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Cellular studies of neuromuscular disorders related to the sarcomeric proteins

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There is no greater wealth than wisdom, no greater poverty than ignorance, no greater heritage than culture, and no greater friend and helpmate than consultation.

Imam Ali, the first of the twelve rightful successors of the holy prophet Muhammad (peace and blessings be upon him and his family)

To Prophet Muhammad
Mercy for all creation
(Peace and blessings be upon him and his family)

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ABSTRACT

Sarcomere is the basic unit of cardiac and skeletal muscle contraction and its proper function requires an invariant organization of this structure. Mutations in sarcomeric proteins are known to cause increasing number of different cardiac and skeletal muscle diseases. The front line in research on muscle diseases is at present to define the genetic background and pathogenesis of these diseases. The potential for development of effective therapies depends on elucidation of the molecular and cellular impact of the mutations on morphological abnormalities and muscle weakness that accompany pathogenesis.

In paper I we identified an unexpected skeletal muscle myopathy in an infant with fatal cardiomyopathy due to a homozygous mutation in *MyBPC3*. The ectopic expression of cardiac MyBPC was restricted to abnormal type 1 muscle fibres, indicating that the muscle pathology was caused by a dominate-negative effect of mutant *MyBPC3*.

In paper II we addressed the expression profile of a panel of sarcomeric components during myogenesis, with a focus on proteins associated with a group of congenital disorders. The analyses were performed in cultured human skeletal muscle myoblasts and myotubes. We identified early expression of certain isoforms involved in congenital diseases, suggesting the possibility of an early role for these proteins as constituent of the developing contractile apparatus during myofibrillogenesis.

In paper III we used human tissue-culture cells as a model to investigate the primary trigger for β-tropomyosin-related myopathies and the basis for the histological changes seen in muscle biopsies of patients. Protein localization and pathobiology caused by dominant *TPM2* mutations were investigated by transfecting human myoblasts and C2C12 with WT and mutant EGFP-fusion β-TM constructs. Abnormal aggregation of β-TM variants and their localization within the thin filaments was observed in myoblasts and differentiated myotubes. We demonstrated that histopathological phenotypes associated with β-TM mutants might be accounted for the variable response to the cellular environment influenced by physiological context, in combination with the time course of expression of mutant protein rather than the alteration of amino acid itself. Our results confirmed that cell cultures of human skeletal muscle are an appropriate tool and environment closer to the reality in human skeletal muscle and more reliably mimic the disease conditions.

In paper IV we identified and characterized a new human protein aggregate myopathy and cardiomyopathy associated with combined mutations in isogenes *TRIM63* and *TRIM54*, encoding muscle specific ring finger proteins, MuRF1 and MuRF3, respectively. Our morphological and cellular investigation suggested that the disease is caused through impaired organization of the microtubule network and sarcomeric M-band proteins. The results from this study have deepened the understanding of pathogenesis of a group of sarcomeric myopathies, which is an essential step towards identifying new therapeutic targets.

Keywords: Myogenesis, myoblast, sarcomeric myopathy, *TPM2*, *TRIM54*, *TRIM63*

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Homa Tajsharghi, Trond P Leren, **Saba Abdul-Hussein**, Mar Tulinius, Leif Brunvand, Hilde M Dahl, Anders Oldfors. Unexpected myopathy associated with a mutation in *MYBPC3* and misplacement of the cardiac myosin binding protein C. J Med Genet 2010;47:575-577

II. **Saba Abdul-Hussein**, Peter F.M. van der Ven, Homa Tajsharghi. Expression profiles of muscle disease-associated genes and their isoforms during differentiation of cultured human skeletal muscle cells. Submitted

III. **Saba Abdul-Hussein**, Karin Rahl, Ali-Reza Moslemi, Homa Tajsharghi. Phenotypes of myopathy-related beta-tropomyosin mutants in human and mouse tissue cultures. Submitted

IV. Montse Olivé, **Saba Abdul-Hussein**, Anders Oldfors, Dieter O. Fürst, Peter F. M. van der Ven, José Gonzalez-Costello, Laura Gonzalez-Mera, Benjamin Torrejón-Escribano, Josefina Alió, Adolf Pou, Isidro Ferrer, Homa Tajsharghi. MuRF1 and MuRF3 mutations cause a new protein aggregate myopathy and cardiomyopathy. Manuscript

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ABBREVIATIONS

ACTA1	Alpha-skeletal actin
ACTC	Alpha-cardiac actin
AFM	Association Francaise contre les myopathies
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphoshate
bHLH	Basic helix loop helix
C2C12	Mouse myoblast cell line
cDNA	Complementary DNA
CK	Creatine kinase
DA	Distal arthrogryposis
DAPI	4',6-diamidino-2-phenylindole
DES	Desmin
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EGFP	Enhanced green fluorescent protein
EM	Electron microscopy
FACS	Fluorescence-activated cell sorting
F-actin	Filamentous actin
FHL1	Four and a half LIM domains 1
Fn	Fibronectin
G-actin	Globular actin
Ig	Immunoglobulin
IL-6	Interleukin-6
L6, L8	Rat skeletal muscle cell line
MCAM	Melanoma cell adhesion molecule
MM14	Murine myoblast cells
MyLC	Myosin light chain

MRFs	Myogenic regulatory transcription factors
MRF4	Myogenic regulatory factor 4
MuRF1	Muscle specific RING finger protein 1
MuRF2	Muscle specific RING finger protein 2
MuRF3	Muscle specific RING finger protein 3
MyBPC1	Slow myosin binding protein C
MyBPC2	Fast myosin binding protein C
MyBPC3	Cardiac myosin binding protein C
MyHC	Myosin heavy chain
MYH1	Adult fast skeletal muscle myosin heavy chain IIx
MYH2	Adult fast skeletal muscle myosin heavy chain IIa
MYH3	Embryonic myosin heavy chain
MYH4	Adult fast skeletal muscle myosin heavy chain IIb
MYH7	Slow/beta-cardiac myosin heavy chain
MYH8	Prenatal myosin heavy chain
NCAM	Neural cell adhesion molecule
NM	Nemaline myopathy
NADH	Nicotineamid adenine dinucleotide hydrogenase
Pax	Paired-box transcription factors
PAS	Periodic acid and Schiff's reagent
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
TPM1	Alpha-Tropomyosin
TPM2	Beta-Tropomyosin
TPM3	Gamma-Tropomyosin
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
TRIM63	Tripartite motif containing 63

TRIM54	Tripartite motif containing 54
TTN	Titin
TNNI1	Slow troponin I
TNNI2	Fast troponin I
TNNI3	Cardiac troponin I
TNNT1	Slow troponin T
TNNT2	Cardiac troponin T
TNNT3	Fast troponin T
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
V-CAM-1	Vascular cell adhesion molecule 1

1 INTRODUCTION

1.1 Skeletal muscle satellite cells

Satellite cells or muscle stem cells derive from the central area of dermomyotome located at the dorsal section of the somites of the paraxial mesoderm [1] . The cells have increased size of nuclear with little cytoplasmic organelle content [2] localized between the basal lamina and sarcolemma of muscle fibres periphery [3] figure (1). Satellite cells are heterogeneous group contain a mixture of stem cells capable of self-renew and committed progenitors responsible for postnatal growth, regeneration and homeostasis repair [4, 5] [3, 6]. The percentage of stem cells is high in newborns but decreases in number with age due to loss of muscle mass "sarcopenia" or muscle wasting caused by muscular dystrophies [1].

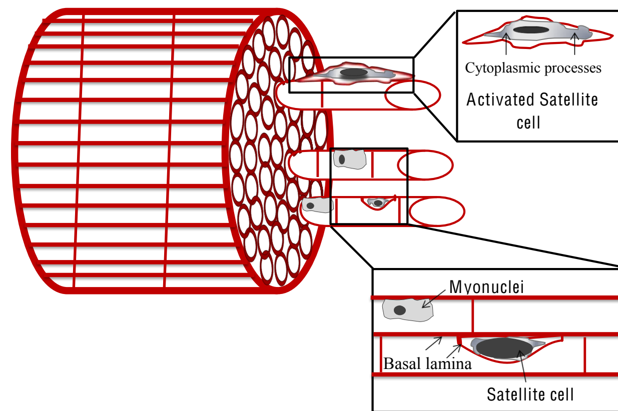

Figure 1.Schematic diagram of an activated satellite cell in regenerating myofibre and a quiescent satellite cell in normal myofibre.

The satellite cells commence from the quiescent stage due to trigger by trauma, such as injure, degenerative disease or weight bearing [3]. The stimulation of myogenic precursors is restrained by proximal signals from muscle niche, microvasculature and inflammatory cells [7]. The activated satellite cells move outside basal lamina and start to cycle, proliferate, differentiate and fuse with each other or to pre-existing myofibres [3, 8]. The process can be summaries as follow, stem cells (fate not acquired) → progenitor (fate acquired) → myoblasts (fate acquired, myogenic genes activated) → differentiation → myofibres [9].

