[115] reveal abnormalities very similar to those found in myotilinopathy. The hallmark of desminopathy, the granulofilamentous material, could also be present in myotilinopathy, zaspopathy and filaminopathy patients, but autophagic vacuoles, myofibrillar degeneration and accumulation of compacted and fragmented filaments and dense material seem to be more consistently found in myotilinopathy and zaspopathy.[111-115] In addition, clusters of cytoplasmic 15-28 nm filaments have been observed in myotilinopathy.[114,116]

In a subset of patients with mutations in SEPN1 gene, muscle biopsies showed distinctive circumscribed hyaline plaques resembling Mallory bodies known in hepatic disorders. Immunohistochemical studies revealed accumulation of desmin, alphaB-crystallin, actin, A-beta-amyloid and many other proteins within the plaques. Z-band streaming, specific minicores, aggregates of intermediate filaments and tubulofilaments in the sarcoplasm and plaques containing Z-disc derived material were observed on EM.[117]

Frequency of Desminopathy among Other Myofibrillar Myopathies

Among 63 patients with myofibrillar myopathy studied in the Mayo clinics, only 6% show mutations in *DES*, while 3% had sequence alterations in *CRYAB*, 10% in *MYOT*, 15% in *ZASP* and 3% in *FLNC*.[15] In an International sample of 52 affected families studied at the National Institutes of Health, 46% showed mutations in DES, 8% in *MYOT* and 2% in *ZASP*; none of the patients had mutations in *CRYAB* and no testing was done for *FLNC* mutations (Goldfarb,

Table 7. Myopathology of myofibrillar myopathy associated with mutations in MYOT, ZASP, SEPN1, FLNC and VCP genes

Mutant Gene (Protein)	Myopathology Features	Electron Microscopy	Reference
MYOT (myotilin)	variation in fiber size, fibrosis, increased number of internal nuclei, rimmed vacuoles, spheroid bodies, inclusions with myotilin, filamin C, α-actinin, ZASP, desmin, alpha-B crystallin, dystrophin, plectin, gelsolin, ubiquitin, prion protein, CD10 kinase, alpha1-antichymotrypsin	GFM, autophagic vacuoles, tubulofilament-like structures, 15-28 nm filaments	111,112,114
ZASP	hyaline, congophilic and amorphous deposits devoid of oxidative enzymes, small vacuoles, increased number of internal nuclei, fiber splitting, necrotic & regenerating fibers, aggregation of myotilin, desmin, alpha-B crystallin, dystrophin, N-CAM, prion protein, plectin, ubiquitin, gelsolin	Z disk streaming, autophagic vacuoles, remnants of sarcomeres, degraded filamentous material	115
SEPN1 (selenoprotein N)	variation in fiber size, increase in internal nuclei, hyaline plaques with reduced or absent oxidative enzyme histochemcial activities, necrosis and regeneration of muscle fibres, endomysial fibrosis, fat cell replacement, desmin, alpha-B crystallin, dystrophin, tau AT100 and AT120, A-beta amyloid, ubiquitin, actin, alpha-actinin, 8-OHdG, beta- and gamma-sarcoglycans, nebulin, SERCA2, telethonin	Z disk streaming, minicores, aggregates of 7-12 and 8-10 nm filaments, 20 nm filaments, 20 nm tubulofilaments in sarcoplasm, Mallory body-like inclusions	117
FLNC (filamin C)	desmin, filamin C, myotilin, dystrophin, sarcoglycans deposits	GFM, rods, Z disk streaming	118
VCP (valosin-containing protein)	marked to moderate to mild myopathic features, rimmed vacuoles, foci of desmin, α-B crystallin, ubiquitin, valosin-containing protein	GFM, destroyed sarcomeres,	119

GFM: granulofilamentous material.

nonpublished results). Relative numbers of myofibrillar myopathies among 23 studied Spanish families were 26% in *DES*, 60% in *MYOT*, none in *CRYAB* and 14% did not show mutations in any gene (Olivé, in preparation). Finally, in a set of 41 patients with myofibrillar myopathy from Britain, 17% had mutations in DES, 7% in *MYOT* and 7% in *ZASP* (Bushby, in preparation). These results, although incomplete, show significant variability in the prevalence of desminopathy among other myofibrillar myopathies.

Treatment

There is no specific treatment for desminopathy, but some of the complications and premature death can be prevented. Early detection and treatment of cardiac arrhythmias and conduction defects is essential since implantation of a pacemaker can be lifesaving. Pacemaker and implantable cardioverter defibrillator (ICD) should be considered in individuals with arrhythmia and/or cardiac conduction defects. Individuals with progressive or life-threatening cardiomyopathy are candidates for cardiac transplantation. Respiratory support, consisting of continuous or bilevel positive airway pressure (CPAP and BIPAP), initially at night and later at daytime, are indicated in patients with hypercapnea and other signs of incipient ventilatory failure. Risk of chest infection should be considered in these patients. Assistive devices should be used in individuals with advanced muscle weakness. Gene and stem-cell therapy is an active area of research that promises effective treatments in the future.

Concluding Remarks

Desminopathy is associated with mutations in *DES*, *CRYAB* and perhaps other genes interacting with desmin. Disease-associated *DES* mutations affect amino-acid residues that are crucial for filament assembly; they render dominant inhibitory effect on desmin function. In humans or transgenic mice, they lead to accumulation of chimeric intracellular aggregates containing desmin and other cytoskeletal proteins. Desminopathy manifests with a variety of phenotypes depending on the type of inheritance and the location of mutations within the relatively large and structurally and functionally complex desmin molecule. Dominant mutations show a wide phenotypic variability which is probably a result of interactions between desmin, other intermediate filaments and chaperones capable of compensating for their detrimental effects. AlphaB-crystallin serves as such a chaperone for desmin but if mutated it may cause a myopathic syndrome identical to those resulting from mutations in *DES*. The current knowledge of the molecular basis of disorders resulting from mutations in *DES* and *CRYAB* genes allows the use of diagnostic genetic testing. In spite of significant progress in the studies of desminopathy there are unresolved problems. It is unclear how misfolded and aggregated desmin triggers the disease development. The muscle-specific proteolytic system involved in degradation of misfolded proteins needs further examination. Mechanisms leading to alternative disease expression in skeletal or cardiac muscle cells also require serious consideration. New technologies will help to solve these problems and facilitate novel specific therapies.

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Chapter 12

Muscular Integrity—A Matter of Interlinking Distinct Structures via Plectin

Patryk Konieczny and Gerhard Wiche*

Abstract

Myocytes are characterized by the presence of highly specialized cytoskeletal structures that are part of regularly spaced functional units distributed over long distances. In this chapter we discuss previously published evidence as well as novel data showing that the proper positioning and architecture of Z-disks and of sarcolemma-associated costameric structures are largely dependent on the cytolinker protein plectin and its associated intermediate filament (desmin) cytoskeleton. Deficiency in either plectin or desmin lead to muscular dystrophies of similar pathology. However, while in the absence of plectin, desmin networks collapse and form aggregates, when desmin is missing, plectin retains its typical localization. This suggests that plectin recruits and anchors desmin filaments to both Z-disks and costameres and thus is a key element for maintaining and reinforcing myocyte cytoarchitecture. We hypothesize that as an essential link of the Z-disk-costamere axis, plectin is likely to play also a crucial role in myofiber signaling.

Cytolinkers and Striated Muscle Fibers

The proper function of striated muscle cells is enabled by highly specialized cytoskeletal systems, such as sarcomeres, peri-sarcomeric arrays, intercalated disks and various subsarcolemmal structures, including costameres, neuromuscular junctions and myotendinous junctions (reviewed by refs. 1,2). Cytolinkers, intermediate filament (IF)-based proteins of usually very large size, together with their associated IF networks are emerging as key players in maintaining the integrity of these structures and facilitating interactions between them.

The cytolinker or plakin protein family encompasses a group of seven proteins: plectin, desmoplakin, bullous pemphigoid antigen 1 (BPAG1), envoplakin, periplakin, actin-crosslinking factor 7 (ACF7)/microtubule-actin crosslinking factor (MACF) and epiplakin (reviewed by refs. 3,4). In general, cytolinkers are multi-modular proteins, containing in variable combinations a plakin domain, plakin/plectin repeat domains, an actin-binding domain (ABD) consisting of two calponin-homology domains, a coiled-coil or spectrin-repeats-containing rod and microtubule-binding domains. These structural elements determine one of the major functions of cytolinkers, i.e., the networking of IFs with other cytoskeletal filaments, filament-anchoring structures and organelles.

Cytolinkers expressed in striated muscle comprise desmoplakin I (332 kDa),[5,6] plectin (>500 kDa)[7,8] and BPAG1b (824 kDa).[9,10] As an obligate component of desmosomes, desmoplakin is abundant at intercalated disks of heart; however, it is not expressed in skeletal muscle. In contrast,

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BPAG1b and especially plectin show a much more abundant expression in both tissues. The important role of muscle-specific cytolinkers is underscored by the fact that targeted inactivation of these proteins resulted in either skeletal or cardiac myopathies,[9,11,12] while mutations in the human plectin gene lead to epidermolysis bullosa associated with muscular dystrophy (EB-MD)[13] and in desmoplakin to dilated cardiomyopathy, wooly hair and keratoderma.[14]

Plectin, a Highly Versatile Cytolinker Protein

Plectin was discovered in the early eighties as a high-molecular-weight protein copurifying and colocalizing with vimentin IFs.[15-17] Since then numerous studies revealed its localization in almost all mammalian tissues.[7,18] Antibodies to plectin were found to decorate cytoskeleton-plasma membrane junctional sites, including hemidesmosomes along the basal cell surface membrane of stratified and simple epithelial cell layers,[7,19] dense plaques of smooth muscle,[7] costameres of skeletal muscle[20,21] and focal adhesion contacts[22] and primary longitudinal adhesion structures[23] of cultured fibroblasts and myotubes. Moreover, in cardiac and skeletal muscle, plectin was identified as a prominent constituent of Z-disk structures, intercalated disks and neuromuscular and myotendinous junctions.[7,24,25]

Plectin forms dumbbell-shaped molecules composed of a central ~200 nm-long α-helical coiled-coil rod domain and flanking globular domains[26] (Fig. 1). Cloning and sequencing revealed that the plectin gene spans 32 coding exons,[27,28] most of which cover the N-terminal part of the molecule, whereas the rod and C-terminal domains are encoded by unusually large single exons. Based on the cDNA-deduced amino acid sequence, plectin was predicted to have a spectrin-type ABD residing at the N terminus[28,29] and 6 tandem repeats at the C terminus.[27]

In vitro binding assays revealed plectin to interact with various IF proteins, including vimentin, glial fibrillar acidic protein, neurofilaments, cytokeratins, the nuclear lamina protein lamin B and desmin,[30-33] with the major IF-binding site residing in its C-terminal repeat 5 domain[34,35] and an additional vimentin-binding site mapping within its ABD[36] (Fig. 1). Both, the central helical rod domain of vimentin and its N-terminal domain have been implicated in binding to plectin.[30,36] Beyond binding to IFs, plectin was shown to crosslink vimentin IFs[30] and to link them with microtubules and microfilaments.[37-39]

Plectin not only integrates IFs with other filamentous systems, but it also anchors them to the proteins of the nuclear envelope, such as lamin B[31] and nesprin 3,[40] to mitochondria (through an unknown mechanism) and to plasma membrane-associated proteins, such as spectrin,[41] integrin β4,[42] dystrophin, utrophin and β-dystroglycan[21] (Fig. 1). Moreover, exceeding its role as a universal cytolinker protein, plectin regulates actin filament dynamics[43] and acts as a signaling platform by scaffolding and influencing the activities of signaling proteins, such as the cytoplasmic tyrosine kinase Fer,[44] the protein kinase C receptor RACK1[45] and the AMP-sensing kinase AMPK[46] (Fig. 1). By controlling IF cytoarchitecture, plectin has been shown to affect signaling pathways involved in cell migration[47] and to have an antagonistic role in oxidative stress- and hyperphosphorylation-mediated alterations of vimentin and keratin networks.[47,48]

A unique feature of plectin is the diversity of transcripts encoding protein variants with differing N terminal structures.[49,50] Out of sixteen alternative exons involved, eleven (1, 1a-1j) are spliced into a common exon 2, three (-1, 0a, 0) precede one of the first coding exons (1c) and two (2α, 3α) are situated between exons encoding plectin's ABD. Furthermore, transcripts (without exon 31) encoding rodless plectin isoforms have been reported.[49,51,52] Plectin isoforms are differentially distributed among tissues, with plectin 1c and 1d being restricted to brain and muscle, respectively and plectin 1a representing the most prominent isoform of skin, lung and small intestine.[50] Moreover, laser scanning fluorescence microscopy of transfected cells and teased muscle fibers revealed distinct subcellular targeting of plectin isoforms. Plectin 1f concentrated at focal adhesion contact sites of cultured cells and costameres of myofibers, while plectin 1b and 1d were targeted to mitochondria and Z-disks, respectively.[21,53]

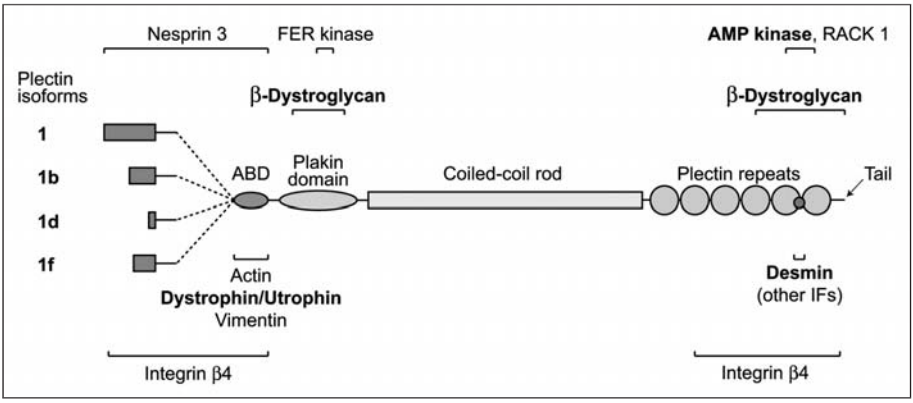

Figure 1. Schematic representation of plectin's multi-modular structure and interaction domains. The central coiled-coil rod is flanked by N-terminal plakin and C-terminal plectin repeat domains. The N-terminal actin binding domain (ABD) is preceded by isoform-specific sequences (dark/red squares) corresponding to the major isoforms expressed in muscle fibers, plectin 1, 1b, 1d and 1f. Known interfaces of plectin with binding partners are indicated above and below the model. Major binding partners in muscle are highlighted.

Mutations in the Plectin Gene Cause Epidermolysis Bullosa (EB)

Based on its widespread occurrence and crosslinking functions, plectin was predicted to play an important role in the maintenance of tissue integrity by reinforcing cell cytoarchitecture. Strong evidence for such a role emerged when plectin deficiency was linked, first, to EB-MD[13,29,54-56] and EB simplex Ogna[57,58] and, more recently, to EB with pyloric atresia.[59] EB is a heterogeneous group of syndromes caused by mutations in proteins stabilizing the association between the epidermis and the underlying dermis. It is characterized by blistering and erosions of the skin and mucous membranes (reviewed by refs. 60,61).

EB-MD results from mutations in the plectin gene, with the majority of them occurring within the rod domain (Table 1). In most cases these mutations lead to premature termination of translation.[61] The expression levels of the truncated mutant polypeptides vary from completely absent to just reduced, compared to normal plectin levels[61,62] and it is conceivable that at least in some cases plectin rodless isoforms are expressed. Furthermore, truncated plectin molecules were demonstrated to have changed biochemical properties, such as increased self-aggregation.[63] In EB-MD patients a range of features characteristic of chronic myopathy were observed, such as variability in the diameter of fibers, pyknotic nuclear clumps and also centrally nucleated, atrophic, split, necrotic and regenerating fibers.[13,55] This phenotype was accompanied by increases in the amount of endomysial and perimysial connective tissue and by increased space between the plasma membrane and the muscle sarcomere.[64] Furthermore, in a few cases, distortions in network organization of desmin IFs and of mitochondria have been reported[55,65] and symptoms of neuromuscular disease have been found.[29,66]

Ablation of Plectin in Mice Results in Structural Aberrations in Muscle Cells

To define more precisely the role of plectin in tissue development and integrity, plectin null mice were generated.[11] Although the tissue development in these mice seemed unaffected, they died 2-3 days after birth, being much smaller than their wild-type littermates and revealing skin blistering due to degeneration of keratinocytes. In addition to blistering, plectin-null mice exhibited multiple structural aberrations in skeletal and cardiac muscles reminiscent of those occurring in EB-MD patients, such as the presence of degenerating fibers, focal disruption of the sarcolemma

Table 1. List of mutations identified in the plectin gene of EB-MD patients

Mutation	Domain	Reference
956ins3	plakin	61
1287ins3	plakin	63
1541ins36	plakin	61
2677del9	plakin	61
2719del9	plakin	56
2745-9del21	plakin	94
Q1053X	rod	95
4359ins13	rod	96
4416delC	rod	96
Q1518X	rod	63
E1614X	rod	61
5069del19	rod	97
5083delG	rod	94
Q1713X	rod	98
5148del8	rod	29
5309insG	rod	61
5588insG	rod	99
Q1910X	rod	54
Q1936X	rod	95
5866delC	rod	56
5905del2	rod	97
5907ins8	rod	13
E2005X	rod	94
R2319X	rod	100
R2351X	rod	98
R2421X	rod	95
R2465X	rod	64
12633ins4	plectin repeats	95
K4460X	plectin repeats	94
13803ins16	tail	65

and myofibrils and partial disintegration of intercalated disks. Also, aggregated mitochondria in the stage of autolysis were found in the damaged fibers.[32] This prompted the notion that muscle performance, but not development, was affected by plectin deficiency, consistent with an important role of plectin in the maintenance of tissue integrity. However, the molecular mechanisms underlying the pathological changes remained elusive.

