

The Z-disc proteins myotilin and FATZ-1 interact with each other and are connected to the sarcolemma via muscle-specific filamins

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Summary

Myotilin and the calsarcin family member FATZ-1 (also called calsarcin-2 or myozenin-1) are recently discovered sarcomeric proteins implicated in the assembly and stabilization of the Z-discs in skeletal muscle. The essential role of myotilin in skeletal muscle is attested by the observation that certain forms of myofibrillar myopathy and limb girdle muscular dystrophy are caused by mutations in the human myotilin gene. Here we show by transfection, biochemical and/or yeast two-hybrid assay that: (1) myotilin is able to interact with the C-terminal region of FATZ-1 and that the N- or C-terminal truncations of myotilin abrogate binding; (2) myotilin can also interact with another calsarcin member, FATZ-2 (calsarcin-1, myozenin-2); (3) myotilin and FATZ-1 bind not only to the C-terminal region of filamin-C containing the Ig repeats 19-24, but also to the other two filamins, filamin-A and

filamin-B, as well as the newly identified filamin-B_{var-1} variant; (4) the binding of myotilin to filamin-C involves binding sites in its N-terminal region, whereas FATZ-1 associates with filamin-C via sequences within either its N- or C-terminal region; and finally, (5) the C-terminal region of filamin-C like filamin-B and filamin-B_{var-1}, shows binding activity with the β 1A integrin subunit. Our findings further dissect the molecular interactions within the Z-disc that are essential for its organization, and provide evidence for a novel connection between Z-disc proteins and the sarcolemma via filamins and β 1 integrins. These data shed new light on the complex organization of the Z-disc that is highly relevant to understanding muscular dystrophies.

Key words: Myotilin, FATZ-1, Calsarcin, Z-disc, Filamins, Integrin

Introduction

In striated muscle cells, the Z-disc constitutes the border of individual sarcomeres, where anti-parallel actin filaments spanning the sarcomeres are crosslinked. The Z-disc represents a highly organized three-dimensional structure containing several proteins assembled in multi-protein complexes (reviewed by Faulkner et al., 2001; Epstein and Davis, 2003). The Z-disc plays a key role in the supramolecular assembly of the sarcomeric unit during myogenesis, in the transmission of the tension generated by various sarcomeres along myofibrils and in the regulation of contractile activity. Z-discs are attached to the sarcolemma and to the extracellular matrix at specific sites called costameres. At these sites, Z-disc-associated linker molecules connect the sarcomere with two transmembrane adhesion molecule complexes, the dystroglycan-sarcoglycan complex and the integrin receptor complex. In recent years, an increasing number of mutations in genes of distinct Z-disc components have been found to be responsible for various forms of muscle disorders, attesting to the importance of these proteins in maintaining the integrity of striated skeletal muscle

cells (reviewed by Dalkilic and Kunkel, 2003). Nevertheless, most Z-disc proteins have only recently been identified and their molecular interactions and functions are far from understood.

Some of the Z-disc proteins including α -actinin 2 (Luther, 2000), telethonin (Mues et al., 1998), ZASP-1 (Faulkner et al., 1999), FATZ-1 (filamin, α -actinin and telethonin binding protein of the Z-disc; also termed myozenin-1 or calsarcin 2) (Faulkner et al., 2000; Takada et al., 2001; Frey et al., 2000), myotilin (Salmikangas et al., 1999) and myopalladin (Bang et al., 2001; Beggs et al., 1992) are primarily or exclusively found at that location. Myotilin is a newly identified 57 kDa protein mainly expressed in striated muscle. The N-terminal half of myotilin contains a binding site for α -actinin (Salmikangas et al., 1999; Hauser et al., 2000). Its C-terminus, which exhibits two Ig-like domains, mediates binding to filamin-C (Van der Ven et al., 2000). Myotilin is able to crosslink actin filaments and to critically affect the assembly of sarcomeres (Salmikangas et al., 2003). Its importance in the maintenance of muscle integrity is further supported by the recent

observation that pathogenic mutations in the myotilin gene cause a subset of myofibrillar myopathies and limb girdle muscular dystrophy (LGMD) type 1A that are characterized by streaming of Z-discs and degeneration of myofibers (Hauser et al., 2000; Hauser et al., 2002; Selcen and Engel, 2004). FATZ-1 is another newly characterized 32 kDa protein with no canonical structural motifs (Faulkner et al., 2000; Frey et al., 2000; Takada et al., 2001). The 75 N-terminal and the 128 C-terminal amino acid residues of FATZ-1, designated CD1 and CD2 regions respectively, contain α -helices, and flank a central glycine-rich domain (Faulkner et al., 2000). FATZ-1 was found to interact in a similar manner to myotilin with both α -actinin 2 and filamin-C as well as telethonin, another protein potentially implicated in LGMD (Frey et al., 2000; Faulkner et al., 2000; Takada et al., 2001; Frey and Olson, 2002). Furthermore, FATZ-1, together with FATZ-2 and FATZ-3, belongs to a novel family of proteins, named calsarcons, able to interact with sarcomeric calcineurin, a calcium/calmodulin-dependent phosphatase involved in the regulation of genes affecting muscle differentiation and the formation of different fibre types (Frey et al., 2000; Dellling et al., 2000; Frey et al., 2004).

A second group of proteins, which are present in the Z-disc but also extend into other portions of the sarcomere or the sarcolemma, include titin (Gautel et al., 1996; Labeit et al., 1997; Maruyama, 1997), nebulin (Jin and Wang, 1991; Kruger et al., 1991; Wright et al., 1993) and the filamins. The filamin family is composed of three isogenes that encode filamin-A, filamin-B and the muscle-specific filamin-C (reviewed by Van der Flier and Sonnenberg, 2001). The N-terminal portion of these ~560 kDa dimeric proteins contains an actin-binding domain (ABD) composed of two calponin homology domains followed by 24 Ig-like repeats. The latter are interrupted by two ~30 amino acid flexible loops, called hinge-1 (H1) and hinge-2 (H2). The H1 loop between Ig-repeats 15 and 16 is absent from some splice variants of filamin-B and filamin-C (Xie et al., 1998; Xu et al., 1998). The ubiquitous filamins A and B are involved in multiple processes, such as the organization of actin filaments, membrane stabilization, and serve as a scaffold for signaling proteins (Calderwood et al., 2001; Van der Flier and Sonnenberg, 2001). Recent studies have indicated that different filamin-A and -B variants have an impact on myogenesis and a distinct potential to bind various integrin β subunits at the sarcolemma (Van der Flier et al., 2002). The importance of these proteins is attested by the observation that mutations in filamin-A and filamin-B genes result in defects of human cortical development and in human skeletal disorders, respectively (Sheen et al., 2001; Krakow et al., 2004). Filamin-C is largely restricted to skeletal and cardiac muscle cells (Maestrini et al., 1993; Xie et al., 1998). It contains a stretch of 81 amino acids in repeat 20, which is not present in the other filamins and is thought to be responsible for its recruitment into the Z-discs. The insert bearing Ig repeat 20 interacts with the C-terminal half of myotilin, and thus, myotilin can form a link between filamin-C and α -actinin (Van der Ven et al., 2000). Interestingly, mutations in genes encoding γ - and δ -sarcoglycans have recently been shown to be responsible for LGMD type 2C and 2F, respectively (Thompson et al., 2000), and are associated with an altered distribution of filamin-C at the sarcolemma, attesting to the functional interplay between myotilin, filamin-C and the sarcoglycans (Thompson et al., 2000).