1.1.1 Muscle regeneration

The regeneration is a multiple wave procedure similar to embryonic muscle development. The process begins with embryonic wave initiation, followed by foetal wave and postnatal wave ending by the growth of musculature to adult size [10]. However, there are few differences between the embryonic muscle development and muscle regeneration, for instance cell niche, anatomy and involvement of regulatory genes [9]. The niche of satellite cells regulates the function of the cells during muscle regeneration through autocrine, paracrine and endocrine mechanisms [4].

The disturbed myofibre undertakes either total autolysis or local in a process that can be divided into two phases, degeneration and regeneration phase [2]. The degeneration phase begins by the premature death of cells in muscle fibres (necrosis) with disordered sarcolemma leading to an increase permeability that causes escalating level of a muscle protein commonly located to the myofibre cytosol, namely creatine kinase (CK). Furthermore, the disrupted sarcolemmal or sarcoplasmic reticulum triggers an influx of Ca^{2+}

causing a loss of Ca^{2+} homeostasis, leading to the activation of Calpains. The Calpains are calcium dependent proteolysis that cleaves myofibrillar and cytoskeletal proteins [2].

The regeneration phase is a highly orchestrated process ensuring that specific genes are regulated in a sequential organized manner. The mechanism involves cell-cell and cell matrix interactions as well as extracellular secreted factors including, trophic factors members of EGF family, interleukin-6 (IL-6) family of cytokines and nitric oxide [2]. The process of regeneration comprising, activation of satellite cells, proliferation, differentiation, up regulation of muscle transcription factors and muscle specific genes (figure 2). Additionally, the muscle repair process involves contribution of non-muscle so called multipotent cells that include stem cells derived from bone marrow, vessel-associated stem cells, neuronal compartment and mesenchyme tissues [2, 10].

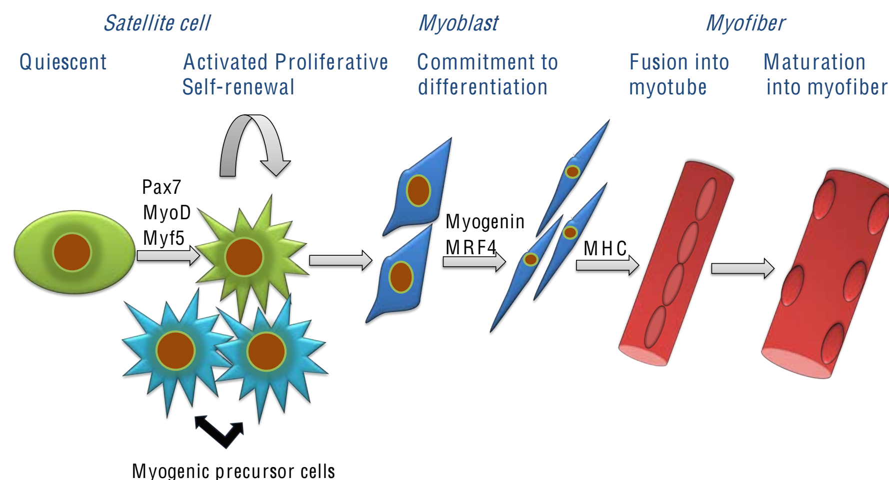

Figure 2. A model of the main events of regeneration in skeletal muscle.

There are two kinds of transcription factors networks controlling muscle development, the paired-box transcription factors and myogenic regulatory transcription factors [10]. The paired-box (Pax) transcription factors are family of proteins working upstream of myogenic regulatory transcription factors and are characterized by the presence of a paired domain. They are important during development through the regulation of cell specification and organogenesis [10]. In several Pax proteins including Pax3 and Pax7, contain a second highly conserved motif, the homeodomain [11]. Pax3 expressed during embryonic development while Pax7 expressed in postnatal development in the resident progenitors population [3].

The myogenic regulatory transcription factors (MRFs) are a superfamily of basic helix loop helix (bHLH) transcriptional regulators with over 400 members that are activated during developmental processes including myogenesis and neurogenesis [11]. Each member of this family has a distinct role [11]. For example, the activation of *MYF5* promotes satellite cells self-renewal, regulates proliferation rate and homeostasis [2], while the activation of *MYOD* promotes differentiation [2]. The expression of *MYOG* and *MRF4* regulates the terminal differentiation program [2]. *MRF4* plays a role in determining fibre phenotype in postnatal life, particularly in maintaining slow phenotype [12].

1.1.2 Myofibrillogenesis

The myofibrillogenesis procedure requires the assembly of structural and regulatory molecules into the sarcomeric contractile units. This involves the function of many different proteins together to convert the molecular interaction of actin and myosin into the mechanical force and movement. The myofibrillogenesis process involves huge alterations of myogenic cells (myoblasts) cytoarchitecture and protein expression. The myoblasts start migration, elongation, extensive cytoskeletal reorganization;

membrane alterations and cytoskeleton proteins changing from non-muscle-specific to muscle-specific protein isoforms. Eventually, the myoblasts either fuse with existing damaged fibres for repair to one another for new fibre formation of mature multinucleated cell [13].

There are mainly two models proposed by Holtzer and Sanger for the formation of contractile myofibrils. Holtzer and colleagues suggested that precursors of mature thin and thick filaments form independently in the myoplasma of developing muscle and at the late stages of development proceed along common filaments [14, 15]. Sanger and colleagues proposed a model were precursors of thin and thick filaments form along the same structure, which together develop into mature sarcomeres [14, 16].

1.1.3 Primary culture of human myogenic cells

There are several myogenic cell lines such as rat L6, L8, mouse C2C12, and murine MM14 myoblasts cell lines that have been frequently used for various *in vitro* studies for example, the characterization of muscle development, a model for human muscle disorders, or for the development of cell- based therapies for muscle diseases. The human myogenic cells (myoblasts) have been used in similar studies but in a limited manner. The limitation is due to the difficulty of isolating a pure primary human myogenic cells (myoblasts) [7]. The human primary myogenic cultures usually prepared from mononucleated cells dissociated from the whole muscle whereby the satellite cells are released from their niche. The cells are detached from the muscle tissue enzymatically and mechanically. The single cells are removed by filtration and plating. Furthermore, due to the presence of non-myogenic mononuclear cells in human skeletal muscle, including adipocytes, fibroblasts and lymphocytes different methods are used to isolate a purer population of myoblasts. Therefore for more specific and efficient isolation of myoblasts, Webster et al. utilized fluorescence-activated cell sorting (FACS) to positively select for cells expressing the human neural cell adhesion molecule (NCAM), that is a cell surface antigen, or melanoma cell adhesion molecule (MCAM), or the vascular cell adhesion molecule 1 (V-CAM-1)[7].

Cultured myoblasts go through three morphological and biochemical distinct developmental stages. The first stage is proliferation, proceeding through at least one round of cell division with high motility rate compared to other cultured cells. The cultured myoblasts have limited proliferation capacity due to sub-optimal levels of growth factors in the growth medium [17]. The second stage is the migration and alignment of single myoblasts into long chains followed rapidly by membrane fusion and the formation of the multinucleate myotubes. Finally, the third stage is the development of the contractile machinery.

The primary myogenic culture has its advantages and drawbacks. An advantage could be the ability of studying the morphological and functional changes occurring during myogenesis which are well characterized and can be observed in primary myogenic culture due to the rapid formation of long multinucleated myotubes from a layer of mononuclear cells (figure 3). A drawback could be that the living functional cells are in a defined environment outside the body and thus the cells are removed from a complex environment of changeable cell populations and variable volumes of extracellular fluids that have a great influence on the cells [7].