The IF Network of Striated Muscle Fibers

The IF network of mature striated muscle fibers is composed of desmin, synemin, paranemin cytokeratins and the IF-like protein, syncoilin. Desmin filaments form the major IF scaffold in striated muscle, surrounding myofibrils at the level of Z-disks and extending to the sarcolemma, intercalated disks and to various organelles, including nuclei, the sarcoplasmic reticulum and mitochondria (reviewed by ref. 67). In humans, mutations in the desmin gene result in idiopathic dilated cardiomyopathy[68] and other skeletal and cardiac disorders.[69,70] Desmin-deficient mice exhibit muscular dystrophy characterized by the loss of muscle integrity,[71,72] very similar to plectin-null mice and patients suffering from EB-MD (Table 2). Also, mitochondrial networks are disrupted and dysfunction and overproliferation of mitochondria in the subsarcolemmal region of exercised

Table 2. Comparison of phenotypes observed in desmin and plectin deficiency

	Deficiency Phenotype	
	Desmin	**Plectin**
Striated muscle		
Myocyte degeneration	+[25,71,72]	+[11,13,25]
Fibrosis	+[71,72]	+[25,64]
Elevated serum CK level	–[75]	+/–[66/25]
Disarrayed myofibrils	+[71,72]	+[25,66]
Sarcolemma detachments	?	+[25]
Costameres	aberrant[76]	aberrant[25]
Mitochondria	dysfunction[74]	dysfunction[25,65]
NMJs	aberrant[77]	aberrant[66]
Heart		
Cardiomyopathy	severe[68,69,71,72,78,79]	mild[11,25,65]
Hypertrophy	+[79]	mild/–[65/25]
Calcium deposits	+[71,72]	–[25]
Dilation of chambers	+[79]	–[25]

mice are observed.[73,74] This phenotype is accompanied by compromised endurance performance, without any significant increase in serum creatine kinase levels[75] and by the loss of the costameric lattice in the majority of myofibers.[76] In addition, desmin was suggested to be important in the process of terminal fiber regeneration, maturation of muscle fibers and maintenance of the NMJ structure.[77] Desmin-deficient hearts are transiently hypertrophic, show extensive cardiomyocyte death, calcium deposits and dilation of cardiac chambers, leading to generalized heart failure.[71,72,78,79] Thus, these hearts exhibit a much more severe myopathy than those from plectin-deficient mice (Table 2). Since dilated cardiomyopathy was implicated to be a disease of intercalated disks,[80] we hypothesize that the mild cardiac phenotype observed in the absence of plectin might be a reflection of a relatively well preserved intercalated disk structure due to the presence of functional desmoplakin-desmin networks. Such an interpretation would be consistent with a recent study by Yang et al[81] where it was shown that overexpression of a desmoplakin mutant protein with an

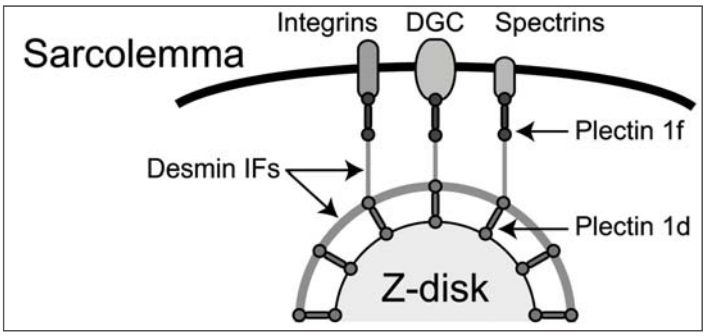

Figure 2. Model depicting plectin isoform-specific functions in muscle. Plectin 1d and 1f target desmin IFs to the Z-disk and to the dystrophin-glycoprotein complex (DGC), respectively. Additional putative interactions of plectin 1f with integrin- and spectrin-based complexes are depicted as well.

incapacitated IF-binding site leads to ultrastructural changes of intercalated disks in mice, paralleled by the detachment of desmin IFs from desmosomes.

Synemin[82] and paranemin[83] copolymerize with desmin IFs.[84] Moreover, synemin was suggested to anchor desmin IFs to Z-disks through its binding partner α-actinin[85] and to the costameric lattice through either vinculin,[85] α-dystrobrevin[86] or dystrophin and utrophin.[87] Interestingly, the desmin-binding protein syncoilin was also indicated in docking desmin IFs to the sarcolemma by association with α-dystrobrevin.[88,89] Independently of desmin IFs, anchorage of the contractile apparatus at the sarcolemma is provided also by cytokeratins 8 and 19.[90] In contrast to the desmin-based cytoskeleton, cytokeratins colocalize not only with costameres overlying Z-disks, but also with those encircling M-lines.

EB-MD as a Disease of Lost Connection between IFs, Z-Disks and Costameres

From early on, plectin was implicated in linking desmin IFs to Z-disks and to the sarcolemma[7,20,21] and, similar to synemin and syncoilin, the protein was shown to directly bind to desmin.[32] Schröder and coworkers in fact revealed that association of plectin with Z-disks precedes the formation of the intermyofibrillar desmin cytoskeleton[51] and that plectin together with vinculin and integrin β1 is a constituent of costameric precursors in differentiating muscle cells.[23] In accordance, it has recently been shown that plectin interacts via multiple binding sites with β-dystroglycan, dystrophin and utrophin, major components of the dystrophin-glycoprotein complex.[21] Taken together, these data suggest that the loss of myofiber integrity observed in the absence of plectin or desmin have a similar etiology, i.e., the absence of a functional IF network system. Unpublished results from our laboratory show that the anchoring of desmin to Z-disks

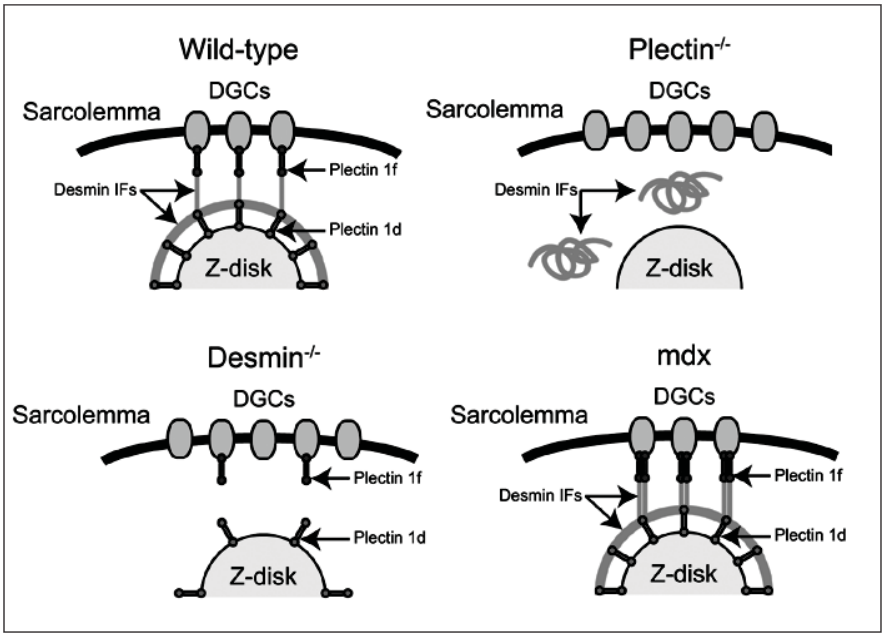

Figure 3. Working models comparing IF network organization in normal (wild-type), plectin$^{-/-}$, desmin$^{-/-}$ and mdx mice. Note loss of IFs linking Z-disks with sarcolemmal DGCs in both, plectin- and desmin-deficient fibers. Also, while the localization of plectin is unaffected, its content is decreased in the absence of desmin. In contrast, linkages are reinforced upon loss of dystrophin.

and to the sarcolemma, is specifically mediated by two distinct isoforms of plectin, plectin 1d and plectin 1f, respectively (Fig. 2). Importantly, these linkages turn out to be dominant over the ones provided by syncoilin and synemin.[25]

The multidomain structure and vast variety of binding partners make plectin a top candidate for coordinating Z-disk functions with the dystrophin-glycoprotein complex as well as integrin- and spectrin-based subsarcolemmal protein assemblies (Fig. 2). The profound alterations of costameric lattice organization in plectin-deficient myofibers,[25] as well as its perturbation under desmin deficiency,[76] support this hypothesis. Moreover, it has recently been revealed that dystrophin deficiency in mice leads to increased levels of sarcolemma-associated plectin 1f and to a redistribution of β-dystroglycan to a costameric location exclusively above Z-disks.[21] The various scenarios of IF network organization in muscle fibers of mice under normal conditions (wild-type) and upon loss of plectin, desmin and dystrophin, respectively, are schematically depicted in Figure 3.

There is increasing evidence that Z-disks, costameres, as well as the Z-disk-costamere axis are involved in myofiber signal transduction.[91-93] By organizing the IF scaffold in myofibers and targeting it to costameres and Z-disks, plectin is likely to play a crucial role in the orchestration of myofiber-specific signaling events. In this case it can be expected that plectin contributes to the pathology of a variety of muscular dystrophies, including those related to defects in dystrophin.[21]

Future Directions

It will be a challenging task for future studies to dissect the molecular mechanisms underlying plectin-dependent signaling cascades in muscle fibers in more detail and define their role in energy homeostasis, stress responses and regeneration. Furthermore, mice with a plectin gene conditionally deleted in striated muscle as well as various plectin isoform-specific knockout mouse models may be useful for developing therapies for plectin-related myopathies.

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Chapter 13

The Sarcomere and the Nucleus:
Functional Links to Hypertrophy, Atrophy and Sarcopenia

Mathias Gautel*

Abstract

Skeletal muscle has a remarkable ability to rapidly adjust to changes in physiological requirements. This includes hypertrophic muscle growth and the atrophic loss of muscle mass, both of which occur in response to hormonal, endocrine and mechanical stimuli. In ageing muscle, sarcopenia (the loss of muscle fibres) can aggravate hormonally and mechanically induced atrophy. Hypertrophy and atrophy are associated with changes in sarcomeric protein composition and metabolic enzymes. The coordinated changes of transcriptional and splice mechanisms, protein turnover and cell fate integrates signalling pathways from hormone and cytokine receptors, as well as the sarcomere itself. This involves a number of proteins that shuttle between sarcomeric and nonsarcomeric localisations and thus convey signals from the contractile machinery to the nucleus. The M-band is emerging as a hub mainly for protein-kinase regulated ubiquitin signalling and protein turnover, whereas the I-band and Z-disk contain stretch-sensitive pathways involving transcriptional modifiers. Disruptions of these pathways can cause hereditary myopathies.

Introduction

Among highly differentiated tissues of the vertebrate body, striated muscle has an outstanding ability to respond to external stimuli and changes in its workload by rapid adaptation of contractile properties, metabolic flow, cell size and electric behaviour. These sweeping changes involve the concerted control of transcription regulation, protein synthesis, protein degradation and metabolic flow, which are only beginning to be clarified. The discovery of major pathways regulating muscle size, fibre type and metabolic state are now emerging and show clear nexuses, where extracellular and intracellular signals converge.

Growth of muscle tissue, or hypertrophy, occurs in response to several stimuli, such as mechanical activity and passive stretch, hormonal stimulation e.g., via steroid receptors, cytokines and growth factors like myostatin or insulin-like growth factor-1. Conversely, the loss of muscle mass, or atrophy, can be triggered by disuse, microgravity, catabolic steroids such as glucocorticoids, cytokines including tumour necrosis factor, genetic factors, acidosis and catabolic nutritional states. The broad range of regulatory external and internal factors suggests that many of these pathways may be converging on a few final common signalling proteins and that considerable crosstalk between these may exist.

Recent progress shows that hypertrophic and atrophic signalling communicates with several "hubs" within the contractile machinery of striated muscle, i.e., the sarcomere itself (Fig. 1).

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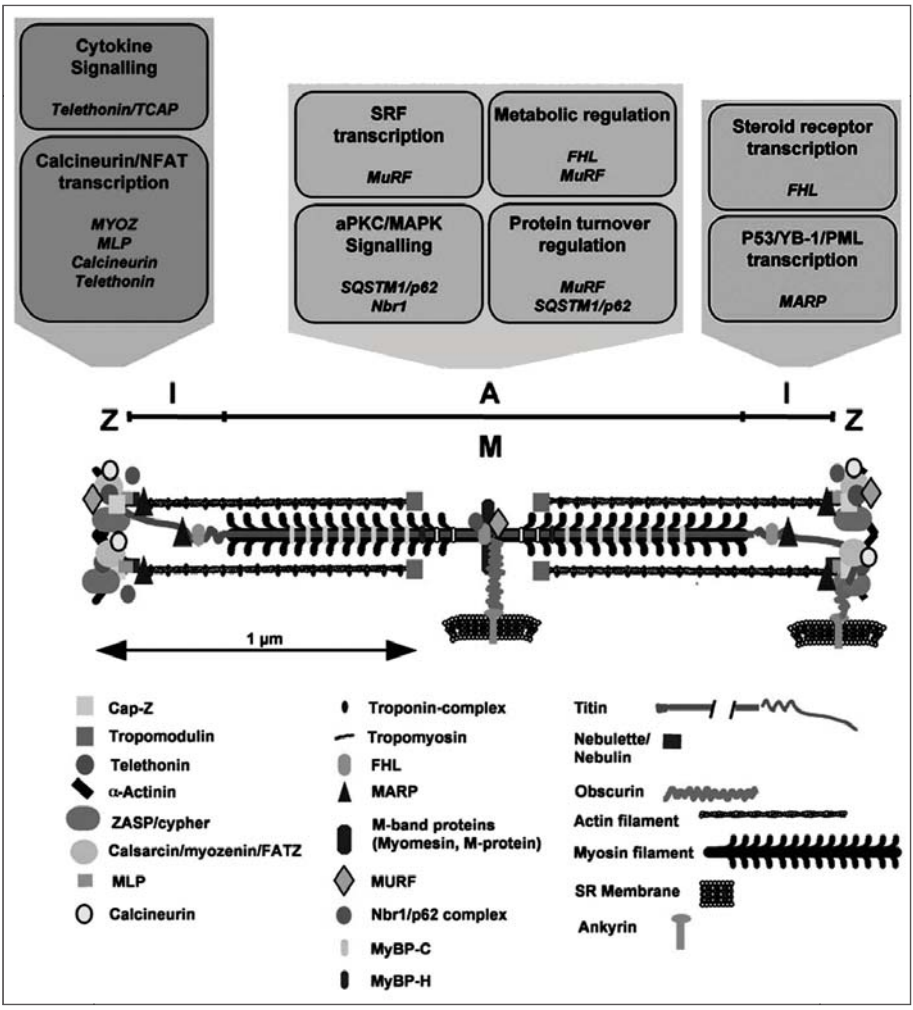

Figure 1. Sarcomeres, the smallest contractile units of striated muscle, are the highly regular, quasi-crystalline arrays of contractile filaments composed of actin and myosin subunits and the elastic third filament, composed of single molecules of titin. Unlike the motor protein filaments composed of actin and myosin subunits, whose main function is the formation of the contractile machinery, the third filament combines multiple functions. Actin and titin filaments are linked by multiple interactions at the Z-disk, most importantly by the actin crosslinking protein alpha-actinin, which also binds to specific sites on titin. Titin filaments are further crosslinked by a small muscle protein, telethonin. In the I-band region, titin is an entropic spring important for keeping A-bands centred during contraction and relaxation. At the M-band, myosin and titin are crosslinked by a family of related M-band proteins, myomesin (MYOM1), M-protein (MYOM2) and a third gene product, MYOM3.[95,137] The Z-disk, I-band and M-band have emerged as organisation centres for signalling modules, highlighted above the sarcomere. The Z-disk unequivocally functions as a calcineurin signalling module but also crosstalks to growth factor/cytokine signalling via telethonin. The I-band contains a module of muscle ankyrin repeat proteins with structural and gene transcription modulating functions and links to androgen receptor signalling. In the M-band, several ubiquitin-linked signalling proteins are targeted to and around the protein kinase domain of titin. They assemble modules regulating protein turnover, SRF-dependent gene transcription, signalling by atypical protein kinase-C and MAP/ERK kinases and metabolic regulation. A color version of this figure is available online at www.eurekah.com.

Mechanical forces seem to play important roles in modulation of the conformation and hence activity of these protein complexes, yet direct links to hormone and cytokine receptors suggest a complex interplay of mechanical and cell surface receptor-linked signalling. As a muscle fibre, on the other hand, is also a highly specialized contractile unit, the interplay between ubiquitous signalling proteins and muscle-specific components gives rise to an intriguingly complex picture. The evolution of the muscle-specific pathways has adopted highly efficient ways to coordinate mechanical work, metabolic flow and external anchorage and communication. Not surprisingly, therefore, many of the protein players discussed in the following have been found to be involved in hereditary myopathies, when genetic defects disrupt their concerted interplay.

Myogenic differentiation, hypertrophy and atrophy result from this complex interplay of multiple transcriptional pathways. Muscle-specific basic helix-loop-helix transcription factors such as Myf5, MyoD, Mrf4 work in cooperation with MADS-box factors like serum response factor (SRF) and myocyte enhancer factor 2 (MEF2) with a more ubiquitous role.[1] During postnatal adaptation of muscle, or muscle remodelling, aspects of early developmental programmes can be reactivated and cooperate with specific factors in determining fibre-type characteristics.[2] During postnatal remodelling and hypertrophy, the transcription factors MEF2 and NFAT play prominent roles.[3,4] Of the pleiotropic factors, SRF is absolutely required for postnatal hypertrophic growth.[5,6] Posttranslational modifiers of histone (histone acetylases and deacetylases)[7] and transcription factors (protein kinases and phosphatases, ubiquitin and SUMO transferases) cooperate in the complex interaction between chromatin structure and transcriptional machinery. Most of these responses are modulated by muscle activity, either by sensing intracellular calcium levels, or mechanical strain. Different compartments of the sarcomere harbour specific signalling modules (Fig. 1), for example a calcineurin module at the Z-disk and an ubiquitin signalling module at the M-band. In the following, these links between sarcomeric compartments and signalling pathways modulating muscle gene expression and their relationships with atrophy, hypertrophy and sarcopenia will be discussed.

Z-Disk Signalling Pathways

The Z-disk is the transverse anchoring plane of actin filaments and the third filament system, composed of the giant elastic blueprint of the sarcomere, titin[8] (also known as connectin; Fig. 1). The Z-disc has a structural role in ensuring sarcomeric stability, but at the same time has emerged as a scaffold for signalling events involving protein kinases, protein phosphatases and transcriptional cofactors. Z-disk structure is modulated by the contractile state of the myofibril,[9] leading to drastic changes in transversal stiffness[10] and hence presumably to significant conformational changes. These changes provide the possibilities for altered protein function and/or contacts within its flexible lattice and suggest that the Z-disk as a functional entity may act as sensor for both active and passive strain. A number of nonstructural proteins localize to the Z-disk and have been implicated in sarcomeric signalling to the nucleus, either in putative strain-sensor complexes or as parts of protein kinase pathways.

Muscle LIM Protein MLP (CRP3)

The 194 residue Muscle LIM Protein (MLP, also known as cysteine and glycine-rich protein 3 or CRP3) was originally described as a regulator of myogenic differentiation,[11] possibly by functional interactions with MyoD.[12] Subsequently, MLP knockout animals did not show a global defect in muscle development, as the initial studies might have suggested,[11] but rather a cardiac phenotype with aberrant intercalated disc morphology and development of dilated cardiomyopathy. This is clearly incompatible with a modulation of the skeletal muscle restricted MyoD function, drawing focus on potential other partners of MLP in striated muscle. MLP is normally associated with the Z-disk,[13] but accumulates in the nucleus after pressure overload[14] or after passive stretch of cultured cardiomyocytes.[15] The Z-disk association is possibly predominantly due to interaction of the cysteine rich proteins with the major actin crosslinker, alpha-actinin.[16,17] Identification of an interaction between MLP and the titin ligand telethonin prompted a concept of an MLP-based

stress sensor defective in cases of dilated cardiomyopathy.[18] Interestingly, mutations in this proposed telethonin-MLP stress sensor have very selective phenotypes, being either cardiac-selective or skeletal muscle specific.[19,20]

A function clearly corroborated on the morphological and signalling level is the requirement of MLP for the Z-disk targeting of the calcium-calmodulin activated protein phosphatase calcineurin (formerly PP2B, now PPP3CA). Calcineurin Z-disk localization is disrupted in MLP –/– mice and their stress-induced calcineurin-NFAT activation is blunted.[21] MLP expression itself can be induced by calcineurin; its co-expression with calcineurin seems to have an additive effect on slow myosin heavy chain expression,[22] in agreement with its upregulation in fast-to-slow fibre type transition[23] and the enhanced MLP expression by contractile activity in skeletal muscle.[24] Analysis of contractility in MLP knockout animals suggest that this protein plays a subtle role in the maintenance of normal muscle characteristics, with some evidence of type I fibre atrophy and changes in resting sarcomere length, as well as in early events during the recovery process of skeletal muscle to injury.[25] MLP may thus play both structural and gene-regulatory roles.

