Title of the Dissertation

"Structural and biophysical studies of myotilin: an actin scaffolding component in the Z-disc of human striated muscle"

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Structure of N-terminal PDZ domain of ZASP in complex with the C-terminal peptide of myotilin: implications for myofibrillogenesis

Manuscript II: in preparation

Biophysical characterisation of FLNc Ig-domain 23-24 harbouring mutations on the dimerisation domain

APPENDIX II

Publication

Pathophysiology of protein aggregation and extended phenotyping in filaminopathy

CURRICULUM VITAE
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Abstract

In the sarcomeric Z-disc of human striated myofibril, diverse array of actin binding and cross-linking proteins are continually being identified. One of such important actin-organising proteins is myotilin, a 57 kDa protein responsible for several myofibrillar myopathy termed myotilinopathies. Current knowledge proposes myotilin as a contributor to the overall stability of actin scaffold in the Z-disc through dimerisation and multiple interactions with five co-located Z-disc proteins. Until now however, the overall assembly of myotilin dimer is unknown and the mechanism underlying dimer formation is obscure.

To address the molecular basis of myotilin dimerisation, and gain insights into the mechanism of myotilin interaction with F-actin and the Z-disc partners, structural and biophysical investigations were conducted. By light and X-ray scattering in solution, we observed the formation of myotilin dimer is concentration dependent in a manner reminiscent of a crowded cellular environment. Combining SAXS, NMR and cross-linking/mass-spectrometry, we revealed for the first time, an antiparallel dimeric structure of myotilin. The dimer interface was mapped to the Ig-2 domain with mutation (K358E/K359E) on the identified interface leading to reduced overall structure stability and aggregate formation. This was further demonstrated in co-sedimentation studies with F-actin, where we propose that isolated myotilin Ig-domains constitute independent F-actin binding modules.

X-ray crystal structure of PDZ-ZASP in complex with myotilin peptide revealed three peptide residues which were located to a conserved binding pocket in the PDZ. In full agreement with the crystal structure, NMR analysis and biophysical data indicate significant stability in the complex typical of a β-augmentation binding mode.

We propose monomeric myotilin is sufficient to cross-link F-actin, and in reference to published evidences on cellular events such as exercise induced myofibrillar remodeling which triggers increased amounts of myotilin, a consequent formation of myotilin dimer would contribute rigor and stability to the actin-scaffold of myofibrillar Z-disc.
Zusammenfassung

In der Z-Scheibe sarcomeric der menschlichen quergestreiften Myofibrillen, Vielfalt von Aktin-Bindung und Vernetzung Proteine werden ständig identifiziert. Eine so wichtige Proteine Aktin-Organisation ist Myotilin, ein 57 kDa Protein für mehrere myofibrillären Myopathie bezeichnet myotilinopathies verantwortlich. Aktuelle Kenntnisse schlägt Myotilin als Beitrag zur Gesamtstabilität von Aktin-Gerüst in der Z-Scheibe durch Dimerisierung und mehrere Interaktionen mit fünf Co-located Z-Scheibe Proteinen. Bis jetzt jedoch die Gesamtmontage Myotilin Dimer unbekannt ist und der Mechanismus Dimer-Bildung zugrunde liegt, ist unklar.

1.0 Introduction

1.1 The vertebrate striated muscle

In higher vertebrates, coordinated voluntary and involuntary movements at the cellular and organismal level are known to be orchestrated by cellular filament-forming proteins, principally actin and myosin, which are organised into bundles called myofibrils. In humans, muscle fibres account for one-third of the average body weight, and depending on the anatomic location and physiological function, human muscles have been sub-divided as smooth- and striated-type (Cooper, 2000a). Under polarised light microscope, sections of human smooth myofibrils lack pattern of transverse stripe (striation), hence called smooth, and they are found predominantly in organs like blood vessels, uterus and gastro-intestinal tracts where slow, sustained contraction is required. Conversely, striated muscles are typically found attached to the skeletal system and in the myocardium of human heart for fast powerful contractions. The striated appearance of myofibrils in skeletal and heart muscles is organised into light and dark shades corresponding respectively to the so-called I-band (isotropic under polarised light) and A-band (anisotropic under polarised light), (Fig. 1.1).

Protein components of striated muscles exist in dynamic complexes, where they are engaged as scaffolds for the assembly, repair and maintenance of the basic contractile unit – the sarcomere. Most prominent pairs of contractile proteins known to contribute elasticity to the striated myofibrils are: actin-nebulin and myosin-titin. Polymeric actin and myosin make up the bulk of striated muscle thin and thick filament systems respectively, with nebulin and titin known as molecular ruler proteins that regulate the length of the respective filaments in developing myofibril (Clark et al., 2002).
Figure 1.1  Views of striation in the sarcomere of vertebrate myofibrils.

(a) Schematic illustration of a piece of muscle fibre built from bundles of myofibrils with the typical striation annotated.

(b) Live imaging of mouse striated sarcomere; raw image (left) and processed image (right) obtained using newly developed 350-µm-diameter microendoscope; (adapted from Llewellyn et al., 2008).

(c) The left image shows an electron micrograph of longitudinal section of fish white muscle. Image on the right is a schematic diagram showing the main components of the sarcomere. A-band comprises bipolar myosin thick filaments which are cross-linked at the M-band. The actin thin filaments of the I-band are tethered at their barbed end to the Z-disc and interdigitates with the thick filaments in the A-band while Nebulin (800 kDa) runs along the thin filaments and overlaps in the Z-disc. Titin runs between the M-line and the Z-disc. Figure adapted from (Luther, 2009).
The highly ordered, compartmentalized structure of striated myofibrils, together with the localisation, interactions and assembly of the plethora of proteins have witnessed major research in the last four decades. The next section thus provides a synopsis of recent research findings which have remarkably enhanced our understanding of the structure of a myofibril from the assembly of the basic functional unit called sarcomere to the disintegration that accompanies malfunctions in a single or multiple components of the sarcomere.

1.1.1 Myofibrillar sarcomere: structure and composition

The myofibrillar sarcomere is the basic repeating contractile unit of a mature striated myofibril, spanning a length of approximately 2 - 2.5 µm (Gautel, 2008). A sarcomere appears as a band-like compartment as seen from an electron micrograph of a longitudinal section of striated myofibril (Fig. 1.2), with the alternating I-band and A-band composed of three giant motor proteins: actin, myosin and titin. Filamentous actin, myosin and titin are assembled in longitudinal register within the sarcomere. Thin filament spans the I-band, comprising unipolar actin filament in association with the ruler protein, nebulin, and other accessory structural and regulatory proteins like tropomyosin, tropomodulin, troponin-complex and titin N-terminal repeats. Actin filament stretches out from its anchor point – the Z-disc (or Z-band) – spanning across the I-band towards the edge of the A-band where it interdigitates partially with the thick filament myosin (Ehler and Gautel, 2008). Muscle contraction occurs when actin and myosin slide past one another at the region of interdigitation resulting in a shortening of the sarcomere and force generation (the famous sliding filament model) (Huxley and Niedergerke, 1954; Huxley and Niedergerke, 1958). Similarly, the A-band is composed mainly of bipolar myosin filaments cross-linked at the middle of the sarcomere known as M-band. At the M-band, accessory proteins like myomesin and M-protein co-locate with myosin. The carboxyl-terminus of titin is anchored to the M-band where it binds to specific constructs of myosin, thus titin covers one half of the sarcomere with its amino-terminus stretching out from the Z-disc, spanning across the I- and A-bands to the M-band. It is therefore not surprising that the third filament system is represented by titin, the largest ever known protein of molecular weight approximating to 3.7 MDa. For this reason as well, titin is regarded as one of the molecular blueprints upon which sarcomere length is defined (Wang, 1996).
Figure 1.2  Compartmentalisation in the structure of a sarcomere with few of the protein components in each compartment listed. Figure adapted from (Laing and Nowak, 2005).
1.1.2 Sarcomerogenesis

The model of events leading to the formation and longitudinal register of supramacromolecular filaments in the sarcomere has been widely studied and debated (Gregorio et al., 1999; Ehler and Gautel, 2008; Sparrow and Schöck, 2009; Kontrogianni-Konstantopoulos et al., 2009).

Five models are currently proposed describing how sarcomere is assembled into a mature myofibril, a process aptly referred to as sarcomero-myofibrillogenesis. Sanger and co-workers provided an excellent review of the first four models (Sanger et al., 2005), while the fifth and most recent model is proposed by Rui and co-workers (Rui et al., 2010). In brief, the first model proposes a “template model” where stress fiber-like structures (integrin adhesion sites) serve as template upon which the coordinated assembly of subsequent sarcomeric proteins is formed. The second model simply proposes an “independent formation and subsequent assembly” of thin and thick filament structures. The third model proposes a “stitch mechanism” mediated by titin between independently formed sarcomeric components of the I-Z-I complexes (Ojima et al., 1999) and the A-band thick filament components. The fourth model, referred to as the “premyofibril model”, proposes that mature myofibrils are preceded by two intermediary structures: premyofibril and nascent myofibril. This model postulates that premyofibril, characterized by band-like patterns of alpha-actinin-2-rich Z-bodies and non-muscle myosin IIB, form at the edges of developing myocytes and later fuse into mature myofibrils (Sanger et al., 2002).

The fifth and recently favoured “two-state” model of assembly is devoid of step-wise intermediary structures or filaments assembly driven by titin (Rui et al., 2010). The model proposes an initial chaotic state of “independently assembled” latent protein complexes comprising I-Z-I structures (at a minimum, containing actin filaments, alpha-actinin-2, ZASP, myotilin, FATZ, nebulin and titin (Ojima et al., 1999)), as well as integrin adhesion components, followed by their subsequent “direct assembly” with myosin thick filament mediated by thin filament associated “troponins-tropomyosin complexes” (Fig. 1.3).
Experimental evidence put forward by Rui and co-workers in support of the fifth model of sarcomero-myofibrillogenesis also shows that the primordial actin cross-linking Z-disc protein – alpha-actinin – exist in complex with ZASP as the vertebrate counterpart, and plays tension-sensor role critical to the stability of the I-Z-I latent protein complexes required for subsequent sarcomere assembly.

This evidence lends further support to the shifting paradigm that the sarcomeric Z-disc and its plethora of scaffolding proteins not only play important anchorage role for muscle stability but also serves as a critical nodal point in sarcomero-myofibrillogenesis upon which slight alteration often leads to numerous disease states (Frank et al., 2006; Knöll et al., 2011).
Figure 1.3  Illustration of myofibril and sarcomere formation in a two-state model proposed by Rui and co-workers (a) Schematic organisation of a myofibril, represented here with two sarcomeres. Thin filaments include actin filaments and their associated proteins such as nebulin, troponins (Tns) and tropomyosin (Tm). Actin filaments are the major components of I-bands, and are cross-linked to Z-bands via α-actinin. Thick filaments are composed of myosin and are connected from the M-band to the Z-band by titin. (b) A “two-state sarcomere assembly” model. Prior to sarcomere formation, various complexes including integrin, tension sensor (alpha-actinin/ZASP/Zipper), the I-Z-I structure, myosin heavy chain (MHC) filament and the Tns-Tm, all are assembled independently. Subsequently, the various complexes coalesce and interact with the integrin pathway responsible for sarcomere stretching. (c) Relationships between the sarcomeric functional complexes. The arrows indicate the interaction among these complexes as determined by the results presented in this study (adapted from Rui et al., 2010).
1.2 Sarcomeric Z-disc structure: protein components and diseases.

All proteins investigated during the period of this thesis work are located to the myofibrillar Z-disc of human striated muscle sarcomere. As such, the following subsections present succinct details of the current knowledge on this dynamic region of the sarcomere.

1.2.1 3-D Structure of vertebrate Z-disc

The Z-disc (Z-line or Z-band) forms the lateral boundary between one sarcomere and the next sarcomere in a mature striated myofibril. Z-discs define the striking regularity and polarity of thin filaments across the length of myofibrils. Several F-actin capping and cross-linking proteins have been located to the sarcomeric Z-disc; notably, alpha-actinin-2, CapZ and filamin-C, as well as filament-scaffolding proteins like myotilin, myopodin and FATZ reviewed by (Frank et al., 2006; Knöll et al., 2011).

Electron micrograph studies of ultrathin sections of intact vertebrate striated muscle specimens have provided us with tremendous insights into the 3-D structure of sarcomeric Z-disc (Luther, 2009). Viewed longitudinally, the Z-disc structure appears as a zigzag interdigitation of actin filaments (Fig. 1.4a). From a transverse view, the Z-disc appears as a square-lattice array (when passive), but adopts the basket weave structure when actively contracting (Fig. 1.4c) (Goldstein et al., 1988). The width (nm) of the Z-disc is known to vary relative to muscle fibre type, as well as the number of layers of alpha-actinin-2 to F-actin cross-links present, which are referred to as Z-links (Luther, 2009). In fast muscle fibres and a two layer Z-disc, the width of Z-disc is about 30 – 70 nm composed of two alpha-actinin-2 particles which cross-link adjacent actin filaments of opposite polarity; notably in this context, both alpha-actinin-2 molecules in the Z-links are estimated to be at distance of 19 nm apart. Conversely, in slow muscle fibres, the Z-disc is about 100 – 140 nm wide with about 3 - 6 layers of Z-links observable (Fig. 1.4b). Overall, the Z-disc of vertebrate sarcomere is revealed as a tetragonal lattice structure based on thin filaments cross-links via alpha-actinin-2 and other scaffolding proteins like myotilin, CapZ, myopodin, ZASP and filamin-C.
Figure 1.4  Schematic representation of (a) longitudinal Z-links generated by repeating units of two alpha-actinin-2 molecules cross-linking F-actin of opposite polarity (adapted from Sjöblom et al., 2008). (b) different layers of Z-links imaged using electron microscopy from fish body muscle, and (c) the Z-disc in a passive state (square lattice), right, and in the active state (basket weave) right (Luther, 2009).
Lateral anchorage of giant filament systems comprising actin, titin and nebulin at the Z-disc has long made this region to be viewed as a plate-like structure which evolved primarily for sarcomere stability. Interestingly however, this functional view of the Z-disc is fast changing with continual discoveries of new Z-disc proteins capable of playing not only scaffolding role but signalling, mechano-transduction and mechano-sensation functions with mutational dysfunction often leading to fatal disease manifestations (Clark et al., 2002; Sheikh et al., 2007; Frank and Frey, 2011). Thus, the next subsections will be dedicated to the primordial protein components of the Z-disc, their receptive structures, biological interactions and associated myofibrillar Z-disc myopathies.

1.2.2 Core Z-disc proteins and associated diseases

As mentioned in the previous section, the Z-disc is composed of numerous actin binding and bundling proteins, as well as network of scaffolding proteins. Bona fide Z-disc proteins include alpha-actinin-2, myotilin, ZASP/oracle/cypher, myopodin, palladin family, FATZ/calsarcin/myozenin, CapZ, telethonin and filamin-C. A number of other proteins have been co-located to the Z-disc, as well as the Z-disc periphery traversing to the sarcolemma and myotendinous junction (Table 1.1, Fig. 1.5 and Fig 1.6). For the dual purpose of brevity and clarity, the primary Z-disc proteins with their Z-disc diseases are chosen for further description.
Table 1.1  Core protein components of the Z-disc and their associated diseases (adapted from Liang and Nowak, 2005).

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>LOCATION</th>
<th>DISEASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-actinin-2</td>
<td>Z-disc</td>
<td>HCM, DCM</td>
</tr>
<tr>
<td>Capz</td>
<td>Z disc</td>
<td>- -</td>
</tr>
<tr>
<td>Myotilin</td>
<td>Z disc</td>
<td>LGMD1A, MFM</td>
</tr>
<tr>
<td>FATZ 2 (Calsarcin 1, Myozin 2)</td>
<td>Z disc</td>
<td>HCM</td>
</tr>
<tr>
<td>Gamma Filamin (Filamin C)</td>
<td>Z disc</td>
<td>MFM</td>
</tr>
<tr>
<td>ZASP/Cypher</td>
<td>Z disc</td>
<td>MFM (Cardiomyopathy)</td>
</tr>
<tr>
<td>Myopalladin</td>
<td>Z disc</td>
<td>- -</td>
</tr>
<tr>
<td>Myopodin</td>
<td>Z disc</td>
<td>- -</td>
</tr>
<tr>
<td>Bcl2-Associated Anthanogene (BAG3)</td>
<td>Z disc</td>
<td>MFM</td>
</tr>
<tr>
<td>Integrin-Linked Kinase (ILK)</td>
<td>Z disc, Costameres</td>
<td>DCM</td>
</tr>
<tr>
<td>Muscle LIM Protein (MLP)</td>
<td>Z disc, nucleus, intercalating disc, costameres</td>
<td>HCM, DCM</td>
</tr>
<tr>
<td>Titin (Connectin)</td>
<td>Extends from Z disc (N terminus) to M line (C terminus)</td>
<td>FHC</td>
</tr>
<tr>
<td>Nebulin (Nebulatte)</td>
<td>Extends from Z-disc (C terminus) to filament tip (N terminus)</td>
<td>Nemaline Myopathy</td>
</tr>
<tr>
<td>Plectin</td>
<td>Z disc periphery</td>
<td>Muscular Dystrophy</td>
</tr>
<tr>
<td>Telethonin (T-cap)</td>
<td>Z disc, at N terminus of titin</td>
<td>LGMD2G</td>
</tr>
</tbody>
</table>

MFM: myofibrillar myopathy; HCM: hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; FHC: familial hypertrophic cardiomyopathy; LGMD: limb girdle muscular dystrophies.
Figure 1.5  Annotated overview of protein localisation and interaction from the core of sarcomere to the periphery, the costamere and sarcolemma. Protein-protein interactions are indicated as solid lines in the Z-disc (light blue background) and in the structures linking Z-discs to cell-cell and cell-matrix-junctions (light brown background). Dual compartment proteins that can shuttle between a cytosolic and a nuclear localisation are linked by dashed lines. (Image credits: MUZIC-ITN http://www.proteopedia.org/wiki/index.php/Group:MUZIC:Interactome)
Figure 1.6 Cardiac Z-disc cytoskeletal structure associated proteins.

An increasing number of molecules have been identified on or in the vicinity of cardiac Z disc/titin cytoskeleton. Many of these proteins have been linked to intrinsic mechanical sensor/signal modulator functions. MYOZ2, myozenin 2 (carsarin 1); Cn, calcineurin; PDZ-3LIM, one-PDZ and three-LIM domain protein (e.g. ZASP); PDZ-1LIM, one-PDZ and one-LIM domain protein (e.g. ALP); MLP/CRP3, muscle-specific LIM protein/cysteine-rich protein 3; FHL2, four-and-a-half LIM protein 2; MAPRs, muscle ankyrin repeat proteins; MURFs, muscle-specific ring-finger proteins (Figure credits: Hoshijima, 2006).
1.2.3 Alpha-actinin-2: structure and interactions

Alpha-actinins belong to the spectrin family of actin binding proteins which includes spectrin, dystrophin, utrophin and fimbrin. In vertebrates, there are 4 ATCN genes which encode four paralogues of alpha-actinin that have been grouped into 2 distinct classes based on tissue localisation: the muscle-specific and the non-muscle alpha-actinins. Alpha-actinin-2 and -3 are muscle-specific isoforms that are located to the sarcomeric Z-disc, while alpha-actinin-1 and -4 are non-muscle forms that are commonly associated with focal adhesion structures. Alpha-actinin-2 adopts an anti-parallel homodimeric, rod-shaped structure of ~35 nm in length (Fig. 1.7). Each protomer of dimeric alpha-actinin-2 is composed of an N-terminal actin-binding domain (ABD) comprising two calponin homology domains, followed by a centrally located rod-domain containing four tandem spectrin-like repeats, and a carboxy-terminal calmodulin homology domain composed of four EF-hands.

The X-ray crystal structures of the individual domains of alpha-actinin-2 have been determined (Djinovic-Carugo et al. 1999; Ylanne et al. 2001; Franzot et al. 2005). And the full length of human alpha-actinin-2 dimer recently yielded its atomic and envelope structures to Djinovic-Carugo and co-workers in opened and closed conformations (manuscript submitted). Besides F-actin cross-linking and binding to titin Z-repeat-7, myotilin, palladin family, ZASP, FATZ, muscle LIM protein (MLP) and CapZ, alpha-actinin-2 also bind a host of other molecules, for example PIP2 and membrane receptors, which are involved in the localisation and regulation of actin filament structure.
Figure 1.7  Domain organisation in the structure of alpha-actinin.

(a) Upper panel is a schematic description of the domain organisation in striated muscle alpha-actinin-2 annotated with interacting protein partners of the Z-disc.

(b) Shows an electron micrograph image of F-actin binding mode by alpha-actinin; polarity of actin filament is marked with single or triple black arrowheads. Binding of alpha-actinin along a single filament is marked with white arrowheads. Figure adapted from Luther, 2009.

1.2.3.1 Alpha-actinin-2 diseases

A lethal point mutation in alpha-actinin-2 (Q9R) has recently been found in an individual affected by dilated cardiomyopathy (DCM) (Mohapatra et al., 2003), and via linkage analysis the gene for alpha-actinin-2 (ACTN2) has also been implicated in hypertrophic cardiomyopathy (HCM) (Chiu et al., 2010). The alpha-actinin-2 Q9R mutation in DCM particularly disrupts the interaction with Z-disc muscle LIM protein (MLP) affecting cellular differentiation. In the HCM case, a missense A119T was identified in alpha-actinin-2, as well as three further causative mutations, T495M, E583A and E628G (Theis et al., 2006; Chiu et al., 2010).
1.2.4 Filamin-C (FLNc): structure and interactions

Three human filamin gene paralogues are currently known encoding structurally homologues proteins: filamin-A (FLNa 278 kDa), filamin-B (FLNb 280 kDa) and filamin-C (FLNc 290 kDa). While FLNa and FLNb are ubiquitously expressed in several human tissues, FLNc is expressed specifically in striated myofibrils. The description here thus focuses on FLNc. Structurally, FLNc is rod-like protein consisting of amino-terminal actin-binding (ABD)-domain, and two rod regions separated by hinges, made of 24 tandem immunoglobulin-like (Ig) repeats. Human FLNc adopt a v-shaped, parallel dimeric assembly (Fig. 1.8) and it is the only isoform known to have an 82-residue intra-domain insert between Ig-repeats 19 and 20. Self-association into dimer is mediated via the very C-terminal Ig-repeat 24. The 82-amino acid insert is believed to be involved in targeting FLNc to the sarcomeric Z-disc (van der Ven et al., 2000a; Gontier et al., 2000; van der Ven et al., 2000b; Popowicz et al., 2006).

The structures of FLNc ABD-domain, the dimeric domain, and a number of tandem or individual Ig-repeats in the rod regions have been determined recently (reviewed by Djinovic and Carugo 2010).

**Figure 1.8** Schematic illustration of parallel dimer of human filamin C.
Each protomer of filamin C dimer comprises an N-terminal actin binding domain (ABD) followed by two C-terminal rod domains (rod 1 and rod 2) separated by hinge 1 as annotated. The rod regions are composed of tandem array of 24 Ig-like domains. The Ig-domain pairs – Ig-16-17, Ig-18-19 and Ig-20-21 – interpenetrate each other forming a compact region as illustrated. The dimer is mediated by the C-terminal end Ig-24 (Figure adapted from Djinovic-Carugo and Carugo, 2010).
FLNc shuttles between the sarcomeric Z-disc and the sarcolemma with multiple interaction partners at the Z-disc including actin filaments, FATZ protein family, myotilin, myopodin, and Xin; and at the sarcolemma both γ-sarcoglycan and δ-sarcoglycans also binds FLNc. Together with recently characterised disease-mutations, the shuttling of FLNc from the Z-disc to the sarcolemma implicate FLNc in important roles for sarcomere formation and integrity as well as Z-disc mechano-stability and mechano-sensing (van der Ven et al., 2000b).

1.2.4.1 Filaminopathies

Mutations in filamin C (FLNc) results in devastating myofibrillar myopathies termed filaminopathies. Hallmark features of this subset of myofibrillar myopathies (Olivé et al., 2008) includes myofibre dissolution with irregular actin bundles, presence of desmin and focal adhesion protein aggregates, as well as Z-disc streaming with concomitant aggregation of Z-disc proteins including alpha-actinin-2 and myotilin (Shatunov et al., 2009; Luan et al., 2010). Patients exhibiting clinical features of FLNc-related filaminopathies have been found to possess several mutations located to FLNc ABD-domain (Duff et al., 2011) as well as the dimerisation domain (Ig-repeat-24) and the rod repeats (Ig-repeat-7-8) (Vorgerd et al., 2005; Löwe et al., 2007; Kley et al., 2012). The disease phenotype, clinical features and the characteristics of FLNc-related filaminopathies has recently been reviewed (Fürst et al., 2013).
One of the focus proteins of this research is the human Z-disc Alternatively Spliced PDZ-domain containing (ZASP) protein, known also as LIM domain-binding protein 3 (LDB-3). Canonical human isoform of ZASP is approximately 78 kDa in size, made of 727-amino-acid, and located to striated skeletal and cardiac myofibrillar Z-disc. ZASP is also independently identified by other researchers as protein Oracle (Passier et al., 2000) and Cypher (in mouse) (Zhou et al., 1999). ZASP is a core component of the sarcomeric Z-disc and a member of the enigma family of proteins (Wang and Su, 2010).

Like most enigma family members, it has an N-terminal PDZ domain and three C-terminal LIM domains (Fig. 1.9) with three isoforms generated by alternative splicing currently identified in human skeletal muscle (Faulkner et al., 1999). PDZ domains are known to target proteins to sites of complex formation, as such ZASP functions most probably as the oracle of Z-disc multi-protein complexes by tethering and regulating interacting proteins via its PDZ and LIM domains (Passier et al., 2000). This is supported by experimental evidences which shows ZASP is co-expressed with alpha-actinin-2 in the early stage of myofibril and sarcomere formation (Sanger et al., 2002; Rui et al., 2010). In striated myofibril, the PDZ domain of ZASP has been shown to interact with alpha-actinin-2 (Zhou et al., 1999). On the basis of interaction with alpha-actinin-2 class I C-terminal PDZ-binding motif (PBM) with last three residues S-D-L, the PDZ domain of ZASP is categorised as a typical class I interaction module (Au et al., 2004; Bezprozvanny and Maximov, 2001). Recent report on the interaction of ZASP PDZ domain with class III PBMs in myotilin and FATZ family which bears last three residues E-E-L and/or E-D-L (von Nandelstadh et al., 2009), suggests that ZASP PDZ domain has dual capacity (or classification), and possible structural plasticity, as it is able to bind both class I and class III motifs from three different co-located proteins – alpha-actinin-2, myotilin and FATZ family.

In addition, there is increasing evidence that ZASP also performs signaling functions; the LIM domain of Cypher (the mouse orthologue of ZASP) binds and directs protein kinase C (PKC) to the Z-disc, with mutation affecting this interaction (Zhou et al., 1999). Apart from the PDZ and LIM domains, the internal
motif (ZASP-like motif), which is sandwiched between the PDZ and LIM domains and encoded by exon 6 in the ZASP gene, confers complementary interaction capability to ZASP as demonstrated with the spectrin repeats of α-actinin-2 (Klaavuniemi and Yläne, 2006). The ZM-motif of skeletal muscle isoforms of human ZASP has also recently been characterised as a novel actin-binding module (Lin et al., 2014).

Together, these indicate that ZASP plays important roles during myofibrillogenesis which is unlimited to signalling and targeting of multi-protein complexes for a physiologically functional myofibrillar sarcomere (Sheikh et al., 2007; Rui et al., 2010).