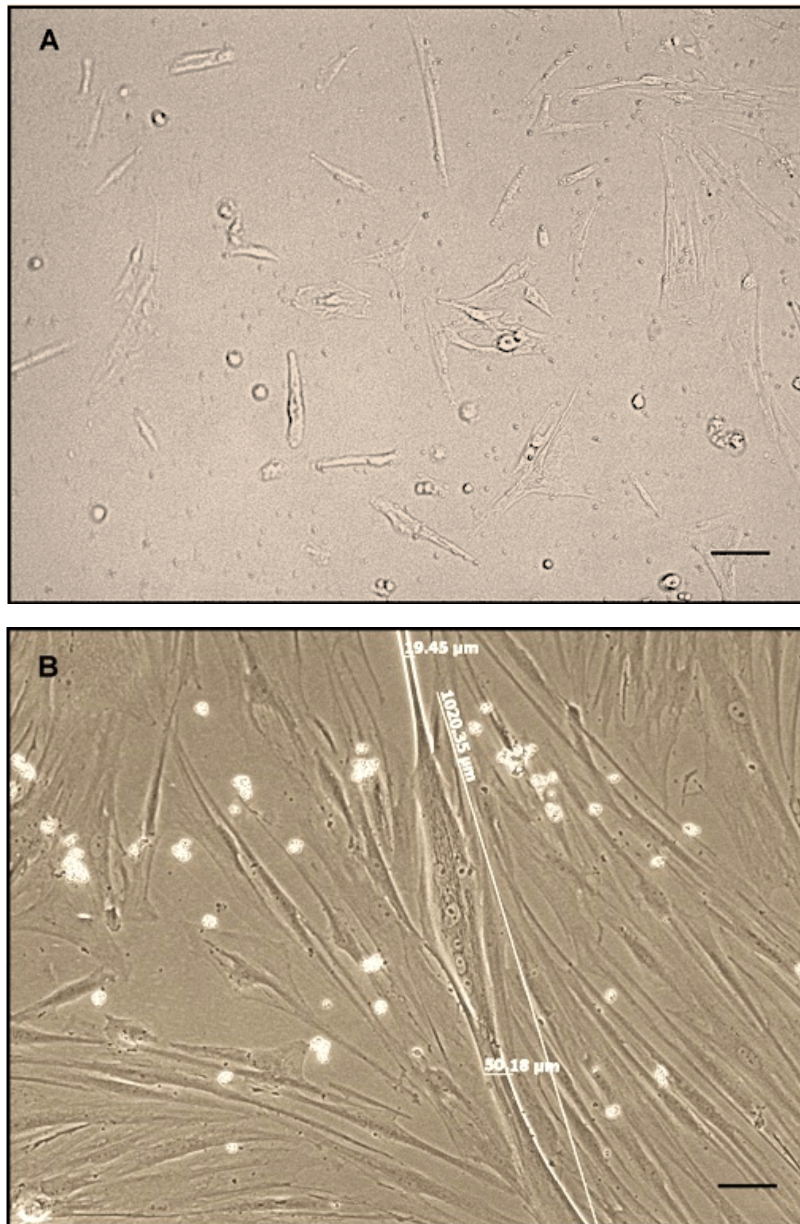

Figure 3. Microscope view using 10x objective of human myoblasts (A) and myotubes after 30 days of differentiating (B) in tissue culture. Scale bar = 200 µm

1.2 Myofibres

The myofibres are long cylindrical structure surrounded by a plasma membrane, sarcolemma and contain up to several thousands of nuclei located peripherally beneath sarcolemma. Muscle fibres are 10 µm in diameter and few millimetres to several centimeters long. Each fibre composes of myofibrils, and cytoskeletal network, that anchor the contractile fibrils to the sarcolemma. Myofibrils are built of repeating contractile units known as sarcomeres. The sarcomere is the most highly ordered macromolecular structure in eukaryotic cells [18] built of thick and thin filaments. This arrangement is responsible for the cross-striated banding pattern observed under light and electron microscopy.

1.2.1 Sarcomere

The sarcomere is between 2,0 - 2,5 µm in length with the Z-disc at their ends and where the thin actin filaments of opposite directions are linked together by alpha-actinin dimers [19](figure 4). There are several proteins located at the Z-disc such as, alpha-actinin, which is an F-actin cross-linking protein

associated with the barded end of the thin filaments. The alpha-actinin is a homodimer made of two elongated subunits with antiparallel orientation and a basic structure similar to that of spectrin and dystrtophin. Another protein located at the Z-disc is CapZ protein that belongs to a member of a family of heterodimeric actin binding proteins called capping proteins present in most tissues that bind to the barded ends of actin filaments. Z-disc is located in the

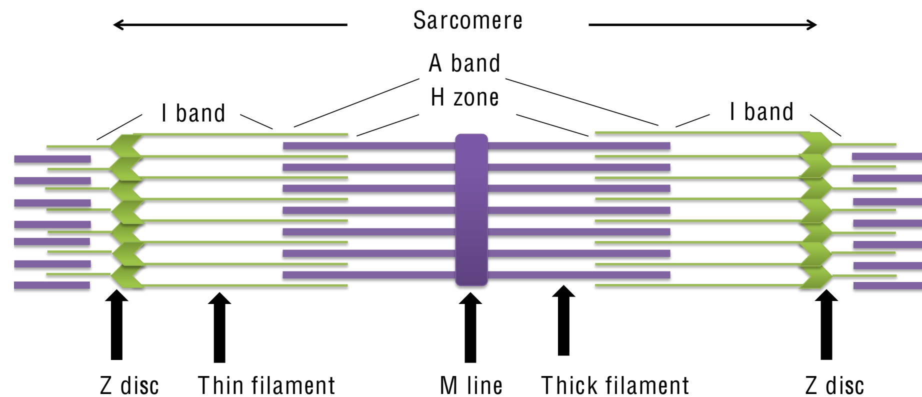

Figure 4. Schematic diagram of a single sarcomere.

middle of the I-band, which appears lighter in a light microscope and contains mainly actin filaments whereas the A-band appears darker due to polymers of myosin molecules. The A-band divided by a light region called H-band. The midline of H-band is the M-line where the thick filaments are anchored by several myosin-binding proteins. The M-line contains other proteins such as myomesin, muscle specific calpain 3, and muscle specific RING finger protein 1(MuRF1). The MuRF1, MuRF2 and MuRF3 proteins are highly homologous ring finger proteins. These proteins have various physiological functions in skeletal and heart muscles [20]. They bind to sarcomeric proteins, cytoskeletal proteins, enzymes involved in ATP production, regulators of nuclear transcription, which in turn modify nuclear translocation, gene expression, and subcellular targeting. The MuRFs proteins play an important role in linking myofibril components with microtubules, intermediate filaments and nuclear factors [21]. Moreover, they function as E3 ubiquitin ligases in ubiquitin mediated muscle protein turnover to regulate protein degradation and gene expression in muscle tissues [21, 22]. Furthermore, MuRF1 and MuRF3 are E3 ubiquitin ligases that catalyse the degradation of slow/beta MyHC and MyHC IIa via the UPS in striated muscles [23]. MuRF1 detected at the Z-line and soluble form in the cytoplasm. MuRF2 is expressed in at least four isoforms in striated muscles and also binds to titin and then disappears as mature myofibrils form [24]. MuRF3 co-localizes with microtubules and with the sarcomeric Z-line and M-line. It is suggested that MuRF2 and MuRF3 are associated indirectly with titin via MuRF1 and the localization of MuRF1 to Z-lines has been speculated to be the result from coiled-coil heterodimerization with MuRF3.

1.2.2 Thin filament

The major components of the thin filaments are actin, tropomyosin, and the troponin complex. The globular actin monomers (G-actin) form a double-helical filament (F-actin) that interacts with myosin with NH2-terminal. There are two actin isoforms in sarcomeric muscles, alpha-skeletal and alpha-cardiac actin encoded by two distinct genes. The structure of the two isoforms is conserved during vertebrate evolution from reptiles to birds and mammals. In humans they are co-expressed in adult skeletal and cardiac muscles. Tropomyosin molecules are assembled in a head-to-tail fashion as homodimers or heterodimers in the major groove of the actin filament forming a filament associated with actin double helix. Tropomyosins are elongated dimeric proteins with subunits consisting of alpha-helical coiled- coils and arranged in parallel orientation. There are three isoforms, α, β and γ, encoded by *TPM1*, *TPM2* and

TPM3 respectively. The relative ratio of alpha to beta-subunits is higher in fast compared to slow muscles and varies among different fast muscles. Each tropomyosin dimer spans seven actin monomers, and each troponin complex is also associated with seven actin repeats. The troponin complex consists of three subunits TnC, the Ca^{2+} binding subunit, TnI, the inhibitory subunit and TnT, the tropomyosin subunit. Troponin constitutes the Ca^{2+} sensitive switch that regulates the contraction of striated muscle fibres. The elongated NH2-terminal half of TnT extends for a considerable length along tropomyosin and spans the head to tail overlap of tropomyosin. There are two TnC genes, coding for TnC-fast and TnC-slow/cardiac isoforms, and three TnI genes coding for TnI-fast, TnI-slow, and TnI-cardiac isoforms. There are three TnT genes each of which generates a large number of transcripts by alternative splicing [24-27].

1.2.3 Thick filament

The thick filament is the bipolar polymer of the motor protein myosin that interacts with actin to produce force and sarcomere/muscle shortening. The thick filament contains myosin, several myosin binding proteins MyBPC, M- protein, and myomesin. The sarcomeric myosin conventional class II myosin has two heads and long tails connected by a neck region and consist of two heavy chains. Myosin is a molecular motor that generates mechanical force from ATP hydrolysis, through the interaction of myosin globular head domain (myosin sub-fragment 1 S1) with actin filaments. Myosin composed of one pair myosin heavy chain (MyHCs) and two pairs of light chains (MyLCs). Human sarcomeric myosin heavy chain (MyHC) isoforms encodes by a multigene family and their expression is developmentally regulated. There are eight known sarcomeric MHCs isoforms, five of which expressed in skeletal muscles fibres. The embryonic MyHCemb and neonatal MyHCneo, are two isoforms expressed during embryonic and perinatal development; MyHC I or β/slow MyHC, expressed in slow skeletal muscle fibres; MyHC IIa and MyHC IIx are fast isoforms expressed in fast skeletal muscle fibres. All myosin-associated proteins have a characterized structure of two types of internal domains of 100 amino acids each domain. These domains are homologous to either immunoglobulin (Ig) or fibronectin (Fn) type III repeats and consist of seven beta-strands arranged in two sheets. There are three major isoforms of MyBPC slow, fast and cardiac each contains several Ig and Fn like repeats and binding sites for myosin that are located in the COOH-terminal. The M-protein and myomesin are both located at the M-line and bind the COOH-terminal of the myosin molecules. The two proteins are similar in size and consist of the same number and sequence of repeated Ig and Fn-like modules [24-27].