Telethonin (TCAP)

Telethonin (also called T-cap), a 167 residue protein encoded by one of the most abundant mRNAs of any sarcomeric protein, links several of the Z-disk signalling proteins and further components.[26] Telethonin can bind to the extreme N-terminus of titin[27,28] in a very tight complex[29] that cross-links two titin monomers. Intriguingly, telethonin also interacts with several other proteins outside the Z-disk and can relocalise in response to stress or atrophy signals. Pathogenic mutations in titin and telethonin appear to influence complex formation and/or its regulation and can cause limb-girdle muscular dystrophy type 2G (LGMD2G) and hypertrophic or dilated cardiomyopathies.[30,31]

Telethonin has also been implicated in sensing mechanical strain, by its interaction with the alpha-actinin binding LIM protein MLP.[18] Neurogenic atrophy of human muscle, where nerve lesions lead to mechanically induced atrophy, causes a remarkable and selective loss of telethonin from the Z-disk,[32] suggesting a more transient interaction with its ligands, including the main binding partner titin. Of all Z-disk proteins studied by live cell imaging, however, telethonin shows the slowest exchange rate.[33] Force-probe molecular dynamics simulations[34] based on the atomic structure of the titin-telethonin complex[29] suggest extraordinary mechanical stability of this structure. A physiological force sensor, however, can only act as such when conformational changes are induced at physiologically relevant force levels. It is unlikely, therefore, that the titin-telethonin complex can play a role as a force sensor; rather, this complex seems to anchor titin filaments near the barbed end of actin filaments.[29] As telethonin was also reported to interact with the calcineurin regulator protein family member calsarcin[35] (also called FATZ or myozenin),[36] the small potassium channel associated protein minK,[37] muscle LIM protein/CRP3[18] and the secreted TGF-beta domain of the muscle growth factor myostatin,[38] telethonin is a protein whose many interactions imply functions other than just titin crosslinking. Modulation of the secretion of myostatin by telethonin, a growth factor controlling the balance between myoblast proliferation and differentiation,[39] could thus be an important factor in determining muscle growth and might be involved in both hypertrophy and sarcopenia. Modulation of these telethonin functions by phosphorylation, by protein kinase D[40] and possibly titin kinase,[41] may play a role in controlling these interactions or protein dynamics. More detailed insights into these mechanisms will now be required to understand the role of mutations in telethonin in muscle signalling and in diseases like limb girdle muscular dystrophy (LGMD)[30] or dilated cardiomyopathy.[18]

Calsarcin/FATZ/Myozenin (MYOZ)

The family of filamin, alpha-actinin, telethonin interacting Z-disk proteins (FATZ) was isolated independently in the laboratories of Georgine Faulkner, Alan Beggs and Eric Olson.[36,42-44] The resulting nomenclature is: FATZ1/calsarcin-2/myozenin-1; FATZ2/calsarcin-1/myozenin-2; FATZ3/calsarcin-3/myozenin-3, encoded on three MYOZ genes. For simplicity, I will use the

MYOZ gene nomenclature. MYOZ2 is expressed specifically in adult cardiac and slow-twitch skeletal muscle, whereas MYOZ1 is restricted to fast skeletal muscle. MYOZs interact with calcineurin, the PDZ-LIM domain protein LDB3[35,44] and the alpha-actinin binding protein myotilin.[45] Calcineurin-A plays important roles in sensing and integrating intracellular calcium concentrations and relaying this information to changes of the phosphorylation level of downstream effector proteins. One of the most important muscle targets of calcineurin is the transcription factor NFAT, whose dephosphorylated form shuttles to the nucleus, while its phosphorylated form is exported from the nucleus and trapped in the cytosol. NFAT-mediated transcription regulates muscle fibre size and is involved in hypertrophic responses, as well as fibre-type differentiation.[3,46]

A knockout mouse model of MYOZ2 showed increased calcineurin signalling in striated muscles, suggesting that MYOZ acts as a brake on calcineurin activity.[47] As a consequence of the abnormal calcineurin activation, MYOZ2 knockout mice showed an excess of slow skeletal muscle fibres, which is in agreement with the established roles of calcineurin/NFAT in activity-dependent regulation of fibre type.[3] Also in agreement with known calcineurin functions as a pro-hypertrophic factor, MYOZ2 knockout activated a hypertrophic gene program in the absence of hypertrophy and showed enhanced cardiac growth in response to pressure overload. These observations suggest that MYOZ family members act as modulators of calcineurin signalling and participate in the transmission of stress signals leading to cardiac remodelling in response to pressure overload in vivo. Interestingly, analysis of patients with hypertrophic cardiomyopathy recently identified myozenin-2 (MYOZ2) as a novel disease gene in this disease.[48] The hypertrophic phenotype of the MYOZ2 mutations may be a human correlate of the observations on MYOZ2 deficient mice and underline the critical role of MYOZ in the interplay of several Z-disk associated signal transducers that control muscle gene transcription.

ZASP/Cipher/Oracle (LDB3)

The alpha-actinin, Z-disk alternatively spliced PDZ-containing protein ZASP/Cypher/oracle (LDB3, LIM domain binding 3) interacts through its PDZ domain with the major Z-disc actin cross-linker, alpha-actinin.[49,50] LDB3 interacted directly with the alpha-actinin rod and competed with actinin-binding LIM protein (ALP) binding to the rod. During inhibition of stress fibre assembly, LDB3 and alpha-actinin colocalization could be partially disturbed, suggesting that LDB3 is bound to alpha-actinin mainly when alpha-actinin is localizing in stress fibres. LDB3 has a conserved sequence called the ZM-motif. LDB3 was also found to interact with Z-disk localized protein kinase C (PKC), identifying it as a cytoskeletal kinase scaffold.[49]

In knockout animals of LDB3, Z-disks were disrupted, leading to a severe myopathy.[51] The postnatal onset of the phenotype suggests that LDB3 is not involved in primary myofibrillogenesis, but may play a role in myofibril turnover and load adaptation.[51] A more dynamic role in cellular communication is also suggested by imaging techniques in living developing skeletal muscle cells.[33] Amongst several Z-disk proteins analyzed by fluorescence recovery after photobleaching (FRAP), LDB3 showed one of the fastest exchange rates together with myotilin. The structural components alpha-actinin and actin, not surprisingly, exchanged somewhat slower. Mutations in LDB3 have been linked to dilated cardiomyopathy and isolated noncompaction of the left ventricular myocardium (INLVM),[52,53] as well as to late-onset distal myopathy.[54] Many point mutations found in the cardiomyopathy patients are located in the internal region of LDB3. However, no evidence was found that human patient mutations in the internal domain would affect the LDB3 colocalization with alpha-actinin, or that the mutations would destabilize the LDB3 protein. How these mutations affect LDB3 function remains currently enigmatic.

TCAP, CRP3, MYOZ and LDB3 thus appear to form a Z-disk calcineurin signalling module (Fig. 2) that may be mechanically modulated. Additionally, telethonin also seems to regulate endocrine functions via the growth and developmental cytokine myostatin (Fig. 1).[38]

I-Band Signalling Pathways

The elastic I-band region of titin is composed of several serially arranged molecular springs of different stiffness and undergoes sequential conformational changes when sarcomeres are stretched

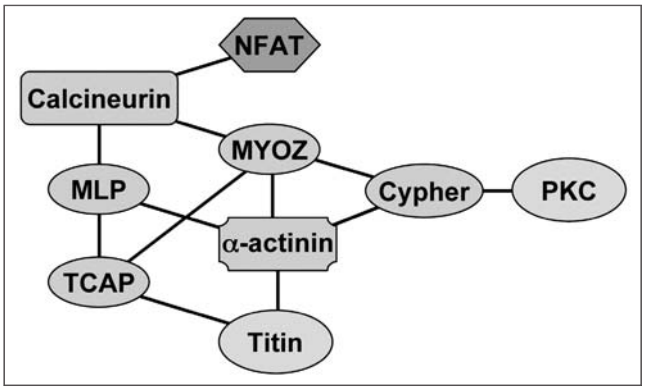

Figure 2. Protein links in the Z-disk calcineurin signalling module. Shown are the known protein interactions between muscle LIM protein (MLP), telethonin (TCAP), myozenin/FATZ/calsarcin (MYOZ), cypher/ZASP (LDB3), protein kinase C (PKC) and the structural links to titin and alpha-actinin. The main functional readout of this module appears to be the activation of NFAT.

or compressed.[8] This predominantly mechanical function serves to centre the A-band in the sarcomere and assure symmetric force production of both sarcomere halves.[55,56] Recently, the I-band region of titin has also emerged to be involved in a number of complex protein interaction networks with proteins implicated in nucleo-cytoplasmic signalling. A number of ankyrin-repeat, LIM, or immunoglobulin domain proteins as well as the calcium-dependent protease calpain-3/p94 were found in this region and are proposed to participate in passive stretch sensing.

Muscle Ankyrin Repeat Proteins

Striated muscles contain a closely related family of three muscle ankyrin repeat proteins (MARPs), the Cardiac Ankyrin Repeat Protein (CARP, ANKRD1 or MARP1), Ankyrin-repeat domain 2/Ankyrin Repeat Protein with PEST and Proline-Rich Region (Ankrd2/Arpp or MARP2) and Diabetes Associated Ankyrin Repeat Protein (DARP or MARP3). CARP was isolated as a cytokine-responsive gene,[57] developmentally regulated by Nkx2.5 and up-regulated in cardiomyocytes by doxorubicin stress.[58-60] These and subsequent analyses suggested MARPs to have a transcriptional role. When over-expressed in cardiomyocytes, CARP suppressed cardiac troponin C and atrial natriuretic factor transcription and inhibited Nkx2.5 transactivation of the atrial natriuretic factor promoter.[59] CARP/MARP3 expression is not restricted to cardiac muscle alone, however, but is also expressed in slow skeletal fibres. Ankrd2 was identified as a stretch-responsive gene product upregulated in stretched muscle.[61] Indeed, Ankrd2 is one of a number of muscle genes upregulated under eccentric exercise, which include muscle LIM protein (MLP), the muscle ankyrin repeat proteins, the actin binding protein Xin and myosin binding protein-H. The expression of some genes, including myoD, myogenin, MLP and CARP, is sensitive to mechanical strain under both isometric and eccentric contractions, while Ankrd2 expression responds only to eccentric contractions.[62,63] MARP are not ubiquitously co-expressed: Ankrd2 shows a definite preference for slow skeletal fibres and the cardiac atria, whereas CARP expression is found throughout all chambers of the heart with weak skeletal muscle expression.[64-67] These expression differences may suggest functions related to energy metabolism, contractile phenotype, or passive mechanical properties. In agreement with multiple functions, MARPs are found not only at different cytosolic locations, but also in the nucleus.[67]

All MARPs contain a binding site for the N2A region of titin within their ankyrin repeat region and in adult heart muscle colocalise with calpain-3 and myopalladin in the I-band.[68,69] The CARP ligand myopalladin was initially identified as a ligand of the SH3 domain of the actin

filament ruler proteins nebulin/nebulette at the Z-disk complex.[68] Subsequent ligand analysis also detected binding of CARP and DARP to the titin I-band sequence within a specific domain called N2A, where myopalladin, calpain 3/p94 and CARP form a complex with titin that may be subject to direct mechanical modulation.[69] Within the sarcomere, MARPs may also interact with the multitasking small protein telethonin.[67] However, in fetal rat cardiomyocytes in culture, CARP and DARP were partly relocalized to the nucleus when the cells were exposed to passive stretch, with DARP also appearing at the intercalated disk.[69] The possible implication of MARPs in modulation of muscle gene expression is underlined by their interaction with several nuclear transcription factors. CARP was found to interact with the transcription factor YB-1.[58] Ankrd2 was found to interact with three transcription factors, YB-1, PML and p53 and to localize to PML bodies in proliferating myoblasts[67] where it modulated their transcriptional activity. This suggests a direct link between the MARP family and transcription programs controlling cell survival and muscle gene expression. The pathway is mechanically modulated, as Ankrd2 undergoes a fourfold increase in expression after passive muscle stretch.[61] Expression of MARPs is reduced in dystrophic muscle,[64] but increased following denervation,[66] in the mouse model of muscular dystrophy with myositis (mdm) due to a titin N2A deletion and in heart failure.[70] These combined observations suggest that MARPs may play structural and signalling roles and could link the elastic I-band region as a passive stretch-sensor to control of nuclear transcription.

Recently, mice have been generated missing all three MARP family members (triple MKO). Surprisingly, these animals showed a relatively mild phenotype, with a trend towards a slower fibre-type distribution, but without differences in muscle fibre size.[71] Subtle differences in mechanical behaviour during eccentric exercise were recorded.[71] The absence of major failure of sarcomere maintenance, myofibrillar gene expression, fibre type differentiation and morphological differentiation suggest that the MARP family may therefore play only subtle roles in skeletal muscle, modulating structural functions and gene transcription.

FHL2

FHL2 (four-and-a-half *L*IM domains) is a modular protein of the LIM domain protein family. This family includes FHL1, FHL2 (also called DRAL), FHL3, FHL4 and ACT (reviewed in refs. 72, 73). FHL interactions are manifold and involve structural, signalling and metabolic proteins.

One of their characteristic properties is a tissue specific expression pattern. FHL2 is predominantly expressed in heart and skeletal muscle.[74] In muscle, FHL2 is involved in communication between the nucleus and the sarcomere. FHL2 was originally described as a protein differentially expressed between normal human myoblasts and their malignant counterparts, rhabdomyosarcoma cells and is positively regulated by the tumour suppressor p53.[74] Several other known interactions link the protein to pathways controlling muscle differentiation: FHL2 is a corepressor of the transcriptional repressor promyelocytic leukemia zinc finger (PLZF),[75] which recruits corepressors as part of the histone deacetylase complex in chromatin remodeling. FHL2 also participates in the regulation of androgen receptor dependent genes, either by direct interaction with the activated androgen receptor (AR), or indirectly by stimulation of Rho signaling pathways.[76,77] FHL2, as well as FHL1, seem to integrate signals at cell contact sites by interacting with several alpha- and beta-integrins in muscle[78] and to induce myogenesis in concert with beta-catenin.[79-82] These interactions link FHL2 to further signalling pathways controlling the myogenic factors MyoD and Myf5[83] and cell survival pathways involving p53 activation in a ternary complex with the homeodomain-interaction protein kinase 2 (HIPK2).[84,85] FHL2 can further act as a co-activator of NFkappaB,[86] a transcription factor essential for the response of muscle to various cell stresses and the initiation of hypertrophic growth.[87] As prolonged NFkappaB activation is also implicated in atrophy,[88] the level and duration of this activation need to be finely tuned and the modulating functions of FHL proteins may play an important role in this. NFkappaB activation involves a further FHL2 ligand, the MAP kinase ERK2. FHL2 in these cases serves a repressor function in

cardiomyocytes through its ability to inhibit ERK1/2 transcriptional coupling.[89] A fairly unequivocal role of FHL2 therefore seems to be that of a brake on ERK1/2 signalling.

In addition to protein signalling, FHL2 was recently implicated in sphingolipid signalling via interactions with sphingosine kinase-1 (SK1), a key enzyme catalyzing the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P).[90] S1P regulates diverse cellular responses, including cell growth, differentiation, proliferation and apoptosis. In cardiomyocytes, the overexpression of FHL2 was found to attenuate the activity and antiapoptotic effects of SK1.[90] Endothelin-1, a potent survival factor in cardiomyocytes, inhibited FHL2-SK1 association and increased SK1 activity. In skeletal muscle, S1P regulates the proliferation and differentiation of satellite cells by inducing quiescent satellite cells to re-enter the cell cycle.[91] It is interesting to speculate that sarcomeric links from FHL2 via SK1 may be involved in S1P signalling to satellite cells, a potential mechanism by which muscle work and metabolic flow could be translated into satellite cell recruitment. Such a mechanism could be interesting in sarcopenia and muscle regeneration.

These diverse interactions are in agreement with the observed multiple localizations of FHL2 in different cellular compartments: the cytoplasm, nucleus, focal contacts, as well as around the Z-disks and the M-bands in cardiac myofibrils.[74] Interaction analysis in cardiac muscle revealed links with two regions of titin, the N2B region, a cardiac-specific insertion in the elastic I-band part of titin and the constitutively expressed Mis2 region of M-band titin.[92]

While mice null for FHL2 show no obvious defects under normal conditions, they display an increased hypertrophic response following beta-adrenergic stimulation, which is in agreement with a function as negative regulator of ERK1/2 activity (reviewed in ref. 92). A high level of homology and identical subcellular localizations of FHL proteins in cardiomyocytes, as well as similar responses from promoter activation assays, indicate redundant functions among the FHL protein family, which may account for the lack of a more pronounced basal FHL2 –/– phenotype. The broad variety of the interaction partners involved in either signalling, modulation of gene expression and metabolic enzymes indicates a role of FHL2 in the integration of signals of different pathways important for the differentiation and maintenance of muscle cells. It is presently not clear how FHL2 interactions with all these very diverse ligands are regulated and how mechanical strain on titin will affect these, to contribute to mechanically modulated muscle gene expression.