Based on their localization at the Z-disc in striated muscle cells and their common binding partners, we decided to test the possibility that myotilin and FATZ-1 associate with each other. Here we present evidence for the interaction between these two proteins by yeast two-hybrid studies, biochemical and cell transfection assays. Furthermore, to better elucidate the interactions implicated in linking myofibrils to the sarcolemma, we performed yeast two-hybrid assays to assess the ability of the C-terminal regions of filamin-A, filamin-B and filamin-C to bind to myotilin, FATZ-1 or the cytoplasmic domain of the β 1A integrin subunit, one of the subunits located at striated muscle costameres. The overall results demonstrate that myotilin and FATZ-1 bind to each other and are potentially connected to the cell membrane by interactions involving distinct filamins and β 1 integrin subunits. The data shed new light on the complex organization of the Z-disc and its connection to the sarcolemma.

Materials and Methods

cDNA constructs

Full-length myotilin, FATZ-1 and FATZ-2 were generated by RT-PCR using human skeletal muscle total mRNA as template. The cDNAs encoding myotilin and FATZ have been previously described (Salmikangas et al., 1999; Faulkner et al., 2000). Reverse transcription was performed with Superscript reverse transcriptase and PCR was performed using proof-reading *Pfu* DNA polymerase (Promega). cDNA fragments were cloned in yeast expression vectors (pAS-21 and pACT2) and pcDNA3-HA (Invitrogen) for eukaryotic expression. Myotilin and FATZ-1 deletion constructs were generated using primers that added appropriate stop codons along with restriction sites allowing subcloning in a PCR reaction with *Pfu* DNA polymerase. Primer design was based on human myotilin, human FATZ-1 and human FATZ-2 sequences (GenBank accession numbers AF144477, AJ278124 and AY013297 respectively). The point mutations in myotilin resulting in either a T57I or S55F substitution were introduced utilizing the PCR overlap extension method with oligonucleotides containing the appropriate mutations. The cDNAs encoding the C-terminal portion of filamin-A, filamin-B, filamin-B_{var1} and filamin-C as well as the cytoplasmic domain of the β 1A integrin subunit (residues 752-798) have been previously described (Van der Flier et al., 2002). cDNA for the C-terminal region of filamin-C, was isolated in a previously described yeast two-hybrid screening utilizing FATZ-1 as bait (Faulkner et al., 2000). This filamin-C fragment was used as a template for the generation of a cDNA fragment that allowed subsequent subcloning in pAS21. All constructs were verified by DNA sequencing.

Cell culture, transfection and immunofluorescence microscopy studies

CHO cells (ATCC) were grown in Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were seeded at 30-40% confluence on glass coverslips 1 day before transfection. Cells were transfected with 1 μ g plasmid constructs for myotilin and/or FATZ-1 using Fugene 6 reagent (Roche) according to the manufacturer's instructions. After 48 hours, cells were washed twice with PBS, fixed in 3.5% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100, processed for immunofluorescence microscopy and viewed using an Axiophot microscope (Carl Zeiss) equipped with a cooled CCD camera.

Frozen 2 μ m sections of human skeletal muscle were immobilized on poly-L-lysine-coated glass slides, fixed with cold acetone, and further processed for immunofluorescence microscopy studies

(Salmikangas et al., 1999; Salmikangas et al., 2003). Specimens were examined using a Leica TCS SP confocal laser-scanning microscope (Leica).

Antibodies

The following immunology reagents were used: mAb anti-T7 tag (Novagen), anti-HA tag mAb HA.11 (Babco); rabbit myotilin antisera 736/948 raised against a synthetic peptide (Salmikangas et al., 1999); rabbit antiserum 151 raised against the first 150 amino acids of mouse myotilin (Monica Moza, University of Helsinki, Finland); TRITC-conjugated anti-mouse IgG2b (Rockland Immunochemicals); Alexa 488-conjugated anti-mouse IgG (Molecular Probes, USA); mouse mAbs directed against the GAL4 activation (AD) or DNA binding domain (BD) (Santa Cruz Biotechnology) and peroxidase-conjugated anti-mouse Ig (Bio-Rad).

Yeast strain transformation and interaction analysis

The *Saccharomyces cerevisiae* strain PJ69-4A (a gift from P. James, Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI) which contains the genetic markers *trp1-901*, *leu2-3*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1-HIS3*, *GAL2-ADE2*, *met2::GAL7-lacZ* (James et al., 1996) was used for all assays. It contains three regulated and selectable reporter genes, *HIS3*, *ADE2* and *Lac-Z*, each driven by a different promoter under the control of *GAL4* transcriptional factor. Cells were co-transformed with two defined constructs in pAS2-1 or pACT2 (Clontech), according to the manufacturer's procedure. Double transformants were selected on agar synthetic complete (SC) medium lacking leucine and tryptophan (SC-LW). For each transformation, eight colonies were arrayed in 96-well microtiter plates and then transferred onto agar SC-LW (positive control), SC-LW without adenine (SC-LWade) and SC-LW without histidine (SC-LWH) supplemented with 2 mM 3-amino 1,2,3-triazole. Growth was determined after 5 days and 10 days incubation at 30°C. Transactivation controls were systematically performed for each construct with the opposite vector without insert.

Quantitative determination of β -galactosidase activities was performed in triplicate by using three independent clones grown in SC-LW with O-nitrophenyl β -D-galactopyranoside (Sigma) as the substrate, according to the manufacturer's recommendations. To ascertain that the transformed yeast expressed the various recombinant proteins, cell extracts were prepared using the trichloroacetic acid method (Clontech Protocol Handbook) and subjected to immunoblotting with antibodies directed against the *GAL4*-AD or *GAL4*-DBD. The apparent molecular weight of the various recombinant proteins corresponded to that predicted based on their cDNA and their expression levels were comparable (not shown). Furthermore, the functionality of each myotilin construct was validated by demonstrating an interaction with other known binding partners (Salmikangas et al., 1999; Van der Ven et al., 2000) (A.T. and Y.G., unpublished observation).