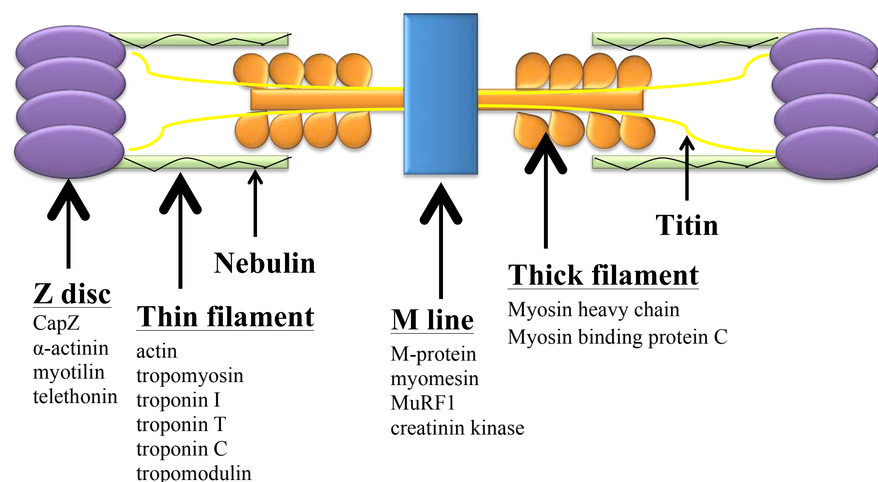

Figure 5. Schematic diagram showing the major components of the sarcomere.

1.2.4 Titin

Titin is a giant protein encoded by a single gene and extends from Z-disc to the M-band within the sarcomere. Titin comprises an inextensible segment at the level of the A-band and an elastic segment at the level of I-band. At the A band, titin binds myosin at the level of the MHC rod and C protein. The COOH-terminal of titin is anchored to the M band and binds both M protein and myomesin. The major part of titin mass consists of repeating immunoglobulin Ig and fibronectin-III (FN-III) domains. It contains 244 recognizable beta-sheet domains of which 112 have been assigned to the immunoglobulin superfamily and 132 to the fibronectin type III superfamily. Both Ig and FN-III domains provide binding sites for diverse proteins, including myofibrillar, membrane components, enzymes and signaling molecules [24-27].

1.2.5 Nebulin

Nebulin is a giant protein presents in skeletal muscle and extends along the thin filaments as a single polypeptide from the Z-disk to the free end of the thin filament. The COOH- region attached to the Z-disc and interacts with alpha-actinin. Nebulin modules have an alpha helical conformation that is probably stabilized by its interaction with actin [27].

1.3 Congenital myopathies

The congenital myopathies are a class of muscle diseases present at birth defined by distinctive morphologic abnormalities in the skeletal muscle. The disease is usually inherited in an autosomal dominant or autosomal recessive fashion, but there are some sporadic cases. The cap disease and nemaline myopathy (NM) are congenital myopathies. The term cap disease is used to describe the abnormal peripheral crescents in the majority of muscle fibers. Caps are present in 20%-70% of muscle fibres, with a higher proportion of fibres involved in the more severely affected patients. There is a sharp delineation between the cap and the adjacent regions of the fibre with normal ultra-structure. These findings have prompted speculation that caps result from failure of myoblast fusion and abnormal protein synthesis during myogenesis [28].

Nemaline myopathy (NM) is characterized by the presence of rod-shaped structured in the muscle fibres. The smallest rods ($<1 \mu m$ long) palisade in the plane of the Z discs. The larger rods are separated from myofibrils and accumulate randomly or in clusters, especially under the sarcolemma and round nuclei. The fibre type 1 predominance in NM is associated with abnormally high expression of fetal myosin and co-expression of fast and slow myosin in some muscle fibres [28].

Distal myopathy is a second class of muscle diseases that is either inherited or sporadic muscle disorders characterized pathologically by myopathic changes in skeletal muscles [28].

2 AIMS OF THE STUDY

- To describe the clinical, morphological and genetic characteristics of a new unexpected myopathy associated with a mutation in cardiac isoform of myosin binding protein C.
- Using cultured human skeletal muscle cells to investigate the expression profile of a panel of sarcomeric components during myoblast proliferation and myotube development with a focus on proteins associated with a group of congenital disorders.
- To assess whether cultured human skeletal muscle cells are appropriate tool to study developmental stages of myofibrillogenesis.
- To investigate whether cultured human skeletal muscle cells can be used as an efficient host for gene transfer.
- To use human cultured skeletal muscle cells as a cellular model to examine the phenotypes of myopathy-related beta-tropomyosin mutants.
- To compare human and mouse cultured skeletal muscle cells as cellular models to investigate the behavior of beta-tropomyosin mutants.
- To describe the clinical, morphological and cellular characteristics of a new human protein aggregate myopathy and cardiomyopathy associated with combined homozygous null mutation in *TRIM63* and a heterozygous missense mutation in *TRIM54*.

3 MATERIALS AND METHODS

Muscle biopsy (Paper I & IV)

A biopsy specimen obtained from the quadriceps muscle from an infant patient at the age of two months. An open muscle biopsy specimen was obtained from the biceps brachii from a 55 years old man. The samples were instantly frozen in isopentane chilled by liquid nitrogen and stored at -80°C.

Primary human muscle cells and C2C12 (Paper II-IV)

Healthy controls of similar age were used in paper I. Enzymatically isolated human satellite cells from a healthy donor were obtained from MYOSIX through a collaborative program with Association Francaise contre les myopathies (AFM). Nine standardized batches of these cells were used in paper II-IV. Skeletal muscle biopsy obtained from the patient was used to isolate muscle satellite cells and cultured as previously described [29]. In addition, a mouse cell line C2C12 myoblasts purchased from American Type Culture Collection (ATCC) was used in paper III.

Electron microscopy (Paper I & IV)

A small piece of the biopsy tissue was fixed in 2% glutaraldehyde and post-fixed with 1% osmium tetroxide and finally embedded in araldite. The ultrathin sections were stained with uranly acetate and lead citrate to be examined with electron microscopy.

Morphological and histochemical analyses (Paper I & IV)

Cryostat sections of frozen skeletal muscle specimens were stained with haematoxylin-eosin, Gomori trichrome, PAS and congo red. In addition, enzyme histochemistry performed for NADH-tetrazolium reductase and myofibrillar ATPase at different pH.

DNA analyses (Paper I & IV)

- *Genomic DNA*

  DNA was extracted from frozen skeletal muscle and or peripheral blood.

- *DNA Sequencing*

  Mutation screening was performed for *MYBPC3* in paper I, *TRIM63* and *TRIM54*, in addition to *MYH7*, *ACTA1*, *TPM2*, *TPM3*, *TNNT1*, *ACTN3*, *ACTN2* and *FHL1* in paper IV. In addition, sequencing of *TRIM63* exon 5 was performed in 200 control DNA samples.

- *Restriction fragment length polymorphism (RFLP) analysis*

  The mutation of *TRIM54* was screened for the patient and eight members of his family, in addition to 100 Swedish and 100 Spanish controls in paper IV.

RNA analyses (Paper I, II & IV)

- *cDNA synthesis*

Total RNA was extracted from skeletal muscle tissue in paper IV, using the RNeasy Mini Kit & I. In addition, total RNA was extracted from primary muscle cultured-cells in paper II and IV, using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Synthesis of first-strand complementary DNA (cDNA) was performed from RNA using Ready-To-Go You-Prime First-Strand Beads.

- *Polymerase chain reaction (PCR) and sequence analyses*

Polymerase chain reaction (PCR) performed on cDNA from patients in order to confirm the appearance of the mutation at the transcript level for *MYBPC3* in paper I and *TRIM63* and *TRIM54* in paper IV. PCR analysis was performed on cDNA extracted from cultured cells in paper II and IV. The expression of a panel of genes including; desmin (*DES*), titin (*TTN*), MyHC isoforms (*MYH1*, *MYH2*, *MYH3*, *MYH4*, *MYH7* and *MYH8*), α-cardiac actin (*ACTC*) and α - skeletal actin (*ACTA1*), tropomyosin isoforms (*TPM1*, *TPM2* and *TPM3*), troponin T isoforms (*TNNT1*, *TNNT2* and *TNNT3*), troponin I isoforms (*TNNI1*, *TNNI2* and *TNNI3*), skeletal muscle myosin-binding protein C isoforms (*MYBPC1*, *MYBPC2*) and cardiac myosin-binding protein C (*MYBPC3*), myogenic factor 5 (*MYF5*), myogenic differentiation 1 (*MYOD1*) and myogenin (*MYOG*) was assessed by PCR and sequence analyses in paper II. The nucleotide sequence determination was carried out by cycle sequencing using an ABI 3730xl DNA sequencer (GATC Biotech AG, Konstanz, Germany).