M-Band Signalling

The Titin Kinase Signalosome

At the M-band periphery, about 50 nm from the central M-band line M1, resides the catalytic kinase domain of titin.[93] Titin kinase is an autoinhibited serine/threonine kinase with superficial homology to the family of calcium/calmodulin regulated myosin light chain kinases (MLCK).[94] Unlike MLCK however, titin is regulated by a dual autoinhibition mechanism that involves a C-terminal extension, blocking the ATP binding site and a tyrosine autoinhibition reminiscent of phosphorylation-regulated kinases.[41] Titin kinase is not activated by only Ca^{2+}/CaM or Ca^{2+}/S100-proteins, unlike MLCK.[41] Weak Ca^{2+}/CaM modulation is conferred only when phosphorylation of the autoinhibitory tyrosine is mimicked by replacement with glutamate, which renders the enzyme constitutively active.[41] However, for access of the autoinhibitory tyrosine, the C-terminal autoinhibitory tail needs to be removed, with no protein factor (including the known calcium binding proteins) having been identified as being able to do so. The relief of intramolecular inhibition is essentially a partial unfolding event of the autoinhibited conformation of the kinase. The folded closed and partially unfolded open states are separated by an energy barrier that can be overcome by ligand binding. In a protein firmly embedded in the contractile machinery and hence exposed to passive and active strain, the energy landscape will also be modulated by mechanical force. In fact, titin is a paradigmatic protein for the study of mechanically induced conformational changes.[8] Contraction induces rapid changes in M-band structure before the much stiffer Z-disk is deformed,[56] suggesting that the M-band is an ideal sensor for active strain.[95]

Recent molecular dynamics simulations suggest that kinase activation may be possible by mechanical forces, implicating titin kinase as an M-band sensor for mechanical stress.[96] A signalling

complex (signalosome) was identified that interacts with an open conformation of the kinase, predicted to be mechanically inducible. A complex of two structurally related ubiquitin-associated zinc-finger proteins, nbr1 and p62, can bind to titin kinase, with p62 also interacting with the ubiquitin E3 ligase MURF2.[97] Nbr1 and p62 can be found at both M-bands and Z-disks. In the absence of mechanical activity, this signalosome dissociated and p62 translocated to the intercalated disk and the cytoplasm.[97] MURF2 translocated to the nucleus, where it can then interact with nuclear partners like SRF. SRF interaction is also reported for MURF3, but could initially not be confirmed.[98] The high homology especially of the RING-B-box domains of all MURFs, which mediate the interaction with SRF, suggests that this interaction may be a redundant function of all MURFs. Nuclear MURF2 led to downregulation of nuclear SRF and its cytoplasmic relocalization, thus suppressing SRF-dependent muscle gene expression.[97] The interaction of MURF2 with SRF and also the as yet uncharacterized interaction of MURF1 with the transcriptional cofactor glucocorticoid modulatory element binding protein-1[99] (GMEB-1) may be functionally related to the ubiquitin associated activity of MURFs during muscle atrophy (Fig. 3).

The TK/nbr1/p62 pathway has multiple potential ramifications and may be highly nonlinear; it could thus potentially integrate and relay multiple signals. P62 is emerging as a central adaptor molecule through participation in several pathways involved in myogenic differentiation as well as the cell stress response. P62 interactions include MAP kinase p38,[100] the MAP kinase-kinase MEK5[101] involved in ERK5-activation, the TNF-receptor associated kinase RIP,[102] atypical protein kinases-C (aPKC),[103] Src-family tyrosine protein kinases like lck[104,105] and insulin receptor/insulin-like growth factor-1 receptor signalling via Grb14.[106,107] Interestingly, titin kinase signalosome components are altered in diabetes in an exercise-dependent way.[108] p62 channels input from a number of these kinases to the activation of NFkappaB.[100,102,109,110] Some of these have multiple roles in cell survival and myogenic differentiation. For example, p38 activates the myogenic transcription factors Mef2 and MyoD by phosphorylation of Mef2C[111,112] and the MEK5/ERK5 kinase cascade is crucially involved in myogenic differentiation and hypertrophic growth via MEF2 activation.[113-116] p62 also interacts with and regulates the orphan-hormone receptor COUP-TFII,[117] which is involved in strain-adaptation of cardiac gene expression and metabolic

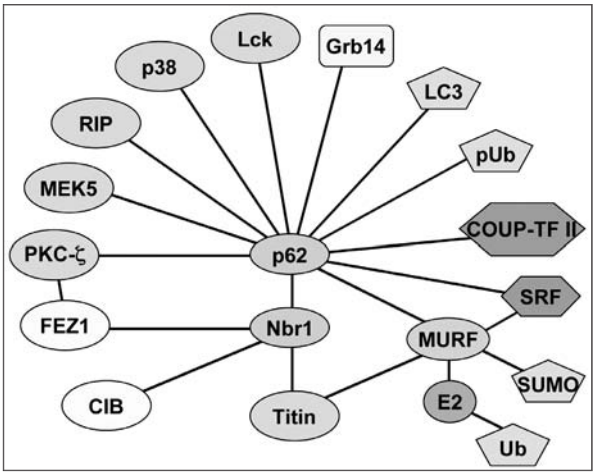

Figure 3. Known interactions of the titin kinase associated, ubiquitin-binding proteins Nbr1 and SQSTM1/p62. Both Nbr1 and SQSTM1 are linked to signalling via the atypical protein kinase C-zeta and bind poly-ubiquitin chains via their C-terminal UBA domain. SQSTM1 also binds to the autophagy label Atg8/LC3 and further links to small protein modifiers exist via the MURFs to SUMO. SQSTM1 interacts with numerous other protein kinases, many of which are relevant for the control of muscle growth and remodelling. For further details, see text.

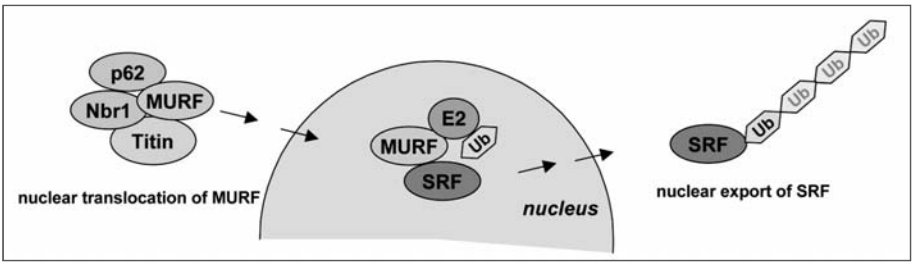

Figure 4. Model of the putative regulatory role of MURF on SRF-dependent gene expression. In active muscle, MURFs are trapped in the sarcomere and associated at the M-band with the titin kinase signalosome. During muscle inactivity or other atrophic signals, this complex dissociates and MURFs translocate to the nucleus. In the nucleus, they employ components of the nuclear ubiquitylation machinery (E2 enzymes, ubiquitin) to ubiquylate SRF. Mono or polyubiquitylated SRF is translocated to the cytosol, reducing SRF-mediated gene expression.

adaptation in skeletal muscle.[118,119] Lastly, p62 can target ligands of its PB1 and ZZ domain region to polyubiquitin chains via its C-terminal ubiquitin-associated UBA domain. This may assemble larger signalosomes via lysine-63 linked polyubiquitin, in analogy to other ubiquitin-mediated kinase signalling pathways.[120,121] Association with lysine-48 linked poly-ubiquitin chains however could target these complexes for proteasomal degradation[122] and, via the interaction of p62 with LC3, to the autophagy of ubiquitylated proteins (Fig. 4).[123]

The potential role of the Nbr1 ligands CIB and ZIP1[124] in muscle is unclear, but both proteins seem absent from the sarcomere. Nbr1 in the sarcomere therefore seems to act mainly as a scaffold to target p62 to titin kinase and to link its activity to p62 and the MURF complex.

MURFs (TRIM)

The RING/B-box/coiled-coil or tripartite motif containing (TRIM) protein family of MURFs (muscle-specific RING-finger proteins) was described initially as a putative ligand of the serum response transcription factor, SRF.[98] Three MURF genes (MURF1/TRIM63; MURF2/TRIM55; MURF3/TRIM54) encode highly homologous proteins that can homo- and hetero-dimerize via a coiled-coil domain,[125] a defining feature of the TRIM protein family. Extensive differential splicing occurs in most MURF genes,[125] leading to isoforms with tissue-specific expression patterns in the case of MURF2.[126] Interactions of MURF1 and MURF2 with domains near the C-terminus of titin lead to association with the M-band.[125,126] However, both MURF1 and MURF3 have also been localized to the Z-disk,[98,125] indicating that the sarcomeric targeting of MURFs is not solely dependent on titin interactions. MURF3 and MURF2 have been found in association with glutaminated microtubules and nascent myosin filaments,[98,126] for which the RING and B-box domains are crucial.[98] A knockout mouse model of the highly homologous MURF1, however, shows no signs of impaired myofibril assembly, but to the contrary, a resistance to both disuse- and steroid-induced atrophy.[127] Similarly, MURF1/3 double knockout animals do not show defects in primary myofibrillogenesis, but rather show a postnatal myosin storage myopathy due to abrogated myosin heavy chain turnover.[128] This suggests that many MURF functions require synergistic action of more than one isogene. It needs to be clarified whether the highly homologous RBCC regions of MURFs lead to functional redundancy or whether they adopt distinct cellular functions.

Similar to related TRIM proteins, where the RING domain is associated with ubiquitin modification,[129] the RING domain of MURFs has been implicated in posttranslational modification by both the ubiquitin, as well as by the ubiquitin-related SUMO systems (for a review, see e.g., 130). MURF1 and 3 have ubiquitin ligase activity in vitro[127,128] and MURF1 is upregulated in atrophic muscle similar to other ubiquitin E3 ligases.[131] On the other hand, the RING domain of MURF1 (sometimes also called SMRZ) interacts with the small ubiquitin-like modifier

Smt3b/SUMO3[132] and all MURFs interact with the SUMO E2 transferase UBC9. In addition, MURF1 also interacts with SUMO isopeptidases, further pointing to a possible involvement of MURFs in the SUMO pathway.[99] Like other SUMO-associated proteins, MURF1 and 2 can also appear in the nucleus,[99,126] specifically under conditions of muscle atrophy for MURF2.[97,126] The interactions with the transcriptional cofactor glucocorticoid modulatory element binding protein-1 (GMEB-1)[99] and with SRF[97] may thus be functionally related to either the ubiquitin or potential SUMO associated activity of MURFs. Given the established E3 ligase activity for MURF1 and 3 in cooperation with the E2 ubiquitin-conjugating enzymes UbcH5a, -b and -c and the near identity of the RING/B-box domains between all MURFs, it is plausible to propose that nuclear ubiquitylation may account for the changes in cellular localisation of SRF (Fig. 4), similar to related nuclear ubiquitin signalling pathways.[133,134]

Up-regulation of MURF1 also inhibited cardiomyocyte hypertrophy via protein kinase C-epsilon activity, by blocking kinase interaction with the scaffolding protein RACK1. This prevented PKC translocation to focal adhesions, an important event in hypertrophic signalling through alpha-adrenergic receptors.[135] MURFs therefore not only associate with different cytoskeleton compartments such as microtubules, Z-disks and M-bands and nuclear proteins, but interact with a host of diverse proteins implicated in metabolic regulation,[136] sarcomere assembly, protein degradation, transcriptional regulation and SUMO-mediated posttranslational regulation. To what extent these highly specialized proteins are indeed redundant remains to be investigated. The localization to Z-disk and M-band, the cytoplasm and the dynamic translocation to the nucleus are aspects of this multifunctionality, which are only now emerging.

Titin, Nbr1, SQSTM1 and MURFs form an M-band associated kinase-ubiquitin signalling module with far-reaching potential ramifications and crosstalk to many other important modulating signalling pathways in muscle. The multicompartment protein FHL2 provides links to both M-band and I-band signalling pathways.

Future Perspectives

Over recent years, the notion that the sarcomere is purely a contractile machine of high order has been expanded by the discovery that multiple signalling pathways not only affect the function of this structure, but that there is active communication, with signals controlling muscle cell proliferation, growth and remodelling emanating from the sarcomere. Protein kinase, protein phosphatase, protein ubiquitylation and sumoylation pathways modulate protein turnover and gene expression patterns, in concert with a number of transcriptional modifiers that are actively exchanged between sarcomere and nucleus. The observation that many of these regulators are targets of hereditary muscle diseases not only highlights their importance, but raises the hope that a detailed understanding of their interwoven functions may result in new therapeutic approaches to ameliorate muscle diseases. However, it is exactly the complexity, redundancy and nonlinearity of sarcomeric signalling that will require concerted efforts on the molecular, cellular, physiological and clinical level to make this potential become clinical reality.

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Chapter 14

Other Model Organisms for Sarcomeric Muscle Diseases

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Abstract

Model organisms are vital to our understanding of human muscle biology and disease. The potential of the nematode *Caenorhabditis elegans*, the fruitfly, *Drosophila melanogaster* and the zebrafish, *Danio rerio*, as model genetic organisms for the study of human muscle disease is discussed by examining their muscle biology, muscle genetics and development. The powerful genetic tools available with each organism are outlined. It is concluded that these organisms have already demonstrated potential in facilitating the study of muscle disease and in screening for therapeutic agents.

Introduction

The major problems in investigating human muscle disease are that the organism concerned is long-lived, experimentally inaccessible during the important events of muscle development and many essential experiments are impossible and/or unethical. Model genetic organisms, including mice (see Chapter by Dr. Nguyen and Dr. Hardeman) provide alternative approaches to understanding aspects of normal and diseased muscle. Genome sequencing of human and the major genetic model animals—mouse, zebrafish (*Danio rerio*), fruitfly (*Drosophila melanogaster*) and the nematode (*Caenorhabditis elegans*) has revealed close homologies in the genes and muscle proteins between these species and humans. Comparisons of muscle structure, function and development also show extensive homologies. However, although their muscles are similar to those from humans they do also differ to varying degrees from human muscle.

We aim to acquaint the reader with sufficient background to appreciate the opportunities that the nonmammalian model genetic organisms offer for study of human muscle genetic diseases and give examples to illustrate what these organisms have already contributed to disease investigations. We argue that there is enormous potential in using these nonmammalian genetic models to investigate human muscle disease that remains to be fully exploited.

The Major Nonmammalian Model Genetic Animals

The three major nonmammalian model organisms for genetic study of development, cell biology, physiology, neurobiology, ageing etc are the zebrafish, the fruitfly and the nematode (usually referred to as the 'worm'). Comparisons of their genomes with humans and their proven success as models with which to illuminate gene function have led to a wider acceptance, that they have important potential for the study of human disease.

Sequence analysis of these organisms' genomes show that a large fraction of their genes have human homologues; 60-70% of human genes have counterparts in *C. elegans* and *D. melanogaster*.

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Having single genes where vertebrates have several paralogues these invertebrate organisms are well suited to identifying the core functions of gene families. The zebrafish has more orthologous genes and is particularly advantageous for dissecting the function of individual members of gene families.

Why, or how, were these organisms chosen and why are they such useful model genetic systems? In each case, the choice was made by an individual investigator. As the models proved amenable, each spawned very large international networks of researchers. It is the communal sharing and developing of genetic resources, tools and information within these networks that make these organisms such powerful tools for genetics research.

Drosophila melanogaster, the fruitfly, was one of the first genetic model organisms. It contributed throughout the 20th Century to most major advances in genetics, especially pioneering genetic approaches to study animal development, cell biology, neurobiology etc. Thomas Hunt Morgan chose it as an experimental genetic organism around 1905 because it was inexpensive and easy to culture, had high female fecundity and flies could be kept in large numbers. Subsequently it transpired that it had only four chromosomes, a smallish genome, mutants were easy to obtain and it was cytogenetically amenable. Its genome (180 Mb) has been fully sequenced and annotated, along with those of 11 close relative species (see Flybase).

Sydney Brenner used similar arguments in the 1970s when initiating research on the nematode, *Caenorhaditis elegans*. This organism can be grown cheaply and easily on *Escherichia coli* lawns in Petri plates. Crosses are easily made and different mutant lines stored by freezing. Since the female worm is a self-fertilizing hermaphrodite, homozygotes are readily produced without the need to mate. Crippled animals survive as homozygotes, a great advantage when studying severe muscle mutations. The genome (97 Mb) is sequenced[1] and contains >19,000 genes. It is unique in that a map of the invariant cell fate of every cell within the nematode is known.[2]

The zebrafish, *Danio rerio* was chosen as a suitable vertebrate model genetic organism by George Streisinger during the 1970s. It has excellent optical properties during early stages. Females have high fecundity and embryos develop rapidly, reaching a motile stage 24 hours after fertilisation. Eggs are fertilised outside the body which facilitates genetic and cellular manipulations. The zebrafish bridges the taxonomic gap between the worm and fly and the mouse. A major advantage of the fish as a genetic model for disease is that its organs and systems are more homologous to those of humans.[3] Although the facilities required for culturing fish are more extensive than for either invertebrate model, sperm can be stored frozen which allows many different mutations and transgenic lines to be kept for long periods and transferred inexpensively.

Importantly, research communities associated with each of these organisms have developed and maintain publicly funded mutant stock collections, sequenced and annotated the genomes and placed all this in open access databases, along with guides to aspects of the organisms' genetics, biology and bibliography. These are accessible at Flybase (http://flybase.bio.indiana.edu/), Wormbase (http://www.wormbase.org/) and ZFIN, the Zebrafish Information Service (http://zfin.org/) and are linked to the major international bioinformatics resources. National and local mutant stock collections are maintained.

What Makes These Special as Model Genetic Organisms for Studies of Muscle?

The simple answer is that compared to mammalian models all stages of muscle development are visible or easily accessible in vivo. In reality, it is a combination of our sophisticated knowledge of and ability to manipulate, their genetics, their overall biology and specifically their muscle biology.

The generation of many separate populations of myogenic cells (myoblasts) is thought to underlie establishment of muscle fibre diversity in species from Drosophila to man.[4,5] Four types of myoblast diversity are particularly significant. First, at, or before, terminal differentiation, myoblasts gain an 'identity' by expressing genes specifically required for the formation of particular muscles or muscle groups and display behaviours, such as adopting specific positions, orientations

or morphologies within the tissue anlagen.[6-10] 'Identity' ensures that muscle fibres interact correctly with connective tissues, become correctly positioned and oriented and make correct paired attachments to skeletal and neural cells. Second, myoblasts that initiate new fibre formation may be distinct from those that contribute to growth. This is best understood in Drosophila, where founder myoblasts, in the embryo and at metamorphosis, express different genes and have different developmental capacities from so-called fusion-competent myoblasts.[6,7] Tissue culture and zebrafish studies suggest a similar system operates in vertebrates.[11,12] In fly embryos only founder cells appear to have muscle identity.[6,7] The situation in vertebrates is unresolved.[4,13] Third, amniote myoblast clones have a cellular memory that determines the contractile protein genes they express and hence the fibre type formed when they terminally differentiate.[14-16] As fibres of similar fibre type exist in most muscles it is entirely unclear how this property relates to muscle identity. Is the slow fibre founder in slow soleus muscle distinct from a slow founder in the mainly fast plantaris? Fourth, myoblast clones, including those from human embryos, differ in their responsiveness to growth factors[17] and capacity for proliferation[18] in ways reminiscent of the classical distinction between stem cells (capable of proliferating indefinitely) and progenitor, or transit amplifying, cells (capable of only a limited burst of proliferation followed by terminal differentiation). Stem cell-derived cells from nonmuscle tissue participate in muscle repair and are particularly important in the context of muscle maintenance.[19,20] Drosophila and zebrafish studies have begun to address these issues in the context of early muscle patterning.

Zebrafish (*Danio rerio*)

Zebrafish contain muscle types characteristic of all the vertebrates. As in mammals, skeletal muscles can be divided into somitic, somite-derived migratory hypaxial (e.g. fin) and head muscles.[21-23] Skeletal muscle can be classified into slow and fast twitch fibres which, in embryos, are topologically separable. Mature fish have a subcutaneous layer of slow-twitch fibres at the dorsoventral midline; most of the rest of the skeletal musculature is fast-twitch. At least six fibre types have been identified in each somite as early as 24 hours postfertilisation.[24-27] Zebrafish cardiac myogenesis produces a heart with only two chambers and relies on many of the same genes as mammalian heart; mutations in genes that cause heart disease also affect fish hearts.[28-29] Zebrafish also have vascular and visceral smooth muscle. Human muscle disease often preferentially affects specific muscle groups or fibre types, providing a major argument for investigating muscle disease in fish.