**Figure 1.9**  Domain organisation and isoforms of human skeletal muscle ZASP.

Gene transcript of ZASP canonical isoform results in a 78 kDa protein composed of an N-terminal PDZ domain followed by a C-terminal region comprising three LIM domains and an evolutionarily conserved 26-residue central motif called ZASP-like motif (ZM) which is encoded by exon 6. The identified regions of interaction with protein partners co-located in the Z-disc are indicated accordingly. The ZM bearing region has recently been characterised as a novel actin-binding domain in skeletal muscle isoforms and confers complementary interacting capability on ZASP within the Z-disc. Alternative gene splicing in exons 9 and 10 generates three isoforms in human skeletal muscle as illustrated in the lower panel (adapted from Lin et al., 2014). The long isoform, either containing exon 10 (1-isoform) or lacking exon 10 (2-isoform), include the N-terminal PDZ domain, internal ZM and three C-terminal LIM domains. The 2-isoform replaces 1-isoform in postnatal skeletal muscle. The short isoform (3-isoform) has a stop codon in exon 9 and lack the LIM domains. Mutations in the ZM (A165V and A147T) has been reported in affected members in multiple families in the United States and Europe with myofibrillar myopathies referred to as Zaspopathies (Griggs et al., 2007).
1.2.5.1 Zaspopathies

Human muscle diseases resulting from aberration(s) in ZASP gene are described as Zaspopathies (Selcen and Carpén, 2008; Griggs et al., 2007; Selcen and Engel, 2005) Two mutations in the ZM-motif (A165V and A147T) have particularly been associated with dilated cardiomyopathy (DCM) and DCM associated with isolated left ventricular non-compaction of the myocardium (INLVM) (Griggs et al., 2007). ZASP has also been shown to be the major Z-disc component to have O-linked-β-N-acetyl glucosamine (O-GlcNAc) modification, with significant modification in diseased states (Leung et al., 2013). This possibly presents ZASP as a prominent marker of cardiac dysfunction and of diagnostic importance. Furthermore, ZASP ablation in mice was shown to be embryonic or perinatal lethal, most likely due to functional failure in multiple striated muscle types that displayed disorganised and constructed Z-discs in skeletal and cardiac muscle – typical of myofibrillar myopathies (Zhou et al., 2001).

1.2.6 Myotilin subfamily: structure and interactions

The major protein of this thesis research is myotilin – an approximately 57 kDa Z-disc protein expressed poorly in human cardiac muscles and richly in skeletal muscles (Salmikangas et al., 1999). Together with palladin and myopalladin, myotilin is the founding member of a small family of structurally and functionally homologues Z-disc scaffolding proteins that regulate actin organisation and dynamics; reviewed excellently by (Otey et al., 2005). Structurally, the domain architecture of myotilin is organised into a unique serine-rich N-terminal half containing a 23-residue hydrophobic stretch. The C-terminal half comprises two tandem immunoglobulin-like (Ig) domains and a short C-terminal tail with PDZ-binding motif (PBM) (Fig. 1.10).

Myotilin binds G-actin and efficiently cross-links F-actin into stiff, stable bundles (Salmikangas et al., 2003; von Nandelstadh et al., 2005). Myotilin has also been reported to interact with five other co-located Z-disc proteins including alpha-actinin-2, filamin C (FLNc), FATZ/myozenin/Calsarcin family, ZASP and MuRF-1/2 (von Nandelstadh et al., 2011; Gontier et al., 2005; Salmikangas et al., 2003).
These published reports mapped the regions where biological interactions occur on myotilin. Binding to alpha-actinin-2 was mapped onto the N-terminal region while most other biological interactions are mediated via the tandem C-terminal Ig-domains; including actin binding and bundling, FATZ-1 and FLNc binding, and self-association into homo-dimer (van der Ven et al., 2000a; Salmikangas et al., 2003; Gontier et al., 2005). The presence of a class III PDZ-binding motif (PBM) at the C-terminal tail of myotilin permits anchorage to the PDZ domain of ZASP (Fig. 1.10) (von Nandelstadh et al., 2009). Individual structures of myotilin Ig-domains have recently been determined by NMR spectroscopy and deposited in the PDB (Fig. 1.11). The first Ig-domain of myotilin with PDB code 2KDG (Heikkinen et al., 2009) is a clear *I*-set (or h-type) Ig-domain with 9 β-strands, while the second myotilin Ig-domain with PDB code 2KKQ is a *C2*-set (or s-type) Ig-domain consisting of 7 β-strands (Bork et al., 1994; Smith and Xue, 1997; Otey et al., 2009).

*Figure 1.10*  Schematic diagram of domain organisation in myotilin.

Myotilin is composed of a unique, pair-wise serine-rich N-terminal half without any known domain classification. The N-terminus is followed by a C-terminal half comprising two tandem Ig-like domains separated by a short linker. Individual structures of the Ig-domains have been determined to atomic resolution and deposited in PDB with codes: 2KDG and 2KKQ. Annotations in the figure highlight the region where seven (7) disease-associated mutations have been reported, and the domain boundaries important for self-association into dimer as well as interactions with other Z-disc proteins. Color legend: Blue – Ig-2 domain; Sand – Ig-1 domain.
Figure 1.11   Cartoon representations of high resolution NMR structures of myotilin immunoglobulin-like (Ig) domains. The first domain (Ig-1), *sand*, is a clear I-set type with 9 β-strands (A-A’-B-C-C’-D-E-F-G) (PDB ID: 2KDG). The second domain (Ig-2), *blue*, is a typical C2-type Ig-like domain with 7 β-strands (A-B-C-C’-E-F-G) (PDB ID: 2KKQ).
1.2.6.1 Myotilinopathies

Seven (7) distinctive missense mutations in myotilin have been associated with human myofibrillar myopathies, and have collectively been described and characterised as myotilinopathies (Olivé et al., 2005; Selcen and Carpén, 2008; Shalaby et al., 2009). Hallmark feature of myotilinopathies is manifested as an ectopic expression with oxidative stress markers (Janué et al., 2007), together with Z-disc streaming following accumulation of dense, filamentous aggregates containing desmin, filamin C, actin filaments, mutant ubiquitin (UBB+1) and p62 (Olivé et al., 2008) Clinically, myotilinopathies are manifested as progressive muscle diseases with late stage onset.

Of the eight mutations reported in myotilinopathies, only R405K locates to the second Ig-domain of myotilin (Shalaby et al., 2009; Keduka et al., 2012), the other mutations are located on the serine-rich N-terminus; reviewed in (Ruparelia et al., 2012). Studies of the expression pattern of myotilin in developing mouse, and during myofibrillogenesis in cultured cells, suggest a late-stage restriction to skeletal muscle and specific localisation to Z-discs with forced early expression resulting in myofibrillar disruption and misalignment of Z-disc structures (Mologni et al., 2001; Mologni et al., 2005). Muscle development in mouse deficient in myotilin has also been reported to progress without disease phenotype, suggesting myotilin is not overtly important for muscle development.

However, the presence of structural and functional homologues of myotilin (palladin and myopalladin) might serve complementary role in the absence of myotilin (Moza et al., 2007). It is thus reasonable to propose that structural or molecular defects in myotilin might underpin the resulting myotilinopathies as has been recently demonstrated in mutant myotilin which shows drastically reduced degradation by muscle ubiquitin proteasome system (von Nandelstadh et al., 2011).

During myofibrillogenesis however, myotilin has been reported to be dynamically co-expressed with alpha-actinin-2 in Z-bodies (Wang et al., 2005; Wang et al., 2011), as well as during myofibrillar remodeling in delayed-onset muscle soreness (DOMS) induced by eccentric exercise (Carlsson et al., 2007). These events indicate myotilin perform roles critical for stability and maintenance of sarcomeric Z-disc during active muscle stress.
1.3 Project aims and objectives

Recent research findings propose that myotilin performs critical roles for sarcomere stability and the maintenance of Z-disc integrity. Evidence from myotilin up-regulation during myofibrillar remodeling and reduced proteasome targeting upon mutation have also indicated roles critical for sarcomere repair and maintenance (Carlsson et al., 2007; von Nandelstadh et al., 2011). How myotilin accomplishes these roles on a molecular scale is however unknown and a distinct role for myotilin in muscle development remain elusive.

Published reports indicate that myotilin exists in two oligomeric states, the monomer and the dimer, and the assembly of myotilin into homodimer is considered as a requirement for the stabilisation of cross-linked F-actin bundles (Salmikangas et al., 2003). However, what regulates and drives the assembly of myotilin from monomer into dimer is poorly understood. Unclear also is the structural assembly of myotilin dimer, which facilitates F-actin cross-link and permits binding to co-localised protein partners in the Z-disc.

Protein aggregation with concomitant myofibrillar Z-disc dissolution is generally known as the hallmark feature of disease-mutations associated with myotilin and filamin C (Olivé et al., 2005; Fürst et al., 2013). What promotes this phenotype at the biophysical and structural level is also very much obscure.

Since the structure of biological macromolecules and the repertoire of biological interactions they engage underpin their biological functions, it becomes pertinent to structurally and biophysically characterise myotilin in order to decipher the structural mechanisms guiding self-assembly into homodimer and the interaction mechanisms with other Z-disc partners.

The structural data would provide insights into how myotilin is functionally integrated in the Z-disc context. Additionally, an understanding of the pathomechanism of myotilinopathies and filaminopathies caused by recently described mutations in the Ig-domains could be gained.
Therefore, the aims of this thesis research were:

- To characterise myotilin structurally and biophysically in order to gain insight into the mechanisms underlying the dimeric assembly, and understand how myotilin dimer is assembled in the context of the Z-disc.
- To characterise myotilin interactions with ZASP-PDZ domain,
- To further delineate myotilin interactions with F-actin.
- To decipher the structural and biophysical bases of disease mutations in FLNc Ig-domains.
2.0 Materials and Methods

2.1 Molecular cloning of protein constructs: ligase-independent method

N- and C-terminal deletion constructs of myotilin were designed from bioinformatics prediction of ordered myotilin boundaries (Fig. 2.1), that would facilitate structural studies by X-ray crystallography. Specific DNA plasmids covering these boundaries were generated by the ligase independent cloning (LIC) method.

Figure 2.1 Order-disorder boundaries per residue of full length myotilin as predicted using PONDR methods (Romero et al., 2001). 36 % of the full length myotilin is predicted disordered with the first 18-to-232 portion predicted as disordered with average prediction strength of 0.6.
Oligonucleotide primers for DNA amplification were designed using protein management information system (PIMS) implemented at the center of optimisation for structural studies (COSS). The primers used are listed in Table 2.1 and stored in the PIMS database. In brief, N-terminal His6-tagged myotilin constructs covering residues outside the predicted unstructured N-terminus were generated from plasmid bearing DNA insert of full length human myotilin, and subcloned into plasmid vector pETM14-LIC or the pETM11 derivative with 3C- and TEV protease cleavage site to facilitate removal of N-terminal His6-tag. Similarly, PDZ domain of ZASP covering residues 1-85 with an N-terminal GST-His6-tag and TEV protease site was cloned into plasmid vector pETM11 (Novagen). The PDZ domain was also sub-cloned into pETM14-LIC vector for expression of N-terminal His6-tagged PDZ-ZASP bearing 3C-protease cleavage site.
<table>
<thead>
<tr>
<th>constructs</th>
<th>region</th>
<th>forward primer</th>
<th>reverse primer</th>
<th>plasmids vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>myotΔ1</td>
<td>250-444</td>
<td>CCACGTTCATTCAAGTGC</td>
<td>TGGAGCTGCGCTAACGTC</td>
<td>pETM14-LIC/6-His-3C</td>
</tr>
<tr>
<td>myotΔ2</td>
<td>226-452</td>
<td>CTCGAAGTTCTTACATGCA</td>
<td>CTTAGGAAGCTGGAAGAGTTF</td>
<td>pETM14-LIC/6-His-3C</td>
</tr>
<tr>
<td>myotΔ3</td>
<td>234-452</td>
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<td>CAAGTTCCTACATCACA</td>
<td>pETM14-LIC/6-His-3C</td>
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<tr>
<td>myotΔ4</td>
<td>234-491</td>
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<td>CTCAGATTGAGCTGCCAAACGCTG</td>
<td>pETM14-LIC/6-His-3C</td>
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<td>AAGTTTCCTTACATGAG</td>
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<td>AAGTTTCCTTACATGAG</td>
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<td>myot.Ig1</td>
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<tr>
<td>(Ig-2)</td>
<td>349-459</td>
<td>ACAGAG</td>
<td>TGGAGCTGGAAGAGTTG</td>
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<tr>
<td>ZASP-PDZ</td>
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<td>AGG</td>
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<td>myotΔ5</td>
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<td>TGGAGCTGGAAGAGTTG</td>
<td>pETM14-LIC/6-His-3C</td>
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<tr>
<td>(K358E/K359E)</td>
<td>250-498</td>
<td>CTCAGATTGAGCTGCCAAACGCTG</td>
<td>TGGAGCTGGAAGAGTTG</td>
<td>pETM14-LIC/6-His-3C</td>
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<td>myotΔ5</td>
<td>250-498</td>
<td>CCACGTTCATTCAAGTGC</td>
<td>TGGAGCTGGAAGAGTTG</td>
<td>pETM14-LIC/6-His-3C</td>
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<td>(R405K)</td>
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<td>GACCCGACGCGGTTA</td>
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</tbody>
</table>

Table 2.1  
Protein constructs and respective oligonucleotide primers.
2.2 Polymerase chain reaction (PCR)

PCR reaction mixture was set up in total volume of 50 μl. The PCR reaction starts with 25 ng template plasmid DNA, 0.4 μM each of forward and reverse primers (Sigma Aldrich) and 1x Phusion Flash PCR Mastermix (Thermo Scientific). The two-step cycling program for DNA amplification excludes an annealing step (Table 2.2) as the ligase-independent primers were designed with average Tm of 69-72 °C.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Time</th>
<th>Temperature °C</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial melt</td>
<td>5 min</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30 sec</td>
<td>98</td>
<td>25-30</td>
</tr>
<tr>
<td>Elongation</td>
<td>30 - 60 sec*</td>
<td>65-72</td>
<td>25-30</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>2 min</td>
<td>65-72</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td></td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

*depending on the size of the insert or vector to be amplified

2.3 Agarose gel electrophoresis and PCR product purification

Linearised PCR products are separated and analyzed in a 1 % agarose gel electrophoresis using 1x TAE buffer, and subsequently purified using DNA extraction kit (GeneJet Gel Extraction: Thermo Scientific) following manufacturer’s instruction.
2.4 DNA manipulation and annealing

For complementary 3’ overhang required for ligase-independent annealing of PCR amplified DNA into plasmid vector (pETM14-LIC), 20 µl reaction mixture containing 0.2 pmol of the amplified DNA and 300 ng of PCR amplified plasmid vector were sequentially treated with T4 DNA polymerase for 30 mins at 22 °C and 75 °C for 20 mins (Table 2.3). Annealing was subsequently set up at room temperature for 5 – 15 mins and the resulting plasmid product used to transform competent DH5α cells.

Table 2.3 T4 DNA treatment recipe for PCR amplified DNA insert and plasmid vector

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NEB Buffer 2</td>
<td>2</td>
</tr>
<tr>
<td>dATP/dTTP (100 mM)</td>
<td>0.5</td>
</tr>
<tr>
<td>DTT (100 mM)</td>
<td>1</td>
</tr>
<tr>
<td>BSA (10 mg/mL)</td>
<td>0.2</td>
</tr>
<tr>
<td>T4 DNA polymerase (3 Units/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>dH2O added to final volume (20 µl)</td>
<td></td>
</tr>
</tbody>
</table>

2.5 Transformation, plasmid DNA extraction and purification

Competent DH5α cells (50 µl) were mixed with the resulting plasmid and standard heat-shock transformation procedure performed. Fully recovered DH5α cells in SOC medium, incubated for 1 hr at 37 °C were then plated on selective LB agar plates and incubated overnight at 37°C. Selective LB medium was inoculated with a single colony from LB agar plate and grown overnight at 37 °C.

The cells were harvested and commercial kit was used for plasmid extraction and purification following manufacturer’s protocol (MiniPrep Kit, Qiagen). Purified
plasmid DNA concentration was estimated at 260 nm with a NanoDrop (ND-1000) spectrophotometer. The DNA sequence was verified using the services of LGC (Agowa) Genomics or Eurofins MWG Operon (Europe).

2.6 Site directed mutagenesis

Forward and reverse primers encoding a change of tandem Lys358, Lys359 to Glu359, GluE359, and Arg405 and Lys405 were designed to generate mutant variants of myotΔ5 (250-498) construct: myotΔ5 K358E/K359E and myotΔ5R405K. The PCR set-up is similar to the wild-type standard PCR. Following PCR amplification, the template plasmid was completely digested with DpnI (Fermentas) for 1 hour at 37°C and transformed into competent DH5α cells. The cells were plated on selective LB agar plates. Plasmid subsequently extracted from cells, purified and sequenced before transformation into expression host.
2.7 Recombinant protein expression and purification

2.7.1 Expression of protein constructs in *E. coli* strain Rosetta (DE3) pLysS

For the expression of all protein constructs in this study, except where otherwise noted, subsequent to DNA insert sequence verification, plasmids encoding the protein of interest were transformed by standard heat-shock procedure into chemically competent bacterial host of choice - *E. coli* strain Rosetta (DE3) pLysS (Novagen). By inoculating freshly transformed cells into 100 mL Luria-Bertani (LB) media supplemented with selective antibiotic (chloramphenicol: 34 µg/mL, kanamycin 50 µg/mL), preculture was grown overnight at 37 °C in a shaking incubator (180 rpm). Subsequently, 10 mL preculture was used to inoculate 1 L of (LB) media (minimum of 6 L per expression) supplemented with selective antibiotics. Cells were grown further at 37 °C to an optical density of 0.5-0.6 at 600 nm. Over expression of recombinant protein was achieved by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by overnight incubation at 20 °C. Cells were harvested by centrifugation and stored in -80 °C until purification.

2.7.2 Purification of 6xHis-tagged myotilin constructs

Harvested cells were suspended and lysed using French press at 277 K in buffer A containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 % glycerol (supplemented with 10 mM imidazole). Purification of myotilin constructs from supernatant resulting from centrifuge-clarified lysate was achieved in two steps.

Using affinity chromatography on Äkta system FPLC (GE Healthcare), 6xHis-tagged myotilin was first bound to HisTrap column (GE Healthcare) by passing the supernatant through the column. This was followed by one or two-step gradient elution of bound protein using buffer B containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 % glycerol (supplemented with 180 mM imidazole) (Sigma-Aldrich). Eluted fractions in the main peak were pooled and examined on SDS-PAGE. The pooled fraction was dialysed at 4 °C in buffer A without imidazole (supplemented with 0.5 mM DTT). Cleavage of affinity tag overnight was achieved using precision protease at 1:50 mass ratio. Cleaved fraction, uncleaved fraction and protease were separated using GSTrap and HisTrap columns.
In a final step, the cleaved protein was loaded on gel filtration column Superdex 75 16/60 (GE Healthcare) pre-equilibrated with storage buffer containing 20 mM HEPES, pH 7.2, 150 mM NaCl, and 5 % glycerol. Main peak fractions from gel filtration were pooled and purity confirmed on SDS-PAGE. Pure protein was concentrated using Millipore ultrafiltration membrane of appropriate molecular weight cut-off, flash frozen in liquid nitrogen and stored in – 80 °C.

2.7.3 Purification of GST-tagged PDZ domain of ZASP

Frozen cells were suspended in buffer A containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 % glycerol and lysed at 277 K using French press. Purification of tag-free ZASP-PDZ from supernatant resulting from centrifuge-clarified lysate was achieved on Äkta FPLC in three steps. First, affinity capture of tagged-ZASP-PDZ via a glutathione-sepharose HP resin (GE Healthcare), with elution of bound protein using buffer A supplemented with 10 mM reduced L-Glutathione (Sigma-Aldrich). Eluted peak fraction is pooled and examined on SDS-PAGE. Second, cleavage of affinity tags is achieved using recombinant tobacco etch virus (rTEV) protease at 1:50 mass ratio, with subsequent removal of GST-His6-tag and protease via repeated affinity capture step. Third, a final polishing step with size exclusion chromatography (SEC) using prep-grade Superdex 75 16/60 (GE Healthcare) in buffer containing 20 mM HEPES, 150 mM NaCl, 5 % glycerol, pH 7.2. Main peak fractions from SEC are pooled and examined on SDS-PAGE to confirm protein purity. Pooled fraction containing pure protein is concentrated using the 3 kDa cut-off Millipore ultrafiltration membrane.
2.8 Biophysical and biochemical analyses of purified proteins and protein interactions

2.8.1 Protein quantification

The concentrations of purified protein samples were determined by measuring the absorption at 280 nm using NanoDrop spectrophotometer (ThermoFisher Scientific) and calculated according to the Beer-Lambert relation based on the molecular weights (MW) and molar extinction coefficients (ε) of the individual constructs (Table 2.4)

Table 2.4 Respective molecular weight and molar extinction coefficient of protein constructs examined in the studies

<table>
<thead>
<tr>
<th>Protein construct</th>
<th>Residues boundary</th>
<th>Molecular weight from Sequence (kDa)</th>
<th>Extinction Coefficient (M⁻¹. cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>myotΔ1</td>
<td>250-444</td>
<td>22.3</td>
<td>24450</td>
</tr>
<tr>
<td>myotΔ2</td>
<td>220-452</td>
<td>26.5</td>
<td>25940</td>
</tr>
<tr>
<td>myotΔ3</td>
<td>234-452</td>
<td>25.0</td>
<td>25940</td>
</tr>
<tr>
<td>myotΔ5</td>
<td>250-498</td>
<td>28.3</td>
<td>27 430</td>
</tr>
<tr>
<td>myotΔ14</td>
<td>349-490</td>
<td>16.4</td>
<td>17210</td>
</tr>
<tr>
<td>Ig-1</td>
<td>250-347</td>
<td>11.3</td>
<td>8730</td>
</tr>
<tr>
<td>Ig-2 (Δ15)</td>
<td>349-459</td>
<td>12.9</td>
<td>15720</td>
</tr>
<tr>
<td>PDZ-ZASP</td>
<td>349-459</td>
<td>9.3</td>
<td>8480</td>
</tr>
<tr>
<td>myotΔ5 (K358E/K359E)</td>
<td>250-498</td>
<td>28.3</td>
<td>27430</td>
</tr>
</tbody>
</table>
2.8.2 Calibration of gel filtration column (Superdex 75 16/60)

Calibration of size exclusion chromatography column Superdex 75 16/60 (GE Healthcare) was performed by dissolving protein standards (GE Healthcare) of known molecular weight in gel filtration buffer A (20 mM HEPES, pH 7.2, 150 mM NaCl, 5% glycerol). Protein markers (100 µL) were loaded on the column through 0.5 mL loop and fractionated at 0.5 mL/min. The retention volume of protein markers was noted on S75 1660 and used to estimate the relative oligomeric state of purified proteins.

2.8.3 Size exclusion chromatography coupled to multi angle laser light scattering (SEC-MALLS)

Robust analysis of protein oligomeric state via absolute molecular weight analysis was performed using SEC-MALLS. Purified protein samples were injected into analytical Superdex 200 10/300 column (GE Healthcare) mounted on HPLC system (Agilent Technologies) pre-equilibrated with 20 mM HEPES pH 7.2, 150 mM NaCl, 5% glycerol. Aliquots of myotilin constructs myotΔ1 (250-444), myotΔ2 (220-452), and myotΔ5 (250-498) with the mutant variant myotΔ5 K358E/K359E ranging in concentration from approximately 0.04 mM to 4.0 mM were injected. Protein eluting off the gel filtration column was fed into a downstream triple-angle laser light scattering detector (miniDAWN TREOS, Wyatt Technology), connected in tandem to a UV detector at 280 nm (Agilent technologies) and a refractive index detector (RI-101, Shodex). Data were analysed using ASTRA software provided by the manufacturer (Wyatt Technologies), and the absolute weight-average molecular weights measured are reported. For approximation of protein concentration on the column, a dilution factor of 10 (±2) was applied consistent with published value (Podzimek, 2011).
2.8.4 Chemical cross-linking of myotilin

Myotilin dimerisation in solution was detected and captured by chemical cross-linking with Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), a zero-length cross linker that is used to couple close proximity carboxyl groups to reactive primary amines. EDC reaction is optimised by adding the water soluble homobifunctional amine-reactive cross linker – sulfo-N-hydroxysulfosuccinimide (s-NHS). Purified myotilin constructs (myotΔ1, myotΔ3, myotΔ5 and myotΔ14) at protein concentration of 1 mg/mL were cross-linked. Premixed cross linkers was added at a final concentration of 10 mM (EDC) and 2.5 mM (s-NHS), and reaction allowed to proceed for 30 minutes at room temperature before being simultaneously quenched and prepared for SDS-PAGE analysis using 5x SDS sample buffer.

2.8.5 Mass spectrometry analysis of cross-linked myotilin

Monomer and dimer bands from cross-linked myotilin samples were excised and subjected to in-gel-digestion using trypsin for subsequent analysis by tandem HPLC-ESI-MS/MS in an LTQ-Orbitrap XL instrument with data-dependent precursor selection of ions of charge ≥3+ for CID construction. Cross-linked candidate peptides were subsequently retrieved by search of precursor masses which were not identified as linear tryptic peptides (OMSSA database search) but which fitted to the sum of two peptides allowing up to two missed cleavages. Cross-linked candidate peptides identified in the dimer band where sorted into categories by profiling. The chromatograms were manually inspected and checked for overlapping isotope patterns and shifts in retention time. Chromatographic peaks were integrated and the ratio of areas in monomer and dimer measurements (Area (M/D)) was used for identification of dimer-specific cross-link peptides.
2.8.6 Circular dichroism

Circular dichroism (CD) spectra were recorded on Chirascan-plus (Applied Photophysics, UK). Purified myotΔ5 (250-498), variant myotΔ5 (K358E/K359E), the Ig-1 (250-347) and Ig-2 (349-459) were dialysed against buffer containing 0.05 M sodium sulphate, 10 mM potassium phosphate, pH 7.2. The samples at a concentration of 0.5 mg/mL were added to 1 mm quartz cuvettes and CD spectra recorded in the spectral range from 190 nm to 260 nm. To assess protein structural stability, CD spectra between 200 – 260 nm were recorded with thermal unfolding of protein samples from 20 - 85 °C using a continuous temperature ramp (1 °C per minute) and a scan time per point of 0.75 sec with 1 nm step size. Protein melting temperatures (T_m) were deconvoluted using Global 3™ analysis software provided with the CD instrument.

2.8.7 Thermal shift assays

For differential scanning fluorimetry (thermal shift assays), SYPRO Orange dye 40x (Invitrogen) was added to 2-5 µg protein sample in a total volume of 15 - 25 µL. Assays were carried out in an IQ5 real-time PCR detection system (BioRad) set up as previously described (Kley et al., 2012).

2.8.8 Isothermal titration calorimetry (ITC)

Interaction between myotilin and ZASP PDZ domain was quantified on Micro-Cal iTC200 machine (GE Healthcare). Myotilin C-terminal hexapeptide (YESEEL) was synthesized and supplied in 25 mg lyophilized form (PSL, Munich). Experiments were performed at 30 °C in gel filtration buffer. Myotilin peptide (0.4 mM) was titrated in 2 µL steps against purified PDZ-ZASP (0.02 mM). Origin software was subsequently used to fit the interaction isotherms in a single site binding mode.
2.8.9 Micro-scale thermophoresis (MST)

To detect and quantify the dimerisation of myotilin at very dilute concentrations, MST (Jerabek-Willemsen et al., 2011) was performed on an NT.115 Monolith instrument (Nanotemper Technologies, Munich). Measurements were recorded at 25 °C using 20% LED and 40% IR-laser power in 20 mM HEPES pH 7.2, 150 mM NaCl, 5% glycerol, and 0.05% Tween 20. Wild-type myotΔ5 (250-498) was fluorescently labelled with Alexa Fluor 647 following manufacturer’s protocol. Unlabelled myotΔ5 was serially diluted from ~10 nM to 450 μM in the presence of 0.5 μM of the labeled counterpart. Thermophoresis was subsequently fitted according to the law of mass action to determine dissociation constant $K_D$ using Nanotemper analysis software v.1.5.41.

