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Titin and Obscurin: Giants Holding Hands and Discovery of a New Ig Domain Subset

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The sarcomere, the fundamental unit of muscle contraction, contains some extraordinarily large polypeptides (0.6–3 MDa). Most of these proteins belong to the titin family, which was initially defined by the sequences of *Caenorhabditis elegans* twitchin [1,2] and human titin [3,4]. These proteins consist of multiple immunoglobulin (Ig) and fibronectin type III domains (Fn3), one or two protein kinase domains and, in some cases, highly elastic unique regions. As exemplified by studies of titin, the Ig domains serve as binding sites for other proteins and help assemble the sarcomere (reviewed in Ref. [5]). The newest member of the titin family is obscurin (Obs) (~800 kDa), which was initially identified as a ligand of a Z-disk portion of titin [6,7], although it primarily localizes to the sarcomeric M-band in mature muscle [6,8]. Using the power of *C. elegans* molecular genetics, we described the essential roles of the Obs homolog, UNC-89, in signaling and scaffolding in the M-band 5 years before Obs was discovered [9]. UNC-89 is required for the assembly or stability of the M-band and for thick filament organization [10–13]. The M-band is the portion of the sarcomere where myosin-containing thick filaments are cross-linked by a protein network that, in vertebrate muscle, consists of, at least, the C-terminal portion of titin, myomesin (also a poly-Ig/Fn3 protein) and Obs. The linkage between myosin thick filaments occurs through antiparallel dimers of myomesin that interact via their C-terminal Ig domains and bind to myosin via their N-terminal Ig/Fn3 domains [14,15]. Obs binds myo-mesin and titin but it is also believed to act as a linker between myofibrils and the SR (sarcoplasmic reticulum). This was first suggested by finding that the C-terminus of some isoforms of Obs interacts with the SR membrane proteins, small ankyrin-1 isoform 5 (sAnk1.5) and ankyrin-2 (Ank2) [16,17]. The Obs knockout mouse supports this model: it shows changes in longitudinal SR architecture even though it has normal sarcomeric organization somewhat surprisingly [18]. The role of Obs in linking the sarcomere to the SR is likely conserved in nematode UNC-89. Although ankyrin-like molecules have not yet been reported to interact with UNC-89, there is genetic evidence for this function: UNC-89 is required for the proper organization of the ryanodine receptor and SERCA, as well as for optimal calcium signaling [19]. Moreover, independent studies on nematode UNC-89 [13] and mouse Obs [20] demonstrate an evolutionarily conserved and novel mechanism by which UNC-89/Obs regulates ubiquitin-mediated protein degradation at the M-band, and this involves inhibition of a cullin 3 complex.

The ultrastructural and molecular architecture of the M-line is not as well described for *C. elegans* muscle as it is for vertebrate muscle. Although *C. elegans* has an obscurin homolog, UNC-89, there are no obvious homologs of myomesin or titin. TTN-1 is a 2.2-MDa polypeptide localized to the I-band with extension into the outer edge of the A-band and can be regarded as a hybrid between invertebrate twitchin and vertebrate titin [21,22]. This is likely due to the much larger sarcomeres of nematode and many other invertebrate muscles. Moreover, some M-line proteins appear to be nematode specific (e.g., UNC-98 [23] and UNC-96 [24]). An interacting network of proteins (including UNC-89) has been described for the M-line, many having vertebrate homologs/orthologs, which link the muscle cell membrane beginning with integrin through proteins that interact directly with myosin heavy chains in the thick filaments [25–27].

Vertebrates, but not invertebrates, contain genes that encode two additional UNC-89/Obs type proteins: SPEG (striated muscle preferentially expressed gene [28,29]) and Obsl1 (obscurin-like 1 [30,31]). SPEG exists in two isoforms, each containing two protein kinase domains, a feature shared with certain isoforms of UNC-89 [32,33] and with certain isoforms of obscurin [34,35]. There are at least three predicted isoforms of Obsl1 (130–230 kDa). The largest of them, Obsl1a, is predicted to have 20 Ig domains and 1 Fn3 domain in an organization that is identical with that of the N-terminal portion of Obs. All Obsl1 isoforms begin with the same N-terminal Ig domain (OL1). Obsl1 mRNAs are expressed strongly in the heart and placenta, in smooth muscle and at lower levels in many other non-muscle tissues [30,31]. Consistent with the broader tissue expression of Obsl1, null mutations in the Obsl1 gene result in cases of “3M growth syndrome”, an autosomal recessive non-muscle disorder [36]. So far, mutations in Obs have not yet been reported to be associated with human muscle disease. Nevertheless, recently various Obs isoforms have been shown by Western blot and immunofluorescence localization to be expressed in non-muscle tissues including brain, skin, kidney, liver, spleen and lung [37]. Moreover, the Obs gene, OBSCN, is frequently mutated in various types of cancers [38,39], and its expression is significantly decreased in breast cancer cells [40] and breast cancer biopsies [41] and is implicated in metastasis [41].