Protein analyses (Paper I-IV)

- *Gel electrophoresis, immunoblotting and chemiluminescent analysis*

Protein samples were extracted from skeletal muscle tissue from a control, a patient with critical ill intensive care unit (ICU) and the patient in paper IV. Proteins from cultured cells were obtained using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), after RNA was recovered from the cell lysate, by acetone precipitation. The protein extracts from either skeletal muscle tissue or cultured cells were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after heating to 100 °C for two minutes in Laemmli Buffer. Separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies against MuRF1, MuRF3 and α-tubulin, and HRP-conjugated secondary antibodies using chromogenic Western Blot Immunodection kit. The chemiluminescent analysis was performed using anti-sarcomeric actin as primary antibodies detected with horseradish peroxidase-labeled secondary antibodies. The immunoreaction was detected with SuperSignal West Femto Chemiluminescent Substrate (Pierce) and captured by ImageQuant™ software in LAS 4000 CCD camera.

- *Immunohistochemical analysis*

The analysis was performed on frozen skeletal muscle sections, which were incubated with antibodies against cardiac MyBPC, embryonic and fetal MyHCs and α-cardiac actin in paper I. In paper IV, a panel of antibodies against sarcomeric and cytoskeletal proteins were used including slow and fast myosin heavy chains, myotilin, filamin C, desmin and αB-crystallin. The immunohistochemistry analysis was performed on proliferating myoblasts and myotubes. The cells were incubated with primary antibodies (Table 1) followed by incubation with EnVision™Flex/HRP and visualized by the indirect peroxidase-antiperoxidase complex method using diaminobenzidine as a chromogen. Nuclei were counterstained with hematoxylin in paper II.

- *Immunofluorescence analysis*

  In papers II, III, IV cells fixed in 4% formaldehyde (methanol-free 16% formaldehyde solution), free aldehyde groups were blocked with 50mM NH_4Cl, permeabilized in 0,1% Triton X-100. Cells were incubated with primary antibodies (Table 2) for 1 h in a humidified chamber at 37°C, followed by incubation with secondary antibodies for 1h in the dark. Finally, the slides were mounted with a coverslip using Prolong Gold antifade reagent with DAPI to highlight cell nuclei.

PRODUCT NAME	PRODUCT CODE	ANTIGEN	DILUTION IMMUNOCYTOCHEMISTRY/ IMMUNOFLUORESCENCE	DILUTION SECONDARY ANTIBODY	COMPANY
Mouse monoclonal to desmin	M0760	Desmin	1:100	1:1000 Anti-mouse Dylight 549	DakoCytomation
Mouse monoclonal [F5D] to myogenin	Ab1835	Myogenin	1:50		Abcam plc
Mouse monoclonal to troponin T, fast	NCL-TROPT	TNNT	1:20		NovoCastra™Lyophilized
Mouse monoclonal to titin	3010-S	Titin	1:50		BioCytex
Mouse monoclonal to sarcomeric actin	M0874	Alpha-skeletal actin	1:10	1:1000 Anti-mouse Dylight 549	DakoCytomation
Mouse monoclonal cardiac actin	M622709	Alpha-cardiac actin	1:20	1:1000 Anti-mouse Dylight 549	Nordic Biolabs Bioreagents
Mouse monoclonal to myosin heavy chain, (developmental)	NCL-MHCd	MyHC-embryonic	1:10	1:1000 Anti-mouse Dylight 549	NovoCastra™Lyophilized
Mouse monoclonal to myosin heavy chain, (neonatal)	NCL-MHCn	MyHC-neonatal	1:10	1:1000 Anti-mouse Dylight 549	NovoCastra™Lyophilized
Mouse monoclonal to myosin heavy chain, (slow)	NCL-MHCs	MyHC-slow	1:250		NovoCastra™Lyophilized
Mouse monoclonal to myosin heavy chain, (fast)	NCL-MHCf	MyHC-fast	1:120		NovoCastra™Lyophilized
Mouse monoclonal to MYH2	N2.261	MyHC-fast IIa+slow	1:120	1:1000 Anti-mouse Dylight 549	Santa Cruz Biotechnology
Mouse monoclonal to sarcomeric tropomyosin	T9283	TPM	1:100		Sigma-Aldrich
Rabbit polyclonal to beta tropomyosin	ARP48224T100	TPM2	1:120		Aviva Systems Biology
Rabbit polyclonal to myosin binding protein C, (slow)	HPA021004	MYBPC1-slow	1:50		Sigma-Aldrich
Rabbit polyclonal to myosin binding protein C, (fast)	SAB2101539	MYBPC2-fast	1:100		Sigma-Aldrich
Mouse	Ab8293	TNNI-	1:500		Abcam plc

monoclonal [12F10] to skeletal muscle troponin I, (slow)		slow			
Mouse monoclonal [2F12A11] to skeletal muscle troponin I, (fast)	Ab119943	TNNI-fast	1:200		Abcam plc
Mouse monoclonal [284(19C7)] to cardiac troponin I	Ab19615	TNNI-cardiac	1:500		Abcam plc
Mouse monoclonal T12 to Z-disc titin		Z-disc	1:20	Polyclonal Anti-Mouse Immunoglobulins/FITC 1:1000	Fürst et al., 1988
Mouse monoclonal T3 to A/I junction titin		A/I junction	1:5	Polyclonal Anti-Mouse Immunoglobulins/FITC 1:1000	Fürst et al., 1988
Mouse monoclonal T30 to A-band titin		A-band	1:5	Polyclonal Anti-Mouse Immunoglobulins/FITC 1:1000	Fürst et al., 1989
Mouse monoclonal T51 to M-band titin		M-band	1:5	Polyclonal Anti-Mouse Immunoglobulins/FITC 1:1000	Obermann et al., 1996

Table 1: Antibodies used for immunocytochemical and immunofluorescence analyses.

Antigen	Species	Company	Ref	Dilution			
				IH muscle sections	IF muscle sections	IF cell culture	WB
MuRF1	Goat	Abcam	ab4125		1:250		1:200
MuRF1	Mouse	Abcam	ab57865		1: 250		
MuRF1	Rabbit	Santa Cruz	Sc-32920			1:50	1:50
MuRF2	Goat	Santa Cruz	Sc-49457			1:50	
MuRF3	Goat	Abcam	ab4351		1:50	1:50	
Alpha-tubulin	Rabbit	Abcam	ab 4074		1:50	1:1000	
Titin T12 (Z-disc)	Mouse		Fürst et al., 1988		Undiluted	1:20	
Titin T3 (A/I junction)	Mouse		Fürst et al., 1988		Undiluted	1:5	
Titin 30 (A band)	Mouse		Fürst et al., 1989		Undiluted	1:5	
Titin 50 (M band)	Mouse		Obermann et al., 1996		Undiluted	1:5	
Myomesin (BB78)	Mouse		Vinkemeier et al 1993		1:5	1:5	
M protein (AA259)	Mouse		Vinkemeier et a.1993		1:2		
TNNI1	Rabbit	Abcam	ab85087		1: 250		
Tropomyosin 3	Sheep	Abcam	Ab78609		1: 200		
Slow myosin	Mouse	Novocastra	NCL-MHCS	1: 50			
Fast myosin	Mouse	Novocastra	NCL-NHCS	1: 50			
MyHC-fast IIa+slow	Mouse	Developmental studies hybridoma bank, Iowa	N2.261			1.120	
α-sarcomeric actin	Mouse	DakoCytomation					1:500
Desmin	Mouse	DakoCytomation		1: 50		1:100	
Myotilin	Mouse	Novocastra	NCL-myotilin	1: 100			
Filamin C	Mouse		RR90 (13)		1:25		
αB-cristallyn	Rabbit	Novocastra	NCL-ABcrys	1: 500			
Telethonin	Goat	Santa Cruz	SC-8726		1:500		
α-actinin	Mouse	Sigma	A-7811		1: 800	1:3000	
FHL-1	Rabbit	Abcam	ab49241		1: 500		
Ubiquitin	Rabbit	DakoCytomation	Z-458	1: 100			

Table 2: Antibodies used in paper IV

- *Light/Fluorescence and Confocal microscopies*

  Immunoreactivity was analyzed by light/fluorescence microscopy using the Zeiss Axio Observer microscope (Carl Zeiss AG, Germany) equipped with 10x, 20x, 40x and 63x oil objectives in paper I-IV. Confocal microscopy Zeiss LSM 510 Meta or a LSM 700 inverted Axio Observer.Z1 microscope were used in paper III and IV.