2.8.10 Co-sedimentation assays

Purified rabbit skeletal actin were polymerized into filaments as previously described (Kostan et al., 2009). Purified myotilin constructs, Ig-1 (250-347), Ig-2 (349-459), myotΔ5 (250-498) wild-type and the mutant variant myotΔ5 (K358E/K359E) were dialysed overnight in G-buffer (5 mM Tris, pH 8.0, 0.2 mM CaCl$_2$, 0.5 mM DTT). Pre-assembled actin filaments at fixed concentrations of 3 μM - 5 μM were used accordingly for the binding and bundling assays. In the binding assays, myotilin samples at concentrations (2 μM – 40 μM) were incubated for 20 min with actin filaments in final volume of 40 μL. Reaction mixtures were subsequently centrifuged in a Beckman TL-100 ultracentrifuge mounted with a TLA-45 rotor at maximum speed (125000xg) for 45 min to 1hr at 20 °C. The bundling assays were carried out in 40 μL volumes using myotilin samples accordingly at concentration range from 2 μM – 200 μM, followed by 20 min incubation and centrifugation at 10000xg for 10 min at 20 °C in an Eppendorf centrifuge. Pellets and supernatants were separated and their volumes equalized with SDS sample buffer. Samples were subjected to SDS–PAGE and proteins were visualized by Coomassie brilliant blue staining and quantified with a densitometer.
2.9 Crystallization and structure solution

2.9.1 Crystallization of myotilin

Pre-crystallization test was performed on purified myotilin constructs (myotΔ1-to-myotΔ5), the Ig-1 (250-347) and Ig-2 (349-490) using concentration range of 3 mg/mL to 60 mg/mL following instructions in the PCT kit (Hampton Research, USA). Several commercial crystallization conditions were screened, assisted by a nanodrop-dispensing robot (Phoenix RE; Rigaku Europe, Kent, United Kingdom) on a 96-well plate formats. Initial hits obtained were scaled up and optimised by manually fine-tuning crystallant conditions in a 24-well plate format using the sitting-drop vapour-diffusion technique. Lead conditions were optimised by fine-tuning crystallant concentration and pH over a narrow range, by varying protein concentration as well as addition of additives like trehalose, maltose, sorbitol and sucrose (5% w/v).

2.9.2 Crystallization of PDZ-domain with myotilin C-terminal peptide

Screening for co-crystals of purified ZASP-PDZ domain with the C-terminal hexapeptide of myotilin was carried out at 22 °C in a sitting-drop vapour-diffusion technique using several commercial screens. Multivariate matrix screening was assisted by a nanodrop-dispensing robot (Phoenix RE; Rigaku Europe, Kent, United Kingdom). Initial hits of the co-crystal grown from 15 mg/mL PDZ-ZASP premixed with excess of myotilin peptide appeared as long, thin, plate-like clusters (Fig. 2a), and were obtained from JCSG+ Suite crystal screen containing 2.4 M sodium malonate, pH 7.0. Crystal optimisation was subsequently achieved by manual refining in a hanging-drop vapour diffusion set-up, using molar ratio 1 (ZASP-PDZ)-to-10 (myotilin-YESEEL) followed by micro-seeding and a controlled rate vapour-diffusion by spreading thin film of Al’s oil over the reservoir (D’Arcy et al., 2007; Chayen, 1997). This approach led to diffraction-quality single co-crystals which appeared after one week in drops consisting of 1.9 M sodium malonate, 0.1 M HEPES, pH 7.0 (Fig. 2b), reproducibly grown in 35% PEG 6000, 0.05 M Imidazole, pH 8.0.
2.9.3 Cryoprotection, data collection and processing.

Prior to being flash cooled in liquid nitrogen, the co-crystals were briefly soaked in excess myotilin peptide and then transferred to a solution containing 18% glycerol as cryoprotectant. The diffraction data set was collected at 100 K on the beamline ID23-1 of European Synchrotron Radiation Facility (ESRF), Grenoble, using a wavelength of 0.90 Å. Diffraction data was processed with XDS package (Kabsch, 2010), converted to MTZ file format using POINTLESS and scaled with SCALA from the CCP4 suite (Winn et al., 2011; Evans, 2006). Data-collection statistics are shown in table 1. Data analysis was performed with phenix.xtriage from PHENIX software package (Adams et al., 2010).

2.9.4 Molecular replacement and structure solution

Initial molecular replacement trials using the structure of ZASP PDZ solved by NMR as a search models failed, probably due to the previously described difficulties such as, for example, accuracy of the atomic positions in NMR models (Chen, 2001). The structure was successfully solved by molecular replacement with the program MOLREP (Vagin and Teplyakov, 2010) using the coordinates of CLP-36 (PDLIM1) (pdb accession code: 2pkt) as a search model. The molecular replacement in P21 space group yields a clear solution with 2 PDZ molecules per asymmetric unit. Structure was refined to a final R/Rfree factors 15.03/17.86.
2.10 Nuclear magnetic resonance spectroscopy (NMR)

All NMR experiments were performed with Varian Inova 600 MHz and 800 MHz spectrometers equipped with $^1$H/$^{13}$C/$^{15}$N triple resonance probes at 25 °C. $^{15}$N labelled myotilin samples (myotΔ1 250-444 and myotΔ5 250-498) were prepared by suspending cells at log phase in $^{15}$N-ammonium chloride and antibiotic supplemented minimal media. Cells were allowed to further grow at 37 °C to optical density 0.5 – 0.6, and $^{15}$N-labelled protein overexpression for 4 hours was induced with 0.5 mM IPTG. Labelled proteins were purified following the procedure described for unlabelled protein samples, except in the final gel filtration step where buffer containing 20 mM Na$_2$PO$_4$, pH 6.5, 0.15 mM NaCl, 0.15% (wt/vol) NaN$_3$ was used. Prior to measurement, D$_2$O to a final concentration of 10% (v/v) was added to samples at 1 mM. 1D $^1$H-NMR spectra were obtained using the WATERGATE method for solvent suppression (Liu et al., 1998); $^{15}$N-HSQC were acquired using Rance-Kay gradient enhancement methods (Kay et al., 2011). Spectra were processed with NMRPipe (Delaglio et al., 1995) and analysed with Sparky software (Goddard and Kneller, UCSF). Chemical shifts observed in $^{15}$N-HSQC experiments were quantified as $\sqrt{(\Delta \sigma(\text{^{15}N})^2+25\Delta \sigma(\text{^1H})^2)}$. 
2.11 SAXS analysis of purified myotilin constructs

Small angle X-ray scattering (SAXS) measurements on purified myotΔ1, and myotΔ2 were collected on Pilatus 1M and 2M pixel detectors respectively at X33BioSAXS at DORIS III and P12 BioSAXS beamline at PETRA III (DESY, Hamburg, Germany) (Roessle et al., 2007), (Round et al., 2008). SAXS data for the myotΔ5 construct and the mutant variant myotΔ5 (K358E/K359E) were collected at the BM29 BioSAXS beamline (ESRF, Grenoble, France) (Pernot et al., 2013). Protein concentrations were determined with Nanodrop UV/Vis spectrophotometer at 280 nm immediately before SAXS measurements. SAXS data were recorded for each concentration points (Table 2.5) using sample-to-detector distance of 2.7 m, 2.8 m and 3.1 m at the respective beamlines (X33, P12, BM29), covering momentum transfer s between 0.01 < 0.6 Å⁻¹ at X33 and 0.01 < 0.5 Å⁻¹ at P12 and BM29 (s = 4πsin(θ)/λ, where 2θ is the scattering angle).

2.11.1 SAXS data processing

The data were processed using ATSAS program package ver.2.4 (Petoukhov et al., 2012). Based on comparison of 8 successive frames with 15 s exposures (X33) or 20 successive frames with 1 s exposures (BM29/P12), sample frames free of radiation damage were processed further. Data from the detectors were normalized to the transmitted beam intensity and averaged, and the scattering of buffer solutions were properly subtracted. The scattering curves were normalized by protein concentration and the forward scattering I(0) and radius of gyration (Rg) were determined from Guinier analysis (as well as P(r) function), assuming that at very small angles (s ≤ 1.3/Rg), where the intensity is represented as I(s) = I(0)exp((sRg)2/3).

Rg and I(0), along with the maximum particle dimensions, Dmax, were additionally estimated by the indirect Fourier transform method implemented in the program GNOM (Svergun, 1992). The molecular mass was calculated from excluded volume (Porod’s law) using the program PRIMUS (Konarev et al., 2003). For globular proteins, this hydrated particle volume in Å³ is around twofold the MM in Da (Mertens et al., 2012).
2.11.2 SAXS *ab initio* structure modelling

Envelope structures were determined *ab initio* using the bead-modeling program DAMMIF (Franke and Svergun, 2009). DAMMIF represents the particle as a collection of densely packed beads randomly assigned to solvent (index = 0) or solute (index = 1), and the particle structure in solution is described by a binary string of length M inside an adaptable and loosely constrained search volume compatible with the maximum particle dimension. Disconnected strings of beads are penalized, and the scattering amplitudes are calculated. Simulated annealing is then used to search for a model that minimizes the discrepancy between the experimental and calculated intensities. The results of 10 independent DAMMIF reconstructions were compared using SUPCOMB20 to determine the most representative (typical) model. Averaged DAMMIF models were also determined using DAMAVER for starting model to refine of envelope structures.

2.11.3 SAXS molecular structure modelling

Rigid-body molecular models were generated using the program CORAL (Petoukhov et al., 2012); a rigid-body modeling program that uniquely combines the features of BUNCH and SASREF in reconstructing pseudo-atomic resolution structures from experimental SAXS data (Petoukhov and Svergun, 2013; Petoukhov and Svergun, 2005). Missing loops, linkers or terminal extensions are interconnected and represented as random polypeptide chains. Ten (10) independent CORAL runs were performed.
Table 2.5  Protein concentration series submitted to SAXS measurements.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Protein sample concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
</tr>
<tr>
<td>myotΔ1 (250-444)</td>
<td>0.04 0.16 0.27 0.52 1.50</td>
</tr>
<tr>
<td></td>
<td>1.0 3.5 6.0 11.5 32.0</td>
</tr>
<tr>
<td>myotΔ2 (220-452)*</td>
<td>0.04 0.23 0.42 0.79 1.81 1.96</td>
</tr>
<tr>
<td></td>
<td>1.1 6.1 11.1 21 48 52</td>
</tr>
<tr>
<td>myotΔ5-wt (250-498)</td>
<td>0.02 0.05 0.18 0.35 0.69 1.52 1.86</td>
</tr>
<tr>
<td></td>
<td>0.5 1.42 5.1 10.1 19.5 42.9 52.6</td>
</tr>
<tr>
<td>myotΔ1-mut K358E/K359E</td>
<td>0.02 0.04 0.1 0.18 0.33 0.69 1.48</td>
</tr>
<tr>
<td></td>
<td>0.5 1.1 2.9 5.1 19.5 19.5 41.8</td>
</tr>
</tbody>
</table>

Buffer: 20 mM HEPES, 150 mM NaCl, 5% glycerol, pH 7.2;

*20 mM MES, pH 6.2, 150 mM NaCl, 5% glycerol
3.0 Results

3.1 Constructs design

For recombinant protein expression and purification, plasmids were designed to cover specific sequence boundaries of myotilin outside the predicted disordered N-terminus. N-terminal 6xHis-tag with precision protease cleavage site upstream was incorporated as illustrated in myotilin constructs (Fig. 3.1).

![Schematic Illustrations of Domain Boundaries](image)

**Figure 3.1** Schematic illustrations of the domain boundaries of full length myotilin and the designed constructs. Full length myotilin has two tandem Ig-domains in the C-terminal half region flanked by a largely disordered N-terminal half and a C-terminal extension bearing PDZ-binding motif. All myotilin constructs were designed to be purified via a precision protease (3C) cleavable affinity tag (6xHis-tag) located at the N-terminus. Residue boundaries of key constructs described in this thesis are shown. The red star on myotΔ5 indicates double lysine mutation introduced to generate a mutant variant myotΔ5 (K358E/K359E).
3.2 Purification of myotilin constructs and PDZ-ZASP

Following overexpression of 6xHis-tagged myotilin constructs in *E. coli* strain Rosetta (DE3) pLysS, and two-step purification via affinity capture of tagged-protein by passing crude extract on HisTrap, subsequent tag-cleavage and removal, and a final polishing step on size exclusion chromatography, approximately 18 mg of myotΔ5 (250-498) was purified (Fig 3.2a). Similar protein yield was purified from myotΔ2 (220-452) and myotΔ3 (234-452) constructs, while slightly lower yield was obtained from myotΔ1 construct (Table 3.1) and (Fig. 3.3 a–b). Significant amount of the mutant variant myotΔ5 (K358E/359E) was lost to aggregation (Fig.3.2b), resulting in varied lower yield; however, sufficiently pure fractions for characterisation was recovered after SEC. The retention volume of each myotilin constructs on Superdex S75 16/60 relative to retention volume of protein markers of known molecular weight indicated that all myotilin constructs eluted as monomers. The individual Ig-domains: Ig-1 (250-347) was purified with a yield of approximately 6 mg per litre culture compared to lower yield purified of the Ig-2 (349-459) approximately 2 mg per litre (Fig. 3.5 a–b).

The PDZ domain of ZASP was expressed in same *E. coli* strain. GST-tagged PDZ-ZASP was captured on GSTrap column, followed by protease treatment and final size exclusion chromatography; the PDZ was purified with a yield of approximately 15 mg per litre culture, and eluted in a single peak corresponding to the monomer size (Fig 3.4 b).
Table 3.1  Total amounts of purified protein per litre volume of expression culture following two-step purification: affinity and size exclusion chromatography.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Purified*</th>
</tr>
</thead>
<tbody>
<tr>
<td>myotΔ5 (250-498)</td>
<td>18 mg</td>
</tr>
<tr>
<td>myotΔ3 (234-452)</td>
<td>12 mg</td>
</tr>
<tr>
<td>myotΔ2 (220-452)</td>
<td>20 mg</td>
</tr>
<tr>
<td>myotΔ1 (250-444)</td>
<td>9 mg</td>
</tr>
<tr>
<td>myotΔ5 – mut</td>
<td></td>
</tr>
<tr>
<td>(K358E/K359E)</td>
<td>4 mg</td>
</tr>
<tr>
<td>PDZ-ZASP</td>
<td>15 mg</td>
</tr>
<tr>
<td>Ig-1 (250-347)</td>
<td>6 mg</td>
</tr>
<tr>
<td>Ig-2 (349-459)</td>
<td>1.7 mg</td>
</tr>
</tbody>
</table>

*From 3g wet weight of *E. coli* cell pellet on average
Figure 3.2  Affinity and SEC purification of myoΔ5-wt and the mutant variant.  
(a) Purification of myoΔ5-wt from 5 mL HisTrap column (left panel) followed by overnight cleavage and gel filtration on column S75 1660 (right panel). The retention volumes of protein markers of known molecular weight are shown by black arrows. Wild-type myoΔ5-wt eluted as monomer (~63 mL). Peak fractions corresponding to target protein in both steps were pooled and analysed for purity on SDS-PAGE; the result indicate high quality purification following gel filtration on S75 1660. (b) Mutant myoΔ5 (K358E/K359E) eluted also as monomer (~63 mL) on S75 1660 with copious amounts of aggregate in void volume. Fractions of the mutant in the corresponding peak analysed on SDS-PAGE equally shows successful purification after S75 1660.
Figure 3.3  Affinity and SEC purification of myotΔ1 and myotΔ2 constructs.  
(a) Purification profile of myotΔ1 (250-444) from 5 mL HisTrap column (left panel) followed by overnight cleavage and gel filtration on Superdex 75 1660 column (S75 1660). The myotΔ1 construct eluted as a monomer (~70 mL). SDS-PAGE analysis of factions in the corresponding peaks at each step of purification indicate high quality purification after gel filtration on S75 1660.  
(b) Purification profile of myotΔ1 (250-444) from 5 mL HisTrap column (left panel) followed by overnight cleavage and gel filtration on Superdex 75 1660 column (S75 1660). The myotΔ1 construct eluted as a monomer (~63 mL). SDS-PAGE analysis of factions in the corresponding peaks at each steps indicate high quality purification following gel filtration on S75 1660.
Figure 3.4  
Affinity & SEC purification of myotΔ3 construct & PDZ-ZASP.

(a) Purification of myotΔ3 (234-452) from 5 mL HisTrap column (left panel) followed by overnight cleavage and gel filtration on Superdex 75 1660 column (S75 1660). The myotΔ3 construct eluted as monomer (~ 65 mL). (b) Purification of GST-tagged ZASP-PDZ on GSTrap column (left panel) followed by overnight cleavage with TEV and gel filtration on S75 1660. PDZ eluted as a monomer (~ 85 mL). Peak factions corresponding to target protein in each step was pooled and analysed for purify on SDS-PAGE.
Figure 3.5  
Affinity and SEC purification of individual myotilin Ig-1 & Ig-2.

(a) Purification of 6xHis-tagged myotilin Ig-1 (250-347) on 5 mL HisTrap column (left panel) followed by 3C-protease treatment overnight and gel filtration on S75 1660. Purified Ig-1 construct eluted as a monomer (~77 mL). Peak fractions corresponding to target protein were pooled and analysed for purity on SDS-PAGE. 

(b) Elution profiles of myotilin Ig-2 (349-459) construct following similar procedure described above for Ig-1. On S75 1660, myotilin Ig-2 (349-459) eluted as a monomer (~75 mL). Factions corresponding to protein peak were pooled and analysed for purity on SDS-PAGE.
3.3 Crystallization of myotilin

Pre-crystallization test (PCT) indicated up to 35 mg/mL of myotilin samples (myotΔ1, myotΔ2 and myotΔ5) in storage buffer 20 mM HEPES, pH 7.2, 5% glycerol, 150 mM NaCl can be screened for crystal growth. Crystal hits were initially obtained from several commercial conditions. The most promising of these conditions frequently contained low molecular weight polyethylene glycol (PEG) as precipitant; thus PEG weights ranging from 3350 to 6000 were selected and further optimised. Precipitant concentration and pH range were fine-tuned, and protein concentrations up to 40 mg/mL were screened. Only the myotΔ1 and myotΔ5 yielded reproducible but non-diffacting crystal hits which appeared very tiny and irregular in shape (Fig. 3.6)

(a)

Condition:
15 mg/mL myotΔ5
20% PEG 3350, 0.1 M MES, pH 6.5
5% maltose as additives (32 days)

(b)

Condition:
15 mg/mL myotΔ5
20% PEG 3350, 0.1 M MES, pH 6.5
5% maltose as additives (32 days)

(c)

Condition:
30 mg/mL myotΔ5
20% PEG 6000, 0.1 M HEPES, pH 7.5
5% sorbitol as additives (32 days)

(d)

Condition:
3 mg/mL myotΔ1
20% PEG 3350, 0.1 M MES, pH 6.5
5% sucrose as additives (16 days)

Figure 3.6 Representative crystal hits obtained from myotΔ1 and myotΔ5 constructs. Images in (a – c) shows non-diffracting micro-crystals obtained from myotΔ5 (250-498); the UV images are shown on the right panel in (a) and (b); the hit in (c) was set up manually in 24-well plate format. (d) Shows micro-crystals obtained from myotΔ1 (250-444) with UV image.
3.4 Biophysical characterisation of purified protein constructs

3.4.1 SEC-MALLS analysis of myotilin constructs (Δ1, Δ2 and Δ5)

The three myotilin constructs eluted as monomers on size exclusion chromatography at relatively low protein concentrations. To understand the mechanism underlying reported dimerisation in myotilin, and determine which protein concentration levels would be appropriate for structural studies by small-angle X-ray scattering (SAXS), the Δ1 (250-444), Δ2 (220-452) and Δ5 (250-498) myotilin constructs were examined at increasing protein concentrations on SEC-MALLS. Analysis of protein concentrations up to 67 μM of the Δ1 (250-444) construct showed no preceding peak or peak shift corresponding to the dimer (Fig. 3.7a). Similarly, at high protein concentration of 152 μM, distinct dimer peak was unobservable for the Δ2 (220-452) construct (Figure 3.7b). However, at protein concentrations up to 161 μM of the Δ5 (250-498) construct, an indistinct dimer peak preceding the monomeric peak could be observed (Fig. 3.7c); this construct was further concentrated up to 406 μM where the dimer peak was clearly observed with the weight-average molecular mass calculated across the peak corresponding to twice the monomer size (Fig. 3.7d). Regression fitting of the peak fractions of monomer and dimer at the different concentration points analysed on Δ5 (250-498) suggested a monomer-dimer equilibrium point around 0.5 mM with fully formed dimer at concentrations above 1.2 mM (Figure 3.7e). This information was subsequently useful in sampling concentration series for solution X-ray scattering experiments.
Figure 3.7 SEC-MALLS of myotilin constructs myotΔ1, myotΔ2, and myotΔ5.

Elution profiles of each construct from Superdex 200 10/300 column are shown at their respective on-column protein concentrations with weight-average molecular mass across the peaks calculated by MALLS. Both myotilin constructs myotΔ1 (250-444) (a) and myotΔ2 (220-452) (b) eluted respectively as monomers. (c) Shows elution profile of myotΔ5 (250-498) with indistinct dimer peak observed (d) Subsequent concentration of the Δ5 (250-498) construct revealed a clear dimer peak. (e) Regression fit of the relative amount of monomers and dimers present in each sample of myotΔ5 loaded indicated dimer will be fully formed above 1.2 mM.
3.4.2 CD analysis of individual Ig-domains of myotilin

Myotilin individual Ig-domains (Ig-1 or Ig-2) were prepared to test whether the individual domains bind or cross-link actin filaments. Following expression and purification of recombinant myotilin Ig-domain constructs, circular dichroism was performed to assess domain fold. CD analysis shows purified individual myotilin Ig-1 (250-347) and myotilin Ig-2 (349-459) are properly folded into β-structures as indicated by CD minima at approximately 218 nm (Fig. 3.8).

![CD spectra](image)

**Figure 3.8** Circular dichroism of purified single Ig-domains of myotilin.
CD spectra were recorded on samples of both constructs at 0.5 mg/mL protein concentrations in buffer containing 50 mM sodium sulphate, 10 mM potassium phosphate, pH 7.2. Both constructs displayed CD minima around 218 nm indicating proper fold into β-structure typical of Ig-like domains.
3.4.3 Stability of myotilin dimer: construct myotΔ5 (250-498).

Microscale thermophoresis (MST) assay was performed on myotilin construct Δ5 (250-498) to determine the dissociation constant of the dimer. Microscale thermophoresis affords the quantification of biological interactions at relatively low solute concentration. From the thermophoretic isotherms derived from the assay using concentration range up to 440 µM, apparent dimer stability of 5.8 ± 0.3 µM was determined (Fig. 3.9).

**Figure 3.9** Myotilin dimer stability assessed by microscale thermophoresis.

Upper panel shows raw data recorded when unlabelled myotΔ5 (10 nM – 440 µM) was titrated into fixed concentration of labelled myotΔ5 (0.5 µM). Lower panel shows interaction isotherms from thermophoresis and temperature jump derived from the raw data fitted according to the law of mass action to yield a dimer $K_D$ (5.8± 0.3 µM).
3.5 Proximal residues mapped on myotilin dimer interface

The dimeric capacity of myotilin constructs was assessed further by chemical cross-linking using zero-length cross-linker (EDC), and the dimer interface was determined concomitantly by mass-spectrometry. Visualisation of cross-linked samples on SDS-PAGE following Coomassie staining revealed sufficient yields of dimer bands in the assay with constructs myotΔ5 (250-498), myotΔ3 (234-452) and myotΔ14 (349-490) (Fig. 3.10a-b). However, myotilin construct myotΔ1 (250-444) lacking N- and C-terminal flanking regions yielded no observable dimer bands under the same experimental condition (Fig. 3.10c). Thus, myotilin constructs myotΔ5, myotΔ3 and myotΔ14 were selected for mass-spectrometry analysis to examine the dimer interface residues.

Following tryptic digestion of monomeric and cross-linked dimer bands, the samples were analysed on tandem liquid chromatography-mass spectrometry (LC-MS/MS) instrument. Resulting peptides were filtered, categorised and quantitatively scored. Of the high scoring dimer-specific cross-linked peptides (Table 3.2), two peptide residues (358-KKVLEGDSVKL-368 and 385-NNEMVQFNTDR-395) recurrently featured in all samples analysed with the site of cross-linker located between residues Lys359 on one protomer and Glu387 of the other protomer (Fig. 3.11). Both peptides were mapped on the recently solved atomic structure of myotilin second Ig-like domain (PDB ID: 2KKQ). In myotilin Ig-2 structure, the first peptide resides on βA and βB strands in one protomer, while the second peptide extends from βC through a connecting loop to βD strands of the other protomer that constitute the cross-linked dimer.
**Figure 3.10** SDS-PAGE analysis of cross-linked myotilin samples assayed using zero-length cross-linker EDC and s-NHS.

(a) SDS-PAGE visualisation of cross-linked dimers from myotilin Δ5 (250-498) (left panel) following 30 mins of reaction with 10 mM/2.5 mM (EDC/s-NHS mix) in 0.1 M MES, pH 6.2, 0.15 M NaCl. Significant yield of stably cross-linked dimer was obtained as highlighted in the dotted box. Assay result from construct Δ3 (234-452) (right panel) is shown following same reaction time and condition (b) Shows assay result of construct Δ14 (349-490) comprising myotilin second Ig-like domain with C-terminal extension, under the same reaction condition as a-b (c) Shows cross-linking of equivalent concentrations (0.05 mM) of myotΔ1 (250-444) and myotΔ3 (234-452) assayed under the same experimental conditions without dimer band obtained in myotΔ1. In all assays, controls were prepared by adding equivalent volume of water instead of cross-linker.

Legend: Arrow pointing D signifies cross-linked dimer band migrating at twice the size of the monomer band which is indicated by arrow M.
Table 3.2  Summary of high scoring, dimer-specific cross-linked peptides from mass spectrometry analyses of chemically cross-linked myotilin samples. The peptides map recurrently to the second Ig-like domain of myotilin (Ig-2). Recurrently featured peptides are highlighted in green and grey.

<table>
<thead>
<tr>
<th>constructs</th>
<th>MH+</th>
<th>z</th>
<th>Sequence(^1)</th>
<th>domain</th>
<th>P-value(^2)</th>
<th>Sequence(^1)</th>
<th>domain</th>
<th>P-value(^2)</th>
<th>P-value(^3)</th>
<th>Intensity</th>
<th>Rel. Int.</th>
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<tbody>
<tr>
<td>myotΔ14</td>
<td>2339.1311</td>
<td>3</td>
<td>K.VLEGDSVK.L</td>
<td>Ig-2</td>
<td>6.00E-08</td>
<td>NNEMVQFNTDR</td>
<td>Ig-2</td>
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<td>3.20E-17</td>
<td>2.30E+06</td>
<td>1</td>
</tr>
<tr>
<td>(349-490)</td>
<td>2083.0358</td>
<td>3</td>
<td>.GPAMPFIYKPSK.K</td>
<td>Ig-2</td>
<td>5.60E-03</td>
<td>DVNK</td>
<td>Ig-2</td>
<td>1.90E-02</td>
<td>2.10E-05</td>
<td>2.40E+06</td>
<td>0.8</td>
</tr>
<tr>
<td>myotΔ5</td>
<td>3730.0198</td>
<td>4</td>
<td>K.VSGLPAPDVSWYLNKR.T</td>
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<td>6.60E-03</td>
<td>LDVTARPQNLPAKQR</td>
<td>Ig-2</td>
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<td>1.60E-06</td>
<td>5.10E+06</td>
<td>1</td>
</tr>
<tr>
<td>(250-498)</td>
<td>949.2017</td>
<td>3</td>
<td>R.LDVTARPQNLPAKQR</td>
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<td>Ig-2</td>
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<tr>
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<td>Ig-2</td>
<td>3.80E-04</td>
<td>3.30E-15</td>
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</table>

1) D, E, K signifies site of cross-linker, 2) Subscore per peptide filtered (<5e-2), 3) P-value for cross-linked peptide.
4) Relative intensity from cross-linked band compared to total band analysed.
Figure 3.11  Mass spectrometry profile of x-linked peptide residues identified from the dimer-specific band.