Interestingly, Obsl1, like Obs (and UNC-89), associates with a cullin complex; Obsl1 interacts with cullin 7, and each protein is crucial for Golgi morphogenesis and dendrite growth of neurons [42]. Also, most patients with 3M growth syndrome have mutations in cullin 7 [36]. Antibodies to Obsl1 localize the protein to M-bands both in neonatal rat cardiomyocytes and in skeletal muscle, and this localization is to the myofibril cores [31]. This is in contrast to the localization of Obs to the periphery of M-bands [31,8,43]. It is likely that UNC-89 in *C. elegans* functions as both Obs and Obsl1. UNC-89 localizes exclusively to M-lines [9,32] and is essential for thick filament organization [12,13] yet is also required for SR organization [19]. *Drosophila* UNC-89/obscurin, like nematode UNC-89, but not vertebrate Obs, is required for the assembly or stability of M-bands and for organization of the sarcomere, and the protein is located throughout the M-band [44]. These functions of UNC-89-like molecules likely segregated to different molecules during evolution. As pointed out by Geisler *et al.*, the gene for invertebrate UNC-89 likely

underwent genomic rearrangement, followed by gene duplications to give rise to genes for Obs, SPEG and Obsl1 in vertebrates [30].

Geisler *et al.* first identified Obsl1 in a database search that looked for proteins similar to obscurin [30], while Fukuzawa *et al.* identified Obsl1 using yeast two-hybrid screens for proteins that interacted with myomesin [31]. Fukuzawa and colleagues found that a linker sequence between Ig domains 4 and 5 (My4-My5) in myomesin interacted with Ig3 of Obsl1 and Obs. Using the N-terminal five Ig domains of Obs as bait (Ig1-Ig5) to screen a library, they fished clones for both myomesin and the C-terminal portion of titin. Domain mapping showed that the minimal region of titin that interacts with Obs or Obsl1 is the C-terminal Ig domain M10; similarly, M10 was found to interact with Ig1 (O1) or OL1. Using tagged recombinant proteins, the authors established that a ternary complex forms among titin M10, myomesin My4-My5, and obscurin Ig1-Ig3. In cultured cardiomyocytes, overexpression of O1, OL1 or titin M10 led to loss of endogenous Obs at M-lines.

At least 127 disease-causing mutations have been reported in patients with cardiomyopathies, purely skeletal muscle diseases, or childhood diseases affecting both cardiac and skeletal muscles [45]. Several missense or deletion/substitution mutations in M10 result in either tibial muscular dystrophy, an autosomal dominant late-onset distal myopathy, or limb girdle muscular dystrophy type 2J, an autosomal recessive childhood-onset disease [46,47]. In a biopsy specimen from a limb girdle muscular dystrophy type 2J patient homozygous for a 4-aa substitution in M10, obscurin lost its sharply defined M-band localization [31], indicating the physiological significance of this interaction for the structural integrity and function of the sarcomere.

To clarify the mechanism by which titin M10 interacts with Obs and Obsl1, two independent laboratories initially reported the crystal structure of M10 bound to OL1 [48,49]. This work has now been completed by Pernigo *et al.* in this issue of the *Journal of Molecular Biology*, where the authors describe the crystal structure of M10 in complex with O1. The structures show that M10:O1 and M10:OL1 complexes are very similar (Fig. 1). In both cases, Ig subunits pack against each other semi-laterally and in antiparallel fashion, forming a chevron-shaped structure. The interface is formed by the parallel packing of the highly conserved β -strand G in O1/ OL1 and β -strand B in M10. This leads to the fusion of the two alternative β -sheets in the bound domains (resulting in an intermolecular β -sheet composed of strands DEBGF C in O1 and DEBG[A']FC in OL1). This mode of protein interaction has been termed *β -sheet augmentation* and it is often found in poly-Ig proteins [50]. In particular, it is observed in terminal Ig domains of filamentous proteins of the cell, mediating assembly (reviewed in Ref. [51]). In addition to M10:O1 and M10:OL1 at the C-terminus of titin, filamentous protein linkage by β -sheet augmentation has also been observed in (i) the titin N-terminus, where two Z1Z2 Ig-doublets sandwich in between the protein telethonin (Tcap) (the latter folds into an antiparallel β -sheet, leading to the formation of a large intermolecular β -sheet across the three components [52]); (ii) the C-terminus of myomesin, where domain Ig13 associates into homodimers [53,54]; and (iii) the C-terminus of human filamin [55,56] and the filamin analog in *Dictyostelium discoideum* [57,58], which support homodimerization (Fig. 2). Curiously, in all these cases, the resulting filament association is antiparallel, irrespective of whether the binding is direct through

homodimerization (filamin and myomesin), indirect and driven by adaptor proteins (titin N-terminus), or it involves two different proteins (titin-Obs/Obs11).