Cell culture (Paper II, III and IV)

- *Myoblasts and differentiation*

  Isolated satellite cells from skeletal muscle were plated on chamber slides using Dulbecco's modified Eagle's medium (DMEM), supplemented with various factors needed for proliferation of cells in culture.

  For differentiation, cells were replenished at 85%-90% confluence with DMEM medium, supplemented with 5% horse serum and further incubated in this medium from 1 day up to 48 days. The medium was changed three times a week.

- *Generation of the enhanced green fluorescent protein (EGFP)-tagged*

  The wild-type *TPM2* (paper III) and wild-type *TRIM63* (paper IV) constructs were generated through amplification of *TPM2* and *TRIM63* cDNA fragments from reverse transcribed human skeletal muscle RNA using specific primers introducing *XhoI* (5') and *EcoRI* (3') restriction sites. PCR products were cloned into a PCRII-Blunt-TOPO-vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Gel purified *TPM2*/ *TRIM63* fragments with *XhoI* (5') and *EcoRI* (3') restriction sites were subcloned into the *XhoI* and *EcoRI* restriction sites of the enhance green fluorescent protein (pEGFP-N1) to generate WT-$TPM2_{\mathrm{EGFP}}$ and WT-$TRIM63_{\mathrm{EGFP}}$ constructs. All *TPM2* mutant constructs were generated in the pEGFP-N1 backbone through site-directed mutagenesis and cloning into E.Coli in paper III. The wild WT-$TRIM54_{\mathrm{EGFP}}$ and p.D106N-$TRIM54_{\mathrm{EGFP}}$ mutant constructs were generated by amplifying *TRIM54* from reverse transcribed patient skeletal muscle RNA using the same restriction sites and method as described above.
- *Transfection of cells*

  C2C12 and human myoblasts in paper III and myoblasts in paper IV were plated on chamber slides in medium without antibiotics one day prior to transfection. Cells were transfected at 85%-90% confluence using Lipofectamine2000. The cells were incubated overnight at 37°C in humidified 5% CO_2 atmosphere.

Diagram presentation of phenotypes (III)

The percentages of the phenotypes were presented in a diagram of all *TPM2* mutant constructs expressed in both human and C2C12 cells before differentiation and after differentiation.

Multiple sequence alignment (IV)

The entire amino acids of beta tropomyosin in human and mouse were aligned using ClustalW in paper III. Residues within MuRF family domain (MFD) of human and mouse MuRF isogenes were aligned using ClustalW in paper IV.

Web-addresses (I -IV)

ClustalW (http://www.ebi.ac.uk/Tools/msa/clustaw2/)

Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (http://evs.gs.washington.edu/EVS/)

NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)

GenBank accession numbers (I -IV)

Gene	Accession	Gene	Accession
MYOD1	NM_002478	*TNNI3*	NM_000363
MYF5	NM_005593	*MYBPC1*	NM_206821
MYOG	NM_002479	*MYBPC2*	NM_004533
TPM1	NM_001018005	*MYBPC3*	NM_0000256
TPM2	NM_003289	*MYH1*	NM-005963
TPM3	NM_152263	*MYH2*	NM_001100112
ACTA1	NM_001100	*MYH3*	NM_002470
ACTC	NM_005159	*MYH4*	NM_017533
DES	NM_001927	*MYH7*	NM_000257
TTN	NM_133379	*MYH8*	NM_002472
TNNT1	NM_001126132	*TPM2*	NM_009416
TNNT2	NM_000364	*TRIM63*	NM_032588
TNNT3	NM_006757	*TRIM54*	NM_187841
TNNI1	NM_003281		
TNNI2	NM_001145841		

4 RESULTS AND DISCUSSIONS

Paper I: Unexpected myopathy associated with a mutation in MYBPC3 and misplacement of the cardiac myosin binding protein C.

Muscle morphology

Muscle biopsy from an infant at the age of two months showed myopathic changes with numerous small fibres and the expression of slow/β-cardiac myosin heavy chain (MyHC) protein in addition to the presence of cardiac isoform of MyBPC. The expression of α-cardiac actin (skeletal muscle developmental isoform of actin) was detected in few of the small fibres. Disorganization of the sarcomeres and partial depletion of thick filaments were the features in these small fibres revealed by electron microscopy. These small fibres were neither regenerating nor immature fibres because of the negative staining for embryonic and fetal MyHC proteins as in healthy controls of similar age.

Genetic findings

Genomic DNA from the patient was used to amplify and analyse the entire coding sequence of *MyBPC3*. A homozygote null mutation c.2882C→T was identified. In addition, the mutation was identified at the transcript level.

Discussion

We reported a fatal cardiomyopathy and an unexpected skeletal muscle myopathy in an infant due to a homozygous mutation in *MyBPC3*. The expression of the mutant gene was detected at the transcript level in skeletal muscle of the patient. The expression of cardiac MyBPC was restricted to small, abnormal type 1 muscle fibres indicating that the muscle pathology was caused by a dominate-negative effect by ectopic expression of mutant *MyBPC3*. Although, the reason for ectopic expression of cardiac MyBPC remains unknown either alteration of a regulatory sequence by the mutation or existence of a second mutation of a regulatory protein in trans might be speculated. Notably, there was no general up-regulation of cardiac muscle protein isoforms.

Paper II: Expression profiles of muscle disease-associated genes and their isoforms during differentiation of cultured human skeletal muscle cells.

RNA expression and sequence analysis

The expressions of transcript of MRFs including *MYOD1*, *MYF5* and *MYOG*, which regulate the expression of muscle specific genes, were detected in human muscle cell cultures both in proliferating myoblasts and multinucleated myotubes after 6 days of differentiation (D6). In addition, the expression of a panel of sarcomeric transcripts were detected in both proliferating myoblasts and differentiated. Furthermore, the expression of all investigated transcripts was confirmed by sequence analysis.

Immunocytochemistry and immunofluorescence analyses

The myogenic identity and stage of differentiation of the mononucleated myoblasts were examined by the expression of desmin and myogenin, respectively. The immunocytochemistry and immunofluorescence analyses revealed the expression of many proteins involved in muscle diseases, including beta-tropomyosin, slow TnI, slow MyBPC, cardiac TnI in human mononucleotide myoblasts. In addition, our

culture technique resulted in the development of striated myotubes and the expression of adult isoforms of the sarcomeric proteins. The mature sarcomeric cross striated pattern of the myofibrils in our cell culture was revealed by a panel of titin antibodies (Z-disc, A/I- junction, A-band and M-band). As an indication of a successful cell differentiation system, a subset of multinucleated myotubes developed into myofibre-like cells with peripheral nuclei.

Discussion

In the previous studies, mammalian or avian cultured myocytes have been used as an experimental model to analyze the expression of myofibrillar proteins during myogenesis in order to understand the mechanisms involved during myofibrillogenesis [30-35]. However, we used cell cultures of human skeletal muscle to study developmental stages of myofibrillogenesis. Our studies on human myoblasts revealed an early expression of beta-tropomyosin, slow TnI, slow MyBPC, cardiac TnI, which may indicate an early role for these proteins as constituent of the developing contractile apparatus during myofibrillogenesis. In turn, this may suggest that in disease states the mechanisms of pathogenesis for each of the mutated sarcomeric proteins might be reflected by altered expression patterns, and/or disturbed assembly of cytoskeletal, myofibrillar structures and muscle development.

Paper III: Phenotypes of myopathy-relate beta-tropomyosin mutants in human and mouse tissue cultures.

The histological behavior of five β-TM mutants associated with different morphological features was assessed by transfection studies in human and mouse cultured myoblasts and myotubes. The mutations investigated were, the E41K mutation associated with a congenital myopathy characterized by either cap structures or accumulation of nemaline rods, the K49del, G53ins and N202K mutations associated with the appearance of cap structures and abnormal coarse-meshed intermyofibrillar network and the E122K mutation which is linked to a non-specific congenital myopathy and type 1 fibre predominance. We were able to transfect human myoblasts relatively easy with high efficiency and each experiment was repeated at least ten times. The filamentous actin was stained with phalloidin to examine the integration and localization of mutant β-TM into endogenous action. Both fluorescent and confocal microscopies were used to examine the efficiency, morphology and cytoplasmic distribution of wild-type and mutant β-TMs. Furthermore, we were able to successfully differentiate transfected human mayoblast into mature myotubes with elongated shape and multiple nuclei and sarcomeric pattern.