The most abundant pair of cross-linked peptides is highlighted in green and red. Both peptides are located within key structural elements on the second Ig-like domain of myotilin (Ig-2) discussed in the next sections.
3.6 NMR analysis of myotilin dimer interface

NMR $^{15}$N-HSQC spectra were recorded for $^{15}$N-labelled myotΔ1 (250-444) and myotΔ5 (250-498) constructs. When comparing $^{15}$N-HSQC experiments of both constructs, large systematic differences were observed attributable to different respective oligomeric states (Fig. 3.12). In full agreement with this observation, again, the $^{15}$N T2 transverse relaxation times varied by about a factor of 2 when comparing the two myotilin constructs.

![Figure 3.12](image)

**Figure 3.12** Superposition of $^1$H-$^{15}$N HSQC NMR spectra collected for myotilin constructs myotΔ1 (250-444) and myotΔ5 (250-498). Peaks in cyan correspond to monomeric myotilin construct Δ1 (250-444) encoding Ig-1 and Ig-2 in tandem without N- and C-terminal extensions, while purple peaks correspond to the myotilin dimeric construct Δ5 (250-498) encoding the tandem Ig-domains with C-terminus extending to the very end. Peak shift differences were weighted and residues exhibiting the largest shifts on the second Ig-domain were plotted against the sequence (see Fig. 3.13)
Variation in NMR spectra between myotΔ1 250-444 and myotΔ5 250-498 were further analysed. Chemical shifts exhibited by residues on the Ig-2 domain of myotΔ5 (250-498) were examined and the most substantial of these shifts concerned residues Lys359, Glu369, Met388, Leu398, Val406 and Ser424 (Fig. 3.13). Notably, residues Lys359, Glu369 and Met388 map within the dimer-specific cross-linked peptides identified in mass spectrometry analysis of cross-linked myotilin samples, thus corroborating evidence that myotilin dimerisation and interface rests on the second Ig-domain (Ig-2).

**Figure 3.13** NMR chemical shift differences on myotilin Ig-2 domain.
Chemical shift variations from the spectrum of $^{15}$N-labeled myotΔ5 (250-498) plotted against the Ig-2 domain sequence. The spectral shift differences were weighted as $\sqrt{\Delta \sigma(15N)^2 + 25\Delta \sigma(1H)^2}$ and residues Lys359, Glu369, Met388, Leu398, Val406 and Ser424 exhibited substantial variation. These residues were subsequently mapped on the NMR structure of the Ig-2 domain (Fig. 3.14).
**Figure 3.14** NMR shifts mapped on myotilin Ig-2 structure (PDB code 2KKQ).
Cartoon representation of myotilin Ig-2 structure with residues exhibiting NMR peak shifts upon dimerisation highlighted and displayed according to their respective B-factors (cyan_green_red). Residues Lys359, Glu369, Met388, Val406, Ser424 and Leu398 displayed the largest peak shifts as seen in Figure 3.13, and locate within the C2-type Ig β-sheet formed by strands A-B-E-C'-F. Residues Lsy359, Glu369 and Glu387 were identified from mass spectrometry of chemical cross-linked myotilin samples and are represented as balls in magenta.
3.7 Assembly of myotilin Ig-2: pairwise structure-based alignment.

Next, we sought to explore myotilin Ig-2 assembly and the dimer interface at the quaternary structure level. For this, 3-D structure neighbours of myotilin Ig-2 domain (PDB code: 2KKQ) were searched using the DALI server (Holm and Rosenström, 2010). DALI search returned Ig-like domains of obscurin-like 1, titin (Ig-152), palladin (Ig-3), and myomesin as closest structural homologs of myotilin Ig-2. Based on overall structural assembly and similarity scores (RMSD 1.4, Z-score 14), the titin-obscurin-like protein complex structure (PDB code: 3kbn/2wwm) was selected for alignment of myotilin Ig-2 dimer (Fig 3.15). First, myotilin Ig-2 domains were superimposed onto the crystal structure of titin-obscurin-like protein complex using pymol, followed by energy minimization to eliminate structural distortions and clashes using CNS energy-minimization algorithm (Brünger et al., 1998). The Ig-2 dimer was subsequently submitted for interface analysis on PISA server (Krissinel and Henrick, 2007).
Pairwise structured-based alignment of myotilin Ig-2 domains on closest structural homologues using DALI server.

Pairwise structure-based alignment of myotilin Ig-2 to structural homologs (titin and obscurin-like protein 1, PDB code: 3kbn). Conserved residues in the alignment are shown in black boxes (~25%) while structurally similar residues are highlighted black in white background. Structural elements of the C2-type myotilin Ig-2 domain are indicated by back arrows. Alignment was output using clustalW and Esprit 3.0 (Gouet et al., 2003 ; Chenna et al., 2003).
**Figure 3.16** Pairwise structure-based assembly of myotilin dimerisation domain.

Cartoon representation of myotilin Ig-2 assembly computed by pairwise structure-based alignment onto the respective structural homologs shown in Fig 3.15. Dimer-specific pair of residues (Lys359-Glu387) identified by mass spectrometry analysis of EDC-sNHS cross-linked myotilin dimer are represented as balls in magenta. The distance between the Ca- Ca of the cross-linked residues measure 17 Å. Residues exhibiting NMR peak variation upon dimerisation as observed from NMR experiments are highlighted in green.
3.7.1 Analysis of the dimer interface residues.

From PISA analysis (Krissinel and Henrick, 2007) of myotilin Ig-2 dimer, the dimer interface is seen to involve approximately 25% residues per promoter with solvation free energy gain on interface formation indicated by a $\Delta'G$ value of -5.7 kcal/mol (Table 3.3), which indicates hydrophobic interface with a $\Delta'G$ P-value\(^1\) of 0.32 implying that the interface can be interaction-specific. Salt bridges are absent, however 8 residues are observed to additionally engage in network of hydrogen bonds, altogether with an interface area of 886.3 Å\(^2\) (12.5 %) (Table 3.4).

In addition, the interface in the dimer assembly is seen to be dominated by residues ranging from Met350 – Glu387 on βA and βB strands of a protomer, and residues Trp420 – Arg437 on βF and βG strands of the other protomer (Fig 3.16). These residues fall consistently within the dimer interface residues identified from experimental analyses by cross-linking/mass-spectrometry (Fig. 3.11) and NMR (Fig 3.14).

Table 3.3 Summary of PISA analysis on myotilin Ig-2 dimer based on structure-based homology.

<table>
<thead>
<tr>
<th></th>
<th>Solvent accessible area (Å(^2))</th>
<th>Solvation energy (kcal/mol)</th>
<th>Interface structures</th>
<th>Interface $\Delta'G$</th>
<th>Interface P-value</th>
<th>Residues involved</th>
<th>NHB</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>interface</td>
<td>total</td>
<td>isolated</td>
<td>$\Delta'G$</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Subunit 1</td>
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<td>7017.4</td>
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<td>-0.2</td>
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<tr>
<td>Subunit 2</td>
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<td>7133.7</td>
<td>-87.1</td>
<td>-5.5</td>
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<td></td>
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<tr>
<td>Overall</td>
<td>886.63</td>
<td>(12.5%)</td>
<td>-81.3</td>
<td>-5.7</td>
<td>0.323</td>
<td>25</td>
<td>8</td>
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</table>

\(^1\)PDBe PISA defines the parameters as:

NHB, number of hydrogen bonds involved in interface; $\Delta'G$ (kcal/Mol) signifies change of the solvation energy of the structure due to the interface formation and a negative value indicates hydrophobic interface; P-value is a measure of interface specificity, showing how surprising, in energy terms, the interface is. P>0.5 means that the interface is less hydrophobic; P < 0.5 indicates interfaces with surprising hydrophobicity implying that the interface surface can be interaction-specific (Krissinel and Henrick, 2007).
Table 3.4 Hydrogen-bonded residues identified from PISA analysis of myotilin Ig-2 dimer.

<table>
<thead>
<tr>
<th>β-strand</th>
<th>Ig-2 (protomer 1)</th>
<th>Dist. (Å)</th>
<th>Ig-2 (protomer 2)</th>
<th>β-strand</th>
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</thead>
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<tr>
<td>B</td>
<td>K367</td>
<td>2.65</td>
<td>T433</td>
<td>G</td>
</tr>
<tr>
<td>E</td>
<td>R405</td>
<td>2.70</td>
<td>F351</td>
<td>loop A</td>
</tr>
<tr>
<td>loop A</td>
<td>Y353</td>
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<td>S357</td>
<td></td>
</tr>
<tr>
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<tr>
<td></td>
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<tr>
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<td>V431</td>
<td>G</td>
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<td>E</td>
<td>T403</td>
<td>2.81</td>
<td>K354</td>
<td></td>
</tr>
</tbody>
</table>
3.8 Analysis of monomeric double mutant: myotΔ5 (K358E/K359E)

Analysis of peptide residues from mass spectrometry of cross-linked dimer as well as NMR analysis revealed two solvent exposed tandem lysine residues (Lys358 and Lys359) located to βA strand on myotilin Ig-2 structure (Fig. 3.14 and 3.16). The βA strand is a conserved structural element of C2-type Ig-domains (Otey et al., 2009). In addition, the βA strand forms a parallel intramolecular β-sheet with βG strand apparently critical to the overall Ig-fold. To assess the relevance of these lysines in myotilin overall assembly, both residues were mutated to generate a variant of myotilin construct myotΔ5 (K358E/K359E).

3.8.1 Mutation on myotilin Ig-2 domain perturbs structure and reduces stability

MALLS analysis was first performed to assess the difference between purified wild-type and mutant myotΔ5. At equivalent concentrations to the wild-type on SEC-MALLS, the mutant variant eluted mainly as monomers with a broad peak observed where the dimer elutes in the wild-type (Fig. 3.17).

Figure 3.17 Comparative SEC-MALLS of wild-type myotΔ5 (250-498) and the mutant variant (K358E/K359E). Both wild-type and mutant eluted predominately as monomers. However, the mutant exhibited an indistinct peak broadening effect in the region where dimer is observed to elute in the wild-type, suggesting structural difference.
To understand in more detail the effects of this mutation at the secondary structure level, circular dichroism was employed. In a comparative circular dichroism (CD) analysis of the wild-type and the mutant myotΔ5, the mutant displayed reduced ellipticity around 240 nm – 222 nm indicating structural differences (Fig. 3.18).

**Figure 3.18** CD spectroscopy of wild-type myotΔ5 (250-498) and mutant variant myotΔ5 (K358E/K359E). Both samples were measured at ~ 20 µM protein concentration in buffer 50 mM sodium sulphate, 10 mM potassium phosphate, pH 7.2. The wild-type and mutant displayed sharp minima around 218 nm typical for proteins with predominantly beta-structures. However, the mutant variant exhibited significantly reduced ellipticity in comparison to the wild-type between the wavelengths 240 – 222 nm indicating structural changes possibly due to mutation introduced.

Difference in structure between the wild-type and mutant was further examined by assessing their respective stability to thermal unfolding. In CD thermal melt experiment, the melting temperature ($T_m$) of the mutant was determined to 39 °C, considerably lower in comparison to the wild-type $T_m$ of 59 °C (Fig. 3.19a). Differential scanning fluorimetry was additionally performed, and in excellent agreement with the $T_m$ values from CD thermal melt, myotΔ5 mutant displayed reduced structural stability with $T_m$ of 41 °C in comparison to the wild-type $T_m$ of 57 °C (Fig. 3.19b).
Figure 3.19  Stability assays of myoΔ5-wt (250-498) and myoΔ5-mut (K358E/K359E).

(a) CD thermal melt analysis of wild-type and mutant samples measured at 20 µM protein concentration in buffer 50 mM sodium sulphate, 10 mM potassium phosphate, pH 7.2. CD spectra between 200 – 260 nm were recorded with 1 nm band size concomitant with thermal unfolding from 20 °C - 85 °C in a continuous temperature ramp mode (1 °C per minute) and a scan time per point of 0.75 sec. Protein melting temperatures (T_m) were deconvoluted using Global 3™ analysis software provided with the Chirascan-plus equipment. The mutant displayed significantly reduced structural stability with T_m 39 °C (right panel) compared to wild-type T_m 59 °C (left panel). Dotted lines indicate the inflection point where T_m is determined.

(b) Thermal shift assays (Thermoflour) of wild-type myoΔ5 and the mutant variant using 2 µg protein samples and 40x Sypro orange (Invitrogen™). Assay was carried out in protein storage buffer A: 20 mM HEPES, pH 7.2, 150 mM NaCl, 5% glycerol) in triplicate measurement. In excellent agreement with T_m values from CD thermal experiment in (a), the mutant displayed decreased structural stability with T_m average value of 41 °C (right panel) compared to the wild-type with average T_m value of 57 °C (left panel). Black arrows indicate the inflection points on each melting curve where T_m values were estimated.
3.9 SAXS analysis of concentration-driven dimer formation in myotilin

Concentration dependent dimer formation in myotilin was investigated in more details using small angle X-ray scattering which is independent of dilution factors associated with SEC-MALLS analysis described in the section 3.4. SAXS measurements were recorded on myotilin constructs: myotΔ1 (250-444), myotΔ2 (220-452) and myotΔ5 (250-498) accordingly at increasing protein concentrations and structural parameters derived from these experiments are summarized in Table 3.5.

By monitoring the maximum X-ray scattering intensities extrapolated to zero angle \( I(0) \) for each myotilin construct at the respective concentration series, followed by Guinier approximation of radius of gyration \( R_g \) (Å) and the molar mass (kDa) from excluded volume, change in oligomeric state was observed in myotΔ5 (250-498) (Fig. 3.20a-b) and myotΔ2 (220-452) (Fig. 3.21a-b) constructs. Radius of gyration \( R_g \) (Å) and the molecular mass estimated for both constructs also increased with protein concentration and corresponds to the size of a dimer at 1.5 mM and above. Further SAXS analysis of oligomeric mixture using the program OLIGOMER confirmed monomer-dimer equilibrium at protein concentrations of 0.4 mM in Δ5 (250-498), and 0.6 mM in the Δ2 (220-452) (Figs. 3.20c and 3.21c).

Interestingly, at equivalent protein concentrations of 1.45 mM, changes in \( I(0) \) and \( R_g \) (Å) were unobservable for myotΔ1 (250-444) construct (Fig. 3.22); confirming this construct is deficient in dimer formation in full agreement with results from chemical cross-linking assay and NMR analysis (see Fig 3.9c).
Table 3.5  Overall structural parameters from solution X-ray scattering data of myotilin constructs.

### myotΔ5-wt (250-498) (concentrations mM)

<table>
<thead>
<tr>
<th>Structural Parameters</th>
<th>0.02</th>
<th>0.05</th>
<th>0.07</th>
<th>0.18</th>
<th>0.35</th>
<th>0.69</th>
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<td>130</td>
<td>145</td>
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<td>160</td>
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<tr>
<td>Rg - Pr (Å)</td>
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<td>30.2</td>
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<td>34.2</td>
<td>35.5</td>
<td>35.9</td>
<td>38.5</td>
<td>38.6</td>
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<tr>
<td>Rg - Guinier (Å)</td>
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<tr>
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<tr>
<td>Mw (kDa) - Porod</td>
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<td>36.99</td>
<td>44.62</td>
<td>48.15</td>
<td>49.97</td>
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</table>

*Mw (kDa) of monomer from sequence: 28.3 kDa; at highest concentration, myotΔ5-wt forms dimer, consistent with SEC-MALLS analysis (Fig. 3.7)

### myotΔ5-mut (250-498) (concentrations mM)

<table>
<thead>
<tr>
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Pronounced aggregates in the mutant (Grey background); ND = not determined

### myotΔ2 (220-452) (concentrations mM)  myotΔ1 (250-444)

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Mw of myotΔ2 monomer from sequence: 26.5 kDa; myotΔ1 Mw from sequence = 22.3 kDa; at highest concentration, myotΔ2 forms dimer while myotΔ1 remains monomeric.
Figure 3.20  SAXS analysis of myotΔ5 (250-498) construct.

(a) Respective concentration series of purified myotΔ5 exposed to X-rays. Small angle X-ray scattering intensities extrapolate to zero angle, $I(0)$, from the lowest to highest protein concentration of Δ5, points evidently to oligomer formation.

(b) Similarly, sigmoidal increase in radius of gyration ($R_g$, Å) with increasing concentration peaked above 1 mM, indicating structural changes probably due to the increase in dimer population.

(c) Analysis of mixture using program OLIGOMER estimated the relative fractions of monomer and dimer present at each respective concentrations measured. The equilibrium position was determined to ~ 0.4 mM which is in full agreement with the concentration where dimer peak was clearly observed in SEC-MALLS analysis.
Figure 3.21 SAXS analysis of myotΔ2 (220-452) construct.
(a) Respective concentration series of purified myotΔ2 exposed to X-rays. Small angle X-ray scattering intensities extrapolated to zero angle, $I(0)$, from the lowest to highest protein concentration of Δ2, also points evidently to oligomer formation.
(b) Significant increase in radius of gyration ($R_g$, Å) with increasing concentration peaked above 1.8 mM protein concentration consistent with structural transition from monomer to dimer.
(c) Using program OLIGOMER relative amount of monomer and dimer present at each respective concentration measured were estimated. The equilibrium position was determined to ~ 0.6 mM.
**Figure 3.22** SAXS analysis of myotΔ1 (250-444) construct.

(a) Respective concentration series of purified myotΔ1 exposed to X-rays. Small angle X-rays scattering intensities extrapolated to zero angle, I(0), from the lowest to the highest protein concentration of Δ1 exhibited no significant change indicating deficiency in dimer formation by the Δ1 (250-444) construct lacking N- and C-terminal extension.

(b) Consistently, the radius of gyration (Rg Å) remained relatively unchanged with increasing concentration up to ~ 1.5 mM. These data in SAXS are in full agreement with the cues obtained previously from cross-linking and NMR chemical perturbation experiments.

Size and shape descriptors for each myotilin construct were calculated by the inverse Fourier transform of the scattering intensity to account for particle inter-atomic distance distribution P(r), thus to derive the maximum dimension, Dmax (Å) and radius of gyration, Rg (Å) values (Fig. 3.23). In all myotilin samples analysed, the P(r) features two maxima at distances of approximately 25 Å and 50 Å. This is typical of elongated particle with periodic domain arrangements (Petoukhov and Svergun, 2013); where in this case, each maxima corresponds to the first and second Ig-domains of myotilin respectively. Comparison of P(r) of the three constructs at equivalent high concentration shows an increase in the second maxima with concomitant change in maximum dimension (Dmax) for myotΔ2 (220-452) and myotΔ5 (250-498), indicating a central two-domain body in that position.
Figure 3.23   SAXS profiles and inter-atomic distances of myotilin constructs.

(a) For each of the three myotilin constructs at low protein concentrations, experimental SAXS pattern measured at 20 °C is shown (upper panel). The corresponding inverse Fourier transform of the SAXS curve into inter-atomic distances \( P(r) \), and maximum dimensions (\( D_{\text{max}} \) Å) are shown (lower panel). All three myotilin constructs adopt monomeric, extended conformation.

(b) Experimental SAXS pattern is displayed for each myotilin constructs at the highest protein concentrations measured at 20 °C (upper panel). The corresponding inverse Fourier transform of SAXS curves into inter-atomic distances \( P(r) \), and maximum dimensions (\( D_{\text{max}} \) Å) are shown (lower panel). Only myotilin constructs myotΔ2 and myotΔ5 form factor indicated possible elongated, overlapping head-head dimeric conformers; myotΔ1 construct, remains monomeric.
### 3.9.1 SAXS analysis shows mutation on Ig-2 promotes aggregation

Overall structural features of the mutant variant myotΔ5 (K358E/K359E) was also probed by solution SAXS analysis. At equivalent concentrations to the wild-type, X-ray forward scattering intensities $I(0)$, $R_g$ and the $D_{max}$ of the mutant sample points evidently to dramatic aggregate formation (Fig 3.24), and the mutant displayed significant increase in scattering intensities with extremely large $R_g$ clearly attributable to aggregates Fig. 3.24 and see Table 3.6 below.

**Figure 3.24** SAXS analysis of monomeric mutant myotΔ5 (K358E/K359E).
(a) Comparative SAXS intensities of wild-type myot Δ5 (250–498) shown in blue, and the mutant variant (K358E/K359E) shown in red at equivalent protein concentrations. The wild-type and mutant displayed identical intensities at equivalent low protein concentrations of 0.05 mM. Above 0.18 mM, the intensity of the mutant significantly increased with the scattering curve skewed upwards.
(b) A non-linear fit in Guinier plot of the mutant at 0.69 mM indicates aggregation when compared to a linear fit in the wild-type at 1.52 mM.

**Table 3.6** SAXS intensities $I(0)$ and radius of gyration (Guinier) of the wild-type and mutant construct myotΔ5 (250–498).

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<tr>
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3.10  Solution SAXS structures of myotilin

3.10.1  SAXS ab initio envelope structure of myotilin

Following indirect Fourier transform of the experimental SAXS data into real space, molecular envelopes of the three myotilin constructs were restored from their respective experimental SAXS curve at low and high concentrations. *Ab initio* envelope model of myotilin construct myotΔ1 (Fig. 3.25), myotΔ2 (Fig. 3.26) and myotΔ5 (Fig. 3.27) consistently revealed elongated shapes corresponding to monomer.

At high protein concentrations of 1.5 mM, the *ab initio* structure of myotΔ1 construct (250-444) remains monomeric and unchanged with Dmax approximately 95 Å (Fig. 3.25b). In contrast, myotilin constructs with residues extending either to the N-terminus (myotΔ2) and/or the C-terminus (myotΔ5) adopted elongated, overlapping head-head dimeric assemblies with maximum dimensions of 150 Å and 160 Å respectively (Figs. 3.26b and 3.27b).

The overall envelope structures of the dimeric constructs (Δ2 and Δ5) were compared using SUPCOMB20 (Kozin and Svergun, 2001). The output metric of normalized spatial discrepancy (NSD), a measure of quantitative similarity between sets of three-dimensional structures where a value > 1 indicates significant geometric difference, shows both myotilin dimeric constructs are similarly assembled with a NSD value of 0.65. Accordingly, the molecular solution structure of the myotΔ5 which extends C-terminally and possesses the PDZ-binding motif suitable for structural studies of myotilin and ZASP-PDZ complex was refined further.
Figure 3.25 Overall envelope SAXS structures of myotilin Δ1 (250-444).

(a) SAXS envelope structure of myotΔ1 reconstructed using DAMMIF. The structure is rotated and translated to give three different views. At low protein concentration (0.18 mM), myotΔ1 adopts an elongated monomeric structure with $R_g$ of 27 Å and a maximum dimension of ~ 95 Å.

(b) Shows envelope structure reconstructed from high protein concentration of myotΔ1. Consistent with unchanged I(0), and $R_g$, the structure of myotΔ1 remains monomeric; indicating this myotilin construct, lacking N- and/or C-terminal extensions, is deficient in dimer formation.

(C) Superposition of both structures at low and high concentrations using SUPCOMB returned normalized spatial discrepancy (NSD) value of 0.72 indicating significant similarities in geometry. Fits of the respective envelope structures to experimental SAXS data are indicated (see Fig. 3.28 for plots).
Figure 3.26  Overall envelope SAXS structures of myotilin Δ2 (220-452).
(a) Three orientation views of envelope structure of myotΔ2 reconstructed using DAMMIF. At low protein concentration (0.4mM), myotΔ1 adopts an elongated monomeric structure with $R_g$ of 32 Å and a maximum dimension of ~ 120 Å.
(b) Envelope structure of myotΔ2 reconstructed from high protein concentration measurement where stable $R_g$ was observed. Consistent with observed changes in $R_g$, the Δ2 construct adopts a head-head dimeric assembly with corresponding maximum dimension of 150 Å. $R_g$ and fits of the respective envelope structures to experimental SAXS data are indicated (see Fig 3.29).
Figure 3.27  Overall envelope SAXS structures of myotilin Δ5 (250–498).

(a) Three orientation views of envelope structure of myotΔ5 reconstructed using DAMMIF. The Δ5 construct of myotilin extends from the first Ig-domain to the very C-terminal end bearing PDZ-binding motif. At low protein concentration, myotΔ5 adopts an elongated monomeric structure with approximate $R_g$ of 30 Å and a maximum dimension of 120 Å.

(b) Consistent with changes in $R_g$ observed with increasing concentration, and in similar fashion as the Δ2 construct, myotΔ5 construct adopts an overlapping head-head dimeric assembly with maximum dimension of 160 Å.

(c) Superposition of dimeric envelope structures of myotΔ2 and myotΔ5 using SUPCOMB20 indicate similarities in shape with a normalized spatial discrepancy (NSD) value of 0.65.
3.10.2 Molecular solution structure of myotilin

Pseudo-atomic molecular solution structure of myotilin was accordingly reconstructed for the monomeric and dimeric conformers and refined against experimental SAXS data using CORAL (Petoukhov et al., 2013). In this approach, contacts from dimer interface obtained by cross-linking mass spectrometry were specified as constraints between the pre-computed NMR structure of myotilin Ig-1 (PDB ID 2KDG) and the Ig-2 (PDB ID: 2KKQ). Unavailable structures of the N- or C-terminal regions were represented as random polypeptide beads. In keeping with prior structural cues obtained from the ab initio envelopes, the molecular structure at low protein concentration was thus reconstructed for constructs myotΔ1 (250-444) (Fig 3.28) and myotΔ2 (220-452) (Fig. 3.29), and at high protein concentration for the myotΔ5 (250-498) construct (Figs. 3.30 – 3.31).