The edge strands of β -sheets have the inherent capability of establishing main-chain hydrogen bonds with exogenous β -strands. Thereby, they mediate protein–protein interactions by mechanisms such as β -sheet augmentation, β -strand insertion, fold complementation or β -strand zippering [50,59]. β -Rich proteins usually protect their edge strands to prevent undesirable interactions. Capping strategies include very short edge strands, loop coverage, charged side chains, β -bulges or the inclusion of residues (e.g., Pro) that twist and deform the edge strand impeding its accommodation into a β -sheet [60]. Multiple variants of the Ig fold are found in proteins that differ widely in β -strand composition, loop architecture and edge-capping strategy, forming the large Ig-like superfamily [61,62]. Intracellular proteins of muscle commonly contain Ig domains of the I(intermediate)-type [63]. I-Ig domains use a split-first β -strand (A-A') to cap the outer edge of the β -sandwich (i.e., the edge containing the N- and C-termini) and a short C'D loop to block the other edge. Typically, β -strands A and A' are very short and each belongs to a different sheet in the fold (ABED/A'GFCC'), being joined by a cross-over connection in the form of a β -bulge or a short loop (as in OL1 in Fig. 1). In this issue, Pernigo *et al.* reveal a new variety of A-A' capping region in the O1 domain from obscurin. Here, β -strand A' does not cross-over to join the opposite β -sheet but remains associated to strand B in the same sheet forming a flexible A'B loop (Fig. 1). The authors term this Ig fold variety the I*-type. Surprisingly, the I* fold alteration is inconsequential for the binding of O1 to titin M10, where it is the natural twist of the intervening β -strands that causes the discontinuation of the interface. This leads to ponder the role of the I* features in the N-terminus of Obs. In this respect, Pernigo *et al.* found that the I* fold is present in eight other Ig domains (belonging to myomesin, Trk kinase and Kirrel3), where it appears to be an adaptation that contributes to support intramolecular or intermolecular interactions via the newly formed A'B loop (Fig. 2d). It is tantalizing to speculate that the M10:O1 complex at the C-terminus of titin might exploit its distinctive I* features in recruiting other proteins to the M-line, reflecting the scaffolding role of the Z1Z2:telethonin complex at the N-terminus of titin in the Z-disk [52]. In such a case, I* features might enable the specific binding of additional, as-yet unidentified M-line proteins by M10:O1 *versus* M10:OL1.

With regard to interactions, the Ig fold is the archetypal binder in nature. In particular, the V(variable)-type Ig domain mediates highly specific protein contacts that support antigen recognition by antibodies. V-Ig domains have three hypervariable loops (BC, C'C and FG) that accommodate large differences in sequence, length and conformation. Thereby, they display an endless variety of binder motifs that form complex, three-dimensional paratopes. The I-Ig domain type from intracellular poly-Ig proteins lacks developed loops [61]. This has been well described for representative Ig domains from titin that have short loops with conserved sequence and structural motifs [64]. Interestingly, protein engineering studies have shown that Ig domains from titin can tolerate the enlargement and diversification of their loops (BC and C'D) [65,66] and can support sequence-specific binding that way [66]. However, one single case of naturally occurring, loop-assisted, sequence-specific protein binding has been reported for an Ig from the titin-like family: that of the titin Ig A169 that