Expression and purification of GST recombinant proteins

Various cDNA fragments encoding full-length myotilin, residues 1-250 and 216-498 and full-length FATZ-1 were subcloned into pGEX-4T glutathione S-transferase (GST) bacterial expression vector (Amersham Pharmacia Biotech). *Escherichia coli* BL21 (DE3, Novagen) was transformed with these constructs or the vector pGEX-2T without insert to express GST alone. β 1A and β 1D cytoplasmic domains were fused with GST in the bacterial expression vector pRP261, a derivative of the pGEX-3X vector (Van der Flier et al., 2002). Expression of GST or GST fusion proteins was induced by 0.5 mM IPTG for 3 hours at 30°C. Bacteria were collected by centrifugation, resuspended in phosphate-buffered saline (PBS) supplemented with 1% Triton X-100 and 5 mM EDTA and lysed by

sonication. Purification of GST fusion proteins was performed as previously described (Geerts et al., 1999). Quantification of the proteins was performed using the Bradford protein assay (Bio-Rad) with BSA as a standard. Prior to performing pull-down assays, beads with immobilized GST-fusion proteins were resuspended in 1 ml CHAPS buffer (25 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, 0.5% CHAPS, pH 7.5), containing 1% heat-inactivated BSA and incubated at 4°C for 1 hour, to reduce non-specific binding to the beads. The same quantity (5 μ g) of the GST fusion proteins (or GST alone) with adjusted bead volumes were used per pull-down assay.

The various N-terminally truncated filamin constructs were expressed in transiently transfected COS-7 cells using the DEAE-dextran method (Van der Flier et al., 2002). Lysates were cleared by centrifugation at 15,000 g for 10 minutes at 4°C, and subsequently diluted ten times in dilution buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 150 mM sucrose, 3 mM MgCl₂). Diluted lysates were incubated overnight at 4°C with 15 μ l glutathione-Sepharose 4B beads containing 50 μ g GST fusion proteins. The beads were washed with the same lysis buffer and centrifuged through a sucrose cushion (10 mM PIPES, pH 6.8, 50 mM NaCl, 800 mM sucrose, 1 mM MgCl₂), and proteins were resolved by SDS-PAGE and immunoblotted with anti-HA antibody. GST fusion protein loading of the beads was checked by Coomassie Brilliant Blue staining, and the filamin fusion proteins were visualized by immunoblotting with enhanced chemiluminescence (Amersham) (Van der Flier et al., 2002).

In vitro transcription/translation

The different constructs in pcDNA3-HA were transcribed and translated in vitro with the kit TNT T7 quick-coupled transcription/translation system (Promega) as indicated in the supplier's protocol.

Affinity precipitations

³⁵S-labeled methionine recombinant forms of FATZ-1 and FATZ-2, myotilin and of various filamins produced by coupled in vitro transcription/translation were diluted in CHAPS buffer and centrifuged for 5 minutes at 10,000 g. The supernatants were pre-cleared by incubation for 1 hour at 4°C with GST immobilized on glutathione-agarose beads. Precleared lysates were then incubated for 16 hours at 4°C with GST-fusion proteins immobilized on glutathione-agarose beads equilibrated in CHAPS buffer. After three washes with CHAPS buffer, bound proteins were eluted in SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Results

Myotilin and FATZ-1 codistribute in the striated muscle Z-discs and myotilin regulates the distribution of FATZ-1 in transfected CHO cells

Previous studies demonstrated that myotilin and FATZ-1 are located in the Z-disc and have common binding partners (Salmikangas et al., 1999; Faulkner et al., 2000). We assayed whether these two proteins are also able to physically associate with each other. Hence, we first investigated the distribution of myotilin and FATZ-1 in human skeletal muscle by confocal immunofluorescence microscopy. These two proteins were found codistributed at the Z-discs in differentiated striated muscle cells (Fig. 1). We then investigated the distribution of myotilin and FATZ-1 when transiently expressed in CHO cells that have a well-organized actin cytoskeleton (Salmikangas et al., 2003). In single-transfected cells, myotilin colocalized with F-actin and evenly decorated the actin filaments inducing prominent actin

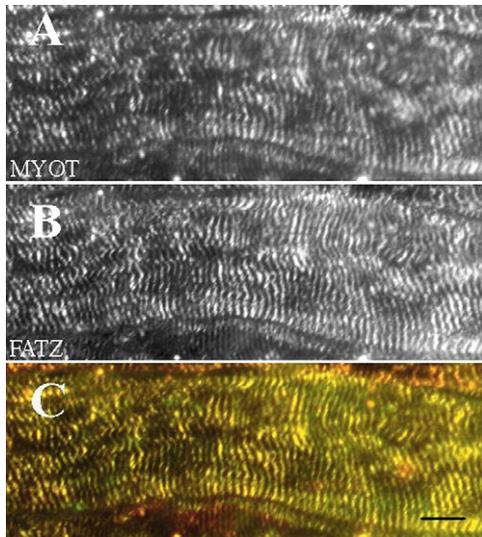


Fig. 1. Expression and localization of myotilin and FATZ-1 in human skeletal muscle was detected using confocal microscopy of skeletal muscle cryosections. Myotilin was stained with a rabbit anti-serum against the N-terminus of myotilin (A) and FATZ-1 with a monoclonal rat antibody (B). The two proteins colocalize at the Z-discs along the skeletal muscle striations (C). Bar, 10 μ m.

bundles, whereas FATZ-1 exhibited a periodical punctuated staining pattern along the stress fibers (Fig. 2A-D). In double-transfected cells, myotilin and FATZ-1 were colocalized. However, in these cells, the periodical punctuated distribution pattern of FATZ-1 was lost and instead, the distribution of FATZ-1 resembled that of myotilin (Fig. 2E-H). This indicates that myotilin can reorganize, either directly or indirectly, the subcellular distribution of FATZ-1. By its ability to bind and bundle actin filaments, overexpression of myotilin causes a dramatic reorganization of the actin cytoskeleton in non-muscle cells (Salmikangas et al., 2003) that may thus result in an impact on the topogenic fate of FATZ-1.