SAXS structure refinement of myotΔ5 at high protein concentration yielded good solution, revealing an elongated, head-head anti-parallel dimer which is assembled via the second Ig-domain (Fig. 3.31). This anti-parallel dimeric arrangement fits the experimental SAXS data to a chi (χ) value of 1.26. In the Δ5 dimer, myotilin C-terminus is slightly extended in contrast to a loosely compact C-terminus observed in the monomeric myotΔ5 construct (Fig. 3.30).
Figure 3.28  Solution SAXS structure of monomeric myotilin Δ1 (250-444).
Upper panel shows cartoon representation of the molecular structure at protein concentration (0.18 mM) docked into the ab initio envelope model with good agreement. Lower panel shows surface representation of the molecular model and the fits of the envelope and molecular models to experimental SAXS data at are plotted. Domain colors: Ig-2 - blue; Ig-1 – sand; N/C-terminal – green beads, envelope model – lime.
Figure 3.29  Solution SAXS structure of myotilin Δ2 (220-452).
Molecular structure of myotilin construct Δ2 (220-452) monomer at sample concentration of 0.04 mM. Cartoon representation of the molecular model is docked into the envelope model with good agreement (upper panel). Two views of a surface representation are shown in the lower panel. Model fits to experimental scattering data are plotted. Domain colors: Ig-2 - blue; Ig-1 - sand; N/C-terminal – green beads, envelope model – orange.
Figure 3.30  Solution SAXS structure of monomeric myotΔ5 (250-498).
Cartoon representation of monomeric myotΔ5 SAXS structure at 0.05 mM protein concentration docked into the respective *ab initio* envelope model with good agreement (upper panel). Surface representation of the structure is shown in the lower panel. Fits of the respective structures to experimental SAXS data are plotted. Domain colors: Ig-2 - blue; Ig-1 – sand; N/C-terminal – green beads, envelope structures – pale blue.
Figure 3.31  Solution SAXS structure of myotilin dimer: myotΔ5 (250-498).
Anti-parallel dimeric assembly of myotΔ5 at 1.5 mM reconstructed using interface data from cross-linking mass spectrometry on pre-computed, individual Ig-domains of myotilin. Surface representation of the structure is shown in the lower panel and fits of the respective model to experimental data are plotted. Domain colors: Ig-2 - blue; Ig-1 – sand; N/C-terminal – green beads, envelope structures – blue.
3.11 Interaction of myotilin with actin filament

Actin-organising properties of Ig-domains, particularly cytoskeletal Ig-domains in the palladin family of which myotilin is a founding member, have been described (Otey et al., 2009; Dixon et al., 2008; Otey et al., 2005). In a previous analysis of myotilin interaction with actin by yeast hybrid assays (von Nandelstadh et al., 2005), the minimal construct observed to bind actin is a construct encoding Ig-2 to the C-terminal tail of myotilin (349-498). To delineate the region of myotilin sufficient for direct F-actin interaction, we prepared myotilin construct myotΔ1 (250-444) encoding the Ig-domains of myotilin in tandem without N-terminal or C-terminal extension, and we tested whether it could bind to F-actin. By F-actin co-sedimentation assay, we observed binding of this myotilin fragment to F-actin indicating either Ig-1 or Ig-2 of myotilin mediate the binding interaction observed (Fig 3.32a). Subsequently, F-actin cross-linking assay was performed with this construct to assess F-actin bundling activity. In the bundling assay at low speed, F-actin co-pelleted with myotΔ1 construct at 0.5:1 molar ratio, in contrast to control experiments where F-actin was not found in the pellet (Fig. 3.32b). This indicated that the Ig-domains of myotilin, in tandem arrangement, constitute two independent actin binding sites; motivating further investigation to dissect the interaction with F-actin by individual Ig-domains of myotilin.
Figure 3.32  Myotilin construct myotΔ1 (250-444) binds and bundle F-actin.
Following co-sedimentation assay of fixed F-actin (10 µM) pre-incubated with increasing concentrations of myotΔ1 (250-444), supernatant (S) and pellet (P) fractions were equalized and analysed by SDS-PAGE with Coomassie staining. (a) Myotilin construct Δ1 (250-444) co-pelleted with F-actin indicating binding activity. (b) This construct also showed F-actin bundling activity as indicated by an enrichment of F-actin in the pellet fraction, whereas in control experiment F-actin was not found in the pellet; indicating that the Ig-domains of myotilin, in tandem arrangement, constitute two independent actin binding modules. Protein amounts in the pellet and supernatant were quantified by densitometric analysis of gel bands.
3.11.1 Assessment of F-actin binding activity: Ig-1 and Ig-2

The capacity of single Ig-domains of myotilin to bind F-actin were first assessed by incubating myotilin constructs Ig-1 (250-347) and Ig-2 (349-459) at increasing concentrations (3 - 18 µM) with fixed amount of pre-assembled actin-filaments (3 µM). Following centrifugation at high speed (100000xg), supernatant and pellet fractions were equalized and analysed by SDS-PAGE using Coomassie Brilliant Blue staining. Both myotilin Ig-1 and the Ig-2 co-pelleted with F-actin to a similar extent as assessed by densitometric analysis of gel bands (Fig. 3.33). In detail, increasing concentration of either myotilin Ig-domains led to their respective enrichment in pellet fraction with F-actin, while in contrast, both myotilin Ig-domains were absent in pellet fraction when incubated alone at the highest concentration assessed. All together this indicates that the individual Ig-domains of myotilin indeed constitute independent F-actin binding modules.

![Figure 3.33](image_url)

**Figure 3.33** F-actin binding by individual myotilin Ig-2 and Ig-1 domain.
SDS-PAGE analysis of supernatant (S) and pellet (P) fractions following high-speed centrifugation (100000xg). Both myotilin Ig-domains co-pelleted with F-actin with increasing concentration (3 – 18 µm). The bound fractions, quantified by densitometric analysis of protein bands on gel and plotted for comparison, show both myotilin Ig-domains bind F-actin.
Assessment of F-actin bundling activity: myotilin Ig-1 and Ig-2

F-actin bundling capacity of single Ig-domains of myotilin was assessed using 5 µM of pre-assembled F-actin with increasing concentrations of myotilin Ig-1 and myotilin Ig-2 (up to 30 µM), under low centrifugation speed (10000xg) for 10 mins. Visualisation of F-actin fractions in the supernatant and pellet by SDS-PAGE shows myotilin Ig-1 and Ig-2 domain lack F-actin bundling capacity (Fig. 3.35). Densitometric quantification of F-actin fraction in pellet indicated the presence of some F-actin in the pellet at higher concentration of myotilin Ig-2 domain; that is, actin to Ig-2 molar ratio above 1:4, which cannot be conclusively interpreted as bundling.

**Figure 3.35** Individual myotilin Ig-1 and Ig-2 domain lack F-actin bundling capacity.

Low speed centrifugation (10000xg) of fixed actin (5 µM) with increasing concentration of individual myotilin Ig-1 and Ig-2 domain (2.5 – 30 µM), followed by SDS-PAGE analysis of supernatant (S) and pellet (P) fraction with Coomassie stain shows Ig-1 and Ig-2 domain of myotilin lack F-actin bundling capacity (upper panel). Densitometric quantification of F-actin amounts in pellet shows some amount of F-actin in the presence of high concentration of myotilin Ig-2.
3.11.3 Comparison of actin-binding sites in palladin Ig-3 and Ig-domains of myotilin.

The recent determination of NMR structure of Ig-3 domain of palladin, a member of myotilin subfamily, and further description of its actin-binding properties (Beck et al., 2013) led us to ask and investigate whether the individual Ig-domains of myotilin (Ig-1 and Ig-2) share similar surface patches that constitute the F-actin binding sites on palladin Ig-3 (PDB code: 2LQR). Structure-based pairwise alignment using DALI server (Holm and Rosenström, 2010) shows that myotilin Ig-2 domain shares similar surface residues with palladin Ig-3 summarized in Table 3.7 and depicted in Fig. 3.34.

![Diagram showing structural alignment of myotilin Ig-1 and Ig-2 on palladin Ig-3.](image)

**Figure 3.34** Structural alignment of myotilin Ig-1 and Ig-2 on palladin Ig-3.
NMR structures of myotilin Ig-2 (blue/2KKQ) and myotilin Ig-1 (sand/2KDG) were aligned on palladin Ig-3 domain structure (red/2LQR). The residues (K15/K18/K51) shown in stick constitute the binding sites of actin by palladin Ig-3. Equivalent residues on myotilin Ig-domains are also shown in stick and highlighted. Myotilin residue K359 on the Ig-2 domain is conserved with residue K18 on one of palladin’s actin-binding sites.
Table 3.7  Analysis of structural equivalent residues on myotilin Ig-2 and Ig-1 by pairwise comparison with the residues that constitute the two actin-binding sites on palladin Ig-3 structure. Residues K15/K18/K51 were identified by (Beck et. al., 2013), to be important for actin bundling by palladin Ig-3. Myotilin Ig-2 has one conserved, equivalent residue on one of the proposed palladin actin-binding sites (highlighted in blue); whereas no conserved or equivalent residues were observed on myotilin Ig-1. Moreover, the Ig-3 of palladin contains lysine-rich motif which is equivalent to the consensus sequence (K-L/K/R-K) in many actin-binding proteins. Similar lysine-rich sequence was identified on myotilin Ig-2 domain in the actin-binding region of palladin Ig-3, thus suggesting myotilin Ig-2 might bundle F-actin in a similar fashion as palladin Ig-3.

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Lysine-rich consensus motif (ABD-3)  Structure-based equivalence  Structure-based equivalence

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3.11.4  F-actin binding activity: wild-type and mutant myotΔ5

In order to assess whether the mutation introduced on Ig-2 of myotilin construct myotΔ5 (250-498) would have deleterious effects on F-actin binding, cosedimentation was performed using wild-type myotΔ5 (250-498) and the variant bearing the double lysine mutation myotΔ5 (K358E/K359E). Assay performed in concentration series shows the wild-type construct binds F-actin and saturates at 1:1 molar ratio consistent with previous published reports (Salmikangas et al., 2003). Regression analysis estimated the binding affinity to a $K_D$ value of 1.74 ± 0.24 µM (Fig. 3.36). The mutant variant Δ5 (K358E/K359E) displayed similar F-actin binding affinity with $K_D$ value of 2.00 ± 0.79 µM (Fig. 3.36b). Interestingly, K359E mutation is on the conserved actin-binding patches identified when compared to Ig-3 of palladin (Table 3.7). However, it is unclear from this assay whether the lysine residues are critical for actin binding, as the binding exhibited by this mutant construct could also be mediated by the presence of myotilin Ig-1.
Figure 3.36  F-actin binding by myotΔ5 (250-498) wild-type and mutant.

Supernatant (S) and pellet (P) fractions of (a) wild-type myotΔ5 and (b) the mutant variant binding to F-actin were analyzed by SDS-PAGE with Coomassie staining. Both wild-type and mutant bind F-actin to similar extent (c) Regression analysis for the binding affinities following densitometric quantitation of the amount of wild-type and mutant co-pelleted with F-actin. This indicated the wild-type and mutant bind F-actin with similar affinity.
### 3.11.5 F-actin bundling activity: wild-type and mutant myotΔ5

F-actin bundling capacity of the wild-type and the mutant myotΔ5 (250-498) constructs were assessed to investigate whether the mutation affects the capability of myotilin to cross-link F-actin. Co-sedimentation assays at low speed shows the wild-type construct bundles F-actin with increasing concentration, whereas the mutant construct displayed haphazard F-actin bundling pattern (Fig. 3.37) At low concentration, the mutant was found in the pellet in high amounts which is attributable to its propensity to form aggregate. Thus, wild-type myotΔ5 (250-498) retains capability to bundle F-actin, and the aggregate promoting mutation on Ig-2 domain causes haphazard bundling of F-actin.

![Bundling assays: wild-type and mutant myotΔ5 (250-498).](image)

**Figure 3.37** Bundling assays: wild-type and mutant myotΔ5 (250-498).

Upper panel shows SDS-PAGE result of the assay with myotΔ5 wild-type and the mutant variant Δ5-mutant (K358E/K359E). Densitometric analysis of gel bands shows wild-type bundles F-actin with increasing concentration. In contrast, the mutant displayed haphazard F-actin bundling attributable to aggregation propensity.
4.0 Discussion

4.1 Myotilin dimerizes in a concentration-driven manner

Previous *in vivo* studies reported myotilin as capable of self-association into homodimer (Salmikangas et al., 1999; Hauser et al., 2000). And the co-existence of two oligomeric species was subsequently demonstrated where the minimal region required for dimer formation maps roughly to the C-terminal of myotilin comprising the tandem Ig-domains together with either the N- and/or the C-terminal flanking regions (Salmikangas et al., 2003; von Nandelstadh et al., 2005). In our characterisation of myotilin constructs by light scattering and X-ray scattering in solution, we observed a striking concentration-dependence of dimer formation. The concentration-driven dimerisation was observable in myotilin constructs bearing either the N-terminal extension (myotΔ2 220-452) or the C-terminal extension (myotΔ5 250-498), but unobservable in the construct bearing only the tandem Ig-domains myotΔ1 (250-444) which does not dimerise. This indicates that the N- and/or the C-terminal flanking regions of myotilin are necessary for dimer formation, and the Ig-domain tandem without terminal extensions is deficient in dimer recruitment. SAXS analysis of $R_g$ and $I(0)$ with increasing concentration in the constructs myotΔ2 (220-452) and myotΔ5 (250-498) shows that the dimer is fully formed at an effective concentration above 1.5 mM (~40 mg/mL), which is a relevant cellular concentration (Wang et al., 2009). Further SAXS analysis indicated the equilibrium mixture of dimer and monomer exist at approximately 0.5 mM, consistent with the concentration where dimer elution begins to appear for myotΔ5 in size exclusion chromatography (SEC).

The confinement of macromolecules within cellular structures often results in significant alterations to macromolecular assembly and reactivity due to high concentrations of local macromolecules present (the so-called “macromolecular confinement and crowding” (Minton, 1992; Minton, 1995). Likewise, macromolecular crowding and protein concentration levels in cells are an intrinsic property and ubiquitous feature which could alter the differential behavior of proteins in different cellular context and in comparison to *in vitro* settings (Minton, 2001; Minton, 2006).
The Z-disc can thus be viewed as a cellular confinement in the muscle sarcomere where total local interacting protein concentrations can reach 400 mg/mL (Uversky et al., 2002; Fulton, 1982). And since cellular confinement and macromolecular crowding can dictate protein interactions and structural assembly, the concentration dependent formation of myotilin dimer revealed in vitro by SAXS analysis is a reminiscence of this physiological phenomenon. Thus, our findings on the concentration-driven dimerisation in myotilin could serve as the mechanism regulating how myotilin performs its role of contributing stability and maintenance of the sarcomeric Z-disc.

This insight is supported by the fact that protein dimerisation provides distinct molecular advantages in several biological events, as excellently reviewed (Marianayagam et al., 2004). Moreover, three key examples stemming from cellular studies of myotilin have further highlighted myotilin expression levels and dynamics in the cell. First, the expression of myotilin in cultured differentiating myocytes as well as studies in embryonic mouse development have shown myotilin to be strictly confined to the latter stage of myofibrillogenesis, and early overexpression of myotilin in cells during this event leads to irregular F-actin bundles (Salmikangas et al., 2003; Mologni et al., 2001). The expression levels of myotilin were noted to increase steadily as myofibril formation progresses, reaching a peak on maturation of myofibrils, and decreasing in other tissues except cardiac and skeletal muscles in mouse. This implies thus that local concentration of myotilin changes dynamically in other to accommodate different cellular requirements like cytoskeletal organisation and tissue remodeling during somatic differentiation. This observation is supported in a second but different cellular context, where markedly increased amounts of myotilin was observed to be important during myofibrillar remodeling following exercise-induced delayed onset muscle soreness (DOMS) (Carlsson et al., 2007); and thirdly, myotilin in Z-bodies of developing skeletal myotubes has been shown to undergo fast dynamic exchange with cytoplasmic pool when compared to a less mobile myotilin in the confinement of mature sarcomeric Z-band (Wang et al., 2005).

Remarkably, in all these cellular events, up-regulation and down-regulation of myotilin concentration appears to be common. Considering our SAXS data in the light of these aforementioned cellular events, where we see evidently that myotilin
The dimer is only fully formed at physiologically relevant high concentrations (~40 mg/mL), thus one could reasonably propose that at low concentrations of myotilin, for example in the early stages of myofibrillogenesis where stress-fibres and the I-Z-I structures form (Ojima et al., 1999; Wang et al., 2005), the prevalence of myotilin monomer selects formation of low order myotilin-complexes, for example between myotilin and one or two of the Z-disc partners co-expressed early (alpha-actinin-2, ZASP and F-actin) as depicted in Figure 4.1. However, upon maturation of myofibril and as the workload is increased in mature Z-disc under active tension during eccentric exercise or during myofibrillar remodeling, an up-regulation of myotilin expression leads to sufficient local amounts leading to effective dimerisation; and dimerisation in turn would provide more molecular interfaces for higher order myotilin Z-disc protein complex assembly (Fig 4.2).

4.2 Myotilin dimer adopts an elongated, antiparallel molecular assembly.

Molecular SAXS structure of myotilin dimer (construct myotΔ5 250-498) derived ab initio and subsequently refined using combined data from chemical cross-linking/mass spectrometry, NMR and structural bioinformatics revealed a head-to-head antiparallel assembly with maximum dimension of approximately 160 Å. The dimer interface lies on the second Ig-domain and is mediated by β-structure elements conserved among many other sarcomeric proteins (Pinotsis et al., 2009). The dimerisation mode of myotilin C2-type Ig-2 protomers generated by structural alignment on the homologous I-set Ig-domains of titin and obscurin-like 1 in the complex form compares well (Fig. 3.16). PISA analysis revealed a hydrophobic interface with specificity (P-value) of 0.32 for myotilin dimer. The titin:obscurin-like 1 complex also display a P-value of 0.32. The titin:obscurin-like 1 complex interface span an area of 669 Å² (12.4%) involving 21 residues per protomer with 12 and 7 residues in hydrogen bond and salt bridges respectively. In contrast, myotilin dimer interface involves 25 residues per protomer with interface area of 886 Å² (12.5%) and 8 residues in hydrogen bonds (table 3.3 and 3.4).

Experimentally determined interface residues by chemical cross-linking combined with mass spectrometry and NMR analysis fall on β-structure elements involved in the interface (Fig. 3.16). The first set of residues was maps on the loop extending into βA and βB strands in one subunit, while the second set of residues maps onto
the βF and βG strands of the other subunit in the assembly. These structural elements constitute the canonical β-sheet network identified in most Ig-domains that mediate protein-protein interactions or self-assembly, and have been proposed as conserved structural elements of C2-set and I-set Ig-domains found in several sarcomeric proteins (Otey et al., 2009; Pinotsis et al., 2009).

Figure 4.1 Actin filaments are assembled in two general types of structures: first, the networks (for example at edges of developing myotubes or stress fibres and focal contacts in sarcolemma or non-muscle cells); second, in registered array of bundles (for example in mature striated myofibrils) (Cooper, 2000b). Illustrated here is a simplistic and reductionist schema of the proposed model of F-actin cross-links in network structures focusing on three of the dynamic proteins observed in premyofibrillar Z-bodies of developing skeletal muscle (Wang et al., 2005): alpha-actinin-2 dimer, ZASP and myotilin; myotilin is presented here in the monomeric form which binds actin via the two tandem Ig-domains as demonstrated in our co-sedimentation experiment. Our in vitro SAXS analysis shows myotilin monomer exists at low local protein concentration, and dimer formation is regulated by increased local total concentration. The dimer has previously been shown to be relevant for contributing strength and rigidity to the force transmission scaffold in the Z-disc of skeletal muscle (Salmikangas et al., 2003) discussed next in Fig 4.2. In the schema above, the N-terminus of myotilin is shown to bind the principal actin cross-linking protein alpha-actinin-2 (Salmikangas et al., 1999), while myotilin C-terminus binds the PDZ domain of ZASP (von Nandelstadh et al., 2009). ZASP in turn connects actin and alpha-actinin-2 via the ZM-motif and
PDZ domain respectively (Klaavuniemi and Ylänne, 2006; Lin et al., 2014); all together, forming stable actin scaffold of early stage premyofibrillar Z-bodies, believed to precede alignment of I-Z-I structures into nascent myofibrils and mature myofibril during myofibrillogenesis (Sanger et al., 2005)

The antiparallel architecture of myotilin dimer as revealed in our SAXS structure provokes key biological insights on how myotilin engages the multiple partners co-located in the Z-disc. One of such plausible insights is in the ‘Z-links’ of F-actin mediated by two molecules of alpha-actinin-2 as revealed in a recent analysis of sarcomeric Z-disc structure by electron microscopy (Luther, 2009). In the Z-links of F-actin, the two alpha-actinin-2 molecules are notably separated by approximately 190 Å in dimension. Interestingly, the dimension of antiparallel myotilin dimer (Δ5 250-498) in our SAXS structure measures approximately 160 Å in Dmax; and since myotilin has been previously reported to bind alpha-actinin-2 via its N-terminal half (Salmikangas et al., 2003), an antiparallel assembly of myotilin via the Ig-2 domain as revealed in our SAXS structure becomes insightful as this would provide two opposing N-terminal interfaces to bridge the gap between two molecules of alpha-actinin-2 in the Z-links, thereby contributing rigor and stability to the pre-assembled filament (see Fig. 4.2).
Figure 4.2 Schematic illustrations highlighting the distinct biological relevance of myotilin in an antiparallel dimeric structure within the context of the sarcomeric compartment – Z-disc. High resolution structures of single myotilin Ig-1 and Ig-2 are currently known. The Z-disc of mature sarcomere can be viewed as a compartment in muscle cells with crowded milieu of proteins of high local protein concentration which favours multiple protein interactions as well as myotilin self-association into dimer. The dimeric assembly of myotilin is revealed in our SAXS analysis (section 3.10) and depicted above. Here, F-actin of opposing polarity from adjoining sarcomeres are stably cross-linked by the principal Z-disc protein, alpha-actinin-2; myotilin is shown here in the antiparallel dimeric structure with the opposing N-termini (structure yet unknown) anchored to alpha-actinin-2, while the C-termini bind the PDZ domain of ZASP. This dimeric assembly, permitting multiple protein interactions contribute stability and rigidity to the overall F-actin scaffold in the Z-disc as experimentally demonstrated in the presence of F-actin severing agent - latrunculin A (Salmikangas et al., 2003).
4.3  Mutation on myotilin Ig-2 domain perturbs structure and promotes aggregation.

In the mass spectrometry analysis of cross-linked myotilin dimer as well as NMR analysis, we identified the residues involved in the interface. The residues fall on structural elements including β-strands (A, B, G and F), and the hinge-like loop connecting βC to βD. The tandem lysines (Lys358/Lys359) identified in the dimer specific interface residues map on βA strand. To assess the relevance of these residues in the overall dimer assembly, both lysines were mutated to glutamates in the construct myotΔ5 generating a variant myotΔ5 (K358E/K359E). The mutation remarkably translated into structural disturbance with reduced protein stability as evident reflected in the unfolding temperature of the mutant which was 18 °C lower than the wild-type. The mutant also displayed large Rg attributable to aggregate formation in SAXS analysis (Fig. 3.24b). With recently reported disease-mutation (R405K) on the Ig-2 domain of myotilin which is characterised by defective dimerisation and aggregation propensity (Shalaby et al., 2009), our biophysical analysis and F-actin co-sedimentation assay of the mutant myotΔ5 (K358E/K359E) which bears double lysine mutation on the Ig-2 domain, shows how important the Ig-2 domain is to the overall biological integrity of myotilin.

4.4  Isolated Ig-like domains of myotilin independently bind F-actin.

Previous studies have demonstrated myotilin is capable of independent direct binding and bundling of F-actin (Salmikangas et al., 2003). The yeast two hybrid screens showed that the minimal construct of myotilin capable of direct actin binding is the Ig-2 domain extending to the very C-terminal end (i.e. 349-498) (von Nandelstadh et al., 2005). We further assessed the regions of myotilin capable of mediating direct F-actin interactions in order to delineate the minimal binding regions and dissect the modes of interaction. In our F-actin co-sedimentation assays with single Ig-domains of myotilin - Ig-1 (250-347) and Ig-2 (349-459) - we observed independent F-actin binding capacity in both constructs.

Analysis of surface patches on both myotilin Ig-domains in comparison to recently described homologous structure of palladin Ig-3, which bundles F-actin via two sites (Beck et al., 2013) revealed only a site on myotilin Ig-2 is conserved for F-actin binding (Fig. 3.34). Overall, our F-actin interaction assays indicate that the Ig-
domains of myotilin constitute two independent F-actin binding modules, and mere isolated myotilin Ig-domains are insufficient for cross-linking F-actin into bundles. Notably, both Ig-domain constructs bind F-actin similarly, implying that myotilin Ig-1 or Ig-2 could separately bind to a helical F-actin. Moreover, F-actin bundling was accomplished when myotilin Ig-domains are in tandem as noted in our experiment with the construct myotΔ1 (250-444). Consistent with previous published reports, the dimeric construct myotΔ5 (250-498) exhibited F-actin bundling with increasing concentration, which supports the notion that myotilin contributes significant stability to F-actin-alpha-actinin-2 complex in the dimeric state (Salmikangas et al., 2003).
5.0 Conclusions

Hitherto, experimental evidence has been lacking on the structure of myotilin dimer, and the mechanisms underlying dimer formation is unknown. In this thesis research, and for the first time, we have revealed the dimeric structure of myotilin and the mechanism driving the assembly using combinatory approaches of structural, biophysical and biochemical techniques. Our SAXS and biophysical characterisation of myotilin evidently shows myotilin exists in the monomeric form at low concentration and the dimer is formed at effective high concentrations that mimic local concentration of the crowded cellular milieu of a sarcomeric Z-disc of striated muscle.

The solution structure of myotilin dimer presented in this thesis revealed an antiparallel architecture with the dimer interface mediated by the second Ig-domain in a head-head arrangement. Extensive biophysical characterisation of a mutant variant Δ5 (250-498) that harbours double lysine mutations (K358E, K359E) on the dimer interface weakened myotilin overall structure and promoted aggregate formation. Our mutational analysis suggest Ig-domains in Z-disc proteins might constitute a mutational hot spot to look out for in patients presenting with myopathy phenotypes as recently identified and characterized in several FLNc Ig-domains (Fürst et al., 2013; Kley et al., 2012). The antiparallel nature of myotilin dimer revealed in our SAXS structure provokes insights into how multiple interactions can be orchestrated by myotilin in the Z-disc, such that it can contribute stability to the Z-disc and/or provide rigor during active sarcomeric stretch. However, it remains to be studied on a structural level how the assembly of full length myotilin will engage alpha-actinin-2 in the presence of F-actin and/or ZASP. Furthermore, characterisation of myotilin C-terminal constructs encompassing only the mere Ig-domain tandem without N- or C-terminal flanks, expands our knowledge on the regions of myotilin sufficient for initiating dimeric assembly at effective high concentration. A further expansion of knowledge from this thesis research is the observation that isolated Ig-domains of myotilin constitute independent F-actin binding modules, indicating monomeric myotilin with the Ig-domains in tandem could bundle F-actin.
The biological relevance of dimeric myotilin on the stability of F-actin scaffold through efficient bundling and multiple interaction is undoubted, as demonstrated in this thesis, and in agreement with previous published reports (Salmikangas et al., 2003; von Nandelstadh et al., 2005).

Our biophysical analysis of interaction between myotilin C-terminal peptide and PDZ-ZASP indicate a 1:1 stoichiometry and micromolar affinity (2.4 µM) within the range reported for most peptide-PDZ domain interactions (Lee and Zheng, 2010), as well as significant increase in thermostability reminiscence of the β-augmentation mode of binding. X-ray diffraction studies of PDZ-ZASP co-crystallized with myotilin C-terminal hexapeptide located three myotilin terminal residues to the canonical PDZ hydrophobic pocket. And further NMR analysis of the peptide binding region revealed myotilin hexapeptide binds in an extended conformation similar to other PDZ-peptide structures which are known to adopt the β-augmentation mode of peptide binding (Pedersen et al., 2014).

Taken together, we thus propose monomeric myotilin is sufficient to cross-link F-actin via the two independent Ig-domains in tandem arrangement, and dimeric myotilin provides distinct biological advantage required for stabilizing actin-scaffold in the myofibrillar Z-disc.

Diverse array of actin binding and cross-linking proteins are continually being identified in skeletal muscle cells. Just as recent research shows that the 132 aa internal region of ZASP, bearing the ZASP-like motif (sZM) conserved in skeletal muscle isoforms, is capable of binding skeletal actin filaments (Lin et al., 2014). This supports the notion that our knowledge on the gigantic architecture of macromolecular assemblies in the striated muscle sarcomere would yet unfold with future discoveries of interaction partners or actin-binding regions in striated cardiac and skeletal muscles.
6.0 References


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APPENDIX I

**Manuscript I: in preparation**

*Acta Crystallographica Section D*  
**structural communications**

**Structure of N-terminal PDZ domain of ZASP in complex with the C-terminal peptide of myotilin: implications for myofibrillogenesis**

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**Synopsis**

Structural and biophysical bases of the interaction between myotilin C-terminal tail and the PDZ domain of ZASP revealed by X-ray crystal structure, isothermal titration calorimetry, differential thermal fluorimetry and NMR.