Importantly for studying human muscle disease, where muscle growth and repair can be major features, zebrafish have a dermomyotome,[25] which contains muscle stem cells[26] and may yield satellite cells. The phasic growth of fish muscle is similar to that in mammals.

Once the early embryonic muscle pattern has been established, vertebrate muscles undergo huge growth. This entails the founding of the correct number of additional muscle fibres in each muscle, which occurs in the early phases of amniote life. Subsequently, the correct number of nuclei must be added to each founded syncitial fibre as the animal grows. In adulthood, this number must be dynamically maintained and adapted dependent on physiological demands. There is evidence from flies, birds and mammals that distinct myoblast populations may influence each of these growth processes.[30-32] Experimental challenge can interconvert some populations.[33] However, the normal embryological origin of vertebrate myoblast populations contributing to muscle growth are uncertain.

The role of myoblast diversity in adult muscle is obscure.[34] Muscle maintenance and repair in the adult can be of several kinds. Mild muscle damage necessitates running repairs to existing fibres, while increased physiological demands enhance muscle mass or alter muscle character and inactivity leads to muscle atrophy. Serious muscle injury or muscle disease can necessitate de novo fibre reconstruction. There is evidence for stem cell populations in adult skeletal muscle.[35] Current interest focuses on the nature, role, origin and capacities of multipotent mesenchymal stem cell populations found within muscle tissue and their relationship to satellite cells, the quiescent myoblasts of adult muscle.[36,37] Change in myoblast diversity is observed in many pathological situations; the extent to which such changes contribute to altered muscle function and disease progression is

unknown. Muscle pathology usually increases 'fibroblast' numbers leading to fibrosis. The origin and relationship of these cells to myogenic cells is unclear.

Amniotes exhibit a great diversity of myogenic routes. During fetal and postnatal myogenesis waves of distinct myoblasts appear and disappear, but their origin and fate are unknown. Molecules that indelibly mark particular cell lineages have not been found. Other myogenic populations exist within adult mammalian muscle. For example, so-called SP cells may be multipotent mesenchymal stem cells,[19] but their normal role is unclear.

Fish myogenesis proceeds in waves similar to those in mammals. Several early myogenic cell populations give rise to diverse early muscle fibres (Fig. 1). The muscle then grows by 'polarized hyperplasia', yielding extra fibres in specific somitic zones, the dorsal and ventral myotomal lips and the 'red muscle rim' between slow and fast muscle regions.[26,38,39] Later 'mosaic hyperplasia' generates new fibres between existing early fibres similar to mammalian secondary fibre formation.[38] Continuous growth of the fish requires fusion of myogenic cells to existing fibres. These may derive from satellite cells (SC) lying under the basal lamina of each fibre, as in mammals and probably contribute to muscle repair. Zebrafish muscle growth and repair is essentially unstudied, although regeneration and growth is known to occur in teleosts.[40]

Zebrafish are readily mutagenized and a wide range of behavioural and developmental phenotypes visually screened for. Large scale screens[41] for mutations affecting muscle development and function are a goldmine for understanding early muscle development. TILLING can be used to create allelic series of mutations in chosen genes. This involves random point (chemical) mutagenesis, followed by DNA sequencing of many fish to identify individuals carrying mutations in target genes. These approaches have tremendous potential to permit detailed analyses of gene function.

Fruitfly (*Drosophila melanogaster*)

All Drosophila muscles are striated. The organism contains many skeletal (attached to the exoskeleton) and visceral muscles, as well as a primitive heart. The musculature of Drosophila has been described in detail[42] and images are available on Flybase.

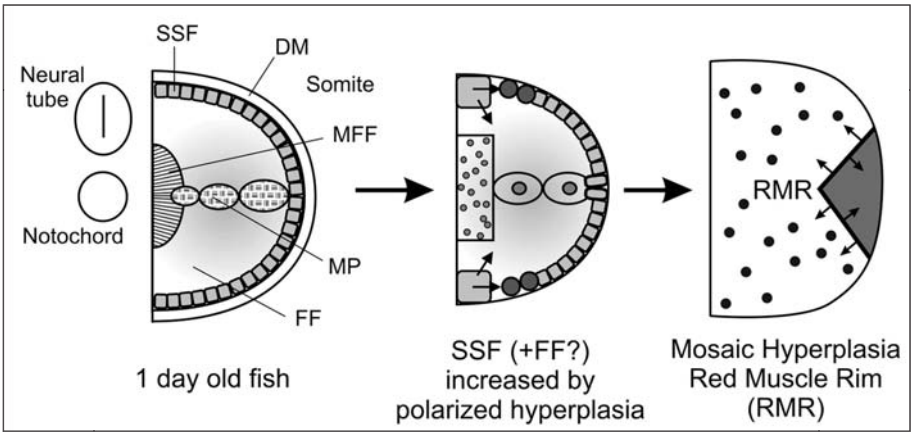

Figure 1. Early myogenesis (left panel) generates four fibre types but leaves undifferentiated cells in the dermomyotome (DM), probably destined to support for later growth. Mononucleate superficial slow fibres (SSF) and muscle pioneer slow fibres (MP) form first, followed by multinucleate fast fibres (FF), a subset of which become specialised as medial fast fibres (MFF). Polarized hyperplasia (middle panel) generates further SSF and FF, probably from DM cells. Mosaic hyperplasia (right panel) arises from precursor cells scattered within the myotome and between the slow and fast regions at the so-called red muscle rim (RMR).

The Drosophila life-cycle requires development of skeletal and visceral muscles twice; once for embryonic/larval development and another during pupal development prior to emergence of the adult (the heart undergoes some development during pupal metamorphosis). Myoblasts that form the embryonic muscles and those that will later differentiate into the adult muscles develop together during early embryogenesis, but while some differentiate immediately to form embryonic/larval muscles, others multiply during larval and early pupal development and then differentiate during metamorphosis to form the adult muscles. No satellite cells have been found in adult flies, though the myoblasts set aside during embryogenesis, only later to form the adult musculature could provide a model system for study of satellite cells.[43] There is an extensive literature on Drosophila muscle development[43,44] and an insightful detailed comparison of Drosophila and vertebrate muscle development.[43] Drosophila embryonic myogenesis is rapid (<<1 day) and easily visible. Mutant studies have identified many genes that are important during myogenesis and revealed cell interactions such as those between founder/pioneer myoblasts that inform studies in the vertebrate models.

Most studies of muscle disease in Drosophila have either focused on the later larval stages (instars) when the muscles are much larger or on the adult, especially the indirect flight muscles (IFM). IFM function can be easily assayed by measures of flight or an elevated wing position phenotype and have been used for fibre physiology/mechanics.[45] The IFM are easily exposed by bisecting the thorax and readily removed for biophysical/biochemical experimentation.[46] The effects of severe mutants can be studied, as flies, at least in the laboratory, do not need flight muscle function for survival and reproduction.

Flies are easily mutagenized by X- and γ-irradiation, various chemicals and, more recently, transposon mutagenesis. For embryonic muscle mutants, screens normally search for recessive lethal mutations which cause embryos to fail to hatch from the egg, followed by inspection to see if they have developed their cuticle and other organs, but remain immotile. Further investigation is then required to identify these mutants as muscle-defective. Muscle mutants in flies were recovered in the IFM by selecting for a dominant flightless phenotype.[47,48] Many muscle protein genes were first identified in this way.

Nematode (*Caenorhabditis elegans*)

The nematode contains a number of muscle systems, but the major set is the bodywall muscles and these have been the target of most genetic studies.[49] The other muscles include those of the pharynx, the 15 diagonal tail muscles with 26 other tail muscles involved in male copulation, 16 sex muscles (of uterus and vulva) in the hermaphrodite, two intestinal muscles and the anal muscles. The body-wall muscles are prominent and the organism small and translucent, so these muscles are readily visualized by polarised light or fluorescent microscopy of the whole organism. Details at the level of the sarcomere are visible. The bodywall muscles occur in 4 strips each consisting of 24 mononucleate, spindle-shaped cells. The first obvious difference is that these muscles contain an obliquely striated array of filaments that for those familiar with vertebrate or arthropod striated muscles is at first glance perplexing, but is wholly analogous to those in the cross-striated sarcomeres of vertebrates and arthropods. A detailed explanation is provided by Waterston[49]in his general account of *C. elegans* muscle. The second difference is that the thick filaments are much thicker than in vertebrates, though less so than in most arthropods including Drosophila. Nematode muscle attachments are more complex. Though the ends of the muscle cells have dense plaques to which the thin filaments attach from only one direction, as in vertebrates and insects, nematode muscle is unusual in that most of the tension generated is transferred directly to the cuticle through a series of lateral attachments at the dense bodies, that are finger-like projections (analogous to Z-discs) into the muscle from the hypodermal side.[49,50] There are similar dense body attachments between the neighbouring muscle cells. The M-lines are seen as finger-like projections that also originate within the muscle cell from the surface closest to the hypoderm.

Random genetic screens using a range of mutagens (X-/γ-rays, chemical mutagens) are readily performed. Muscle mutants were recovered in the first screens for nematode uncoordinated

(*unc*) mutants. Some of these mutants affect the nervous system, but many muscle genes were first identified on this basis[49] and are still actively studied. More recently, severe muscle mutants have been recovered by arrested development with specific phenotypes e.g., the *pat* mutants.[51] The ability to grow worms and flies in large numbers, quickly and cheaply not only facilitates the recovery of primary mutations, but also allows screening for secondary mutations in other genes (intergenic), that either reduce the severity of the primary mutation (suppressors), or enhance it (enhancers).

Genomes/Genetic Technologies of the Model Organisms

The combination of recombinant DNA technology with transgenesis techniques has transformed our ability to manipulate the genomes of model organisms. This has led to the generation and application of an almost bewildering array of powerful molecular tools.

Stable genomic transgenesis is an important process required of model genetic organisms in the current era. Unlike the mouse, in none of these three model genetic organisms is it yet possible to target specific genes for replacement by homologous recombination. Gene replacement thus requires transgenesis of mutant gene copies into genomes containing 'null' mutations for the gene of interest. In *C. elegans* for genes already containing a transposon ('tagged') it is possible to copy a modified version of the gene directly into the native chromosome; similarly in Drosophila an inserted transposon (P-element, see below) can be replaced at a high frequency with another P-element, that could contain a novel version of the same gene.

The nematode genome contains a number of transposable elements including Tc1 and Tc3, members of the Tc1/mariner transposon superfamily.[52] Mutations due to transposon insertion have been developed into 'gene-tagging' (also used in Drosophila and zebrafish) whereby mutations with specific phenotypes are recovered and the genes identified using PCR techniques to obtain the insert flanking sequences. These mutations may be unstable or have weak phenotypes. Stable mutations can be recovered by remobilising the original transposon by imprecise excision that removes flanking genomic DNA creating deletions detectable using PCR methods. More recently, a significant number of *C. elegans* genes have been tagged using the *Drosophila Mos1* transposon, which is more stable than Tc elements.[53] Nematode transgenesis is routinely achieved by non-integration of plasmid vectors by either DNA injection or sometimes particle bombardment[54] and allows a wide variety of gene manipulations to look at the in vivo effects. These include gene 'rescue' (with wild type gene copies) of mutants, insertion of dominant negative mutations made in vitro, expressing gene and protein fusions, to investigate gene promoters using promoter-reporter constructs and ectopic expression of genes. Not only do these allow genetic investigations, but also provide a wide variety of powerful microscopical tools for investigating the organismal cell biology through the expression of protein reporters such as fluorescent tags (e.g. GFP, YFP etc.), enzymes and epitope tags etc.

In Drosophila a stable transgenesis system has been developed using the P-element, a native transposon, though other native transposons have also been developed as vectors. P-element vector DNA is injected into the posterior part of preblastoderm eggs along with a source of transposase (either the enzyme or a defective P-element able to express functional transposase). A variety of P-element vectors are available and whole genes can be inserted for e.g., 'rescue' experiments, to make promoter-reporter constructs for in vivo gene expression analysis, insert in vitro mutagenized genes, or other DNA sequences. Specific P-elements (and some other tranposons e.g., piggyBac) have been made for a range of applications. For instance, they are used for insertional mutagenesis[55] (Berkeley Drosophila Genome Project http://www.fruitfly.org/) and to generate deletions by imprecise excision of a P element insert or by recombination between two inserts. A novel approach is to 'enhancer trap' different genes (using reporter gene sequences—GFP, lacZ, etc.) by relying on insertion near a gene enhancer to overcome the weak endogenous expression of the reporter gene in the P-element. Protein 'trapping' can be achieved by random insertion of a P-element containing GFP as an exon into a genomic intron that leads to the appearance of green fluorescent embryos or tissues. This allows rapid screening for useful protein-GFP fusions.[56]

These activities have produced massive collections of gene knockouts, deletions,[57] enhancer traps, expression reporters etc.

Arguably, the most powerful application of P-element transgenesis has been the development and use of the GAL4-UAS system.[58] In this system transgenes expressing the yeast GAL4 transcription factor 'drive' expression of any other transgenic sequence inserted downstream of the yeast UAS sequence, the normal GAL4 binding target. Large collections of GAL4 lines expressing GAL4 from gene-specific promoter sequences, or from 'enhancer traps' now exist. Simply by crossing an appropriate (by expression level/pattern) GAL4 line with any of the different UAS construct lines expressing a vast array of heterologous proteins (GFP, GFP-protein fusions, dominant negative alleles, human disease proteins etc.) one can control the ectopic expression of the protein of choice to interfere with the system, to follow gene expression patterns or to determine the cellular locations of proteins of interest.[58]

Zebrafish transgenesis is based on either retroviral infection or on the ubiquitous Tc1/mariner transposons. Retroviral systems are used for efficient insertional mutagenesis,[59] enhancer trapping[60] and for a variety of other transgenic purposes. Retroviral systems are relatively laborious. Use of the Tc1/mariner transposon to achieve transgenesis has great potential to exploit the breadth of applications demonstrated by the Drosophila transposon systems.

The discovery that short double-stranded RNA (dsRNA) molecules can cause specific degradation of the corresponding mRNA was made in the nematode.[61] This technique, known as RNAi, is now used widely to 'knockdown' mRNA levels in many different systems. Its use has been developed further in *C. elegans*, in Drosophila and more recently in the zebrafish. Originally applied to worms by injection,[61] it is now routinely achievable by soaking worms in dsRNA, or by feeding dsRNA in liquid culture, but most easily by feeding worms on *E. coli* containing plasmids with the specific dsRNA sequence.[62] A large library of *E. coli* strains containing different dsRNAs covering the nematode genome is available; almost any gene may be screened using the appropriate clones.[63] In flies the major technique is to make transgenic lines with specific dsRNA sequences downstream of the UAS sequence. This allows one to drive RNAi expression in different tissues, dependent only on the availability of a transgenic GAL4 driver. This is a very effective and flexible approach. A large available collection of transgenic UAS-RNAi strains has recently been made.[64]

In the zebrafish, morpholinos, short chemically modified RNA molecules that bind to mRNA provide an effective 'knock down' approach.[65] Recently, RNAi technology has become available through injection of dsRNA.[66]

Model Organism Studies of Human Muscular Disease

There is already considerable model organism muscle research that is of relevance to the study of human muscle disease, including both heart and skeletal muscle. The use of zebrafish genetics to study congenital heart disease has been reviewed,[29] as has the potential of the Drosophila heart as a model system for human heart disease.[67]

Mutations of the myosin heavy chain can cause skeletal muscle myopathy and hypertrophic cardiomyopathy. Many myosin mutations are known in nematodes and flies. Trangenesis of a mutated copy of the nematode *unc-54* (MyHC-B) gene into an *unc54* null strain to study the effects of the human (skeletal) myosin heavy chain IIa E706R mutation that leads to contractions of the joints and a progressive muscle weakness was able to show that the mutant affects myosin function rather than causing a structural effect.[68] At least one Drosophila mutant myosin suppressor of hypercontraction occurs in the myosin 'hypertrophic cardiomyopathy loop'.[69]

Arthrogryposis is a complex, rare human congenital disorder exhibiting multiple joint contractures, usually of the distal limb. It is often associated with congenital muscular dystrophy, congenital myopathies (central core, nemaline), myasthenic syndromes, intrauterine viral myositis and mitochondrial disorders. Two types of arthrogryposis are caused by mutations of the fast twitch muscle-specific TnI (*TNNI2*) or the *TPM2* tropomyosin gene.[70] Tropomyosin/troponin complex mutations are known in nematodes, flies and zebrafish and are usually associated with hypercontraction, a phenotype where normally developed muscles undergo severe contractions

causing muscle damage and dysfunction. Sarcomeric troponin mutations in *C. elegans* e.g., in the TnT (*CeTnT-1*) gene can cause detachment of body wall muscles.[71,72] Mutants of the single Drosophila TnI and TnT genes cause IFM hypercontraction and muscle detachment.[72,73] IFMs appear especially sensitive to hypercontraction. The *held-up²* troponin I mutation at a highly conserved alanine residue (from humans to flies) causes IFM hypercontraction before adult emergence. Adult flies are initially normal, but flightless; then hypercontraction produces progressive crippling due to leg muscle detachment.[74]

Drosophila single myosin heavy chain *Mhc* gene mutations can cause hypercontraction.[75,76] The *Mhc⁶* and *Mhc¹³* myosin rod mutations do so by reducing thick filament mechanical stability.[75] In zebra fish *silent heart* mutants of the *TNNT2* troponin T, gene affect the heart.[28]

Suppressor mutations of the Drosophila *hdp²* (TnI) mutation have been used to investigate hypercontraction and the function of the troponin/tropomyosin complex in the activation of muscle contraction.[69,74,77,78] Suppressors were found intragenically (a second TnI mutation that 'corrects' the effects of the first),[77] in the *Tm2* tropomyosin gene[74] and in the *Mhc* motor domain.[69,78] Suppression by *Mhc* mutations is probably due to reduced contraction force.[69] Certain mutations in the IFM-specific *Act88F* actin gene suppress *hdp²* hypercontraction (Haigh, Cammarato and Sparrow, unpublished). Suppression studies are a powerful way to study the functional relationships of interacting muscle proteins. They may be invaluable in exploring how genetic variance impacts the severity of specific human muscle disease mutations.