Phenotypes observed in human cells transfected with mutant β-TM$_{EGFP}$

While the wild-type β-TM showed good incorporation into the stress fibres in myoblasts and sarcomeric thin filaments in myotubes, various phenotypes were observed in human myoblasts and myotubes transfected with mutant β-TMs. The E41K mutation induced a remarkable levels of diffuse localization and reduce incorporation in myoblasts, a perinuclear aggregates was the dominate phenotype in myotubes. The K49del- and E122K-β-TM$_{EGFP}$ showed similar behaviors in transfected myoblasts and myotubes. The mutants were incorporated into clouds in random locations in the nucleus and cytoplasmic, in addition to occasionally formation of intranuclear rod-shaped structures. Although the mutant K49del-β-TM$_{EGFP}$ and E122K- β-TM$_{EGFP}$ showed good incorporation into the pericellular area, the cytoplasmic rod-shaped structures were observed after differentiation of the transfected myoblasts. Formation of endogenous actin aggregates and poor incorporation into filamentous structure of stress fibres was the dominant features of the G53ins-β-TM mutant in the transfected myoblasts. Nice integration of the mutant TM into sarcomeric structures was found in transfected myotubes with G53ins-β-TM mutant, although the mutant produced diffuse cytoplasmic staining at the far end of the myotubes. Finally, the N202K mutant formed clouds around the nucleus and in the cytoplasm with diffuse staining

and formation of small aggregates. In transfected myotubes the thin filaments appeared extremely thickened, resulting in huge accumulations of polymerized actin that disrupt actin filaments.

Phenotypes observed in C2C12 transfected with mutant β-TM$_{EGFP}$

Transfection of mutant β-TMs into C2C12 was performed to compare the behaviour of the mutants in two different cell models. In contrast to human cells, where the various β-TM mutants behaved differently, the corresponding β-TM mutants resulted in similar features in transfected C2C12 cells. Intense EGFP aggregates co-localized with endogenous actin, mainly at the pericellular area of the cells, was the most striking abnormality by all β-TM mutants in C2C12 myoblasts and myotubes.

Discussion

The *TPM2* mutations caused different morphological phenotypes in transfected cells, suggesting that mutations affect specific TM-binding interaction and/or function that invoke distinct structural changes within α-helical coiled-coil dimer or thin filament. Furthermore, the β-TM mutants behaved differently in human transfected myoblasts and myotubes. This may indicate that defects observed in transfected myoblasts are in vitro defects rather than direct association to the molecular mechanisms of the mutant isoforms.

Although, our human tissue culture model did not recapitulate the pathological features observed in patient muscle biopsies the results may demonstrate a primary trigger for myopathy associated with *TPM2* mutations. This may not be surprising, because the phenotypes observed in muscle biopsies from NM patients either taken from multiple sites or at different time points, are not similar[36-39]. Changes in morphological features are not only influenced by the mutations in different cellular environment. Other variable factors including hormone statue, compositions of sarcomeric proteins and TM isoforms, dynamic turnover, filament stoichiometry, ratio of the mutant variant, could also determine the differences between different cellular environments. This is further supported by the differences observed between phenotypes in transfected human and C2C12 cells. Thus, specific morphological abnormality might be associated with specific mutants under similar cellular conditions, but also influenced by underlying physiological contexts, which determine the cellular condition.

Paper IV: MuRF1 and MuRF3 mutations cause a new protein aggregate myopathy and cardiomyopathy.

Patient

A 60-year old man was diagnosed with hypertrophic cardiomyopathy at the age of 20 years and he developed progressive muscle weakness in the four limbs, rigidity of the spine, scapular winging and mild respiratory involvement.

Muscle morphology

Muscle biopsy revealed fibre size variability, with atrophic in some fibre, mild endomysial fibrosis and predominance of type I fibres with distinctive subsarcolemmal homogeneous accumulations. The accumulations seemed as single or multiple well defined lesions in edges of muscle fibre. The inclusions were faintly basophilic on HE stain, light green with the modified trichrome, and were negative with PAS and congo red staining. The immunoractivity of the inclusions was positive for slow myosin, myomesin, M-line protein, MuRF3 and faint FHL immunoreactivity in fibre regions corresponding to the inclusions.

The inclusions were negative for filamentous actin, desmin, myotilin, c filamin, dystrophin, telethonin, α-actinin, or tropomyosin.

Electron microscopy

The inclusions were abnormally oriented, consisting of fragments of sarcomeres with preserved A-bands and M-lines but lacking I-bands and Z-lines with varied length and width under the sarcolemma. Additionally, the thick filaments were scattered, fragmented and randomly oriented in some fibres.

Genetic findings

The sequencing of genomic DNA from a patient from a three-generation family from Spain revealed a homozygous nonsense mutation in *TRIM63* and a heterozygous missense mutation in *TRIM54*. The appearance of both variants was examined in seven additional members of the family by sequencing and RFLP analyses. Several members of the family carried either *TRIM63* or *TRIM54*. The RFLP and sequence analyses excluded the *TRIM63* and *TRIM54* sequence variants in 400 control chromosomes.

RT-PCR, sequencing, immunoblotting and immunofluorescence studies on muscle tissue and cultured myoblast

The transcript level of *TRIM63* was not detected in muscle tissue or cultured cells from the patient. Additionally, the expression of MuRF1 at the protein level was examined by immunoblotting and immunofluorescence. The western blot analysis indicated the absence of full size or truncated protein in the patient. The immunoblot analysis further indicated an increased in the content of MyHCs in the skeletal muscle tissue of the patient. This result may imply that the degradation of MyHCs via UPS is disrupted due to the absent of MuRF1 expression. The immunofluorescence confirmed the results obtained from immunoblot analysis of skeletal muscle tissue and cultured myoblasts from the patient.

The expression of MuRF3 was confirmed by immunofluorescence and immunoblot analyses of skeletal muscle tissue as well as the cultured myoblasts from the patient. MuRF3 was accumulated with other proteins in the subsarcolemmal regions of many fibres.

The immunostaining of α-tubulin in skeletal muscle and cultured myoblasts from the patient showed abnormal and scattered formation of aggregates in the periphery of fibres and severe distraction of the microtubule network. However, the level of the α-tubulin expression in patient muscle tissue was similar to that in the normal control muscle tissue, indicating that the mutation in MuRF3 affects the structure and organisation of microtubule rather than the expression level.

The immunostaining of several sarcomeric proteins revealed the presence of the individual proteins, however the normal myofibrillar organization was lacking. The myofibrillar showed a primitive pattern of localization resembled to that of nascent myofibrillar.

Transfection assay

The transfection of wild-type *TRIM63* was performed to examine whether the abnormal structure of microtubule network and defects in myofibrillogenesis are associated with the absence of MuRF1. Transfection of the wild-type *TRIM63* resulted in nuclear and cytoplasmic localization of the fusion $MuRF1_{EGFP}$ protein and formation of microtubule networks. Furthermore, transfection of wild-type *TRIM63* induced differentiation and elongation of myoblasts from the patient.

Transfection assay in the human myoblasts was performed to assess the effects of *TRIM54* mutation. While the transfection of the wild-type *TRIM54* was well-tolerated, the mutation induced formation of abnormal cytoplasmic thickened filamentous structures. The filamentous structures co-localized with the endogenous MuRF1 and microtubule filaments, indicating the pathogenic effect of the mutant MuRF3.

Discussion

The three MuRF genes, *TRIM63*, *TRIM55* and *TRIM54* encode highly homologous ring finger proteins, MuRF1, MuRF2 and MuRF3 respectively. These proteins have various physiological functions in skeletal and heart muscles [20]. The MuRF proteins play an important role in linking myofibril components with microtubules, intermediate filaments and nuclear factors [40]. Moreover, they function as E3 ubiquitin ligases in ubiquitin mediated muscle protein turnover to regulate protein degradation and gene expression in muscle tissues [21, 22]. MuRF1 and MuRF3 are E3 ubiquitin ligases that catalyse the degradation of slow/beta MyHC and MyHC IIa via the UPS in striated muscle[23].

The muscle pathology of the patient showed subsarcolemmal inclusions corresponding to thick filament-associated proteins including slow myosin, myomesin and M-line protein, and absent of thin-filament and Z-line-associated proteins. In addition, EM analysis confirmed the results obtained from the muscle morphological analyses, indicating that inclusions contain fragments of sarcomeres with preserved M lines and A-bands but lacking Z-lines and I-bands. The accumulations of the proteins found in the muscle biopsy may reflect a defective degradation system that is likely to be caused by deficient E3 ligases MuRF1 and MuRF3.

Our *in vitro* studies highlighted the essential role of MuRF1 in the formation of stable microtubule networks and myoblasts differentiation by acting as the transient scaffolds of myofibril assembly. The transfected myoblasts from the patient with the wild-type *TRIM63* resulted in the formation of microtubule networks, in addition to promotion of myogenesis. These results indicate the association of deficient MuRF1 expression with abnormal formation of microtubule structures and defect in myofibrillogenesis.

Previous studies have revealed the association of MuRF3 with formation of cellular microtubule, myogenic differentiation and myotube formation [40-42]. Our result from cellular studies further confirmed the importance of MuRF3 for the stability and formation of microtubule structure. Expression of mutant MuRF3 in control myoblasts resulted in formation of cytoplasmic aberrant filamentous structures, demonstrating a dominant negative effect of the *TRIM54* mutation.

The clinical, morphological, genetic and cellular models, in this study suggest that both MuRF1 and MuRF3 proteins functionally cooperate in striated muscle.