Myotilin forms a complex with both FATZ-1 and FATZ-2

The ability of myotilin to associate with FATZ-1 was first tested using various binding assays. Wild-type myotilin, the N-terminal and the C-terminal halves of myotilin were expressed as fusion proteins with GST (GST-myotilin¹⁻⁴⁹⁸, GST-myotilin¹⁻²⁵⁰ and GST-myotilin²¹⁶⁻⁴⁹⁸, respectively) and immobilized on glutathione-agarose beads. HA-tagged recombinant forms of FATZ-1 and FATZ-2, produced by *in vitro* transcription and translation were tested for their ability to bind myotilin (Fig. 3A-B). The results show that FATZ-1 strongly bound to GST-myotilin¹⁻⁴⁹⁸, but only marginally to GST-myotilin¹⁻²⁵⁰ and myotilin²¹⁶⁻⁴⁹⁸ and not to GST alone. In analogy to FATZ-1, FATZ-2 (calsarcin-1), another calsarcin member, was also found to associate with GST-myotilin¹⁻⁴⁹⁸ (Fig. 3C-D). To confirm the functional activity of myotilin constructs, their ability to interact was tested. In line with previous studies (Salmikangas et al., 2003), myotilin was shown to dimerize in GST pull-down assays (not shown). In addition, the N-terminal construct was shown to bind to α -actinin and the C-terminal construct to actin (not

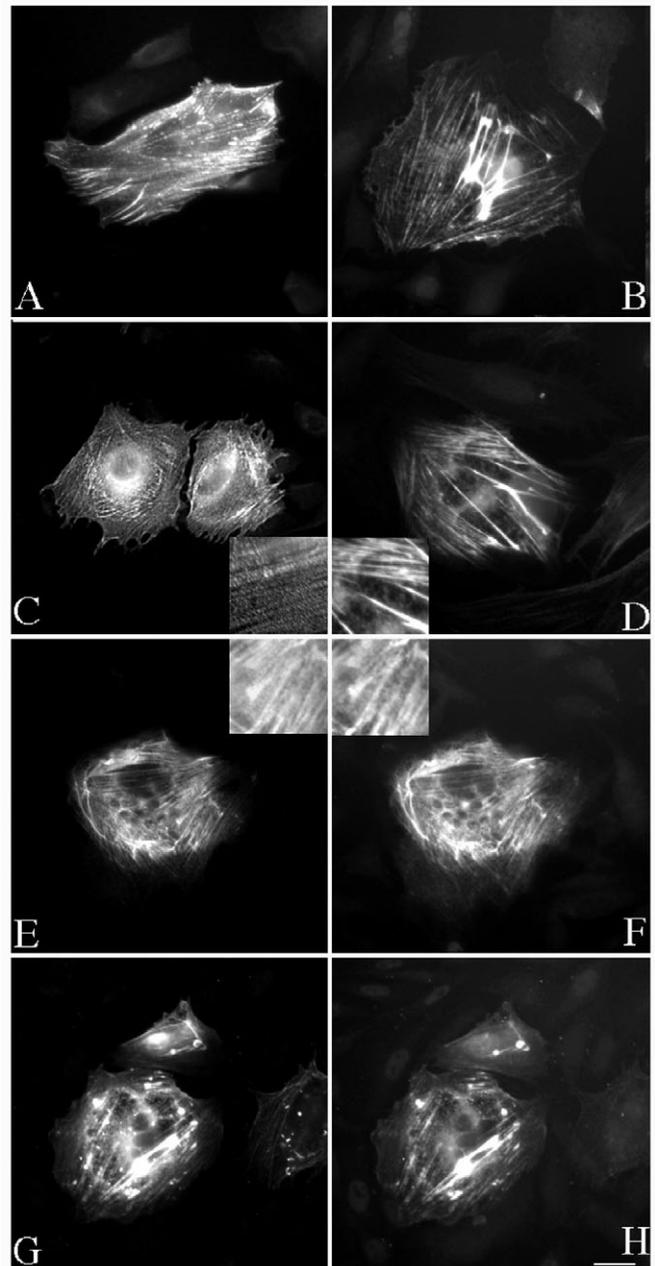


Fig. 2. Expression of myotilin reorganizes FATZ-1 in double-transfected CHO cells. CHO cells were transfected with HA-tagged FATZ-1 (A,C), with T7-tagged myotilin (B,D) or with both constructs (E-H). Expressed proteins were detected with the corresponding anti-tag antibodies. In single-transfected CHO cells, FATZ-1 has a periodical staining pattern along the stress fibers (see inset in C), whereas myotilin decorates the filaments evenly and bundles them (B,D and inset). In double-transfected cells, in which FATZ-1 (E,G) and myotilin (F,H) were coexpressed, both proteins show a high degree of colocalization and the periodical staining of FATZ-1 is lost. Bar, 5 μ m.

shown). These results indicate that myotilin is able to form a complex with both FATZ-1 and FATZ-2 and that the entire myotilin molecule is important and necessary for efficient binding.

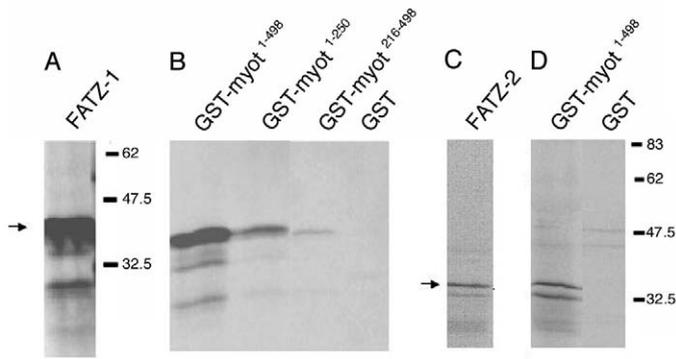


Fig. 3. FATZ-1 forms a complex with myotilin in vitro. ^{35}S -labeled HA-tagged recombinant form of FATZ-1 (A) and FATZ-2 (C) were generated by coupled in vitro transcription/translation and analyzed by SDS-PAGE and autoradiography. GST-fusion proteins of myotilin immobilized on glutathione-agarose beads were incubated with either ^{35}S -labeled FATZ-1 (B) or FATZ-2 (D). Arrows indicate the protein of interest. After washing, bound proteins were analyzed by SDS-PAGE and autoradiography. Molecular mass markers are indicated in kDa.

Characterization of sequences within myotilin and FATZ-1 important for binding

To verify the interaction and further map the binding regions between myotilin and FATZ-1, a series of myotilin and FATZ-1 constructs truncated at their N- or C-terminus were tested in yeast. First, we confirmed the ability of either myotilin¹⁻⁴⁹⁸ or myotilin²¹⁶⁻⁴⁹⁸ to self-dimerize via its C-terminal Ig containing region (not shown). Yeasts co-transformed with GAL4-BD-

FATZ-1 and GAL4-AD-myotilin showed significant growth on the selection markers HIS3 and ADE2, indicating that the His and Ade reporter genes in these yeast cells were activated by a direct interaction between the FATZ-1- and myotilin-GAL4 fusion proteins (Fig. 4). Similar binding activities were observed when GAL4-AD-myotilin constructs carrying either a T57I or S55F substitution, as identified in patients with LGMD1A (Hauser et al., 2000, 2003), were used. We next tested GAL4-AD-myotilin constructs encompassing distinct portions of the serine-rich N- and the C-terminal half of myotilin. Neither of the constructs interacted with FATZ-1, indicating either that only the entire molecule can bind or that truncation destroyed the binding site, possibly by affecting its conformation and/or the dimerization potential of the protein (Salmikangas et al., 1999; Salmikangas et al., 2003) (Fig. 4). Finally, deletion constructs of FATZ-1¹⁶³⁻²⁹⁹ interacted with myotilin, whereas protein fragments encompassing CD1 or GRD of FATZ-1, GAL4-BD-FATZ-1¹⁻⁷⁵, GAL4-BD-FATZ-1¹⁻¹⁷¹ or GAL4-BD-FATZ-1⁷⁵⁻¹⁷¹ did not bind (Fig. 5). This indicates that the CD2 domain of FATZ-1 is sufficient for the myotilin-FATZ-1 interaction. Unfortunately, the construct containing GRD-CD2 transactivated, precluding the analysis of its binding properties in yeast two-hybrid assays. Finally, FATZ-2 was also able to directly interact with myotilin in yeast two-hybrid assays (Fig. 5).