Many, mostly dominant, congenital myopathy mutants are known in the human *ACTA1* gene.[79] A unique feature of the Drosophila IFM for the study of these diseases is that they express all their sarcomeric actin from a single gene, *Act88F,* that is expressed (almost) solely in these muscles.[80] *Act88F* null mutations are viable, so mutated *Act88F* transgenic copies can be used to study effects on muscle structure and function and the mutant actin can be purified for biophysical and biochemical experiments.[81] Since the *Act88F* and *ACTA1* proteins show 94% residue identity one can study the congenital actin myopathies using the Drosophila IFMs.[82] Biopsies from human disease patients show a range of subcellular effects—nemaline rods, intranuclear rods, homogenously staining regions containing large concentrations of thin filaments, zebra bodies[83] and congenital fibre type proportion (CFTD).[84] Transgenesis of *Act88F* genes containing *ACTA1* nemaline mutations all affect muscle structure and function (Sevdali and Sparrow, unpublished). Effects range from mild disturbances of sarcomeric structure to complete absence of thin filaments, 'zebra bodies' (comparatively rare in human biopsies) (see Fig. 2), some nemaline-rod like structures (smaller than usually seen in humans) and novel Z-disc structures enclosing other sarcomeric proteins. Some mutations reduce fibre diameter but since all IFM fibres are equivalent whether these mimic CFTD effects is unclear. Studies of early IFM myogenesis reveal at least two pathways to the fibre defects; most of the actin mutants cannot assemble normal sarcomeres during development; others form sarcomeres normally, but cannot maintain structural integrity during use (Sevdali and Sparrow, unpublished). Nemaline mutations occur throughout the actin molecule with little evidence of clustering of mutant residues with respect to phenotypic effects.[79] The IFM system allows exploration of the relationships between mutant residues, the biochemical interactions of actin and effects on sarcomerogenesis and structural integrity. 'Zebra bodies' also occur in the IFM of Drosophila troponin T-mutants.[73]

This example highlights an issue of working with a model system for human muscle disease. The Drosophila IFM express a single sarcomeric actin from the outset; during early human skeletal myogenesis the *ACTC* cardiac isoform is expressed, switching to the *ACTA1* skeletal isoform during late foetal stages. One can analyse the basic molecular lesion in Drosophila without complications from other isoforms.

Only one well-known human muscle disease (but not a sarcomeric muscle disease) has been focused on in all three model genetic organisms—Duchenne muscular dystrophy. We describe these studies to illustrate the benefits in applying these approaches to the investigation of human muscle diseases, including the sarcomeric diseases.

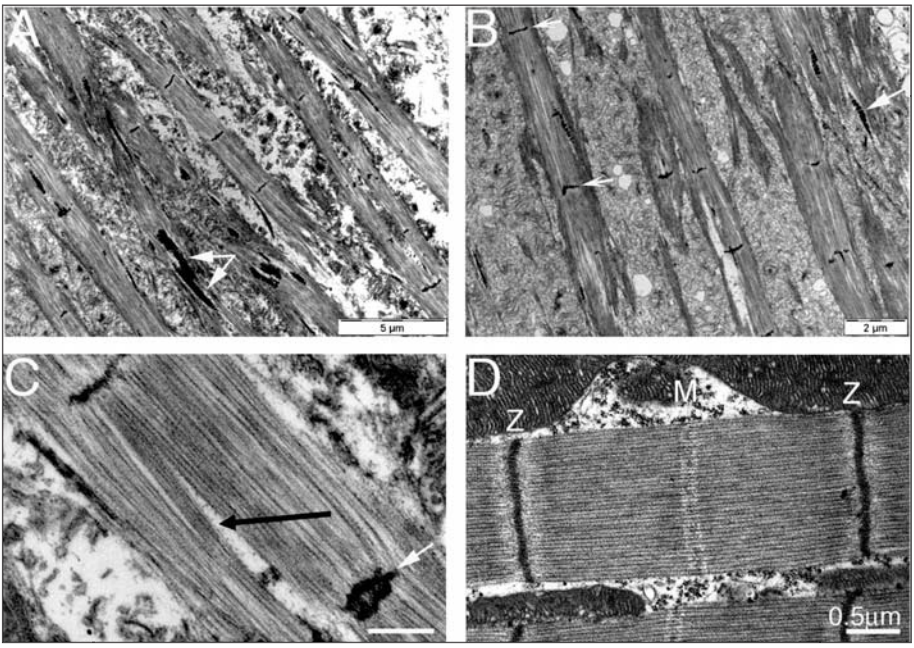

Figure 2. Electron micrographs of flight muscle sarcomeres from *Act88F-G15R* homozygous mutant (A-C) and wild type flies (D). In panel A and B aberrant myofibrils are seen; there is a lack of regular structure; Z-discs never extend across the myofibril width and large arrays of 'zebra-bodies' comprising stacks of short sections of Z-disc-like material, spacing by about 200 nm, are seen mostly within the muscles. In this and other mutants they are also found separated from the myofibrillar materials. Panel C highlights other aspects of these mutant sarcomeres including longitudinal gaps (black arrow) and dark staining material which appears to be split or duplicated Z-discs (white arrow). Panel D, wild sarcomere shows the very regular structure and much reduced I-band width, typical of this muscle type. EMs courtesy of Vikash Kumar.

Muscular Dystrophy

Duchenne muscular dystrophy (DMD) in humans is due to mutations in the gene that encodes dystrophin. Dystrophins and dystrophin-like proteins are widely distributed in animals.[85] The dystrophin-glycoprotein complex (DGC) includes dystrophin, as well as transmembrane and cytoplasmic proteins. Homologues of this complex are known in all three model organisms.

C. elegans possesses single conserved dystrophin-like (*dys-1*) and dystrobrevin-like (*dyb-1*) genes.[86] Conserved homologues have been found also for dystroglycan, delta/gamma-sarcoglycan, syntrophin and divergent, but related, proteins to α- and β-sarcoglycans.[87] Mutations inactivating the *dys-1* gene cause only mild muscle degeneration, but cause behavioural phenotypes of hyperactivity, unusual movements of the head and a tendency to hypercontract[88,89] that are also seen in mutants of other genes encoding DGC components.[86,89] In combination with a weak *hlh-1* gene mutation, the *dys-1* mutation produces a time-dependent muscular dystrophy. The *hlh-1* gene encodes a MyoD homologue. In *mdx* mice MyoD mutations also enhance the severity of dystrophy.[90] Dysfunction of the *dys-1* mutants is partly rescued transgenically by human dystrophin[88] revealing a functional homology. This *C. elegans* genetic combination can thus be used as a model system for human muscular dystrophy.

Microarray studies of two dystrophin mutants, *dys-1(cx35)* and *dys-1(cx18)* revealed expression changes for many genes.[91] Comparison with microarray analyses of human dystrophic patients

showed many similarities but some significant differences probably because of important differences in DGC composition in the two organisms.[91]

Characterisation of the *C. elegans* syntrophin gene[89] illustrates where the relative genetic simplicity and the tools available underpinned substantial progress in understanding a specific gene/protein of the DGC. Syntrophin is a DGC component and searching across the *C. elegans* genome revealed two nematode genes with homology to a human syntrophin sequence. A transgenic *syntrophin-1* (*stn-1*) promoter-GFP reporter construct was made and revealed a nerve/muscle-specific expression pattern, consistent with syntrophin localisation in humans. An *stn-1* intragenic deletion mutation, ordered from the Gene Knockout Consortium, showed phenotypic effects typical of *dys-1* gene mutants. Using transgenesis expression of a wild-type *stn-1* gene controlled by a muscle-specific promoter in a *stn-1* knockout mutant fully rescued the phenotype, indicating that the mutant effects were caused by an absence of protein in the muscles, rather then the nerves. Although the *stn-1* knockout behaves like the *dys-1* and *dyb-1* mutants, its mild effects on muscle degeneration were not potentiated by *hlh-1*.

Proposals that elevated Ca^{2+} levels increased the progression of the disease in *mdx* mice have not been directly tested in the *mdx* mouse but were addressed in *C. elegans* by using a gain of function mutation of the *egl-19* calcium channel or knocking it down with RNAi-mediated inhibition.[92] These studies showed that increased calcium is bad for dystrophic muscle.

C. elegans can be used, quickly and inexpensively, to screen for unknown genetic/biological interactions or as an early stage screen for chemicals that may lead to therapy in humans. This was demonstrated in successful screens for molecules, including serotonin and prednisone, that alleviate the symptoms of the dystrophin mutations.[88,93,94] Identifying potential drugs for therapeutic development also highlights cellular processes for further investigation. A screen for mutations with a phenotype similar to *dys-1* recovered a number of mutations including *dyb-1* encoding a homologue of dystrobrevin[95] and the SLO-1 gene.[96] SLO-1 regulates neurotransmitter release in nematode motorneurons and had not previously been implicated in dystrophic effects. The double *dys-1; slo-1* mutant worm does not have an additive phenotypic effect, suggesting that this channel is involved in the same pathway as the nematode dystrophin homologue. Random mutagenic screens have successfully identified mutations that suppress the phenotypic effects of *dys-1* mutants, thereby uncovering new genes likely to be involved in dystrophy,[95] including the *dyc-1* gene.

The *D. melanogaster* genome contains one or two genes for most of the essential components of the DGC, including dystrophin, dystrobrevin, dystroglycan, syntrophin and sarcoglycans, but no sarcospan homologue.[97] This is in contrast to the genetic complexity and potential redundancy found with the multiple isoforms of vertebrate DGC components. The conservation of the DGC genes together with their smaller number suggests that the fly system, like that of the nematode, will be a relatively simple, but good model for its human counterpart. Detailed examination of the single dystrophin/utrophin gene homologue shows substantial sequence and predicted structural homology to the human products.[98] However, this Drosophila gene shows a highly complex structure expressing at least six dystrophin-like isoforms, DLP1-3 (long forms), Dp186,[97-99] Dp205 and Dp117,[100] with different expression patterns.[98,101] Only the DLP2 isoform is known to be expressed in muscle fibres and at muscle attachment sites.[98,101] As in vertebrates, the dystrophin gene products are expressed in nerves and muscles and recent genetic studies have begun to illuminate the gene complexity and the functions of its products.[100,102] Transposon mutagenesis reveals that absent or reduced expression of the large gene products expressed in the musculature does not cause degeneration of larval body wall muscles. However, neurophysiological studies showed that the DLP2 dystrophin isoform is required postsynaptically to maintain normal levels of neurotransmitter release.[102] This indicates a novel role for dystrophin in neuromuscular function, though its relevance to human disease is unknown. Using the *Drosophila* GAL4—UAS system to drive expression of RNAi constructs specifically directed against all dystrophins in the muscles and tendon cells did not compromise myogenesis or muscle attachment to tendon cells, but caused a progressive degeneration of larval and adult muscles. Reduction of the Dp117 isoform alone induced this degeneration. Clearly the ability to manipulate gene expression of a complex gene in

Drosophila has revealed at least two roles for dystrophin gene products—maintaining synaptic homeostasis and preserving the structural stability of the muscle.[100]

The zebrafish genome contains 29 genes orthologous to those in humans known to cause dystrophy and databases show transcripts for 28 of these genes.[103] There is a high level of conservation, enhancing the potential of zebrafish for studies of muscular dystrophies. RNAi against the Duchenne/dystrophin gene (*dmd*) produces muscle disease phenotypes in embryos showing one can target the range of different transcripts produced by the gene.[66]

Zebrafish mutants are known which exhibit various phenotypes of dystrophy, one of which is a premature termination mutation in the dystrophin gene,[104] where muscle degeneration becomes apparent as soon as three days after egg fertilization.[105] The *sapje* mutation in the fish orthologue of the human DMD gene causes a degeneration phenotype similar to human muscular dystrophies.[106] The mutation reveals that degeneration effects are due to failure of the muscle end attachments in embryonic muscle, indicating that dystrophin is required for the formation of stable muscle attachments.[106] Such an involvement of dystrophin in vertebrate myotendinous junctions was proposed earlier from studies of *mdx* mice.[107] This zebrafish data is the first direct evidence for this dystrophin function and highlights the importance of studying human orthologues in different models.

Human mutations of DGC components have effects on other tissues,[108,109] including the nervous system. The effects of mutations in DGC complexes are being explored in other tissues within model genetic organisms. Gain-of-function mutants in Drosophila dystroglycan, laminin A and dystrophin genes affect epithelial cells and oocytes and have been characterised using genetic mosaic analysis and RNAi methods.[110] Using Drosophila dystrophin mutants, dsRNA constructs and a range of GAL4/UAS lines, it has been shown that dystroglycan and dystrophin are required in the brain for targeting neurons and glial cells for correct neuronal path-finding.[111]

Conclusions

As genetic systems for studying muscle development and functions in their own right, all three of these organisms have made fundamental contributions to muscle biology. Studies of disease-causing genes and their human mutations in these model genetic organisms are being increasingly used to address questions specifically related to understanding human muscle disease. This suggests that muscle biologists working with these organisms now have the genetic tools to do so, as detailed above, but also that clinically focused muscle scientists are beginning to see the potential of such studies.

An issue of working with a model system for human muscle disease is how well the model system recapitulates the human disease. Neither nematode nor Drosophila muscle has any regeneration capacity in response to dysfunction and neither organism has an adaptive immune response. These can be considered advantages and disadvantages. The model system will not recapitulate the whole disease and in the examples studied to date do not. The defects seen are related, but different. However, this is an advantage, allowing investigation of the basic mutant molecular lesions without complications from repair or immune responses.

In the context of sarcomeric muscle disease, the value of these model organisms will be judged by contributions they make. In this chapter we have sought to illuminate what these model organisms have to offer. The choice of a model organism for studies of human muscle disease must be a balance of different advantages and disadvantages. Thus while zebrafish muscle has much closer homologies to humans it does not yet have the various sophisticated transgenic techniques and resources available in Drosophila, or, as especially in the nematode, the rapidity of screening large numbers of organisms for muscle mutations, RNAi effects across the whole transcriptome, or for chemicals that suppress the phenotype in disease models.

Mammalian genomes contain only slightly larger numbers of genes than these model organisms, yet mammalian genomes produce very many different proteins from them. Comparing homologous muscle protein genes shows that those of nematode and Drosophila, while also complex, are less so than those found in fish or mammals. The fish genes are only slightly less complex than those in mammals. This makes detailed genetic analysis much easier in the invertebrate models.

In conclusion, these three genetic models have enormous potential in the study of human sarcomeric muscle disease. The literature increasingly contains reports of human muscle disease mutations introduced into these model genetic organisms. This approach promises to be especially informative. The European Union recently funded a large research network Myores on 'Multi-organismic approaches to muscle development and disease' (www.myores.org).

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Chapter 15

Therapeutic Approaches for the Sarcomeric Protein Diseases

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Abstract

No curative treatment currently exists for patients with skeletal myopathies caused by defects in sarcomeric proteins though symptomatic treatments including orthoses, night-time ventilation, or mechanical ventilation can provide major benefits. The molecular genetic discovery era has enabled many families to know which gene and precisely which gene defect their family, or in some cases only their affected child has. This knowledge has enormously increased the accuracy of genetic counselling and in some cases can enable prognosis, which helps families to make better-informed life decisions. However, symptomatic treatments and molecular genetics do not help the patient's skeletal muscle problems. The patients with skeletal muscle sarcomeric protein diseases, (from severely affected patients with shortened lifespan, through to the more mildly affected patients), would all benefit from more effective or curative treatments, as would their parents and families. This chapter outlines the experimental therapeutic strategies that have been investigated for other muscle diseases (predominantly the muscular dystrophies, towards which the majority of research emphasis has been focussed) and those that are beginning to be investigated for sarcomeric diseases. It analyses which of these approaches might be applicable to the different skeletal muscle sarcomeric protein diseases.

Introduction

It was twenty years ago that the first gene was identified for a muscular dystrophy, specifically mutations in the dystrophin gene were shown to cause Duchenne muscular dystrophy (DMD) and the allelic, milder disease Becker muscular dystrophy (BMD).[1] Since this breakthrough, the genetic causes of many other muscle diseases have also been elucidated (see Neuromuscular Disorders GeneTable at http://194.167.35.195:3000/). Despite immense research efforts in the quest for therapies and cures for DMD, the only currently successful clinical treatments are the use of corticosteroids, physiotherapy, orthopaedic input, cardiac surveillance and ventilatory support.[2] These interventions did not arise from the knowledge of the genetic cause and although they do positively influence the quality of life of patients and their lifespan,[2] they are not cures. However, of the many different experimental approaches based on the genetic cause that have been attempted for the therapeutic treatment of DMD and other dystrophies, a few have led to clinical trials. There is great hope that one of these approaches, or perhaps a combination of these approaches, will lead to a significant improvement in the clinical phenotype of DMD patients in the near future.

The first skeletal muscle diseases shown to be caused by a defective sarcomeric protein were identified in 1995, with calpain-3 mutations unveiled as the cause of limb girdle muscular dystrophy

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2A (LGMD2A)[3] and mutations in slow α-tropomyosin-3 as the genetic cause of nemaline myopathy.[4] There are now around twenty different sarcomeric proteins known to be involved in skeletal muscle disease, producing phenotypes which include LGMD, myofibrillar myopathies, congenital myopathies and distal myopathies (for a comprehensive list of the sarcomeric protein genes and which diseases they are known to cause see Table 1).

In stark contrast to the sarcolemmal muscular dystrophies, very little published research currently exists for the experimental treatment of sarcomeric protein diseases (exceptions are L-tyrosine[5] and exercise[6] for nemaline myopathy, but these approaches are not based on the genetic cause of the disease).

Hopefully the lessons learnt from the research into therapies for the sarcolemmal muscular dystrophies will be applicable to sarcomeric protein diseases (especially perhaps those that have a dystrophic phenotype) and thus expedite the successful development of treatments for the sarcomeric protein diseases.

Experimental Treatments for Sarcolemmal Muscular Dystrophies

Dystrophin is a 427 kDa protein that provides a mechanical link between the extracellular matrix and the internal cytoskeleton of a skeletal myofiber. Amongst other roles, it protects the muscle cell from membrane damage caused by muscle contraction. At a genomic level dystrophin is the largest human gene, encompassing 2.3 Mb, or 1.5% of the X chromosome. Seventy-nine exons encode the full-length 427 kDa protein, with a cDNA of approximately 14 kb.

Many other muscular dystrophies are caused by mutations in genes that code for proteins that are also involved in the link between the cytoskeleton and the basal lamina. Fragility of the cell membrane (sarcolemma) occurs due to the absence or diminished presence of often not only the protein produced by the defective gene, but also its binding partners. Due to the weakened sarcolemma, muscle fibres die and cycles of degeneration and subsequent regeneration of skeletal myofibers occur. DMD is a progressive, fatal myopathy and although not all other muscular dystrophies are fatal, they too are progressive as the regenerative capability of skeletal muscle declines with time (discussed below). Many of the sarcomeric protein skeletal muscle diseases are not progressive, nor are there significant signs of degeneration and regeneration. Obviously the sarcomeric proteins are not membrane bound, but are instead within or associated with the sarcomere, located internal to the muscle fiber within the sarcoplasm. Many of the diseases caused by defective or absent sarcomeric proteins lead to pathologies that are characterised by protein aggregates (e.g., myfibrillar degradation products in MFM, nemaline rods in nemaline myopathy, myosin in hyaline body myopathy and actin filaments in actin myopathy). Therefore it cannot be presumed that those therapeutic strategies which prove to be successful for the muscular dystrophies will necessarily be applicable to the skeletal muscle diseases caused by defective sarcomeric genes, even the subset which do lead to a dystrophic phenotype. However one point is certain, the hurdles faced as to how to deliver treatment to skeletal muscle, the most abundant tissue of the body, remain the same for all skeletal muscle diseases.