5 GENERAL CONCLUSIONS

Mutations in sarcomeric proteins are important causes of cardiac and skeletal muscle diseases. Given their structural and functional roles in sarcomere contraction, it is not surprising that defects in sarcomeric proteins will affect muscle function. However, the primary effect of defective sarcomeric proteins on sarcomere formation and contractility, and their relation to muscle cell morphology are not completely understood for a number of the diseases. In this thesis, the disease-causing genes in an infant with a new unexpected myopathy and a family with cardiomyopathy and skeletal myopathy and their consequences are described. In addition, we used human tissue-culture cells to understand the pathogenesis and cellular mechanisms underlying a group of sarcomeric myopathies.

In paper I, we investigated an infant with a fatal cardiomyopathy and an unexpected skeletal muscle myopathy. In this patient, we identified a homozygous mutation in the gene encoding the cardiac isoform of myosin binding protein C (*MyBPC3*). The expression of cardiac MyBPC, restricted to abnormal muscle fibres, indicated that the muscle pathology and unexpected skeletal myopathy was caused by a dominate-negative effect by ectopic expression of mutant *MyBPC3.*

Many of the sarcomeric components of striated muscle exist as families of similar isoforms, which are developmentally regulated and differentially expressed in a tissue-specific manner in cardiac, slow and fast muscle. An important step in trying to understand how mutations in specific sarcomeric proteins might affect muscle function is to address the sequential employment and functional compensation of different isoforms of sarcomeric proteins during myogenesis. In paper II, we investigated the expression profile of a panel of sarcomeric components with a focus on proteins associated with a group of congenital disorders. We demonstrated that human skeletal muscle cultured cells are an appropriate tool to study developmental stages of myofibrillogenesis. The early and uniform expression of several of sarcomeric proteins suggested their impact on the developmental mechanisms involved in the initial stages of myofibril assembly, differentiation and formation of muscle. Many proteins involved in muscle diseases are detected in the stages of skeletal muscle cell differentiation that can be reached *in vitro*. This indicated that myoblasts isolated from patients with a mutation in one of the investigated sarcomeric genes might be an invaluable tool to analyze the effects of these mutations on sarcomere structure and formation. It would provide us new insights into development of muscle to indicate whether these diseases are disorders of myofibrillogenesis and muscle development.

Transfection of mouse myoblasts has extensively been used to explore effects of sarcomeric gene mutations, because human myoblasts are more difficult to transfect. However, the use of human cells would be more relevant when studying human myopathies. In paper III, we established means to successfully transfect human myoblasts. We used this model to examine the sole consequences of amino acid alteration in the mutated beta tropomyosin, associated with different morphological features, and to find hints about which properties of the mutants are likely to cause the morphological changes. Data from our human tissue culture model suggested the properties of β-TM mutants, which may be considered as the basis for the histological changes seen in muscle biopsies of patients and give us clues regarding the primary trigger for myopathy. We demonstrated that compared to mouse myoblasts (C2C12), human cells are environment closer to the reality in human skeletal muscle and more reliably mimic the disease conditions. In addition, we have demonstrated that histopathological phenotype associated with β-TM mutants may be accounted for the variable response to the cellular environment influenced by physiological context, in combination with the time course of expression of mutant protein rather than the alteration of amino acid itself.

In paper IV, we investigated a patient in a three-generation family with hypertrophic cardiomyopathy and progressive muscle weakness. Sequence analyses demonstrated that the patient was homozygous for a MuRF1 null mutation and heterozygous for a MuRF3 mutation. Muscle specific ring finger proteins MuRF1 and MuRF3 encoded by *TRIM63* and *TRIM54*, respectively, are microtubule-associated proteins that localize to the sarcomeric M-band and Z-line. Association of MuRF with microtubules is required for its stability and myogenesis. MuRF1 and MuRF3 both are E3 ubiquitin ligases that mediate degradation of MyHCs via the ubiquitin proteasome system. The muscle pathological features included subsarcolemmal deposition of myosin and aberrant distribution of microtubules. Electron microscopy revealed peculiar accumulation of thick filament-like sarcomere fragments lacking Z-line and thin filaments beneath the sarcolemma and between apparently normal myofibrils. Differentiation of cultured myotubes from patient indicated perturbed myofibrillogenesis and an abnormal organization of the microtubule network. Transfection with wild-type MuRF1 induced organization of normal microtubule network and initiation of myofibrillogenesis in cultured myoblasts from the patient. Transfection of control myoblasts with mutant but not wild-type MuRF3 induced the formation of cytoplasmic irregular filamentous structures that co-localized with endogenous MuRF1 and alpha-tubulin demonstrating the pathogenic effect of MuRF3 missense mutation.

Clinical, genetic and cellular investigations suggested that both proteins functionally cooperate in striated muscle. MuRF1 and MuRF3 work together in muscle differentiation, likely through stabilization of microtubule, regulation of protein degradation and maintaining titin M-line integrity. In addition, the hypothesis from paper II was confirmed in paper IV, where myoblasts isolated from patients with mutations in genes expressing muscle specific proteins showed to be an invaluable tool to analyze the effects of these mutations on sarcomere structure and myofilaments formation.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Det finns olika former av muskelsjukdomar, och gemensamt för dessa är att de förorsakar muskelsvaghet och därmed försämrad rörelseförmåga. De flesta studerade muskelsjukdomar orsakas av mutationer som stör viktiga muskelproteiner, men just vilket protein som är skadat är i många fall ännu okänt. Vidare, för sjukdomar där mutationen är känd, behövs fortsatt forskning för att förstå hur mutationen påverkar muskelfunktionen och sjukdomsförloppet. Varje muskel uppbyggd av ett antal muskelbuntar som omges av ett bindvävslager och i sin tur består varje muskelbunt av ett visst antal muskelceller, som kallas muskelfibrer. En muskelcell är uppbyggd av mindre komponenter, nämligen muskelfibriller som ligger vid sidan om varandra och är tvärrandiga i normalt mikroskop. Fibrillerna är uppbyggda av långa kedjor av sarkomerer, den kontraktila delen av cellen som konstruerad av två typer av filamenter den tjocka filament (myosin filament) och den tunna filament (aktin filament).

Skelettmuskulatur har förmåga att förnya sig i samband med fysisk skada eller muskel atrofi vid en muskelsjukdom. Formningen av muskulära vävnaden är en komplex process som sätter igång med hjälp av satellit celler och äger rum vid vuxen muskel regenerering och kännetecknas av vissa ändringar i muskel cell morfologi och cytoarkitektur. Satellit celler är en inaktiv muskel stem cell som ligger mellan basmembranet och muskelfibriller. De kan utvinnas från muskelbiopsier och användas som ett värdefullt verktyg för cell kultur studier av sjukdomarnas patogenes eller de inledande skedena av muskelutvecklingen.

I två delarbeten i avhandlingen har vi identifierad genetiska orsaker till två nya hjärt- och muskelsjukdomar. Vi identifierade ett oväntat skelett muskelsjukdom förorsakad av en recessiv trunkerade mutation som stör den hjärtmuskel varianten av myosin bindande protein C (MyBP-C). MyBP C som är viktig för sarkomerstrukturen. Vi dessutom identifierade och karakteriserade en ny hjärt- och muskelsjukdom förorsakad av mutationer som stör funktionen och struktur av två varianter av muskel RING zinc finger protein familj (MuRF). Identifiering av genetiska orsaker till nya muskelsjukdomar är grunden till diagnostik, inklusive prenataldiagnostik och behandling.

I studie av normal muskelbiologi i odlade humana muskelceller, vi följde cellerna från ”stamcell-stadiet” mot differentiering till kontraktila muskelceller. Resultatet från sådan studie visade i vilken ordning olika former av sarkomerproteiner byggs in i sarkomeren, vilket kunde vidare klargöra betydelsen av enskilda proteiner för sarkomerbildningen när muskel sjukdomen är orsakad av defekt i dessa proteiner.

Genom genetisk manipulation etablerade vi sjukdomsmodellen för tropomyosin (TM)-relaterade sjukdomar i humana muskelceller. Vi fokuserade på muskelsjukdomar som är förorsakad av mutationer som stör funktionen eller struktur av beta-TM. TM ger stabilitet till aktinfilament i sarkomeren, och genom interaktion med andra proteiner är den central för att kontrollera kalciumrelaterad muskelkontraktion. Mutationer i beta-tropomyosin (beta-TM) leder till olika kliniska och morfologiska fenotyper. Vi ville uppnå en bättre förståelse av underliggande sjukdomsmekanismer och hur mutationerna leder till sjukdomsutveckling och studerade hur mutationerna påverkar sarkomerbildningen. Humana muskelceller odlades och transfekterades med beta-TM som bär på liknande mutationer som orsakar muskelsjukdomar hos patienter. Vidare transfekterade celler differentierades för att kunna studera effekten av den specifika mutationen under muskelcellutvecklingen. Målet var att kartlägga hur muskelsvikt uppstår på molekylär och cellbiologisk nivå för att kunna förstå patogenesen av dessa sjukdomar. Vi kunde identifiera de första molekylära ändringarna som händer i sarkomerstrukturen.

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