Myotilin and FATZ-1 directly interact with all three filamins

Both myotilin and FATZ-1 bind filamin-C via sequences contained within either its Ig-like repeats 19-21 (Van der Ven et al., 2000) or repeats 20-24 (Faulkner et al., 2001), respectively. Therefore, using in vitro binding assays, we first tested the ability of these proteins to bind to the two other human filamins, filamin-A and filamin-B as well as filamin-B_{var-1}, a newly identified filamin-B variant, from which a 41-amino acid stretch is lacking between repeats 19 and 20 (Van der Flier et al., 2002) (Figs 6, 7). Therefore, we expressed full-length myotilin and FATZ-1 as fusion proteins with GST, GST-myotilin and GST-FATZ-1, respectively and immobilized them on glutathione-agarose beads. HA-tagged recombinant filamin-A, filamin-B, filamin-B_{var-1} and filamin-C containing repeats 19-24, obtained by in vitro transcription and translation were tested for their ability to bind to either myotilin or FATZ-1. As illustrated in Fig. 6, all filamin variants containing repeats 19-24 bound to either myotilin or FATZ-1 (Fig. 6B,C) but not to GST alone. The results indicate that both FATZ-1 and myotilin are able to form a complex with different filamins.

GAL 4 AD Fusion		GAL 4 DNA-BD Fusion	
		FATZ-1 ¹⁻²⁹⁹	
Myotilin ¹⁻⁴⁹⁸	1 498	+	
Myotilin ^{S55F}	1 498	+	
Myotilin ^{T57I}	1 498	+	
Myotilin ¹⁻²⁵⁰	1 250	-	
Myotilin ¹⁻¹⁰⁰	1 100	-	
Myotilin ¹⁻³⁶¹	1 361	-	
Myotilin ²¹⁶⁻⁴⁹⁸	216 498	-	
Myotilin ⁷⁹⁻⁴⁹⁸	79 498	-	

Fig. 4. Yeast two-hybrid survey of the sites in myotilin mediating interaction with FATZ-1. The PJ69-4A yeast strain was co-transformed with pACT2 plasmids encoding various deletion mutants of myotilin in fusion with GAL4-activation domain (AD) and pAS2-1 plasmids encoding FATZ-1 fused at its N-terminus with GAL4-DNA-binding domain (BD). The N-terminal serine-rich domain and the two Ig-like domains of myotilin are represented by a dark grey box and the black loops, respectively. Growth was estimated after 5 to 10 days of incubation at 30°C. +, growth; -, no growth, on selective media. The results indicate an interaction between myotilin¹⁻⁴⁹⁸, myotilin^{S55F} or myotilin^{T57I} and FATZ-1, whereas shorter myotilin constructs did not interact. Transactivation controls were systematically performed for each construct with the opposite vectors without insert. Each experiment was repeated at least twice.

Fig. 5. Yeast two-hybrid survey of the sites in FATZ-1 mediating interaction with myotilin. Experiments were performed as described in Materials and Methods and Fig. 4. CD1 and CD2 regions of FATZ-1 are represented by dark grey boxes, whereas the glycine-rich domain is depicted as a white box. +, growth; -, no growth, on selective media. FATZ-1⁷⁵⁻²⁹⁹ and FATZ-1¹⁶³⁻²⁹⁹ caused auto-transactivation (TA) on both selective media. *FATZ-1¹⁶³⁻²⁹⁹ as GAL4-AD fusion protein was not transactivating. The results indicate an interaction between FATZ-1¹⁻²⁹⁹ or FATZ-1¹⁶³⁻²⁹⁹ and myotilin.

	GAL 4 DNA-BD Fusion		GAL 4 AD Fusion	
			Myotilin ¹⁻⁴⁹⁸	
FATZ-1 ¹⁻²⁹⁹	1		299	+
FATZ-1 ¹⁻⁷⁵	1		75	-
FATZ-1 ¹⁻¹⁷¹	1		171	-
FATZ-1 ⁷⁵⁻¹⁷¹			75 171	-
FATZ-1 ⁷⁵⁻²⁹⁹			75 299	TA
FATZ-1 ¹⁶³⁻²⁹⁹			163 299	+*
FATZ-2 ¹⁻²⁶⁴	1		264	+

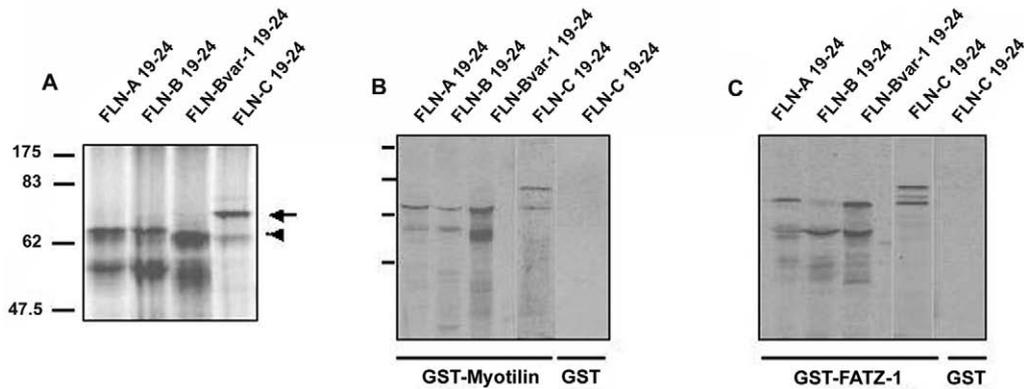


Fig. 6. Binding of filamin isoforms and variants to GST-myotilin and GST-FATZ-1 fusion proteins. (A) HA-tagged recombinant form of filamin-A (FLN-A), filamin-B (FLN-B), filamin- $\beta_{\text{var-1}}$ (FLN-B_{var-1}) and filamin-C (FLN-C), containing repeats 19-24 were generated by coupled in vitro transcription/translation and analyzed by SDS-PAGE and autoradiography. The positions of FLN-A, FLN-B and FLN-B_{var-1} (arrowhead), and FLN-C (arrow) are indicated. (B,C) GST-fusion proteins immobilized on glutathione-agarose beads were incubated with the various radiolabeled filamin recombinants. After washing, bound proteins were analyzed by SDS-PAGE and autoradiography. Filamin-A, filamin-B, filamin-B_{var-1} as well as filamin-C containing repeats 19-24 bind to GST-myotilin but not to GST alone (B). Filamin-A, filamin-B, filamin-B_{var-1} as well as filamin-C containing repeats 19-24 bind to FATZ-1 but not to GST alone (C). The grouping of images was arranged from two different gels in B and in C. Note that filamin-B in the presence of FATZ-1 showed a tendency to proteolytic degradation under the conditions of the GST pull down experiments. Molecular mass markers are indicated in kDa.