**Abstract**

Z-disc alternatively spliced PDZ-containing protein (ZASP) is essential for the assembly of myofibrils and the integrity of Z-disc actin-scaffold in human cardiac and skeletal muscles. Mutations in the actin-binding and LIM domains of ZASP have been shown to cause PKC-mediated severe myopathies. Multiple Z-disc proteins have been reported to bind the N-terminal PDZ domain of ZASP via distinct C-terminal motifs. In particular, the binding of class III motifs of myotilin and FATZ family have recently been described, suggesting ZASP tether these proteins together with α-actinin-2 to form multi-protein complexes in the Z-disc. However, our knowledge on the structural basis and biophysical aspects of these binding interactions is limited. Here, we characterised the binding of myotilin class III peptide motif to PDZ-ZASP and determined the crystal structure of the complex to 1.4 Å resolution, revealing three peptide residues bound to the PDZ. NMR binding studies validate the peptide binding site and shows that myotilin C-terminal tail binds PDZ-ZASP in an extended β-augmentation mode contributing stability to the overall fold of PDZ-ZASP.
1. Introduction

Sarcomere is the basic repeating block of a muscle cell, composed of series filaments that are necessary for muscle contraction. Z-disc is the boundary between two adjoining sarcomeres and is the smallest functional units of the sarcomere. The major component of the Z-disc is the muscle specific α-actinin-2 isoform, which cross-links antiparallel actin filaments from two adjacent sarcomeres. Apart from its major function of cross-linking F-actin, α-actinin-2 is a protein-protein interaction hub of the Z-disc and is involved in a complex interaction network (Sjöblom et al., 2008). Amongst its numerous binding partners are ZASP and myotilin.

Human sarcomeric Z-disc alternatively spliced PDZ-containing protein (ZASP), known also as Oracle and the LIM domain-binding protein 3 (LDB3) is a 78 kDa ortholog of murine protein Cypher localized in striated skeletal and cardiac muscle fibres (Faulkner et al., 1999; Zhou et al., 1999; Passier et al., 2000). As a member of the Enigma family of striated muscle proteins, ZASP possesses an N-terminal PDZ domain (referred hereafter to as PDZ-ZASP) and three C-terminal LIM domains with three alternatively spliced isoforms currently described in human skeletal muscle (Fig. 1) (Wang and Su, 2010). Very few PDZ domain-containing proteins are present in striated muscle, and since PDZ domains are known to engage in well-conserved domain-motif interactions that regulate and tether proteins to sites of complex formation, ZASP is believed to function most probably as the oracle of Z-disc multi-protein complexes by engaging in multiple interactions at the early stage of myofibril assembly (Wang et al., 2005; (Rui et al., 2010). ZASP binds via its PDZ domain specific C-terminal motifs α-actinin-2, myotilin and FATZ protein family of proteins, which are all co-expressed early in formation of muscle Z-disc (von Nandelstadh et al., 2009; Zhou et al., 1999). Co-expression of ZASP with these proteins in developing muscle cell indicates that ZASP is important for the subsequent formation of stable macromolecular assembly in mature Z-discs. In addition, the central ZASP-like motif conserved in all isoforms of ZASP has recently been shown to bind F-actin (Lin et al., 2014) with mutations in this region responsible for ZASP-related myofibrillar myopathies termed zaspopathies (Griggs et al., 2007). The third LIM domain of ZASP or the
mouse ortholog cypher have also been shown to perform signalling function by binding and directing muscle protein kinase C (PKC) to the Z-disc with mutation on the LIM domain resulting in fatal dilated cardiomyopathy (Arimura et al., 2004). Recent reports of cardiomyopathy-related mutations in ZASP and severe muscle defects associated with the ablation of ZASP in developing mice further support the biological relevance of ZASP in muscle fibers (Zhou et al., 2001; Griggs et al., 2007). Thus ZASP plays important regulatory and stability roles during myofibrillogenesis and the sustenance of multi-protein complexes in sarcomeric Z-discs.

Apart from α-actinin-2, myotilin also binds to the ZASP via the same binding module – the PDZ domain. Myotilin is a 57 kDa Z-disc protein expressed poorly in human cardiac muscles and richly in skeletal muscles (Salmikangas et al., 1999). Together with palladin and myopalladin, myotilin is the founding member of a family of structurally and functionally homologues Z-disc scaffolding proteins that regulate actin organisation and dynamics (Otey et al., 2005). Myotilin is composed of a unique serine-rich N-terminal half containing a 23-residue hydrophobic stretch. The C-terminal half comprises two tandem immunoglobulin-like (Ig) domains and a short C-terminal segment with the PDZ-binding motif (PBM) (Fig. 1). Myotilin binds G-actin and efficiently cross-links F-actin into bundles (Salmikangas et al., 2003; von Nandelstadh et al., 2005). Myotilin has also been reported to interact with five other co-located Z-disc proteins including α-actinin-2, filamin C (FLNc), FATZ/myozenin/calsarcin family, ZASP and MuRF-1/2 (von Nandelstadh et al., 2011; Gontier et al., 2005; Salmikangas et al., 2003). Three types of interaction involving PDZ motifs have been described in detail (Hung and Sheng, 2002). The first two types, class 1 and 2, involve binding of carboxy-terminal protein sequences through either an -x-(S/T)-x-hydrophobic or an -x-hydrophobic-x-hydrophobic sequence motif. The third type of recognition involves -x-D/E-x-hydrophobic sequence motifs. A large body of research exists on how PDZ domains, structurally simple protein interaction modules, can achieve effective ligand discrimination, and at the same time occasionally also be promiscuous, with one domain capable of binding multiple targets. PDZ promiscuity is characterised by the fact that some PDZ domains have the ability to bind peptide sequences that belong to more than one class (Beuming et al., 2005).
The solution structure of PDZ-ZASP was solved and its interaction with human α-actinin studied, using nuclear magnetic resonance (NMR) spectroscopy (Au et al., 2004). This study showed that ZASP PDZ is a classical class 1 PDZ domain, which recognizes the carboxy-terminal sequence of α-actinin-2 calmodulin-like domain with high micromolar affinity. von Nandelstadh et al., 2011 recently showed that PDZ-ZASP also binds to the enigma and FATZ families of proteins, which both display type III sequence motifs at their C-termini, and that phosphorylation in the binding motif modulates binding.

A high-resolution structural interpretation of PDZ-ZASP domain function should provide insights into the structural basis of ligand specificity and promiscuity that dictate the diversification of biological function. Here, we report the crystal structure of PDZ-ZASP, co-crystallized with the C-terminal hexapeptide of human myotilin. NMR binding studies, which indicate that myotilin peptide binds the PDZ domain in an extended conformation reminiscent of a β-augmentation mode, support the atomic structure of the complex. Biophysical analysis further characterises and quantifies interaction and together with comparative structural analysis offers molecular basis for PDZ-ZASP promiscuity with implications on Z-disc genesis and its ultrastructure.
2.0 Experimental procedures

2.1 Protein expression and purification
The PDZ domain of human striated muscle ZASP (encompassing residues 1-85) was recombinantly expressed from plasmid vector pETM-11, carrying DNA insert encoding an N-terminal glutathione S-transferase (GST) tag and an additional 6-His-tag with recombinant tobacco etch virus (rTEV) protease site. The plasmid was transformed into E. coli strain Rosetta (DE3) pLysS (Novagen). Cells were grown in Luria-Bertani medium supplemented with 34 µg/mL chloramphenicol and 50 µg/mL kanamycin to an optical density 0.5 - 0.6 at 600 nm.

Overproduction of PDZ-ZASP was achieved by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by overnight incubation at 293 K. Harvested cells were suspended in buffer A containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 % glycerol and lysed at 277 K using French press. Purification of GST-free PDZ-ZASP from supernatant resulting from centrifuge-clarified lysate was achieved on Äkta FPLC in three steps: (i) affinity capture of GST-His-6-ZASP-PDZ via a glutathione-sepharose HP resin (GE Healthcare), with elution of bound protein using buffer A supplemented with 10 mM Glutathione reduced (GSH) (Sigma-Aldrich); (ii) cleavage of affinity tags using recombinant tobacco etch virus (rTEV) protease at 1:50 mass ratio, with subsequent removal of GST-His6-tag and protease via repeated affinity capture step, and (iii) a final polishing step with size exclusion chromatography (SEC) using Superdex 1660 column (GE Healthcare) in a buffer B containing 20 mM HEPES, 150 mM NaCl, 5 % glycerol, pH 7.2. Fractions containing the pure protein were pooled and concentrated using 3 kDa cut-off Millipore ultrafiltration membrane. The purity of PDZ-ZASP construct after the size exclusion chromatography was assessed by SDS –polyacrylamide gel electrophoresis (Fig. S1).
2.1 Static light scattering
Homogeneity and molecular mass of recombinant purified PDZ-ZASP was evaluated by loading 100 µl of 1 mg/ml purified sample on Superdex 200 10/300 column pre-equilibrated with SEC buffer. Protein eluting off the column was fed into a downstream triple-angle laser light scattering detector (miniDAWN TREOS, Wyatt Technology), connected in tandem to a refractive index detector (RI-101, Shodex) (Fig. S2). Absolute molecular mass was determined using Astra software (Wyatt Technologies).

2.2 Isothermal titration calorimetry (ITC)
Interaction between myotilin and PDZ-ZASP was quantified by Micro-Cal ITC200 (GE Healthcare). Myotilin C-terminal hexapeptide (YESEEL) was synthesized and supplied in 25 mg lyophilized form (PSL, Munich). Myotilin peptide (0.4 mM) dissolved in gel filtration buffer B (20 mM HEPES, pH 7.2, 0.15 M NaCl, 5% glycerol) was titrated in 2 µl steps against purified PDZ-ZASP (0.02 mM) in buffer B, and reaction were allowed proceed at 30 °C. Origin software was used to fit the binding isotherms which yield stoichiometry, affinity and binding parameters.

2.3 Thermal shift assays and circular dichroism
Differences in structure and stability between single PDZ-ZASP and peptide bound PDZ-ZASP was assessed using circular dichroism and differential scanning fluorimetry (Thermoflour). Purified PDZ-ZASP at 0.5 mg/ml was dialysed in 0.05 M sodium sulphate, 10 mM potassium phosphate, pH 7.2 prior to CD analysis. PDZ-ZASP-peptide complex was prepared by adding 10-20 fold molar excess of myotilin peptide in buffer B to the purified PDZ-ZASP. CD spectra between 200 – 260 nm were recorded on a pi-star CD instrument (Applied Photophysics). For Thermoflour assay, SYPRO Orange dye 40x (Invitrogen) was added to 3 µg purified PDZ-ZASP in buffer B pre-mixed with 10-20 fold molar excess of myotilin C-terminal hexapeptide in a total volume of 15 µl. Assays were carried out using IQ5 real-time PCR detection system (BioRad) set as previously described (Kley et al., 2012).
2.4 Nuclear Magnetic Resonance Spectroscopy

NMR experiments were performed with Varian Inova 600 MHz and 800 MHz spectrometers equipped with $^1$H/$^{13}$C/$^{15}$N triple resonance probes at 25 °C. $^{15}$N-labelled ZASP-PDZ domain were prepared as previously described (Au et al, 2004) and purified by SEC as final step in buffer containing 20 mM Na$_2$PO$_4$, pH 6.5, 0.15 mM NaCl, 0.15% (wt/vol) NaN$_3$. Prior to measurement, D$_2$O to a final concentration of 10% (v/v) was added to samples at 1 mM. 1D $^1$H-NMR spectra were obtained using the WATERGATE method for solvent suppression (Liu et al., 1998); $^{15}$N-HSQC spectra were acquired using Rance-Kay gradient enhancement methods (Kay et al., 2011). Myotilin peptide (20-fold molar excess) was sequentially titrated into $^{15}$Nlabelled PDZ-ZASP samples from 1 mM to 0.5 mM. Spectra were processed with NMRPipe (Delaglio et al., 1995) and analysed with Sparky software (Goddard and Kneller, UCSF). Chemical shifts observed in $^{15}$N-HSQC experiments were quantified as $\sqrt{(\Delta \sigma(15N)2 + 25\Delta \sigma(1H)2)}$.

2.5 Crystallization

Screening for co-crystals of purified ZASP-PDZ domain with the C-terminal hexapeptide of myotilin was carried out at 22 °C in a sitting-drop vapour-diffusion technique using several commercial screens. Multivariate matrix screening was assisted by a nanodrop-dispensing robot (Phoenix RE; Rigaku Europe, Kent, United Kingdom). Initial hits of the co-crystal grown from 15 mg/ml PDZ-ZASP premixed with 20 fold molar excess of myotilin peptide appeared as long, thin, plate-like clusters (Fig. S3A), and were obtained from JCSG+ Suite crystal screen containing 2.4 M sodium malonate, pH 7.0. Crystal optimization was subsequently achieved by manual refining in a hanging-drop vapour diffusion set-up, using molar ratio 1 (PDZ-ZASP) to 10 (myotilin-YESEEL), followed by micro-seeding and a controlled rate vapour-diffusion by spreading thin film of Al’s oil over the reservoir (D’Arcy et al., 2007; Chayen, 1997). This approach led to diffraction-quality single co-crystals which appeared after one week in drops consisting of 1.9 M sodium malonate, 0.1 M HEPES, pH 7.0 (Fig. S3B), reproducibly grown in 35% PEG 6000, 0.05 M imidazole, pH 8.0.
2.6 Cryoprotection and data collection

Prior to being flash cooled in liquid nitrogen, the co-crystals were briefly soaked in excess myotatin peptide and then transferred to a solution containing 18% glycerol as cryoprotectant. The diffraction data set was collected at 100 K on the beamline ID23-1 of European Synchrotron Radiation Facility (ESRF), Grenoble, using a wavelength of 0.90 Å. Diffraction data was processed with XDS package (Kabsch, 2010), converted to MTZ file format using POINTLESS and scaled with SCALA from the CCP4 suite (Winn et al., 2011; Evans, 2006). Data analysis was performed with phenix.xtriage from PHENIX software package (Adams et al., 2010). Data-collection statistics are shown in Table 1.

2.7 Molecular replacement and structure solution

Initial molecular replacement trials using the structure of ZASP PDZ solved by NMR as a search models failed, probably due to the previously described difficulties such as, for example, accuracy of the atomic positions in NMR models (Chen, 2001). The structure was successfully solved by molecular replacement with the program MOLREP (Vagin and Teplyakov, 2010) using the coordinates of CLP-36 (PDLIM1) (pdb accession code: 2pkt) as a search model. The molecular replacement in P2$_1$ space group yields a clear solution with 2 PDZ molecules per asymmetric unit. The structure was refined to Rwork and Rfree factors of 15.0% and 17.9%, respectively. Refinement statistics is presented in Table 1.
3.0 Results and Discussion

3.1 Biophysical characterisation of myotilin – PDZ-ZASP interaction

*Myotilin peptide stabilizes overall fold of PDZ-ZASP*

Comparison of differential thermal unfolding properties of free PDZ-ZASP and peptide bound PDZ-ZASP revealed approximately 10 °C increase in the melting temperature of the peptide-bound PDZ-ZASP. This increase in thermostability of the PDZ domain in the presence of myotilin peptide suggests stabilization of the structure consequent to complex formation (Fig. 4A).

*Analysis of structural changes in peptide bound PDZ-ZASP*

To investigate whether binding of PDZ-ZASP by myotilin C-terminal peptide results in secondary structure changes that might explain the increase in T_m observed, we used circular dichroism. When comparing the CD spectra of PDZ-ZASP-myotilin peptide complex to free PDZ-ZASP, a spectral shift towards 218 nm was observed. This shift towards minima typical for β-strands suggests an increase in β-content (Fig. 4B) (Gopal et al., 2012; Kelly and Price, 2000; Johnson, 1988), spurring motivation for high resolution structural analysis.

*Quantification of binding affinity*

Affinity of the binding interaction between myotilin C-terminal hexapeptide (YESEEL) and the PDZ domain of ZASP was quantified by isothermal titration calorimetry. Integration and fitting of the binding isotherms derived from sequential titration of myotilin C-terminal peptide into recombinant purified PDZ-ZASP revealed binding stoichiometry of 1:1 with $K_D$ (2.4 ± 1.5) μM (Fig. 4C).
3.2 Structure determination and analysis

**Twinning analysis**

PDZ-ZASP co-crystallized with myotilin C-terminal peptide (YESEEL) and diffracted X-rays to 1.4 Å resolution. The crystal belonged to primitive monoclinic space group P2₁ with unit cell dimension a = 28.90, b = 95.50, c = 28.90, α = γ = 90°, β = 120°. Cell content analysis suggested two PDZ molecules in asymmetric unit with the Matthews coefficient of 1.9 Å³/Da (corresponding to solvent content 35.2%) (Matthews, 1968).

Data processing with XDS and subsequent data analysis with the program POINTLESS gave the highest score for the C-centered orthorhombic C222₁ space group. Indexing and integration was possible in both primitive monoclinic space group P2₁ and C-centered orthorhombic C222₁ with unit cell dimensions a = 28.90, b = 95.50, c = 28.90 Å, β = 120° and a = 28.90, b = 50.06, c = 95.50 Å, respectively. The overall scaling statistics were only slightly worse for the space group C222₁ (Table 1). Subsequent analysis clearly revealed P2₁ as the true space group.

Data analysis with phenix.xtriage detected the presence of pseudo-merohedral twinning in P2₁ with twinning operator h+l, -k, -l or its equivalent –h-l, -k, l. The estimated twin fraction is about 0.36 by Britton plot (Fisher and Sweet, 1981) and about 0.37 by cumulative distribution of H (Yeates, 1988). The a and c axis of an orthorhombic cell are identical to a and b of monoclinic, respectively. The b axis of orthorhombic cell fulfills the conditions: \( b_{ortho} = 2c_{mono} \cos(b_{mono} -90) \). The twinning operator in pseudo-merohedral twins introduces additional symmetry, which misleads the correct space group determination. The identical cases of twinning has been previously reported with the same length of a and c axis or with ccosβ = -a/2 in monoclinic P2₁ crystals (Yang et al., 2000; Rudolph et al., 2004; Reinhard et al., 2012).

**Crystal Structure of PDZ-ZASP in complex with myotilin C-terminal peptide**

Crystal structure of the complex revealed three C-terminal myotilin peptide residues (EEL) bound to the PDZ domain. The peptide residues, which display the type III motif, locate between the second β-strand (β2) and third α-helix (α3) of the
PDZ-ZASP, consistent with the region where C-terminal peptides of the most interaction partners of PDZ domains are observed (Pedersen et al., 2014; Lee and Zheng, 2010). The side-chain of the very C-terminal leucine residue (commonly termed p(0) site) of myotilin forms hydrophobic interactions with the PDZ domain residues Phe15 and Leu17 located on the second β-strand and with Ile69 on the third α-helix, which form a hydrophobic pocket (Fig. 2). The carboxylate group forms hydrogen bonds with the peptide nitrogen atoms of the carboxylate-loop residues Phe15 and Trp17 (Doyle et al., 1996; Songyang et al., 1997), which side chain in turn forms aromatic stacking with Phe15. Bound peptides that form complex structures with PDZ domains typically have 4 or 9 residues (Lee and Zheng, 2010; Bezprozvanny and Maximov, 2001). In our case, we see convincing electron density for the only three amino acid residues of the bound myotilin peptide. Analysis of crystal packing contacts revealed, that the binding site in both molecules in the asymmetric unit is partially occluded by symmetry related molecules, preventing also location of side chains of the preceding glutamic residues at positions p(1) and p(2).

**NMR analysis of ZASP-PDZ and myotilin peptide interaction**

In order to validate the binding site of myotilin peptide observed in the crystal structure of the complex, NMR titration analysis was performed. When excess myotilin peptide was titrated into 15N-labelled PDZ of ZASP, the region where the peptide binds on the PDZ structure displayed large differential NMR chemical shifts (Fig. 3A). The most significant chemical variations in the PDZ domain concern residues Arg16, Leu17, Ile32, Thr33, Gln66 and Ile69 (Fig. 3B). These residues map around the region where three myotilin peptide residues were observed in the X-ray crystal structure of the complex (Fig. 3C), suggesting that myotilin peptide binds in an extended conformation.

The structural analysis of PDZ-ZASP interaction with α-actinin-2 (Au et al., 2004), showed it recognizes type I motifs, which in the case of α-actinin-2 corresponds to peptide sequence GESDL. Our crystal structure, together with our NMR data show that the binding pocket can as well accommodate type III motifs (ESEEL in the case of myotilin), as suggested by (von Nandelstadh et al., 2009). Structural analysis suggest that a longer side-chain, Glu rather than Ser at position p(2), can
be accommodated in the binding pocket, with the Glu side-chain potentially making a stabilising electrostatic interaction with the Lys70 residue. This might provide additional stabilising interaction which plausibly explains the basis for higher affinity of myotilin ($K_D = 2 \, \text{uM}$) compared to $\alpha$-actinin-2 ($K_D = 38 \, \text{uM}$). Notably, the binding interaction between the PDZ-ZASP and myotilin could likely be modulated by phosphorylation, as a change in affinity has been reported with other enigma family PDZ domains (von Nandelstadh et al., 2009). This suggests phosphorylation as a potential regulator of the binding and affinities between PDZ-ZASP and the interaction partners in the Z-disc.
4. Conclusions

Implications for myofibrillogenesis

Cellular events leading to the formation of myofibrils have extensively been studied and described (Rui et al., 2010; Ehler and Gautel, 2008; Sanger et al., 2005). In developing skeletal myotubes, myotilin, ZASP and FATZ have recently been reported to undergo dynamic exchange in the presence of α-actinin-2 and F-actin, which together constitutes pre-myofibrillar I-Z-I complexes (Ojima et al., 1999) that precedes the assembly of F-actin into arrays at the Z-disc of mature myofibril (Wang et al., 2005). In these studies, myotilin and ZASP were particularly noted to exhibit the fastest dynamic exchange compared to α-actinin-2, which interestingly, is relevant to the higher binding affinity and structural stability observed in our biophysical analysis of myotilin-PDZ-ZASP complex in comparison to α-actinin-2-PDZ-ZASP complex. The structural analysis of PDZ-ZASP interaction with myotilin and α-actinin-2 motifs, thus informs on the plausibility of protein tethering role for PDZ-ZASP during myofibrillogenesis. Moreover, ZASP has recently been shown to bind skeletal F-actin via its central region where mutations leads to disease phenotypes (Lin et al., 2014), and since PDZ-containing scaffolds assemble specific proteins into large molecular complexes at defined locations in the cell, thus the notion and importance of a higher order Z-disc actin-scaffolding network orchestrated by ZASP is insightful; involving myotilin, FATZ and centered around α-actinin-2, for the proper assembly and physiological function of striated myofibrillar Z-disc.

Based on our biophysical data and the integrated structural analysis, we predict that the class III PDZ-binding motifs of the FATZ family members that share a conserved sequence with myotilin C-terminal motif, could exhibit a common binding mechanism to PDZ-ZASP as described in this study. However, it would be interesting to decipher the binding affinities and mimic competition events in the cellular environment to fully grasp the mode of selectivity and understand how PDZ-ZASP engages the multiple Z-disc partners.
Acknowledgment

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References


FIGURE LEGENDS

Figure 1  Schematic illustration of domain organisation in the canonical isoform of human ZASP. Gene transcript of ZASP canonical isoform (upper panel) is composed of an N-terminal PDZ domain followed by a C-terminal region comprising three LIM domains and an evolutionarily conserved 26-residue central motif called ZASP-like motif (ZM), which is encoded by exon 6. The ZM has recently been characterised as a novel actin-binding domain in skeletal muscle isoforms and confer on ZASP a complementary interacting capability within the Z-disc (Lin et al., 2014). Mutations in the ZM (A165V and A147T) has been reported in affected members in multiple families in the United States and Europe with myofibrillar myopathies referred to as Zaspopathies (Griggs et al., 2007). Myotilin in turn is composed of a unique, pair-wise serine-rich N-terminal half without any known domain classification. The N-terminus is followed by a C-terminal half comprising two tandem Ig-like domains separated by a short linker. Annotations in the figure highlight the domain boundaries important for self-association into dimer as well as interactions with other Z-disc proteins.

Figure 2. Structure of PDZ-ZASP in complex with myotilin peptide. Co-crystals of PDZ-ZASP and myotilin C-terminal peptide diffracted X-rays to 1.4 Å resolution. left panel shows cartoon representation of PDZ-ZASP structure with the three terminal residues (EEL) of myotilin peptide (red) lodged between the β2 and α3 structures of the PDZ. B-factors of residues from NMR chemical shifts are displayed on the PDZ-ZASP structure; the largest chemical shifts are seen as red and the residues concerned map around the β2 and α3 structures, thus validating the peptide binding site as well as indicating an extended peptide conformation. Right panel shows enlarged view of the structure with highlights of PDZ residues Phe15, Leu17 and Ile69 which forms a hydrophobic stack around the terminal leucine of myotilin.
**Figure 3** NMR analysis of the binding between PDZ-ZASP and myotilin C-terminal peptide.

(A) Superposition of $^1$H-$^{15}$N HSQC NMR spectra of native PDZ-ZASP (red peaks) and the PDZ-ZASP titrated with 20 fold molar excess of myotilin C-terminal hexapeptide (purple).

(B) Chemical shift variations of the spectrum of $^{15}$N-labeled PDZ-ZASP upon titration with 20 fold molar excess of myotilin peptide (YESEEL) plotted versus the ZASP-PDZ sequence. The structural elements of the PDZ domains are annotated.

(C) Residues exhibiting large peak shifts on titration of myotilin peptide are mapped onto the structure of PDZ-ZASP in cartoon representation, with the respective B-factors of concerned residues displayed. These residues include Arg16, Leu17, Ile32, Thr33, Gln66 and Ile69 around the α3 helix as well as β2 and β3 strands, validating the binding region of myotilin peptide observed in the crystal structure and suggesting an extended, β-augmentation conformation.

**Figure 4A** Class III PDZ-binding motif of myotilin contributes stability to the overall PDZ fold.

(a) Thermal unfolding curve of PDZ-ZASP alone with an average melting temperature (Tm) of 51°C. (b) Thermal unfolding curve of PDZ-ZASP premixed with molar excess of myotilin C-terminal peptide; approximately 10°C increase in Tm was observed (c) Comparison of the melting profiles of free PDZ and peptide-bound PDZ-ZASP.

**Figure 4B** Secondary structure analysis of peptide bound PDZ-ZASP.
Circular dichroism (CD) spectra were recorded on 0.5 mg/ml purified PDZ-ZASP alone (green curve). Following addition of molar excess of myotilin C-terminal peptide, CD spectra was recorded for the peptide-bound PDZ-ZASP (white curve). In the presence of myotilin C-terminal hexapeptide (YESEEL), a shift in CD minima for PDZ-ZASP was observed towards the region typical for β-structure (around 215-218 nm).
**Figure 4C.** Quantification of binding affinity.
Binding isotherms (*upper panel*) derived from sequential titrations of 20 fold molar excess of myotilin C-terminal peptide (YESEEL) into purified PDZ-ZASP (20 µM). Integration of isotherm peaks and fitting was achieved in single site binding mode using MicroCal Origin software. Micromolar affinity (2.4 µM) was calculated with a stoichiometry of 1:1.

**Figure S1.** SDS–PAGE analysis of purified recombinant PDZ-ZASP
Recombinantly expressed PDZ-ZASP was purified to 95% quality with a yield of 15 mg per 1 l bacterial culture. M, molecular-weight markers (in kDa); lane 1, GST-tagged PDZ-ZASP eluted from a GSTrap column; lane 2, tag-free ZASP-PDZ purified by gel filtration.