Gene Replacement Therapy: Adding a Functional Version of the Gene

For recessive diseases where no functional protein is produced, gene replacement therapy (that is delivering a normal copy of the defective gene) is an obvious route to pursue. Gene replacement therapy can be achieved by the use of either viral or plasmid vectors containing the cDNA of the gene (or in some cases a reduced cDNA if the gene of interest is too large to fit within the capacity of the vector), or by using normal or genetically modified cells such as myogenic and satellite cells (both precursors of the myogenic lineage), or more pluripotential stem cells as the vehicle. For patients where no protein product was made, the possibility of an immunological reaction against the previously missing gene product needs to be considered, no matter which gene replacement approach is utilised.

For those diseases caused by dominant mutations, where normal protein from the wild-type allele of the gene of interest is produced along with defective protein from the mutant allele,

Table 1. Sarcomeric protein genes that have been demonstrated to cause skeletal muscle disease

Gene	Gene Symbol (OMIM #)	~ cDNA Size (kb)	Disease Caused (OMIM #)	Dominant or Recessive?	Alternative Splicing?	Number of Coding Exons	Alternative Isoform?
Actin (skeletal muscle α-)	*ACTA1 102610*	1.2	Congenital myopathy with excess thin filaments, NEM3 161800; CFTD 255310; Congenital myopathy with cores	Mainly dominant, few recessive	No	6	Actin (cardiac muscle α-)
Calpain-3	*CAPN3 114240*	0.5	LGMD2A 253600	Recessive	Yes	7	Calpain-1, calpain-2
Cofilin-2	*CFL2 601443*	0.5	NEM7 610687	Recessive, only 1 family so far	No	4	Cofilin-1 (non muscle)
Crystallin (α-B-)	*CRYAB 123590*	0.5	MFM/α-B-crystallinopathy 608810	Dominant	No	3	
Desmin	*DES 125660*	1.4	Desmin-related MFM 601419	Dominant and recessive	No	9	
Filamin C	*FLNC 102565*	8.2	Filamin C- related MFM 609524	Dominant	No	48	Filamin A, filamin B
Myosin heavy chain (β cardiac)	*MYH7 160760*	5.8	MPD1 160500; hyaline body myopathy 608358	Dominant mainly dominant	No	38	MYH1 (adult fast), MYH2 (adult), MYH3 (embryonic), MYH4 (fetal), MYH8 (perinatal), MYH6 (cardiac α–)
Myosin light chain-2 (slow cardiac)	*MYL2 160781*	0.5	CMH10 608758 with myopathy	Dominant	No	7	MYL1 (skeletal), MYL3 (skeletal slow)

continued on next page

Table 1. Continued

Gene	Gene Symbol (OMIM #)	~ cDNA Size (kb)	Disease Caused (OMIM #)	Dominant or Recessive?	Alternative Splicing?	Number of Coding Exons	Alternative Isoform?
Myotilin/titin immunoglobulin domain protein	*MYOT/TTID 604103*	1.5	LGMD1A 159000; MFM/myotilinopathy 609200; SBM 182920	Dominant Dominant Dominant	No	9	
Nebulin	*NEB 161650*	20	NEM2 256030	Mainly recessive	Yes	183	
Plectin	*PLEC1 601282*	13.7	LGMD with EBS 226670	Dominant and recessive	Yes	32	
Telethonin	*TCAP 604488*	0.5	LGMD2G 601954	Recessive		2	
Titin	*TTN 188840*	Variable, up to 100.3	TMD 600334; LGMD2J 608807	Recessive Recessive	Yes	Variable, up to 312	
Tropomyosin (slow α-)	*TPM3 191030*	Variable, 0.7 to 0.8	NEM1 609284	Mainly dominant	Yes	Variable, 8 to 10	Tropomyosin (cardiac α-)
Tropomyosin (β-)	*TPM2 190990*	0.85	NEM4 609285; DA1 108120; Cap disease	Dominant	Yes	9	Tropomyosin (cardiac α-)
Troponin-I	*TNNI2 191043*	0.5	DA2B 601680	Dominant	No	5	Cardiac troponin-I
Troponin-T (slow skeletal type 1)	*TNNT1 191041*	0.5 to 0.7	NEM5 605355	Founder mutation, all recessive	Yes	Variable, up to 12	Cardiac troponin-T
Z band alternatively spliced PDZ motif-containing protein	*ZASP 605906*	Variable, up to 2.2	ZASP-related MFM 609452	Dominant	Yes	Up to 13	

AD: autosomal dominant; CFTD: congenital fiber type disproportion; CMH: hypertrophic cardiomyopathy; DA: distal arthrogryposis; DM: distal myopathy; EBS: epidermolysis bullosa simplex; DRM: desmin-related myopathy; LGMD: limb girdle muscular dystrophy; MFM: myofibrillar myopathy; NEM: nemaline myopathy; OMIM: Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/sites/entrez?db = omim); SBM: spheroid body myopathy; TMD: tibial muscular dystrophy.

introducing more normal protein may not necessarily be beneficial. Most dominant diseases are thought to arise from the presence of the defective protein through a negative gain of function or a poison polypeptide. Many of the diseases caused by mutations in the genes coding for sarcomeric proteins are dominant and therefore it is unknown whether increasing the amount of wild-type protein will alleviate the disease symptoms. Delivery of a wild-type protein, by whatever method, may only be applicable to those sarcomeric protein diseases caused by a lack of wild-type protein e.g., calpain-3 in LGMD2A. However some evidence exists that changing the ratio between the amount of wild-type and mutant protein may aid in lessening the phenotypic severity of dominant disease, e.g., siRNA experiments (discussed later) which selectively reduce the amount of protein expressed from the mutant allele, can lessen the disease phenotype in vitro.[7,8]

Plasmids

Plasmids are the simplest form of vector delivery and can be easily grown in bulk and purified. Naked plasmid vectors were shown to be taken up by mouse muscle fibers and plasmids containing dystrophin were successfully transferred into the mdx mouse (mdx mice are negative for dystrophin due to a spontaneous nonsense mutation in exon 23 of the dystrophin gene), e.g.[9] However the intramuscular delivery of naked plasmids was demonstrated to be too inefficient and hence research into how to increase the efficiency through the use of ultrasound, electrical fields, perfusion or electroporation was conducted. The most promising of these approaches were intra-arterial injections[9] which are less invasive, less labour intensive and more likely to deliver to multiple muscles and have even been demonstrated on non-human primates.[10]

Although plasmids have been shown to persist for long periods of time in animals (e.g., up to a year[11]) there is a sustained loss of plasmid over time. Therefore strategies to help the plasmid persist for even longer times, to allow fewer re-administrations are being explored. One such approach is targeting the plasmid for integration into the host mammalian genome by including the sequence for a co-expressed integrase protein within the plasmid along with an integrase recognition signal. This technology was applied to mdx mice and it was shown that the levels of dystrophin expression increased with time.[12]

The safety and efficacy of plasmids in humans was investigated in a Phase I clinical trial of 9 boys with either DMD or BMD. Either one or two injections (two weeks apart) of a plasmid containing the whole dystrophin cDNA were delivered to a single radialis muscle in each patient. Biopsies were taken 3 weeks after the first injection and dystrophin expression was detected in six out of the nine patients.[13] Dystrophin expression was completely around the sarcolemma in up to 6% of myofibres and partial in 26%.[13] Most importantly no adverse affects were seen and no cellular or humoral responses against dystrophin were detected.[13]

The benefit of plasmid vectors over viruses is the ability to perform repeated administrations in a safe manner, without the need for immunosuppression, which raises the possibility of treating more muscle fibres. The clinical trials performed to date are promising, especially if the uptake of the plasmids can be improved in the future, as well as their longevity once within a cell. Delivery of plasmids containing the full length cDNAs of most of the genes coding for sarcomeric protein diseases is theoretically possible, except for perhaps nebulin and titin, due to their size (see Table 1). Therefore for recessive disease caused by an absence of any functional protein, plasmid delivery of normal copies of this gene should be helpful. However, there remain problems with efficiency and the benefit of introducing more wild-type copies of a gene in dominant disease remains to be determined.

Viruses

The dystophin cDNA is too large for many viral vectors, so many internally deleted cDNAs based on either those identified in mild BMD patients or those that were artificially created and tested in vivo in the mdx mouse have been utilised e.g., the microdystrophin gene has reduced central spectrin repeats but intact amino and carboxy terminal domains. Gutted adenoviral vectors, which have large capacity and can encompass the full length dystrophin cDNA became popular, until the unexpected sudden death of a patient after adenoviral delivery of ornithine carbamylase,

possibly from toxic shock syndrome.[14] After this, the adeno-associated virus (AAV), a member of the Parvoviridae family and not associated with human disease, became the vector of choice for future testing. AAV cannot replicate by itself, but instead it requires a second "helper" virus to be co-infected along with it for replication to occur. AAV can however only accommodate 4.7 kb of inserted DNA from the gene of interest.

AAV has been the most important virus for potential therapeutic use for skeletal muscle diseases as it has been shown to effectively transduce both skeletal and cardiac muscle of different ages after intravenous injection (e.g., AAV2 and AAV6). Vascular delivery is possible with certain AAV serotypes, such as AAV6 and AAV8, as they have the capability to cross the vascular endothelial barrier, thus negating the need for permeability enhancing agents (such as histamine or vascular endothelial growth factor, VEGF) or other methods such as high infusion pressures. AAV can also persist as episomes for long times in the nucleus. AAV1 has been the serotype of choice for studies into the LGMDs, although some studies indicate that any therapeutic benefit is only seen if delivery of the virus is before the onset of pathology.[15] One reason for this could be that increased fibrotic tissue and inflammatory cells might prevent effective transduction by AAV vectors as the early requirement for viral transduction is not as crucial for dystrophies with less fibrosis and inflammatory infiltrate.

Only a mild, yet perhaps significant immune response is mounted after AAV delivery, possibly due to the capsid proteins inducing a humoral immune response. However some humans have already been infected with wild-type AAV, such as AAV2.[16] Wang and colleagues have shown that short term immunosuppression after AAV vector delivery to a dog model leads to longer-term expression of the transgene.[17] As stated above, the presence of previously absent proteins can induce an immune response themselves (e.g., in the mouse[15] and dog[17] when α-sarcoglycan was expressed under a CMV promoter). One avenue to reduce immune responses to AAV is to utilise specific skeletal and cardiac muscle promoters, such as the modified creatine kinase promoter, which can restrict expression to these tissues.

In order for viral delivery to be successfully translated to the clinic, producing large quantities of high titre virus at realistic cost is necessary, as well as the development of methods to use viruses of lower titre. Another limitation of AAV has been the small insert size that it can incorporate. AAV plasmids do not have the capacity to contain the cDNA of many of the larger sarcomeric proteins that have been associated with disease, such as nebulin, titin, plectin and myosin and unlike dystrophin, the studies have not been performed (nor have humans been identified) to determine whether shortened mini-cDNAs for these genes retain significant function of the translated proteins. Recent strategies to overcome the size limitation, such as trans-splicing (where a gene is divided in two and inserted into two separate AAV vectors), have been successful however.[18] Contrastingly, small cDNAs such as those for actin, calpain-3 and desmin (in fact the cDNAs for the majority of the sarcomeric genes so far associated with disease), could currently easily be encompassed by AAV vectors. Therefore, if the progress of AAV delivery for membrane associated proteins continues, it would be warranted to pursue this therapeutic method for the smaller sarcomeric genes which lead to recessive disease in particular, with the potential to treat dominant disease if sufficient dilution of the defective protein by wild-type is effective in ameliorating symptoms. AAV clinical trials are currently being conducted with γ-sarcoglycan for patients with limb girdle muscular dystrophy 2C (phase I/IIa using AAV1; http://www.genethon.fr/index.php?id=49&L=1) and with a minidystrophin for Duchenne muscular dystrophy patients (phase I/IIa using AAV2; http://genetherapy.unc.edu/clinical.htm).

Myoblasts and Stem Cells

Heart disease can be caused by mutations in the parallel set of genes coding for cardiac sarcomeric proteins e.g.[19] These diseases can be treated by heart transplants when they are severe or at end-stage. Theoretically skeletal muscle transplants,[20] could be implemented in a similar fashion, although the thought of attempting to replace a majority of the skeletal muscle present in a person, along with the associated risks, is daunting. Perhaps such surgery, including tendon

transplants, would only ever be applicable for a certain given muscle or a small number of muscles in a particular region of the body, such as in the hand or at the wrist or ankle. It is probably more realistic to focus on the delivery of the building blocks of skeletal muscle rather than transplants of whole skeletal muscle.

Skeletal muscle is comprised of postmitotic nuclei, with the level of normal turnover of these nuclei being very low. The regenerative capacity of skeletal muscle means that the fusion of resident myoblasts (mononucleated muscle cells) or satellite cells (which are stem cell like cells located between the basal lamina and the sarcolemma) leads to the formation of new muscle fibres during the early phases of dystrophic pathology. However the proliferative potential of satellite cells is eventually exhausted and the skeletal muscle becomes replaced by connective tissue. Enticing results were achieved from the initial experiments trialling the transfer of enzymatically dissociated skeletal muscle-derived cells from normal mice into the skeletal muscle of mdx mice.[21] These cells were not grown in culture and would have consisted of a mixture of cell types including myoblasts, muscle-derived stem cells (MDSC), fibroblasts and muscle vessel cells.[22]

Further studies with cultured myoblasts were not as promising, due to immune rejection by the recipient, the limited survival of the myoblasts (at least 75% cell death after transplantation) and a restricted area of dispersal from the injection site.[23] A recent 18 month follow up of an immunosuppressed DMD patient who had received normal donor myoblasts by multiple high density injections (1mm apart or less) showed that 34.5% of myofibers in the region of the injection were positive for dystrophin.[24] This is a promising finding apart from the need for concentrated injections. If the hurdle of overcoming the inability to deliver myoblasts via the circulation could be achieved, then myoblast therapy might become an attractive option. Presently the injection delivery regime could only be utilised to assist in the function of a limited number of vital muscles.

The muscle satellite cells are the major skeletal muscle stem cells that maintain and repair skeletal muscle. Greater success has been achieved with the use of satellite cells that have not been cultured, that is cells removed from the host source and immediately grafted e.g.[25] Upon activation, satellite cells divide to produce what are known as muscle precursor cells (MPC). MPCs in turn proliferate and then commit to differentiating and fuse to become myotubes, themselves capable of becoming myofibers. Each myofiber may have a few satellite cells which can generate greater than one hundred myofibers, as well as replenishing the satellite cell population.[26]

Muscle stem cells have been shown to proliferate longer than myoblast cells and additionally they are self-renewing and immune-privileged. After intra-arterial injection, muscle stem cells have been shown to have widespread dispersal, migrating from the circulatory system into muscle.[27] The downside of intravenous injection is that stem cells can become confined in other organs before reaching the skeletal muscles. Another form of muscle-derived stem cells are both CD34 and Sca 1 positive, located in the interstitial space and can differentiate into both muscle and myeloid cells.[28] Nonmuscle-derived myogenic precursors which have been studied include embryonic stem cells, bone marrow derived myogenic precursors, pericytes, CD133+ progenitor cells and mesoangioblasts.

Mesoangioblasts are blood vessel wall-associated stem or progenitor cells. Mesangioblasts have been successfully delivered systemically via intra-arterial injection to both dystrophic mice[29] and dogs[30] and moreover an improvement in the structure and the function of the muscle was subsequently reported (up to 70% of the fibres being dystrophin positive.[30]) Although some questions have arisen about the methodology of the experimental design, e.g.,[31] similar findings have been achieved with the mdx/utrophin$^{(-/-)}$ mouse.[32] Human adult skeletal muscle has been utilized to isolate mesoangioblasts which were shown to contain a subpopulation of pericytes that can in turn be cultured and expanded in vitro and have the potential to differentiate into multinucleated skeletal muscle cells.[33]

Myoblasts and stem cells may be more beneficial for the dystrophic sarcomeric protein diseases than the nondystrophic diseases, as the degeneration of muscle fibers allows for more efficient uptake of the introduced cells. It is possible that the level of survival of muscle or stem cells after introduction into a patient, or an animal model of nondystrophic sarcomeric diseases would be

negligible, unless the muscle is first damaged. If the delivery of muscle and/or stem cells is optimised for the treatment of muscular dystrophies then it might be valid to investigate a damage protocol for those diseases where the muscle fibers do not normally die, in order to facilitate the subsequent repopulation of normal muscle fibers. It may however be difficult to convince a parent of a child with a nondystrophic sarcomeric disease to give permission for their child's muscle to be damaged.

Aminoglycoside Antibiotics

It had been previously observed that the use of aminoglycoside antibiotics (e.g., gentamycin, negamycin) could promote translational read-through of stop codons. As premature stop codons in the dystrophin gene account for 5-15% of DMD cases, this therapeutic avenue was aggressively pursued. Hopeful outcomes were shown in mdx mice, but trials with DMD and BMD patients did not result in any dystrophin expression.[34] These outcomes were partly attributed to batch variation in the production of the aminoglycosides. In the hope of developing a drug that would not have the side effects of aminoglycosides (e.g., ototoxicity and nephrotoxicity) and which might have greater potency, a high throughput screen was performed by PTC Therapeutics for other compounds that would also promote read-through of nonsense mutations and the resulting lead drug was named PTC124. Dystrophin levels up to 20 to 25% of control mice were achieved in mdx mice after PTC124 delivery.[35] Two separate phase I trials were conducted and showed that PTC124 levels that were efficacious in mice did not produce any deleterious outcomes in humans, although some mild side-effects at the highest dose were experienced. A phase IIa trial in DMD boys has recently been undertaken in the United States of America (http://clinicaltrials.gov/ct/show/NCT00264888?order=3). Concerns should lie as to which other genes are also altered by the read-through of stop codons and the long-term effects of delivering such drugs to patients.

Theoretically PTC124 could be applied to any of the sarcomeric protein diseases caused by nonsense mutations. For example, nemaline myopathy caused by the recessive nonsense mutation of troponin-T, which causes the nemaline myopathy peculiar to the Old Order Amish population.[36] Testing this theory in an animal model would be desirable but not currently possible as no model is presently available. Muscle cells cultured from affected patients could be treated and analysed to determine whether read-through of the troponin-T nonsense mutation occurs. If the clinical trials of PTC124 continue to show few deleterious effects, then perhaps patients with the Amish nemaline myopathy could be given PTC124 in the hope of providing functional improvement for what is currently a lethal condition within 2 years of birth. Some patients have nonsense mutations in other sarcomeric protein genes, such as actin and tropomyosin and although these only make up a small percentage of the mutations in these genes, it would be worthwhile testing PTC124 for these patients. However whilst the dystrophin protein levels produced from treatment by PTC124 are in the order of 20 to 25% of normal levels and expressing dystrophin at approximately 20% of endogenous levels in mouse models has been shown to be sufficient to prevent most dystrophic clinical symptoms[37] providing read-through of one of the sarcomeric proteins to this level may not prove therapeutic.

Precise Correction of the Mutation

DNA fragments containing the wild-type sequence have been trialled for correcting a precise mutation in the dystrophin gene. Single-stranded short-fragment homologous replacement (ssSFHR) using a 603 bp PCR product was shown to repair the genetic mutations in 15-20% of mdx myoblasts in vitro.[38] However despite surviving for up to three weeks after intramuscular injection, the transfected cells did not express any full-length dystrophin. Unfortunately this method has not as yet been improved upon.