Fig. 7. Characterization of the interaction of different filamin isoforms with myotilin, FATZ-1 and the $\beta 1A$ integrin subunit in yeast. +, growth; -, no growth, on selective media, tested as described in Materials and Methods and Fig. 4. Ig-like repeats are depicted as light grey boxes. H2 box corresponds to the second hinge region. Both myotilin and FATZ-1 interact with the C-terminal region of all the filamins tested. $\beta 1A$ interacts with all filamins except filamin-A.

	GAL 4 AD Fusion		GAL 4 DNA-BD Fusion			
			Myotilin ¹⁻⁴⁹⁸	FATZ-1 ¹⁻²⁹⁹	$\beta 1A$ ⁷⁵²⁻⁷⁹⁸	
Filamin-A ¹⁹⁹⁵⁻²⁶⁴⁷	1995		2647	+	+	-
Filamin-B ¹⁹⁹⁵⁻²⁶⁰²	1995		2602	+	+	+
Filamin-B _{var-1} ¹⁹⁹⁵⁻²⁶⁰²	1995		2602	+	+	+
Filamin-C ¹⁹⁹⁵⁻²⁷⁰⁵	1995		2705	+	+	+

GAL 4 DNA-BD Fusion		GAL 4 AD Fusion	
		Filamin-C ¹⁹⁹⁵⁻²⁷⁰⁵	
Myotilin ¹⁻²⁵⁰	1  250	+	
Myotilin ²¹⁶⁻⁴⁹⁸	216  498	-	
FATZ-1 ¹⁻⁷⁵	1  75	+	
FATZ-1 ⁷⁵⁻¹⁷¹	75  171	-	
FATZ-2 ¹⁶³⁻²⁹⁹	163  299	+*	

To confirm these interactions by another approach, we further tested the ability of myotilin and FATZ-1 to bind to the various filamin constructs by yeast two-hybrid analysis (Figs 6, 7). The results show that both myotilin and FATZ-1 can directly associate with the C-terminal regions of all tested filamins containing repeats 19-24. Finally, as an extension to previous studies (Faulkner et al., 2000; Van der Ven et al., 2000), we characterized the binding sites for filamin-C on myotilin and FATZ-1 (Fig. 8). The results show that both the N- and C-terminal regions of FATZ-1, as well as the N-terminal region of myotilin can mediate the association with the C-terminal region of filamin-C containing repeats 19-24.

Filamins can connect the Z-disc proteins myotilin and FATZ-1 to the sarcolemma via binding to the β 1A integrin subunit

The C-terminal regions of filamin-A, filamin B and filamin-B_{var-1} have recently been shown to have different binding affinities for various integrin β subunits, including the β 1A and β 1D variants, which are localized at the cell membrane in striated muscle cells and expressed in a developmentally regulated manner (Van der Flier and Sonnenberg, 2001). Therefore, we assessed whether filamin-C also had the ability to bind to the β 1A integrin subunit. Yeast two-hybrid findings indicate that the region of filamin-C encompassing repeats 19-24 associates with the cytoplasmic domain of β 1A (Fig. 7). In line with previous findings, both filamin-B (19-24) and filamin-B_{var-1} (19-24) bound to β 1A, whereas filamin-A (19-24) did not (Van der Flier and Sonnenberg, 2001).

Finally, as the 81-amino acid insertion in Ig repeat 20 specific for filamin-C was previously found to contribute to binding myotilin (Van der Ven et al., 2000), we assessed its impact on β 1A binding. Therefore, we first tested in yeast three filamin-C constructs containing repeats filamin-C (19-21), filamin-C (20-21) and filamin-C (21-23). Although filamin-C (19-21) and filamin C (20-21) showed binding activity with β 1A, filamin C (21-23) did not (Fig. 9A). The interaction between β 1A and the repeat 20 containing filamin-C (19-21) could be confirmed in a GST-pull down experiment (Fig. 9B-D). The filamin C (19-21) construct expressed in transfected

Fig. 8. Characterization of the interaction of the C-terminal region of filamin-C with deletion mutants of myotilin and FATZ-1 in yeast. +, growth; -, no growth, on selective media, tested as described in Materials and Methods and Fig. 4. The N-terminal regions of both myotilin and FATZ-1 interact with the C-terminal region of filamin-C. *As FATZ¹⁶³⁻²⁹⁹ fused with the GAL4-BD caused auto-transactivation on both selective media, binding of FATZ¹⁶³⁻²⁹⁹ to filamin-C was verified utilizing GAL4-AD protein of FATZ-1.

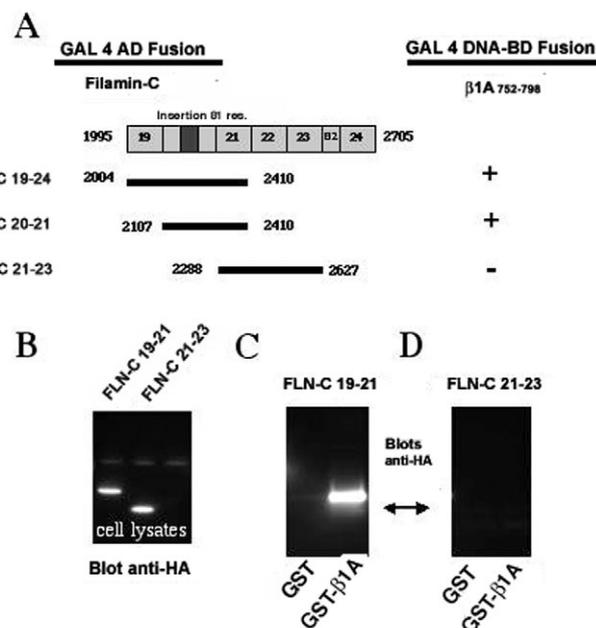


Fig. 9. Binding of the C-terminal region of filamin-C with the cytoplasmic domain of the β 1A integrin subunit in yeast two-hybrid assays and pull-down experiments. (A) +, growth; -, no growth, on selective media in yeast two-hybrid assays, tested as described in Materials and Methods and Fig. 4. Although filamin-C (19-21) and filamin C (20-21) bound to β 1A, filamin C (21-23) did not. (B) Detection by immunoblotting of the expressed products from cDNA constructs for HA-tagged filamin-C (19-21) and filamin-C (21-23) in lysates of transiently transfected COS-7 cells. Pull-down assay with GST- β 1A or GST immobilized on glutathione beads containing either HA-tagged filamin C (19-21) (C) or filamin-C (21-23) (D) fusion proteins. Immunoblot detection of bound filamin-C (19-21) to GST- β 1A. No binding was observed with filamin-C (21-23).