**Figure S2.** Analysis of protein homogeneity and molecular weight.
Analytical size exclusion chromatography coupled to light scattering revealed a single, symmetric protein peak with 98% scattered intensity from the PDZ-ZASP. Molecular mass across the peak was calculated to 9.3 kDa.

**Figure S3.** Co-crystals of PDZ-ZASP with myotilin hexapeptide.
(A) Co-crystals of ZASP-PDZ and myotilin peptide (YESEEL) initially obtained in 2.4 M sodium malonate, pH 7.0 (B) Optimized single co-crystal of the complex grown using crystal seeds and thin film of Al’s oil over the reservoir in 1.9 M sodium malonate, 0.1 M HEPES, pH 7.0.
FIGURES

**FIGURE 1**

**FIGURE 2**
FIGURE 4B

FIGURE 4C

N = 1.05 ± 0.06
K_D = 2.4 ± 1.5 (μM)
ΔH = -1.56 ± 0.1 (kcal/mol)
ΔS = -25.7 (kcal/mol)
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<td></td>
<td>C222₁ (incorrect)</td>
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Where $\bar{I}_{(hkl)}$ is the mean intensity of multiple $I_{i(hkl)}$ observations of the symmetry-related reflections, N is the redundancy.

⁴Rfree is the cross-validation Rfactor computed for the test set of reflections (5 %) which are omitted in the refinement process.
Biophysical characterisation of FLNc Ig-domain 23-24 harbouring mutations on the dimerisation domain.

METHODS

Biophysical characterisation

For the analysis of purified wild-type FLNc d23-24 and the mutant variants FLNc d23-24Δ2 with deletion mutation starting at position T2697 and FLNc d23-24Δ3 bearing K2701_G2703del mutation, circular dichroism (CD) spectroscopy and thermal shift assays were performed as previously described (Kley et al., 2012). Size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) was performed as described (Materials and Methods) in a buffer system containing 0.1 M potassium chloride and 10 mM potassium phosphate, pH 7.3. The protein concentration injected was 1 mg/mL; the sample load 50 - 70 µL. Astra software (Wyatt technology) was used for data analysis.

Structural analysis of recombinant proteins

For structural analysis of the mutant constructs FLNc d23-24Δ2 and FLNc d23-24Δ3 to understand the effect of mutations at the molecular level, we used the previously determined high resolution crystal structures of FLNc Ig-23 (Sjekloča et al., 2007), the dimerisation domain Ig-24 (Pudas et al., 2005) and the SAXS structure of both domains in tandem FLNc Ig-23-24 (Sjekloča et al., 2007).
RESULTS AND DISCUSSION

In accordance with the known structures of FLNc d23-24 (Sjekloća et al., 2007), the wild-type protein exhibited a high proportion of β-strands, evidenced by a minimum in the CD spectra at 218 nm and a positive ellipticity around 205 nm (Fig. 2a). The variant FLNc d23-24Δ3 harbouring p.K2701_G2703del mutation exhibited comparable CD spectra to the wild-type construct, whereas the variant FLNc d23-24Δ2 bearing deletion mutation starting at position T2697 exhibited decreased ellipticity at 218 nm suggesting a change in the three-dimensional structure of the mutant domain 24 due to the deletion of β-structure elements.

When comparing the melting temperatures obtained from thermal shift assay of the three variants, the wild-type had an average melting temperature (Tm) of 58 °C while the mutant variant FLNc d23-24Δ3 showed a melting temperature of 56 °C (Fig. 2b). Interestingly, the deletion mutant FLNc d23-24Δ2 showed a considerably decreased average melting temperature of 49 °C.

Mutation in the construct FLNc d23-24Δ3 was located to the solvent exposed loop connecting βE into βF of domain 24 (Fig. 3a). The three-residue deletion K2701-G2703 does not present dramatic biophysical changes in the variant FLNc d23-24Δ3 as observed in the CD spectra and melting temperature (Tm) when compared to the wild-type construct (Fig. 2a-b). This is unsurprising because the mutation is located on a loop that can accommodate mutational or structural changes with little or no impact on the overall domain structure. In contrast, the variant FLNc d23-24Δ2 harbours deletion mutation located to structural elements βF and βG strands in the dimerisation domain 24 (Fig. 3b). The βF and βG together with βC form one of the two extended canonical antiparallel β-sheets in the C-set domain 24 of FLNc (Pinotsis et al., 2009). In particular, βC strand precisely engage in inter-molecular β-sheet interactions with the antiparallel βC strand of the other protomer, thereby constituting the dimer interface (Pudas et al., 2005). Thus, the deletion mutation disrupted the intramolecular β-sheet network therefore destabilizing the structural elements (βC and βD) and consequently the dimer interface (Fig. 3b). This is notably reflected in the decreased Tm of this variant FLNc d23-24Δ2 (49 °C) and its characteristic asymmetric monomeric profile seen on multi-angle laser light scattering coupled to chromatography (SEC-MALLS) (Fig. 1).
FIGURE LEGENDS

Figure 1  SEC-MALLS profile of the deleterious mutant variant FLNc d23-24Δ2 which eluted asymmetrically as monomer (red) in contrast to the dimeric wild-type-FLNc d23-24 (blue).

Figure 2  Comparative structural and biophysical analyses of the wild-type and mutant FLNc d23-24 variants. (A) Circular dichroism (CD) spectra of the three variants. (B) Differential thermal shift analysis of the wild-type and mutant variants with the respective melting temperatures (Tm) indicated.

Figure 3  Structural analysis of wild-type and mutant FLNc variants bearing mutations on dimerisation domain 24 (A) Cartoon representation of domain 24 in variant FLNc d23-24Δ3 bearing deletion mutation starting at position 2701 (residues KDG). The deletion, shown in stick and colored blue, lies on the solvent exposed loop which connects βE into βF (B) Domain 24 of the wild-type fragment (left) and the variant FLNc d23-24Δ2 (right). The deletion of βF and βG strands (highlighted in green) in the mutant had deleterious consequences on the dimerisation interface.
FIGURES

**FIGURE 1**

- **Log (kDa)**: $2 \times 10^4$, $5 \times 10^4$, $1 \times 10^5$
- **RI**: 0.0, 0.3, 0.5, 0.8, 1.0
- **Time (min)**: 24, 28, 32, 36
- **FLNc d23-24Δ2**
- **FLNc d23-24WT**
FIGURE 2
References


APPENDIX II

Publication

Pathophysiology of protein aggregation and extended phenotyping in filaminopathy
Pathophysiology of protein aggregation and extended phenotyping in filaminopathy

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**[Mutations in FLNC cause two distinct types of myopathy. Disease associated with mutations in filamin C rod domain leading to expression of a toxic protein presents with progressive proximal muscle weakness and shows focal destructive lesions of polymorphous aggregates containing desmin, myotilin and other proteins in the affected myofibers; these features correspond to the profile of myofibrillar myopathy. The second variant associated with mutations in the actin-binding domain of filamin C is characterized by weakness of distal muscles and morphologically by non-specific myopathic features. A frameshift mutation in the filamin C rod domain causing haploinsufficiency was also found responsible for distal myopathy with some myofibrillar changes but no protein aggregation typical of myofibrillar myopathies. Controversial data accumulating in the literature require re-evaluation and comparative analysis of phenotypes associated with the position of the FLNC mutation and investigation of the underlying disease mechanisms. This is relevant and necessary for the refinement of diagnostic criteria and developing therapeutic approaches. We identified a p.W2710X mutation in families originating from ethnically diverse populations and re-evaluated a family with a p.V930_T933del mutation. Analysis of the expanded database allows us to refine clinical and myopathological characteristics of myofibrillar myopathy caused by mutations in the rod domain of filamin.](http://brain.oxfordjournals.org/)**
C. Biophysical and biochemical studies indicate that certain pathogenic mutations in FLNC cause protein misfolding, which triggers aggregation of the mutant filamin C protein and subsequently involves several other proteins. Immunofluorescence analyses using markers for the ubiquitin–proteasome system and autophagy reveal that the affected muscle fibres react to protein aggregate formation with a highly increased expression of chaperones and proteins involved in proteasomal protein degradation and autophagy. However, there is a noticeably diminished efficiency of both the ubiquitin–proteasome system and autophagy that impairs the muscle capacity to prevent the formation or mediate the degradation of aggregates. Transfection studies of cultured muscle cells imitate events observed in the patient’s affected muscle and therefore provide a helpful model for testing future therapeutic strategies.

**Keywords:** myofibrillar myopathy; filaminopathy; filamin C mutation; immunoglobulin-like domain; limb-girdle myopathy

**Abbreviation:** PDB = protein structure database

# Introduction

Filamin C-related myopathies specify diseases caused by mutations in the **FLNC** gene located within the chromosomal band 7q32–q35 and expressed predominantly in skeletal and cardiac muscles. The encoded filamin C protein (FLNC) contains an N-terminal actin-binding domain followed by a semiflexible rod comprising 24 highly homologous immunoglobulin-like domains (Xie et al., 1998; van der Flier and Sonnenberg, 2001). The carboxy-terminal immunoglobulin-like domain is required and sufficient for dimerization (Himmel et al., 2003; Pudas et al., 2005; Sjekloča et al., 2007), which is the molecular basis for filamin’s actin cross-linking activity. In the sarcomere, FLNC cross-links actin in the Z-disc region and additionally binds myotilin, FATZ/calsarcin/myozenin, myopodin and Xin (Faulkner et al., 2000; van der Ven et al., 2000; Gontier et al., 2005; van der Ven et al., 2006; Linnemann et al., 2010). At the sarcolemma, FLNC interacts with sarcoglycan γ and -α, two components of the dystrophin–dystroglycan complex (Thompson et al., 2000). Deficiency of FLNC in mice results in severe muscle defects and perinatal lethality, indicating a critical role for FLNC in muscle development and maintenance of muscle structural integrity (Daïkilic et al., 2006).

The first filamin C-related myopathy was described in 2005 when a nonsense mutation (c.G8130A, p.W2710X) in the FLNC dimerization domain was shown to cause a disease in a large German family characterized by muscle weakness of predominantly limb-girdle distribution and typical myofibrillar myopathy features on muscle biopsy (Vorgerd et al., 2005). Myofibrillar myopathy is a clinically and genetically diverse group of progressive devastating hereditary skeletal and cardiac myopathies. Thus far myofibrillar myopathy has been associated with mutations in seven genes (DES, MYOT, LDB3, CRYAB, BAG3, FLNC and FHL1), but a significant number of patients with myofibrillar myopathy failed to show disease-causing mutations in these genes (Selcen, 2008; Olivé et al., 2011; Selcen et al., 2011a, b). The most important common feature of these diseases is the disintegration of myofibrils and formation of desmin-positive protein aggregates within muscle fibres (Nakano et al., 1996; Schröder and Schoser, 2009). The p.W2710X mutation in FLNC impedes its ability to dimerize (Vorgerd et al., 2005; Löwe et al., 2007). Instead, the mutant protein acquires a strong tendency for uncontrolled aggregation, resulting in the deposition of massive protein aggregates that, in addition to FLNC, attract multiple other proteins including desmin and other Z-disc-associated proteins. These events ultimately lead to disintegration of myofibrils (Vorgerd et al., 2005; Löwe et al., 2007). A haplotype-sharing set of further German families also carrying the p.W2710X FLNC mutation were described soon after the first report (Kley et al., 2007), and an identical mutation was found in three kindships of the Mayo myofibrillar myopathy cohort that were not described in detail (Selcen, 2011a).

More recently, two families with filamin C-related myopathy harbouring mutations in FLNC rod domains have been reported: an internal 12-nucleotide deletion (c.2997_3008del, p.V930_T933del) (Shatunov et al., 2009) and an 18-nucleotide deletion/6 nucleotide insertion (p.K899_V904del/V899_C900ins) (Luan et al., 2010), both in immunoglobulin-like repeat 7. Clinical and pathological characteristics of the disease associated with these mutations were similar to the p.W2710X German phenotype, including characteristic features of myofibrillar myopathy, but no exhaustive comparison was provided and the disease pathomechanisms were not investigated.

Additionally, three distantly related families were described, in which a deletion (c.5160delC, p.F1720LfsX63) in exon 30 encoding FLNC immunoglobulin-like domain 15 triggers a frameshift, nonsense-mediated decay and haploinsufficiency, affecting primarily distal muscles of the upper and lower limbs. Although histological evaluation indicated disease-associated myofibrillar abnormalities, desmin-positive protein aggregates required for diagnosis of myofibrillar myopathy were not detected, probably because no truncated mutant protein is expressed. Muscle imaging revealed a pattern of muscle involvement different from that associated with p.W2710X FLNC mutation (Guergueltcheva et al., 2011). Finally, mutations located in the N-terminal actin-binding domain of FLNC (p.A193T and p.M251T) have also been associated with a distal myopathy characterized by a disease onset in the third decade of life, distal muscle weakness (primarily intrinsic hand muscles), and non-specific myopathic abnormalities with no features of myofibrillar myopathy (Duff et al., 2011). Although aggregation of mutant FLNC with F-actin was observed in transfection experiments in cultured cells, no aggregates were found in muscle biopsies of patients carrying these mutations.

This clearly differentiates the two latter syndromes from myofibrillar myopathy-associated pathologies caused by mutations...
occurring in FLNC immunoglobulin-like domains 7 and 24 and indicates that, as has previously been shown for FLNA and FLNB, different mutations in FLNC can lead to distinct disease phenotypes.

This requires a critical re-evaluation of the class of diseases caused by mutations in immunoglobulin-like domains of FLNC. We present here a comparative analysis of filamin C myopathy phenotypes associated with myofibrillar myopathy pathology and caused by mutations in FLNC rod domains including two novel p.W2710X families of distinct ethnic backgrounds and the re-evaluated p.V930_T933del family (Shatunov et al., 2009). We also assessed the patho-mechanisms of these phenotypes by analysing protein degradation machineries and investigating pathogenic effects of transient expression of FLNC mutants in murine myoblast cell lines.

Materials and methods

Newly identified and re-evaluated filaminopathy kindreds

We identified a large East European kindred originating from a rural region of Macedonia in which 18 members were known to be affected with a skeletal and cardiac myopathy. This kindred is referred to as Family I (Fig. 1). The second family with three affected individuals is of Chinese origin and referred to as Family II (Fig. 1). Using complementary DNA sequencing, a mutation in exon 48 of FLNC first described by Shatunov et al. (2009) was uncovered in Families I and II. In addition, three patients from a German family carrying the p.V930_T933del mutation in FLNC first described by Shatunov et al. (2009) were re-examined for this report (Family III in Fig. 1). Basic information on clinical examination, neurophysiological studies and muscle imaging is provided in the online Supplementary material. Genetic studies were approved by the Institutional Review Board of the National Institute of Neurological Disorders and Stroke in Bethesda, Maryland. Diagnostic criteria and investigation procedures were agreed upon by the participating neuromuscular centres under the leadership of the European Neuromuscular Centre (Goebel and Fardeau, 2004; Goebel et al., 2008).

Myopathological and double immunofluorescence analyses

Skeletal muscle biopsies were performed in two patients from Family I, one patient from Family II, three patients from Family III and 10 existing muscle samples from the previously reported German p.W2710X families (Kley et al., 2007) were re-examined and re-evaluated for comparative analysis. Previously established procedures for light and electron microscopy analyses (Olivé et al., 2005) were used. Double immunofluorescence staining was performed on 4 µm frozen serial sections of muscle biopsies from two patients from Family III with p.V930_T933del mutation and two patients with p.W2710X mutation. Primary antibodies used in this study are listed in Table 1. A newly developed FLNC-specific rabbit polyclonal antibody and a mouse monoclonal myotilin antibody were used to localize areas of protein aggregation in double staining experiments with mouse antibodies and rabbit or guinea pig antibodies, respectively. Isotype-specific secondary antibodies conjugated with fluorescein isothiocyanate (Dako), DyLight 488, Cy3 or Texas Red (Dianova) were applied according to the recommendations of the manufacturer. Adjacent serial sections were stained with Gomori trichrome and Haematoxylin and Eosin.

Cloning of full length and truncated filamin C constructs

Full-length FLNC complementary DNA clones in pEGFP-N3 or pEGFP-C2 (Clontech) were obtained as described (Duff et al., 2011). The c.2997_3008del (p.V930_T933del, d7ΔVKYT) mutation was introduced in the complementary DNA encoding full-length FLNC in pEGFP-N3 using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) and the following oligonucleotides: ACAACCAT GACTACTCTACTAGTGTCCACAGC and CTGCTGACACGAGT CAGGAGTAGTCATGGTTGT. The c.8130G > A (p.W2710X) mutation was introduced into full-length FLNC clones by exchanging the complementary DNA encoding wild-type immunoglobulin-like domains 23–24 with the truncated variant (Löwe et al., 2007) using a unique BspEI restriction site within the complementary DNA sequence encoding immunoglobulin-like domain 23.

For biochemical analyses complementary DNA fragments encoding wild-type and mutant c.2997_3008del, d7ΔVKYT immunoglobulin-like domains 7–8 or 5–9 were amplified from the patient’s complementary DNA using primers F(GTGCGGGACTTTGAGATCAT) and R(ATTCACCAAAGGGGCTCT) or F(CACATCCTGCCCGCCCCACCT) and R(AGGCCCCAATGTTGGCCTTGA), respectively. Fragments were cloned into prokaryotic expression vectors pET23-EEF or pET23-T7 enabling expression of fusion proteins carrying a C-terminal His-tag and a C-terminal EEF- or N-terminal T7-immunotag, and into pGEX-6P3 enabling expression of glutathione S-transferase-fusion proteins (Amersham). Integrity of all constructs was verified by sequencing (LCG Genomics).

Expression and purification of recombinant proteins

Protein expression in Escherichia coli BL21(DE3) CodonPlus cells (Stratagene) and purification of His-tagged proteins was performed essentially as described previously (Linnemann et al., 2010). Glutathione S-transferase-tagged proteins were purified using ProtiOn® Glutathione Agarose 4B according to the manufacturer’s instructions (Macherey-Nagel). After purification, the glutathione S-transferase tag was removed using PreScission Protease (Amersham Biosciences) according to the recommendation of the manufacturer. Subsequently, the glutathione S-transferase tag was removed from the protein solution together with the glutathione S-transferase-tagged PreScission Protease, by affinity chromatography using ProtiOn® Glutathione Agarose 4B.

Biophysical characterization of recombinant proteins

Circular dichroism spectroscopy was performed subsequent to dialysis of purified proteins (wild-type d7–8 and mutant d7–8ΔVKYT) against 0.1 M potassium chloride and 10 mM potassium phosphate, pH 7.3. The samples at a concentration of 0.5 mg/ml were added to 1 mm quartz cuvettes and circular dichroism spectra recorded in the spectral range from 195 to 260 nm. For differential scanning fluorimetry (thermolfluor) stability assays, SYPRO® Orange dye (40 × , Invitrogen) was
Table 1  Primary antibodies used in double immunofluorescence studies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone/code</th>
<th>Source</th>
<th>Company</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>19S regulator</td>
<td>AH1.1</td>
<td>Mouse (mAb)</td>
<td>Abcam</td>
<td>1/100</td>
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<tr>
<td>20S subunits</td>
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<td>Rabbit (pAb)</td>
<td>Thermo Scientific</td>
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<td>Rabbit (pAb)</td>
<td>Abcam</td>
<td>1/100</td>
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<td>ab47124</td>
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<td>Abcam</td>
<td>1/2000</td>
</tr>
<tr>
<td>CHIP</td>
<td>PC711</td>
<td>Rabbit (pAb)</td>
<td>Calbiochem</td>
<td>1/1000</td>
</tr>
<tr>
<td>Filamin C</td>
<td>FLNC d16-20</td>
<td>Rabbit (pAb)</td>
<td>Custom-made (BioGenes)</td>
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</tr>
<tr>
<td>Hsp20</td>
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</tr>
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<td>1/50</td>
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<td>Novocastra</td>
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<td>1/200</td>
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<td>Novocastra</td>
<td>1/20</td>
</tr>
<tr>
<td>p53</td>
<td>PAb 240</td>
<td>Mouse (mAb)</td>
<td>Abcam</td>
<td>1/250</td>
</tr>
<tr>
<td>p62</td>
<td>GP62-N</td>
<td>Guinea pig (pAb)</td>
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<tr>
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<td>Rabbit (pAb)</td>
<td>Abcam</td>
<td>1/50</td>
</tr>
<tr>
<td>Ubiquitin + 1</td>
<td>4083</td>
<td>Mouse (mAb)</td>
<td>Abcam</td>
<td>1/25</td>
</tr>
<tr>
<td>VCP</td>
<td>5</td>
<td>Mouse (mAb)</td>
<td>ABR</td>
<td>1/500</td>
</tr>
</tbody>
</table>

mAb = monoclonal antibodies; pAb = polyclonal antibodies.
added to 5μg protein sample in a total volume of 25μl. Assays were carried out in an iQ™5 real-time PCR detection system (BIO-RAD). Temperatures varied from 20 to 95°C with a fluorescence excitation at 470 nm and detection at 555 nm.

**Structural analysis of recombinant proteins**

To understand the effect of mutations at the molecular level, we searched for closest structural homologues in the protein structure database (PDB) employing PSI-Search, and the PHyre server (Kelley and Sternberg, 2009). Three structural homologues were identified: immunoglobulin-like domain 13 of human FLNC (PDB code: 2D74; sequence ID: 38%), immunoglobulin-like domain 14 of human FLNC (PDB code: 2D7M; sequence ID: 38%) and immunoglobulin-like domains 5–6 of *Dictyostelium discoideum* FLNC (PDB code: 1QFH; sequence ID: 23%). The structure and 2D maps were used for subsequent structural analyses of the deletion mutant FLNC d73VKYT.

**Limited proteolysis**

Proteolytic susceptibility was investigated using the endopeptidase thermolysin (Sigma). Recombinant proteins were diluted to 10μM in 50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0. 10 μg/ml thermolysin was added and the mixture was incubated at 37°C for 15 minutes. The reaction was stopped by adding 0.2 vol. 5 × SDS sample buffer. The samples were analysed by SDS–PAGE on 10% polyacrylamide gels.

**Cell culture and transfection studies**

C2 and C2C12 cells were cultured to 60–80% confluence in six-well plates (TPP) in Dulbecco’s modified Eagle medium supplemented with 15% foetal calf serum, 4 mM l-glutamine, 1% non-essential amino acids and 2 mM sodium pyruvate (Invitrogen). Subsequently, the cells were transfected with wild-type or mutant FLNC constructs using Lipofectamine® LTX and Plus Reagent according to the manufacturer’s instructions (Invitrogen). Aggregation was evaluated 12 and 24h after transfection by live cell imaging using an IX51 microscope (Olympus) or an LSM710 confocal microscope (Zeiss). Unpaired t-tests were used for statistical analysis.

**Results**

**Myofibrillar myopathy patients with filamin C mutations exhibit a uniform phenotype**

A comparative analysis of clinical data of the 25 newly identified patients (18 from Family I, three from Family II and four from Family III) is presented and compared with data on 41 previously reported patients (Table 2). The average age of disease onset was 39–56 years and did not vary significantly between families. The most frequent initial symptom was bilateral weakness in the proximal lower limb muscles manifesting as difficulty with rising from a chair and climbing stairs. Other muscle groups became involved upon disease progression, primarily proximal muscles of the upper limbs and later distal muscles, neck flexors, abdominal/paraspinal and rarely, facial muscles. Muscle weakness slowly progressed to inability to walk. Respiratory weakness developed with disease progression and was a frequent cause of death. A number of patients in each studied family had cardiac abnormalities. Sudden cardiac arrest was presumed in at least three patients from the Chinese family (Luan et al., 2010) and in two from the Macedonian family. Three patients from the same Chinese family suffered from unexplained chronic gastroenteritis before the development of neurological symptoms (Luan et al., 2010). A similar phenotype was noted in one of the newly identified Macedonian patients. Creatine kinase levels were elevated in the majority of cases.

Muscle imaging pattern of lipomatous alterations in lower limb muscles in three patients from Family III carrying the p.V930_T933del mutation (Fig. 2) was similar to that in 10 previously reported p.W2710X patients (Fischer et al., 2008). On the thigh level, adductor longus and adductor magnus, semimembranosus and biceps femoris were most severely affected in the p.W2710X patients, although the involvement of rectus femoris was more pronounced in the carriers of the p.V930_T933del mutation. In the lower legs, the soleus showed distinct alterations in all patients irrespective of the mutation and the medial head of the gastrocnemius was always more affected than the lateral head. Table 3 provides a comparison of myopathological findings in patients with p.W2710X, p.V930_T933del and p.K899_V904del/V899_C900ins mutations in FLNC. The histological features are similar and seem to be independent of the individual mutation. Some phenomena such as rimmed vacuoles may not be detected in early stages of the disease. The most important finding is the presence of myofibrillar polymorphous aggregates (Supplementary Fig. 1) appearing as single or multiple plaque-like formations within the cytoplasm, as convoluted serpentine inclusions of varying thickness or spheroid bodies. Details of histopathological findings are provided in the online Supplementary material. Ultrastructural examination reveals major myofibrillar abnormalities, including accumulation of fine thin filaments emanating from the Z-disc that coalesce into electron dense inclusions underneath the sarcolemma or between the myofibrils often surrounded by groups of mitochondria (Fig. 3). Many fibres contain large areas occupied by granulofilamentous material interspersed with remnants of filaments and small nemaline rods. Additional findings include tubulofilaments measuring 18 nm and large autophagic vacuoles containing myelin-like figures and cellular debris. In general, the range of myopathological changes in these filaminopathy patients overlap significantly with phenomena described in other subtypes of myofibrillar myopathy.

**Double immunofluorescence studies indicate an impairment of protein degradation in abnormal fibres**

To analyse skeletal muscle samples from patients with myofibrillar myopathy with FLNC mutations for disturbances of protein quality control mechanisms, we studied the expression and distribution patterns of heat shock proteins (Fig. 4), markers for...
a BAG3-mediated degradation pathway called chaperone-assisted selective autophagy (Fig. 5), the autophagic-lysosomal pathway (Fig. 6), and the ubiquitin–proteasome system (Fig. 7). Findings of our immunolocalization studies were similar in all patients independent of the FLNC mutation (p.V930_T933del or p.W2710X). Figures 4–7 show the results in a patient with p.V930_T933del mutation. It is noteworthy that increased immunoreactivity of all proteins listed above was only detected in abnormal fibres containing protein aggregates (identified by modified Gomori Trichrome staining and immunostaining for FLNC or myotilin) in at least a subset of serial sections, but not in normal looking myofibres.

Immunofluorescence studies revealed increased immunoreactivity for heat shock proteins Hsp20, Hsp27, Hsp40 and Hsp60 in abnormal fibres, predominantly located within protein aggregates. In contrast, immunoreactivity for Hsp90 was especially enhanced in the periphery of aggregates (Fig. 4). Chaperone-assisted autophagy components (Hsp22, Hsp70 and p62) showed strong immunoreactivity either within or outside of the protein aggregates (CHIP), while BAG3 was found at both locations (Figs 5 and 6).