Inducing a single base alteration has been performed through the use of oligonucleotides such as chimeraplasts (double-stranded RNA-DNA chimeric oligonucleotides) and oligodeoxynucleotides and has been demonstrated at the genomic, mRNA and protein levels both in vitro and in vivo.[39] One benefit of these methods is that they have the potential to correct the mutation permanently

and precisely. Limitations include converted cells being restricted to the area surrounding the injection site of the oligonucleotides, a relatively low number of cells being repaired and an unknown toxicity of the oligonucleotides to humans. Progress is being made however on scaling up the production of the synthetic oligonucleotides.[39]

Due to the current inefficiencies in these methods they are not the most promising strategies for therapeutic approaches. If delivery difficulties could be overcome however, the founder troponin-T mutation in the Amish would again be one disease in which to investigate these methods since all affected individuals have the same mutation. Using one of these methods to correct mutations for a gene such as actin for which there over one hundred different mutations are known would be daunting.

Antisense Oligonucleotides

Protein expression can be altered through hybridisation of antisense oligonucleotides (AOs) to target mRNA sequences such as exon/intron boundaries, translation inhibition codons and sequences downstream of the initiation codon. Approximately 65% of patients with DMD have out of frame deletions whilst about 10% have duplications and hence many researchers have tried to harness antisense oligonucleotides to induced exon skipping or to redirect splicing in order to produce a functional dystrophin protein (albeit less functional than the wild-type dystrophin protein).

Antisense oligonucleotides were first investigated as a genetic therapy of DMD due to the identification of revertant fibres, which are rare fibres that express dystrophin protein through skipping of the mutation-containing exon.[40] Successful antisense oligonucleotide induced skipping of exons, leading to restoration of the dystrophin reading frame, has been demonstrated in cultured mdx myotubes,[41] mdx mice,[42] cultured muscle cells derived from the dystrophic dog model,[43] DMD patients[44] and human muscle explants.[45] These promising findings led to studies of double- and multi-exon skipping, with researchers trying to develop strategies that could be utilised for a large percentage of patients, such as skipping exons 45 through to 55, which could theoretically be beneficial for 63% of DMD patients.[46] Research has moved from administering AO to mouse and human cells in culture through to successful direct injection of AO (either intravascularly or intraperitoneally) into whole mice, with muscle function improvement being demonstrated. Antisense oligonucleotides have now been designed to skip over each of the human dystrophin exons in vitro, except for the first and the last exons.[47]

The delivery of AO would have to be regularly repeated to patients as they are not permanent. The treatment only lasts for the half-life of the induced proteins (plus how long the AO last for, which depends on the chemistry used). The safety and efficacy of intramuscular delivery of AO to humans patients is being tested by two separate groups, one in The Netherlands[48] (http://prosensa.eu/Pdf/NEJM%2027DEC07.pdf) and the other in the UK (http://clinicaltrials.gov/ct/gui/show/NCT00159250?order=1). The results from the clinical trial conducted in The Netherlands with the antisense olignucleotide known as PROO51 have only recently been released. Twenty-eight days after four patients were injected with PROO51 into their tibialis anterior muscles, biopsies were taken. All patients showed no adverse reactions to the injections and additionally expression levels of dystrophin in the range from 3 to 12% of controls (normalised to 17 to 35% when compared to the amount of laminin-α2 present).[49] As dystrophin expression was limited to the treated area, no functional improvement in muscle strength was observed, but gives some reason for optimism that this may occur in future trails using systemic delivery.

Antisense oligonucleotides could be applied to those sarcomeric genes in which skipping an exon containing a mutation would not in itself lead to disease. It is difficult to imagine that removing an exon from small genes such as actin and cofilin-2 would be possible whilst still retaining the function of these proteins, as the deleted exon would account for a large proportion of the overall protein (see Table 1). It might be possible to apply antisense oligonucleotides to the larger genes such as β-cardiac myosin, nebulin, plectin and titin, provided the exon to be skipped does not code for an essential portion of the normal protein. Antisense oligonucleotides may also have the

potential to be applied to alternatively spliced genes, such as tropomyosin or *ZASP*, to enhance the inclusion of an alternate exon, rather than the one containing a mutation, again so long as the mutation was not in an essential exon.

Proteasome Inhibitors

Proteasome inhibitors have been investigated as a therapy for DMD on the basis that if the defective dystrophin protein can be prevented from being degraded, then it may provide enough function to lessen the severity of the disease. The systemic use of a proteosome inhibitor, MG-132, to block the proteosomal-dependent degradation pathway of dystrophin and in turn the dystrophin associated proteins, was explored in the mdx mouse. Delivery of MG-132 was found to be effective in decreasing the amount of damage to the muscle membrane and increasing muscle integrity.[50] The dystrophin protein present after treatment was found to be missing the carboxy-terminal domain due to the presence of the nonsense mutation in exon 23 and hence was a truncated form. However, restoration of the dystrophin associated proteins still occurred. These researchers then utilised skeletal muscle explants from both Duchenne and Becker patients and treated them with MG-132 in culture for 24 hrs and showed that this compound restored dystrophin and its associated proteins in this system.[51] More recently localised treatment of the gastrocnemius muscle in mdx mice using two dipeptide boronic acid inhibitors (Velcade and MLN273), which are known to block the proteasomal-dependent degradation pathway, led to restoration of dystrophin and its associated proteins but moreover reduced the activation of nuclear factor-κB, a protein implicated in the inflammation response.[52] Velcade has already been approved for human use, albeit for the treatment of multiple myeloma.

The recessive LGMDs caused by defective/absent sarcomeric proteins may benefit from the blocking of the proteosomal-dependent degradation pathway as they too can lead to reduced levels of their protein binding partners. However most of the nondystrophic sarcomeric protein diseases do not exhibit a reduction or absence of protein, instead, as stated, they aggregate proteins. Therefore it is probably unlikely that by blocking the proteosomal-dependent degradation pathway, even if it is the pathway being used for degradation by the sarcomeric proteins, any benefit whatsoever would be provided.

Upregulation Therapy

Increasing or reactivating the expression of an alternative gene to replace a defective gene's function is known as upregulation therapy. One of the benefits of this approach is that no immune response should be mounted against a protein previously seen by the body. In a variety of gene families there exists gene redundancy, including transitions from fetal to adult/mature isoforms during development. Whilst the developing or immature tissue might be protected from a disease due to the expression of the immature isoform, disease results when the switch occurs to the mature isoform. Gene redundancy provides a potential therapeutic strategy for such diseases through the pharmacologically-induced re-expression or upregulation of functionally related genes.

The original focus for upregulation therapy for DMD was the autosomal paralogue called utrophin.[53,54] Utrophin is the fetal form of dystrophin and shows great similarity to dystrophin at both the gene sequence and protein structure level and therefore shares most of the same protein binding partners as dystrophin in skeletal muscle. Utrophin is localised to the sarcolemma during skeletal muscle development but after reaching maximum levels at around 17 to 18 weeks, expression declines and instead dystrophin replaces utrophin at the sarcolemma.[55] Utrophin becomes confined to the neuromuscular and myotendinous junctions in normal, mature skeletal muscle.

Transgenic over-expression of a truncated utrophin in mdx mice lead to utrophin localisation at the sarcolemma, prevented the onset of dystrophic pathology usually seen in the absence of dystrophin and additionally led to a functional improvement.[56] Expression of full-length utrophin also rescued the disease phenotype in mdx mice and restored the DAPC by creating a link between this complex and the actin filaments at the costamere.[57] These experiments proved the proposed theory that the dystrophic process caused by an absence of dystrophin could be prevented if the

normal levels of utrophin expressed in postnatal skeletal muscle could be increased. It was also determined that prevention of the dystrophic process would occur if the levels of utrophin were increased after birth rather than before birth,[58] which would be highly desirable for translating such a treatment to DMD patients.

It is theoretically possible to increase the levels of utrophin by two different approaches, either by delivering additional utrophin genes via for example viruses or plasmids or by using a drug to increase native utrophin. Although the utrophin cDNA sequence is shorter than that for dystrophin, it is still large and as such provides hurdles for viral delivery. However viral delivery has been used successfully to improve the phenotype in mice[59] and dogs.[60] In order to identify small and diffusible compounds/drugs that upregulate utrophin, which would allow for bodywide delivery, two approaches have been investigated. One was to conduct detailed analysis of utrophin promoters with the prospect of subsequently being able to design drugs to enhance utrophin levels (reviewed in ref. 53). The other method has been to screen libraries of molecules in a high throughput manner by using a reporter assay to determine whether any drug increases utrophin expression and to subsequently determine the mechanism by which this occurs (as outlined in ref. 54). The company Summit PLC (http://www.summitplc.com) has been screening for compounds which upregulate utrophin and has performed preliminary safety and toxicity tests in zebrafish for a lead compound SMT C1100. The company is aiming to perform Phase I clinical trails with SMT C1100 in the second half of 2008.

Some of the sarcomeric proteins which when defective lead to disease, do belong to gene families that either have isoforms that are expressed during development and/or are expressed in cardiac muscle or other muscle fibre types (Table 1). It may be possible to harness one of these other isoforms to compensate for an absent protein, e.g., utilise another tropomyosin isoform to compensate for recessive nemaline myopathy caused by mutations in slow α-tropomyosin in a similar fashion to utrophin compensating for dystrophin (see Table 1 for other examples of genes with possible alternate isoforms). Patients with no skeletal muscle actin caused by recessive null mutations in *ACTA1* are severely affected at birth and all retain expression of cardiac actin,[61] the fetal isoform in skeletal muscle, which is normally switched off around birth in humans.[62] Those patients with the best clinical outcome express the most cardiac actin in their skeletal muscles, suggesting that if the expression levels of cardiac actin might be elevated further, then the disease phenotype could be lessened even more.[63]

Manipulating Pathways Altered in Muscle Disease Pathobiology

Increasing the expression of other genes, not homologues of dystrophin, has also been shown to be effective in decreasing the effect of dystrophin absence in either cultured myotubes or mdx mice. These include a disintegrin and metalloprotease *ADAM12*,[64] α7β1 integrin,[65] nitric oxide synthetase (*NOS*),[66] insulin-like growth factor I (*IGF-1*),[67] synaptic CT GalNAc transferase (Galgt2),[68] calpastatin[69] and L-arginine, which is a NOS substrate.[70] Alternatively, rather than increasing the expression levels of a compensatory protein, blocking other proteins by using specific antibodies or inhibitors has been shown to be efficacious, namely blocking tumour necrosis factor-α,[71] myostatin[72] or nuclear factor κB.[73] These approaches prove to be effective despite them not necessarily providing a replacement for the absent dystrophin protein, but instead by acting on pathways altered during the dystrophic processes, such as necrosis, fibrosis, regeneration, adhesion and cell stability.

Will manipulating the processes involved with dystrophy assist in the amelioration of clinical symptoms of the sarcomeric protein diseases? For those defective/absent sarcomeric proteins that lead to LGMD then the chances are likely, as they too have the hallmark dystrophic features of degeneration, regeneration, inflammation etc. For the nondystrophic diseases caused by mutant/defective sarcomeric proteins, it is probably unlikely that molecules that reduce inflammation for example would have any benefit.

As indicated above with the dystrophies, the myostatin and IGF-1 signalling pathways (which are involved in controlling skeletal muscle growth) can be manipulated to assist suppression of

the dystrophic processes by making skeletal myofibers larger. In the nondystrophic sarcomeric protein diseases, the muscle fibers do not usually die but instead accumulate aggregates of protein. Therefore increasing the size of the muscle fibers would not seem to have any obvious benefit e.g., if the disease is caused by aggregates in a normal sized myofiber then the disease might just as likely be caused by a larger sized myofiber with aggregates, unless the proportion of aggregates to normal sarcomeres per myofiber could be decreased. However, two lines of evidence suggest otherwise. Firstly the data described in the Chapter by Dr. Edna Hardeman indicate that by crossing mutant skeletal actin knockin mice (which are a model of nemaline myopathy) with transgenic mice that overexpress IGF-1, the severity of the disease decreases. Secondly, a patient with nemaline myopathy caused by a skeletal muscle actin mutation was reported to be a competitive long-distance cyclist even though he had nemaline rods in 98% of his fibers, (patient 4, ref. 74). This man had a slow fiber (type I) predominance and approximately 90% of his myofibers were hypertrophic. Therefore, although the vast majority of his muscle fibers contained nemaline rods, presumably his disciplined exercise training, which increased his muscle fiber size, was assisting in keeping his muscle strength function good. It remains to be empirically determined through the use of animal models of other nondystrophic sarcomeric protein diseases whether manipulating the muscle fiber size can assist in decreasing the pathology associated with the disease.

The therapeutic use of dietary L-tyrosine supplementation for nemaline myopathy has been indicated through observations by patients, parents of patients and clinicians[5] although in-depth animal or human studies have not as yet been reported. After L-tyrosine supplementation patients are reported to have improvement in bulbar function and energy levels and in the case of an adolescent, an increase in strength and exercise tolerance.[5] Nemaline myopathy patients do not manifest a deficiency in L-tyrosine and it is not known how L-tyrosine may ameliorate the clinical features. It is hypothesised however that a possible mechanism could be increased peripheral catecholamine, leading to vasoconstriction and a subsequent decrease in the amount of saliva produced.[5]

Exercise

There is controversy as to whether it is better for patients with muscular dystrophy to avoid exercise in the hope of reducing damage to muscle fibers and subsequent degeneration, due to the limited capacity of muscle satellite cells to regenerate. However, there is understanding that exercise might be beneficial to some patients with skeletal muscle diseases, depending on the underlying biological cause. Many authors recommend that a comprehensive study needs to be conducted with various groups of patients with skeletal muscle diseases and various types of exercise, e.g., low impact versus high impact, in order to determine whether any levels of exercise may in fact be beneficial. For example aerobic cycling training for thirty minute intervals has been shown to improve work capacity of patients with LGMD2I[75] however this cannot be directly translated to patients with other forms of LGMD or indeed other skeletal muscle diseases.

No study has been performed for the effects of exercise on patients who have sarcomeric protein diseases. The evidence from patient 4,[74] the competitive long-distance cyclist already mentioned, suggests that some forms of exercise can be beneficial for certain patients. This patient remaining physically active at a high level may suggest that regular, low-impact exercise might be beneficial for patients with nondystrophic sarcomeric protein skeletal muscle disease. Additionally, studies with a mouse model of one of the sarcomeric protein diseases (nemaline myopathy caused by an α-tropomyosin-3, M9R mutation), showed that 4 weeks of treadmill exercise did not lead to muscle damage, nor did it increase the amount of nemaline bodies present.[76] Furthermore, muscle weakness in this mouse model induced by disuse could be alleviated by endurance exercise.[6] These investigations suggest that exercise is probably not detrimental for human patients with nemaline myopathy and may in fact contribute to muscle strength improvement.

RNA Interference

RNA interference occurs in all living cells and is a biological process that involves the control of gene silencing. Downregulation of mRNA levels can occur by cellular nucleases, activated through

the detection of sequence homology between the mRNA molecule of interest and a respective small interfering RNA or siRNA.[77] When a particular gene is known to be pathogenic, then RNA silencing can theoretically be used to silence this gene. With the progression of predictive software for the design of siRNAs,[78] researchers are investigating RNA interference as a therapeutic approach for a wide variety of diseases, including neurodegenerative diseases such as Alzheimer's disease,[79] motor neurone disease, the spinocerebellar ataxias and Huntington's disease.[80] A variety of mouse models have been utilised for these studies, with mixed results.[81]

Although delivery of the siRNA molecules has been a hurdle,[77] some success has occurred with delivery using lentiviral vectors which express the siRNA e.g.,[82] delivery via hydrodynamic tail vein delivery[83] and by harnessing synthetic vehicles.[84] One aspect to consider is that of off-target effects, in other words the effect of knocking down a gene other than the target gene.

Allele specific silencing in vivo by siRNAs can occur, as demonstrated for example by specific knockdown of only the mutant allele of α-synuclein[7] and superoxide dismutase[8] in mouse models of Parkinson's disease and motor neurone disease. These data indicate that the use of siRNAs could be amenable to sarcomeric protein diseases that are caused by dominant mutations. However not all regions within a target gene are amenable to efficient knockdown by siRNAs[78] with the efficiency of RNA interference determined by the local RNA structure of the region where the siRNA is to bind.[85] Therefore not all dominant mutations within a given gene can be successfully targeted by siRNAs due to the structure of the RNA at that location. Fortunately computer programs are able to largely predict the likelihood of efficient binding, meaning that failure in silico greatly prevents failure in vitro and saves much time and expense. For those defective genes leading to sarcomeric protein disease that have multiple identified mutations, it is probably not feasible to design a panel of different siRNAs to cover the majority of mutations, particularly if only one or a few patients have this mutation. Moreover it is probable that a certain percentage of these mutations will not be amenable to binding by a siRNA. Where single mutations affect a significant number of patients, computer modelling followed by empirical experimentation in vitro may be warranted.

Conclusions and Future Applications

Despite it being twenty years since the Duchenne muscular dystrophy gene was discovered with no successful treatment based on this knowledge clinically available, the current era is the great age of possible effective therapies for skeletal muscle diseases. There are exciting advances in many approaches and some are now in clinical trials. Our expectations are almost certainly aimed too high if we believe that any single therapeutic approach will be an ultimate panacea. Even an increase in muscle function/strength of say 10% may be significant to patients and their families. Many of the approaches currently under investigation attack the problem from different directions. This raises the possibility for combinatorial therapies, with each contributing an improvement which might be additive. Already the literature is scattered with aspects from various technologies being combined, such as antisense sequences being expressed by an AAV vector,[86] autologous muscle precursor cells being transfected with a plasmid prior to delivery,[87] or transduced with a lentivirus[88] and plasmids expressing a microdystrophin fused to a C'terminal version of the herpes simplex virus protein to enhance spread from the transfected cell.[89]

The fundamental problem with treating skeletal muscle disease remains that skeletal muscle makes up as much as 40% of a person's entire body mass and thus the devil is in the delivery of any of the currently experimental treatments. Pharmaceutical-based therapies, including antisense oligonucleotides, should be able to easily reach all skeletal muscles and in some cases the heart, whilst other experimental approaches such as myoblast therapies have greater problems of access. Ultimately the severity or mildness of the disease in a particular patient will dictate the riskiness of the proposed therapy that would be ethical. The progress, effort and investment in therapies for DMD and other muscular dystrophies should make it much easier and faster to develop treatments for the later-discovered sarcomeric protein diseases, which in contrast have had considerably less attention focused on them. If safe and efficient AAV delivery into human skeletal muscle is achieved for example, then it theoretically should be relatively straightforward for many other

skeletal muscle proteins, including the sarcomeric proteins of small to average size, to be delivered in a similar fashion. However, as discussed throughout this chapter, consideration needs to be taken when translating approaches used for recessive disease to diseases caused by dominant mutations. Therefore developing appropriate cell culture and animal models of sarcomeric protein diseases to enable testing of various therapeutic approaches shown to be effective for the muscular dystrophies is highly desirable. This should facilitate patients with sarcomeric protein diseases being able to benefit from muscular dystrophy breakthroughs as quickly as possible.

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