has a unique 9-residue loop protrusion between β -strands A and A' that dictates binding to the E3 ubiquitin-ligase MuRF1 [67]. In stark contrast, the mechanism of β -sheet augmentation is main chain driven and has poor sensitivity to sequence composition. Accordingly, the complexation of Z1Z2/telethonin has been shown to be sequence independent, with specificity in molecular recognition achieved by the spacing and mutual geometry of the binding strands, β -strands G in both Z1 and Z2 [52]. Dimerization of myomesin Ig13 has also been found to be largely main chain dependent, although in this case, two salt bridges (D1580-K1588' and K1588-D1580') were shown to be critical for interface formation [53]. In M10:OL1 and M10:O1, the mutagenesis of several interface residues in M10 (R98E and D61R in Ref. [49]; A25K, A25E, D60R and L61R in Pernigo *et al.* in this issue), O1 (R15F in Ref. [48]) and OL1 (F17R in Ref. [48]) failed to abolish complexation. Only the introduction of a bulky side chain in the interface, O1 A94Y and OL1 A96Y, succeeded in disrupting the complex (Pernigo *et al.* in this issue). This suggests that also the M10:O1/OL1 assembly is largely dictated by intermolecular β -sheet formation and not dependent on specific contacts. In agreement with this deduction and even though O1 and OL1 only share 38.5% sequence identity, M10:O1 and M10:OL1 complexes displayed very similar affinity values measured by isothermal titration calorimetry (Ref. [49]; Pernigo *et al.* in this issue) and an undistinguishable response to force as monitored by single-molecule atomic force spectroscopy [AFM (*atomic force microscopy*)] [48]. This indicates that titin M10 is not able to discern between these two Ig partners *in vivo* but that specificity in the binding is likely governed by cellular localization. Obs localizes to the myofibril periphery, while Obsl1 is in the myofibril core. As both titin and Obs/Obsl1 are non-diffusible proteins, cellular compartmentalization will play a big role in ensuring the correct spatial-temporal occurrence of the complexation, preventing competition for binding to titin M10.

β -Sheet interactions are optimally suited to support filament assembly and sarcomeric anchoring *in vivo*. First, such interactions are mechanically strong and withstand the pulling forces that develop in the sarcoskeleton during muscle function. Second, they offer an enhanced resistance to point mutations that could otherwise lead to disease by debilitating the sarcomere ultrastructure. AFM measurements on the C-terminal homodimer of myomesin Ig13 and the N-terminal titin Z1Z2/telethonin complex revealed mean dimer dissociation forces of ~137 pN [68] and ~700 pN [69], respectively. Considering that Ig domains typically unfold at pulling forces of 100–250 pN [70], we can regard these complexes as mechanically very stable. In contrast, the unbinding forces of the M10:O1 and M10:OL1 complexes are weak, in both cases, ~30 pN [48]. This suggests that, on their own, the M10:O1 and M10:OL1 complexes contribute little to maintain the structure of the sarcomere in the face of contraction/relaxation. This mechanical weakness is likely to reflect both the small β -sheet interface in the complexes and the poor orientation of the subunits in terms of pulling geometry. It has been shown that the orientation of secondary structural elements relative to the applied force vector is a determinant of mechanical strength [71,72]. The removal of strands from a β -sheet is greatly eased when a peeling force is applied rather than a shear-like extension. In rough terms, longitudinal shearing requires a breaking force approximately equivalent to the force needed to rupture one bond multiplied by the number of bonds to be broken, while the application of force orthogonally to the β -strands (peeling)

loads each hydrogen bond in turn, bonds fail consecutively at a lower force and pass the load to the next bond (in a zipper fashion). Shear-like pulling geometries were used in the AFM evaluation of Z1Z2: telethonin [69] and the myomesin Ig13 dimer [68], but a peeling-like effect applied to M10:O1/OL1 complexes [48]. In either of the cases, AFM geometries might represent a subset of the complex and variable mechanical scenarios to which these protein interfaces are subjected in the sarcomere. Thus, it would be interesting to explore the mechanical landscape of the several complexes to establish their active force range.

In conclusion, biochemical, structural and mechanical data shed now light on the molecular networking of titin, obscurin, myomesin and myosin in the sarcomeric M-line. Undoubtedly, data by Pernigo *et al.* constitutes a substantial step toward unraveling the mechanical and scaffolding roles of titin and Obs in such an intricate network, as well as the significance of such roles for sarcomere structure and function.

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Fig. 1.

Crystal structures of titin M10 in complex with Obs O1 and Obsl1 OL1. Complexes (PDB codes 4C4K and 2WP3) are displayed superimposed on the Obs/Obsl1 partners. For visual clarity, only one copy of M10 is displayed. The structural conservation of the Obs/Obsl1 domains is very high, with a significant divergence only present in β -strand A' and the subsequent AB loop. The β -hairpin FG mediating the interaction in Obs/Obsl1 is one of the most conserved regions in these domains (strictly conserved residues are in boldface; the NxxG signature motif of this hairpin as described in Marino *et al.* [64] is in red). The AB loop, despite its different conformation, retains a close sequence resemblance.

Fig. 2. Crystal structures of filamentous assemblies. Deviant N-terminal features in Ig domains are highlighted in cyan. In Obs and myomesin, cyan indicates the β -hairpin AB (and the loop it forms) as characteristic of the new I*-Ig set. In *D. discoideum* filamin (DdFilamin), the dimerizing Ig has an incomplete set of strands so that the colored β -strand in N-terminal position is B. PDB accession codes are as follows: (a) 4C4K, (b) 1V05, (c) 1YA5, (d) 2Y25 and (e) 1WLH.