COS-7 cells bound to a GST fusion protein containing the cytoplasmic domain of the β 1A integrin subunit, but not to GST alone. Together, the data suggest that repeat 20, containing the 81-amino acid insertion is important for the binding of filamin-C to the β 1A subunit.

Discussion

The Z-disc plays a key role in the architectural organization of the sarcomeric unit of striated muscle cells and serves as a site that coordinates various cellular signaling pathways. Although several Z-disc proteins have recently been characterized, the intricate molecular interactions, which mediate the assembly of the Z-disc during myofibril formation, its regulation and the linkage of proteins to the sarcolemma remains largely unknown. The present study sheds new light on the complex organization of the Z-disc by demonstrating that the Z-disc protein myotilin is able to bind to both FATZ-1 and FATZ-2 as demonstrated by biochemical and yeast two-hybrid assays. Furthermore, our findings indicate that myotilin and FATZ-1 not only bind to filamin-C as previously reported, but also to the other two filamins, filamin-A and filamin-B, as well as to the newly identified filamin-B_{var-1} variant (Van der Flier et al., 2002). Finally, the three filamins, as well as the filamin-B_{var-1} have the distinct ability to associate with the β 1A integrin subunit and may serve as a link between Z-disc proteins and the sarcolemma (Fig. 10).

Myotilin binds to both FATZ-1 and FATZ-2: novel interactions contributing to Z-disc assembly

The ability of the two Z-disc proteins myotilin and FATZ-1 to interact with each other is supported by several lines of evidence: (1) the coexpression of myotilin and FATZ-1 in transfected non-muscle cells alters the subcellular redistribution of FATZ-1, and leads to a high degree of colocalization with myotilin; (2) FATZ-1 is able to bind to GST-myotilin, indicating that these two proteins can form a complex *in vitro*; and, finally (3) myotilin directly binds FATZ-1 in yeast two-hybrid assays.

FATZ-1 seems to associate with myotilin by means of its CD2 C-terminal region, as deduced from the results obtained by yeast two-hybrid analysis. Instead, the entire molecule of myotilin appears to contribute to the interaction with FATZ-1, as both N- or C-terminal truncations abrogate binding. Finally, our results from yeast and biochemical assays further demonstrate that myotilin can also directly interact with FATZ-2 (calsarcin-1), another member of the calsarcin protein family that has high homology with FATZ-1, particularly in its N- and C-terminal regions (Frey et al., 2000).

Interactions of myotilin and FATZ-1 with other Z-disc proteins

Previous findings indicate that myotilin can dimerize via its two Ig-like domains (Salmikangas et al., 1999; Salmikangas et al., 2003). The potential for homotypic interaction probably confers to myotilin the ability to participate, in addition to FATZ-1 and FATZ-2, in multiple molecular interactions with different sarcomeric proteins. The serine-rich N-terminal half of myotilin binds to α -actinin 2 (Hauser et al., 2000). Specifically, the region encompassing residues 79-150 of myotilin was found to contain the minimal sequences required for association with the C-terminal region of α -actinin 2. Here we further demonstrate that the N-terminal half of myotilin can also interact with filamin-C repeats 19-24 in yeast. Our study thus extends the findings of a previous report indicating that the C-terminal region of myotilin containing the two Ig-like

domains is able to bind to the unique Ig repeat 20 of filamin-C (Van der Ven et al., 2000). When compared to the deletion constructs used in the latter study, it is likely that the presence of domain 24 in the filamin-C-construct that is important for dimerization (and thus proper folding) of the molecule (Gorlin et al., 1990; Pudas et al., 2005) affects its binding properties. At present, it is not known whether the two interactions, myotilin¹⁻²⁵⁰ with filamin-C repeats 19-24 and myotilin²¹⁶⁻⁴⁹⁸ with filamin-C repeat 20 (Van der Ven et al., 2000) can occur simultaneously. If so, they may act synergistically to strengthen the interaction.

Recently, four single amino acid substitutions in the N-terminal region of myotilin (S55F, T57I, S60C, and S60F) were shown to cause myofibrillar myopathy and/or LGMD1A (Hauser et al., 2000; Hauser et al., 2002; Selcen and Engel., 2004). The fact that deletion of the first 78 N-terminal residues of myotilin abrogated binding to FATZ-1 indirectly suggested that the N-terminal region of myotilin is important for binding. We did not however find evidence that the substitution S55F or T57I would affect the ability of myotilin to bind to FATZ-1. As these sites are potential phosphorylation sites, further understanding of the posttranslational modulation of myotilin and more dynamic binding assays are needed to exclude the impact of these sites on the interaction. It is plausible that the missense mutations in myotilin affect the complex set of interactions among Z-disc proteins and the maintenance and structural integrity of Z-discs, as attested by the extensive streaming of Z-discs and disorganization of sarcomeric striations observed in LGMD1A patients (Hauser et al., 2002). Similar alterations are observed with mutations in another Z-disc component, telethonin, which is indirectly connected to myotilin via FATZ-1 (Faulkner et al., 2001; Frey and Olson, 2002).

The two calsarcin proteins FATZ-1 and FATZ-2 appear to have overlapping functional properties, based on their common ability to bind, in addition to myotilin (this study) and calcineurin, also to α -actinin, filamin-C and telethonin (Faulkner et al., 2000; Frey et al., 2000; Frey and Olson, 2002). Specifically, the CD2 region of FATZ-1 appears critical not only for binding to α -actinin and calcineurin (Faulkner et al., 2000; Frey and Olson, 2002), but also to myotilin and filamin-C, as confirmed in this study. As an extension to previous studies utilizing the C-terminal extremity of filamin-C lacking domains 19-20 (Faulkner et al., 2000), here we found that the CD1 region of FATZ-1 also contributes to its interaction with filamin-C domains 19-24 (Faulkner et al., 2000; Frey and Olson, 2002).

Although it is not yet clear whether the binding sites for myotilin on FATZ-1 involve the same set of sequences, co-immunoprecipitation studies utilizing *in vitro*-translated products have provided evidence that the association of α -actinin and filamin-C to FATZ-1 is competitive (Takada et al., 2001). The presence of two distinct binding sites on the N- and C-terminal region of both myotilin and FATZ-1 for filamin-C however raises the possibility that myotilin, FATZ-1 and filamin-C can form a ternary complex by means of multiple simultaneous interactions. Attempts to confirm this idea by biochemical approaches were hampered by our inability to produce sufficient amounts of soluble FATZ-1 in a bacterial expression system (not shown).