Strong immunoreactivity for the autophagy markers LC3 and LAMP2 was more frequently observed between rather than within the aggregates. HDAC6 and VCP also displayed increased

Table 2 Comparative clinical analysis of 66 filaminopathy patients with myofibrillar myopathy phenotype

<table>
<thead>
<tr>
<th>Reference</th>
<th>Family I</th>
<th>Family II</th>
<th>Family III</th>
<th>Kley et al., 2007</th>
<th>Luan et al., 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inheritance pattern</td>
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<td>AD</td>
<td>AD</td>
<td>AD</td>
<td>AD</td>
</tr>
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<td>China</td>
<td>Germany</td>
<td>Germany</td>
<td>China</td>
</tr>
<tr>
<td>Studied patients</td>
<td>18</td>
<td>3</td>
<td>4</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>Onset age, mean (range), years</td>
<td>41 ± 4 (34–52)</td>
<td>56 ± 1 (55–57)</td>
<td>48 ± 12 (34–60)</td>
<td>44 ± 6 (24–57)</td>
<td>39 ± 3 (35–40)</td>
</tr>
<tr>
<td>Gender (female/male)</td>
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<td>1/2</td>
<td>3/1</td>
<td>22/9</td>
<td>3/7</td>
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<td>Initial symptoms</td>
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<tr>
<td>Back pain</td>
<td>0/9</td>
<td>0/1</td>
<td>0/4</td>
<td>11/26</td>
<td>5/10</td>
</tr>
<tr>
<td>Proximal LL weakness</td>
<td>8/8</td>
<td>3/3</td>
<td>4/4</td>
<td>23/26</td>
<td>10/10</td>
</tr>
<tr>
<td>Advanced illness</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Skeletal myopathy</td>
<td></td>
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<tr>
<td>Limb girdle weakness</td>
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</tr>
<tr>
<td>LL alone</td>
<td>0/8</td>
<td>3/3</td>
<td>0/4</td>
<td>4/28</td>
<td>0/10</td>
</tr>
<tr>
<td>P &gt; D, LL &gt; UL</td>
<td>3/8</td>
<td>0/3</td>
<td>4/4</td>
<td>20/28</td>
<td>10/10</td>
</tr>
<tr>
<td>P &gt; D, LL = UL</td>
<td>0/8</td>
<td>0/3</td>
<td>0/4</td>
<td>2/28</td>
<td>0/10</td>
</tr>
<tr>
<td>P &gt; D, LL &lt; UL</td>
<td>3/8</td>
<td>0/3</td>
<td>0/4</td>
<td>1/28</td>
<td>0/10</td>
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<tr>
<td>D &gt; P, LL</td>
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<td>0/3</td>
<td>0/4</td>
<td>1/28</td>
<td>0/10</td>
</tr>
<tr>
<td>Trunk/abdominal muscle weakness</td>
<td>6/8</td>
<td>0/3</td>
<td>2/3</td>
<td>10/28</td>
<td>2/10</td>
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<td>Winged scapula</td>
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<td>0/1</td>
<td>3/3</td>
<td>6/28</td>
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<td>0/3</td>
<td>0/3</td>
<td>5/28</td>
<td>0/10</td>
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<td>0/3</td>
<td>1/3</td>
<td>0/28</td>
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<td>14/30</td>
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<td>1/3</td>
<td>0/3</td>
<td>3/9</td>
<td>1/6</td>
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<tr>
<td>LV hypertrophy</td>
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<td>0/3</td>
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<td>3/9</td>
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<td>n.k.</td>
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<td>1/1</td>
<td>3/3</td>
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<td>2/2</td>
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<td>4/4</td>
<td>0/1</td>
<td>2/2</td>
<td>16/16</td>
<td>2/2</td>
</tr>
<tr>
<td>NCS abnormality</td>
<td>n.d.</td>
<td>0/1</td>
<td>0/2</td>
<td>0/14</td>
<td>0/2</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheelchair dependency</td>
<td>2/8</td>
<td>0/3</td>
<td>2/4</td>
<td>4/31</td>
<td>n.r.</td>
</tr>
<tr>
<td>Respirator dependency</td>
<td>1/8</td>
<td>0/3</td>
<td>0/4</td>
<td>3/31</td>
<td>n.r.</td>
</tr>
<tr>
<td>Death before age 65 (range, years)</td>
<td>8/9 (47–61)</td>
<td>1/2 (62)</td>
<td>n.k.</td>
<td>4/9 (54–64)</td>
<td>6/6 (59–60)</td>
</tr>
</tbody>
</table>

AD = autosomal dominant; CK = creatine kinase; CM = cardiomyopathy; D = distal; Ig = immunoglobulin; LL = lower limbs; LV = left ventricular; NCS = nerve conduction study; P = proximal; nd = not done; n.r. = not reported; n.e. = not extractable; n.k. = not known.

*presumed; **includes atrial flutter, decreased ejection fraction, mitral/aortic valve regurgitation, palpitations, ST segment depression; number of studied patients is indicated as denominator.
immunoreactivity in abnormal fibres, particularly around and in the space between protein deposits. The most distinctly enhanced p53 immunoreactivity was detected between aggregates and in sub-sarcolemmal regions. Immunoreactivity for p62 was markedly increased within protein aggregates (Fig. 6). Abundant reactivity for 19S proteasome, 20S proteasome, ubiquitin, mutant ubiquitin (UBB + 1) and atrogin 1, all markers of ubiquitin–proteasome system, was observed around and between aggregates, but also, especially ubiquitin, within protein deposits (Fig. 7).

### Biophysical characteristics

In order to investigate potential alterations of biophysical and biochemical properties caused by the p.V930_T933del mutation, we first analysed the secondary structure of the mutant d7-8ΔVKYT by circular dichroism spectroscopy of the bacterially expressed recombinant protein. In accordance with the established crystal structures of immunoglobulin-like domains of filamins (Djinović-Carugo and Carugo, 2010; Nakamura et al., 2011), the wild-type
proteins exhibited a high proportion of \( \beta \)-strands, as evident by a minimum in the circular dichroism spectra at 218 nm and a positive ellipticity at 205 nm. The spectrum of d7-8\textbackslash\textsuperscript{VKYT} was significantly different from that of the wild-type protein, in that the mutant showed a blue shift, displaying a minimum in circular dichroism spectra at 208 nm and a positive ellipticity at 200 nm (Fig. 8A). Apart from that, the maximum amplitude of the mutant protein was highly increased. These changes in circular dichroism spectra are a characteristic sign of a higher proportion of unfolded or disordered structures, indicating significant changes in the 3D structure of the mutant domain. Apart from that, the spectrum displays an increase of magnitude in the region typical for \( \alpha \)-helices, with the exception of the high-energy spectral range, suggesting formation of a short \( \alpha \)-helical region.

To test whether this alteration undermines protein stability, we compared thermal stability (thermofluor) of a recombinant bacterially expressed fragment comprising immunoglobulin-like domains 7–8 to the corresponding fragment harbouring the deletion mutant (d7–8\textbackslash\textsuperscript{VKYT}) using fluorescence-based thermal shift assays. This revealed a melting temperature of 69 °C for the wild-type fragment, whereas the mutant variant showed a considerably decreased average melting temperature of 56 °C (Fig. 8B).

The second test for protein stability, limited thermolysin protease digestion, revealed a significantly increased susceptibility of mutant recombinant FLNC fragments d7–8\textbackslash\textsuperscript{VKYT} (not shown) and d5–9\textbackslash\textsuperscript{VKYT} (Fig. 8C). Whereas a major fraction of the wild-type fragments was still intact after 60 min of incubation with the enzyme, the mutant proteins were already almost completely digested after

Figure 3 Ultrastructural analysis of skeletal muscle from the proband of the Macedonian family. (A and B) Fine filaments emanating at the Z-line level that coalesced into electron-dense inclusions under the sarcolemma or between the myofibrils are surrounded by groups of mitochondria (B). (C–E) Collections of tubulofilaments (right upper corner in C), granulofilamentous material and small vacuoles (left lower corner in C); small rod bodies at the periphery of a fibre region harbouring granulofilamentous material (D), fine electron-dense granulofilamentous material, vacuoles and sparse mitochondria between normal myofibrils (E). (F) Abnormal fibre region containing remnants of filaments, small rod bodies, and prominent electrondense inclusions. Scale bars: C = 0.5 \( \mu \text{m} \); B, D and E = 1 \( \mu \text{m} \); A, F = 2 \( \mu \text{m} \).
5–10 min, similarly to our previously described experiments with the p.W2710X mutant FLNC fragments (Löwe et al., 2007).

**Actin-binding capacity**

Since FLNA was recently shown to bind to F-actin not only through its actin-binding domain, but also through its first rod domain (immunoglobulin-like domains 1–15) (Nakamura et al., 2007), we hypothesized that the mutation in immunoglobulin-like domain 7 might alter actin-binding capacities of FLNC. In our actin co-sedimentation assays using FLNC immunoglobulin-like domains 5–9 we could not, however, detect any binding of either the wild-type or the mutant variant to F-actin (results not shown). This renders it highly improbable that an alteration of

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**Figure 4** Immunolocalization of heat shock proteins in skeletal muscle cryosections from a filaminopathy patient from Family III (p.V930_T933del mutation). Serial cryosections were double-stained with antibodies recognizing the indicated heat shock proteins (Hsp) and either FLNC (Filamin C) or myotilin to localize protein aggregates. For comparison, trichrome and haematoxylin and eosin (H&E) stained sections are shown at the top. Scale bar = 50 μm.
the binding capacity of the p.V930_T933del mutant to F-actin plays a significant role in the pathomechanism of filaminopathy caused by this deletion mutation or any other mutation in this part of FLNC.

Transfection studies

We have previously shown that expression of mutant p.W2710X variants of mini-FLNC (actin-binding domain + immunoglobulin-like domains 15–24) result in spontaneous aggregation of the mutant but not the wild-type protein (Löwe et al., 2007). These experiments were performed in non-muscle cells. To investigate the behaviour of mutant FLNC proteins in muscle cells, we transiently transfected C2 and C2C12 cell lines with constructs encoding N- or C-terminal EGFP-fusion proteins of full-length wild-type, p.W2710X or p.V930_T933del FLNC variants and analysed aggregation at different time points after transfection by using live cell microscopy. In both cell lines the expression of mutant FLNC induced the development of cytoplasmic aggregates (Fig. 9). Analysis of the number of transfected cells containing filamin aggregates showed that in comparison with cells transfected with wild-type FLNC, transfection with p.V930_T933del FLNC yielded 1.6–3.7 times more cells with aggregates, while transfection with p.W2710X FLNC resulted in an 8 to 30 times increased number of cells containing aggregates (Table 4). All differences were statistically significant, indicating that expression of mutant FLNC is sufficient for the increased formation of protein aggregates in muscle cells. Noteworthy, transfection with the p.W2710X mutant resulted in up to 11.4 times more cells with aggregates than the p.V930_T933del mutant.

Figure 5  Immunolocalization of the components of chaperone complexes involved in chaperone-assisted selective autophagy in skeletal muscle cryosections from a filaminopathy patient (p.V930_T933del mutation). Serial cryosections were double-stained with antibodies recognizing the indicated chaperone-assisted selective autophagy pathway markers and either FLNC or myotilin to localize protein aggregates. For comparison, trichrome and haematoxylin and eosin (H&E) stained sections are also shown. Scale bar = 50 μm.
Figure 6  Immunolocalization of markers of autophagy and associated proteins in skeletal muscle cryosections from a filaminopathy patient with p.V930_T933del mutation. Serial sections were double-stained with antibodies recognizing the indicated heat shock proteins and either FLNC or myotilin to localize protein aggregates. For comparison, trichrome and haematoxylin and eosin (H&E) stained sections are also shown. Scale bar = 50 μm.
Discussion

We provide here analysis of phenotypic characteristics and the pathomechanisms of myofibrillar myopathy caused by mutations in immunoglobulin-like domains of FLNC in new and newly re-evaluated families and compare our results to recently reported data. This analysis is needed for the progress of future studies in the field of filaminopathies, including prospects in differential diagnostics and treatment strategies.
Comparative clinical and myopathological characteristics of myofibrillar myopathy associated with filamin C rod mutations

The initial identification of a mutation in FLNC causing myofibrillar myopathy (Vorgerd et al., 2005) induced worldwide genetic testing for FLNC mutations in patients with various types of muscle disease. Subsequently, families with filamin C myopathy were identified in Europe, Asia, USA and Australia (Kley et al., 2007; Shatunov et al., 2009; Luan et al., 2010; Duff et al., 2011; Guergueltcheva et al., 2011; Selcen, 2011a). We include in this report new families from Macedonia and China harbouring the p.W2710X mutation in the dimerization domain of FLNC that is identical to the mutation previously described in German families (Kley et al., 2007). These observations have now established that the p.W2710X mutation is the cause of filaminopathy in genetically unrelated families originating from different ethnic groups, which implies that FLNC codon 2710 is a mutational hotspot. We also report the results of re-evaluation of a German family with an FLNC p.V930_T933del mutation. The phenotypic features of myofibrillar myopathy caused by FLNC rod mutations, either p.W2710X (occurring in immunoglobulin-like domain 24) or p.V930_T933del (occurring in immunoglobulin-like domain 7), are markedly homogeneous, presenting with limb-girdle muscular...
dystrophy distribution of muscle weakness, involving distal muscles in the course of illness, and showing pathological phenomena similar to the previously described in other myofibrillar myopathy subtypes (Vorgerd et al., 2005). Less than 5% of patients show distal lower limb weakness as the first clinical sign. This phenotype differs, however, from those caused by c.577G>A (p.A193T) and c.752T>C (p.M251T) mutations occurring in the actin-binding domain, or the c.5160delC (p.F1720LfsX63) mutation in the rod domain of FLNC leading to haploinsufficiency; both account for distal myopathy regularly starting in the upper limbs without obvious histological changes typical of myofibrillar myopathy (Duff et al., 2011; Guergueltcheva et al., 2011).

New data on p.W2710X and p.V930_T933del mutations strongly confirm cardiac and respiratory muscle involvement that shortens life expectancy to a significant degree. Diagnostic criteria of this type of disease, in comparison with data available for FLNC-associated distal myopathy, are summarized in Table 5. If these criteria are applicable to a patient under evaluation, it is advisable to perform sequence analysis of FLNC. Since exon 48 is a hot spot for mutations, we recommend sequencing this exon first, followed by a full FLNC sequencing in case of a negative result. It is very important to use appropriate primers to avoid the interference with a pseudogene (pseFLNC) that may lead to misinterpretation of sequencing data (Odgerel et al., 2010; van der Ven et al., 2010). A mutation in the actin-binding domain of FLNC should be considered in patients with distal myopathy, especially if thenar atrophy is the first clinical symptom, and the family history is consistent with an autosomal dominant pattern of inheritance.

A further helpful contribution to differential diagnosis in this group of disorders is the characterization of muscle imaging. Lipomatous muscle alterations in the lower extremities observed in patients with p.W2710X and p.V930_T933del mutations closely resemble the patterns previously described in patients with myofibrillar myopathy and allow the use of an algorithm we recently developed for the differentiation of myofibrillar myopathy subtypes (Kley et al., 2007; Fischer et al., 2008; Wattjes et al., 2010). Muscle imaging is also useful for selecting an affected muscle for a diagnostic biopsy.

The most characteristic myopathological finding in myofibrillar myopathy-associated filaminopathy is the presence of polymorphous aggregates appearing at light microscopy as single or multiple plaque-like formations, convoluted serpentine inclusions or
spheroid bodies within the cytoplasm of affected myofibres and, at the ultrastructural level, as electron-dense inclusions or larger areas of granulofilamentous material interspersed with remnants of filaments and small nemaline rods. Accumulating aggregates eventually leading to myofibrillar destruction is a major feature of myofibrillar myopathy (Nakano et al., 1996; Schröder and Schoser, 2009; Selcen, 2011a).

Table 4 Statistical analyses of data from transfection studies

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Vector</th>
<th>FLNC insert</th>
<th>12 h after transfection</th>
<th>24 h after transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GFP expressing cells (%)</td>
<td>Cells harbouring aggregates (%)</td>
</tr>
<tr>
<td>C2</td>
<td>pEGFPC2</td>
<td>Wild-type</td>
<td>12.3</td>
<td>4.9</td>
</tr>
<tr>
<td>C2</td>
<td>pEGFPC2</td>
<td>p.V930_T933del</td>
<td>12.6</td>
<td>10.5**</td>
</tr>
<tr>
<td>C2</td>
<td>pEGFPC2</td>
<td>p.W2710X</td>
<td>11.4</td>
<td>45.4**</td>
</tr>
<tr>
<td>C2</td>
<td>pEGFPN3</td>
<td>Wild-type</td>
<td>25.5</td>
<td>0.9</td>
</tr>
<tr>
<td>C2</td>
<td>pEGFPN3</td>
<td>p.V930_T933del</td>
<td>27.1</td>
<td>2.3**</td>
</tr>
<tr>
<td>C2</td>
<td>pEGFPN3</td>
<td>p.W2710X</td>
<td>18.5</td>
<td>26.3**</td>
</tr>
<tr>
<td>C2C12</td>
<td>pEGFPC2</td>
<td>Wild-type</td>
<td>29.8</td>
<td>4.1</td>
</tr>
<tr>
<td>C2C12</td>
<td>pEGFPC2</td>
<td>p.V930_T933del</td>
<td>27.3</td>
<td>14.8**</td>
</tr>
<tr>
<td>C2C12</td>
<td>pEGFPC2</td>
<td>p.W2710X</td>
<td>20.8</td>
<td>47.7**</td>
</tr>
<tr>
<td>C2C12</td>
<td>pEGFPN3</td>
<td>Wild-type</td>
<td>33.9</td>
<td>0.8</td>
</tr>
<tr>
<td>C2C12</td>
<td>pEGFPN3</td>
<td>p.V930_T933del</td>
<td>34.0</td>
<td>1.4*</td>
</tr>
<tr>
<td>C2C12</td>
<td>pEGFPN3</td>
<td>p.W2710X</td>
<td>23.7</td>
<td>6.4**</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.001 (comparison p.V930_T933del/wild-type and p.W2710X/wild-type). More than 50,000 green fluorescent protein (GFP) expressing cells have been evaluated for statistical analyses.

Table 5 Main characteristics of filaminopathies

<table>
<thead>
<tr>
<th>Inheritance pattern</th>
<th>FLNC rod domain mutations associated with MFM phenotype</th>
<th>Distal myopathy caused by FLNC actin-binding domain mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autosomal dominant</td>
<td>Autosomal dominant</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>Fourth to sixth decade of life</td>
<td>Proximal lower limb weakness</td>
<td>Third to fourth decade of life</td>
</tr>
<tr>
<td>Proximal upper limbs, distal limbs and trunk muscles</td>
<td>Involvement (weakness) of proximal upper limbs, distal limbs and trunk muscles</td>
<td>Calf muscle weakness, proximal muscle weakness</td>
</tr>
<tr>
<td>Thigh: semimembranosus, adductor magnus and longus, biceps femoris, vastus intermedius and vastus medialis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower leg: soleus, gastrocnemius medialis, tibialis anterior</td>
<td>Frequent</td>
<td>Insufficient data (reported in two out of 13 patients)</td>
</tr>
<tr>
<td>Regular in advanced illness</td>
<td>Thigh: semimembranosus, semitendinosus, biceps femoris, adductor magnus</td>
<td></td>
</tr>
<tr>
<td>Muscle imaging of lower limbs (most affected muscles)</td>
<td>Thigh: semimembranosus, semitendinosus, biceps femoris, adductor magnus</td>
<td></td>
</tr>
<tr>
<td>Normal up to 10× increased</td>
<td>Up to 2.5× increased</td>
<td></td>
</tr>
<tr>
<td>Non-specific changes, dystrophic pattern in advanced disease</td>
<td>Non-specific changes, dystrophic pattern in advanced disease</td>
<td></td>
</tr>
<tr>
<td>Polymorphous cytoplasmic protein aggregates (plaque-like formations, convoluted serpentine inclusions, spheroid bodies)</td>
<td>No myofibrillar myopathy-typical protein aggregation</td>
<td></td>
</tr>
<tr>
<td>Rimmed vacuoles</td>
<td>Areas lacking oxidative enzyme activity (moth-eaten appearance)</td>
<td></td>
</tr>
<tr>
<td>Core-like lesions, Type I fibre predominance</td>
<td>EM: no myofibrillar pathology</td>
<td></td>
</tr>
<tr>
<td>EM: myofibrillar disintegration, deposits of granulofilamentous material, tubulofilamentous inclusions, nemaline rods, autophagic vacuoles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Insights into pathomechanisms of myofibrillar myopathy associated with filamin C mutations

Mutant FLNC interferes with protein homeostasis thus launching a process that results in the formation of massive protein aggregates. It has been shown that the cell reacts to this process in
multiple ways. Molecular chaperones bind non-native proteins and facilitate folding of newly translated or damaged proteins into protein complexes (Bukau and Horwich, 1998; Frydman, 2001; Young et al., 2004; Hartl and Hayer-Hartl, 2009). Other chaperones determine whether misfolded or aggregated proteins are subsequently degraded by the ubiquitin–proteasome machinery or through the autophagy pathway (Massey et al., 2006; Arndt et al., 2007, 2010; Carra et al., 2008; Gamerdinger et al., 2009). Impairment of protein degradation plays a pivotal pathogenic role in protein aggregate diseases including many neurodegenerative diseases that are characterized by intracellular accumulation of altered or misfolded proteins (de Pril et al., 2006; Rubinsztein, 2006; Lehman, 2009; Xilouri and Stefanis, 2011). Abnormal expression of proteins involved in protein degradation was observed in some subtypes of myofibrillar myopathy (Olivé et al., 2008), but specific and detailed knowledge of the mechanisms of disease in FLNC-associated myofibrillar myopathy is still lacking. We performed extensive immunofluorescence studies of several protein degradation components in muscle tissue sections of patients with p.W2710X and p.V930,T933del FLNC mutations.

Our results indicate that muscle fibres react to aggregate formation with a highly increased expression and accumulation of chaperones and proteins involved in proteasomal protein degradation and autophagy including chaperone-assisted selective autophagy. However, these compensatory responses do not warrant efficient protein degradation. The detection of abundant mutant ubiquitin UBB+1 in abnormal fibres suggests ubiquitin–proteasome system dysfunction, as noted in other subtypes of myofibrillar myopathy (Olivé et al., 2008), but specific and detailed knowledge of the mechanisms of disease in FLNC-associated myofibrillar myopathy is still lacking. We performed extensive immunofluorescence studies of several protein degradation components in muscle tissue sections of patients with p.W2710X and p.V930,T933del FLNC mutations.

Our data suggest that the diminished functionality of both ubiquitin–proteasome system and autophagy impairs the capacity of molecular chaperones, whose expression levels are increased in abnormal fibres, to prevent the formation or mediate degradation of aggregates. This also seems to be true of chaperone-assisted selective autophagy, an autophagy pathway dedicated to the degradation of Z-disk proteins like FLNC. This chaperone machinery consists of BAG3, Hsp22, Hsp70 and CHIP and is essential for Z-disk maintenance (Arndt et al., 2010).

In addition to the p.W2710X mutation, we present here for the first time data providing insights into the pathomechanisms of the p.V930,T933del mutation located in immunoglobulin-like domain 7 of FLNC. Structural analysis based on the similarly structured immunoglobulin-like domain 14 of human FLNC (PDB code: 2D7M) identified the location and function of the deleted residues (930VKYT933) within immunoglobulin-like domain 7. These residues reside in β-strand E, which is sandwiched by β-strands B and D, and are involved in a network of interactions stabilizing the immunoglobulin-like fold. The hydrophobic residues V (930) and Y (932) normally stabilize the core of the domain through hydrophobic interactions with neighbouring buried residues. The main chain atoms of residues VKY are also involved in a hydrogen bonding network that stabilizes the β-sheet formed by β-strands B, E and D. Deletion of these four residues therefore has a dual destabilizing effect: first, it disrupts the hydrophobic core of the immunoglobulin-like fold and concomitantly loosens the β-sheet that is critical for immunoglobulin-like domain integrity; this is clearly reflected in the circular dichroism spectra and thermal stability assays. Second, the deletion results in exposure of the hydrophobic core of the domain and leads to non-specific protein–protein interactions resulting in protein aggregation.

These results, together with the previously obtained biochemical and biophysical data on the effects of the p.W2710X mutation (Vorgerd et al., 2005; Löwe et al., 2007), uncover the pathway by which both the p.W2710X and the p.V930,T933del mutations cause a moderate to pronounced spontaneous aggregation of mutant FLNC upon transient expression in murine myoblasts. The efficiency of the transfection of C2 and C2C12 cells and the high proportion of transfected cells that show aggregates make them an excellent model for studies of the effects of pharmacological substances on protein aggregation in muscle cells. The establishment of stably transfected cells might further improve the applicability of this cell culture model for these purposes.

To summarize, our results of functional and immunolocalization studies suggest that pathogenic myofibrillar myopathy-causing mutations in the rod of FLNC promote misfolding of affected immunoglobulin-like domains, thereby inducing aggregation of the mutant protein. Our clinical and histological data, especially the late disease onset and the finding that patients in early disease stages show only mild protein aggregation, indicate that muscle fibres can compensate for this effect for long periods of time by mechanisms including the aforementioned protein degradation strategies. Proteasomal and autophagic degradation pathways were shown to decline in relation to oxidative stress and mitochondrial alterations accruing with ageing (Combaret et al., 2009; Wohlgemuth et al., 2010). Both will attenuate protective mechanisms, resulting in manifest aggregation of mutated FLNC and triggering the accumulation of other proteins. As a consequence, Z-disk proteins entrapped in aggregates will disturb general Z-disk protein homeostasis and lead to focal myofibril destruction. These assumptions are consistent with findings in other protein aggregation disorders (Grune et al., 2004; Lehman, 2009; Riederer et al., 2011).
Implications for future therapeutic strategies

At present, therapy of filaminopathy is limited to symptomatic treatment. Data presented here underline the importance of regular cardiac and pulmonary evaluation in order to prevent complications and premature death by timely pacemaker implantation or respiratory support, as indicated. We also advise our patients to perform regular aerobic exercise training and to try creatine supplementation since this has proven to be safe and beneficial in various muscle diseases (Olsen et al., 2005; Ormgaard et al., 2005; Dawes et al., 2006; Jeppesen et al., 2006; Sveen et al., 2007, 2008; Kley et al., 2011).

The finding that increased immunoreactivity of various components of the protein degradation machinery occurs only in muscle fibres harbouring protein aggregates raises one important possibility: early induction of e.g. chaperones or specific E3 ligases may be able to prevent protein aggregation. If confirmed, this may offer new therapeutic approaches. Results of studies in other myofibrillar myopathy subtypes support this hypothesis: over-expression of αβ-crystallin significantly reduced aberrant protein aggregation in cultured HEK293 cells expressing mutant desmin (Wang et al., 2003). Hsp70 and αβ-crystallin also significantly reduced desmin aggregation and attenuated the induction of ubiquitin–proteasome system malfunction in cardiomyocytes expressing mutant desmin (Liu et al., 2006). In cell models of αβ-crystallinopathy, over-expression of Hsp22, Hsp25, Hsp27, Hsp70 and wild-type αβ-crystallin efficiently prevented aggregate formation (Chávez Zobel et al., 2003; Sanbe et al., 2007). Finally, in a mouse model of αβ-crystallinopathy (HSBP8 R120G transgenic mice), treatment with geranylgeranylactone, a potent heat shock protein inducer, and over-expression of Hsp22 reduced aggregate formation and improved cardiac function and survival (Sanbe et al., 2009).

A simple over-expression of proteins involved in protein degradation may, however, not be sufficient to eliminate formed aggregates, as implied by the massive expression of such proteins in abnormal fibres (Figs 4–7). The cell culture models presented here currently seem to be most appropriate to test such strategies before testing them in animal models or patients.

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Supplementary material

Supplementary material is available at Brain online.

References


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PhD research investigated the molecular structure, biological interactions and function of three muscle proteins associated with muscular dystrophies, primarily using X-ray methods, biophysical and bioinformatics techniques.

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Masters Research | Advisor: Dr Guido Drago
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2008 Recipient: Developing Solutions Scholarship, Nottingham University, UK.

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Brain;135(9):2642–2660. doi: 10.1093/brain/aws200