Finally, it is conceivable that these associations between

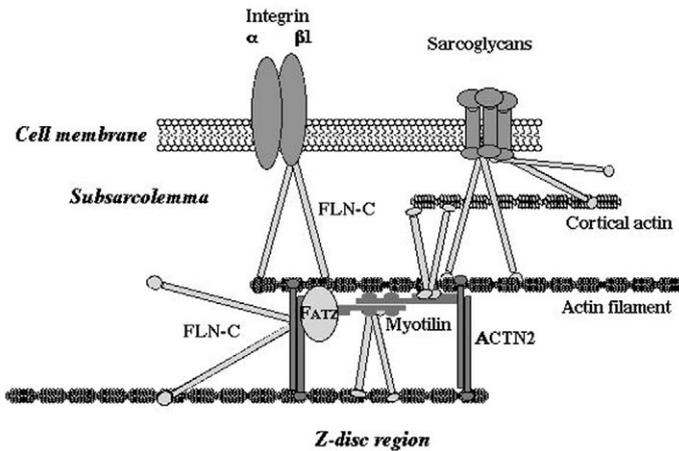


Fig. 10. Schematic representation of two potential mechanical linkages between the contractile apparatus and the sarcolemma. The C-terminal region of filamin-C (FLN-C, represented as tail-to-tail associated dimer) binds to the $\beta 1$ integrin cytoplasmic domain and both γ and δ sarcoglycans associated with the dystroglycan complex at the sarcolemma. The N-terminal region of FLN-C contains an actin-binding domain, which may associate with both sarcomeric actin filaments and cortical actin in the sub-sarcolemmal region, serving thus as a nexus between actin filaments and costameric sites. A number of Z-disc proteins are depicted. Myotilin represented hypothetically as an antiparallel dimer can bind to FATZ-1, FLN-C and α -actinin 2 (ACTN2). FATZ-1 can also bind to myotilin, FLN-C and ACTN2. It should be noted that in some cases, where there is more than one protein partner, competition and/or simultaneous binding could occur. Furthermore, it is likely that certain interactions occur only at distinct stages of development.

myotilin, FATZ-1 and filamin-C have an impact, either directly or indirectly, on calcineurin function, by affecting either its potential to associate with other Z-disc proteins, its subcellular localization and/or calcineurin-mediated signaling (Frey et al., 2000; Frey and Olson, 2002; Frey et al., 2004). The recent observation that FATZ-2 (calsarcin-1)-null mutant mice show enhanced calcineurin activity with activation of the hypertrophic gene program and an increase of cardiac mass affirms the importance of calsarcins in modulating calcineurin signaling in striated muscle (Frey et al., 2004).

Linkage of myotilin and FATZ-1 with filamin-A, filamin-B and filamin-C

As both myotilin and FATZ-1 interact with filamin-C (Faulkner et al., 2000; Takada et al., 2001; Van der Ven et al., 2000; Luther, 2000; Frey and Olson, 2002) (this study), we assessed their ability to bind to the C-terminal regions of two distinct filamin paralogues, filamin-A and filamin-B, which differ from filamin-C by the lack of 81 amino acids in repeat 20 (Van der Flier and Sonnenberg, 2001). These filamins are expressed during *in vitro* myogenesis of striated muscle cells and their mRNAs are subject to alternative splicing (Van der Flier and Sonnenberg, 2001; Van der Flier et al., 2002). Our yeast two-hybrid assays and *in vitro* binding studies show that both myotilin and FATZ-1 associate with filamin-A and filamin-B as well as the filamin B_{var-1} variant which lacks a 41-amino acid stretch between repeats 19 and 20. The latter has recently been

shown to be involved in the organization of the actin cytoskeleton and affects myotube differentiation (Van der Flier et al., 2002). Interestingly, the binding site in myotilin resided in the N-terminus, whereas previously myotilin was shown to interact with domain 20 of filamin C via its C-terminal half (Van der Ven et al., 2000). The present finding has been confirmed in an independent interaction screen, in which myotilin¹⁻⁷⁸ bait defined an interaction with filamin C domains 19-24 (A.T. and O.C., unpublished data). Thus, myotilin appears to contain two filamin binding sites. Based on the complex expression pattern of the different filamins during myogenesis, it is likely that certain interactions are only functional at distinct stages of development and have a variable impact on myofibril assembly.

Filamins serve as a bridge between Z-disc constituents and the $\beta 1$ integrin subunit at the sarcolemma

Filamin-C has been shown to associate with both γ - and δ -sarcoglycans, two proteins of the dystrophin-associated protein complex (reviewed by Thompson et al., 2000). These interactions provide a means by which Z-disc components are mechanically linked to costameric sites. Our results provide evidence for a new mechanical nexus between Z-discs and sarcolemma by showing that filamin-C and filamin-B including the newly identified filamin B_{var-1} variant, are able to interact with the $\beta 1A$ integrin. This widely expressed β integrin subunit is implicated in the migration of myotomal myoblasts, development of cardiac muscle and myogenesis and heterodimerizes with the various integrin α -subunits found in striated muscle cells (Van der Flier et al., 1997; Wigmore and Duglison, 1998; Baudoin et al., 1998; Cachaco et al., 2003). However, as $\beta 1A$ is completely displaced postnatally by the highly homologous $\beta 1D$, the detected interaction between filamins and $\beta 1A$ may be relevant only during certain stages of myogenesis (Van der Flier et al., 1997). In this context, recent findings indicate that the filamins have a distinct ability to bind to various β integrins (Calderwood et al., 2001; van der Flier et al., 2002; Travis et al., 2004) (our unpublished observations). Thus, the interaction of filamins and β integrin subunits might be regulated in an isoform- and variant-specific manner during development. In this context, it is worth noting that the unique insert bearing the Ig-like domain 20 of filamin-C, which was shown to be important for the myofibrillar targeting of the protein and for its binding to myotilin, also contributes to binding of filamin-C to $\beta 1A$ as inferred from our yeast and pull-down assays. Collectively, these data indicate that filamins can serve as a link between myofibrils and the sarcolemma by binding distinct protein complexes containing either sarcoglycans (Thompson et al., 2000) or $\beta 1$ integrin subunits (this study). It should be noted that a recent study has indicated that calpain 3, a muscle-specific calcium-dependent protease, is able to cleave the C-terminal region of filamin-C, affecting its binding to sarcoglycans (Guyon et al., 2003). Calpain 3 may thus regulate the interaction of filamin-C not only with sarcoglycans, but also with $\beta 1$ integrin subunits and distinct Z-disc proteins (see above).

In conclusion, our study further dissects the complex molecular interactions within the Z-disc and its connection with the sarcolemma by demonstrating an interaction between myotilin and FATZ-1 and a novel mechanical link between Z-

disc proteins and the sarcolemma involving filamins and integrins.

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