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Different obscurin isoforms localize to distinct sites at sarcomeres

Amber L. Bowman[a], Aikaterini Kontrogianni-Konstantopoulos[a], Sara S. Hirsch[a], Sarah B. Geisler[b], Hugo Gonzalez-Serratos[a], Mark W. Russell[b], Robert J. Bloch[a,*]

[a] *Department of Physiology, University of Maryland School of Medicine, 655 W. Baltimore St, MD 21201, USA*
[b] *Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, MI, USA*

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Abstract **We used four antibodies to regions of obscurin isoforms A and B, encoded by the *obscurin* gene, to investigate the location of these proteins in skeletal myofibers at resting and stretched lengths. Obscurin A (~800 kDa) which was recognized by antibodies generated to the N-terminal, Rho-GEF, and the non-modular C-terminal domain that lacks the kinase-like domains, localizes at the level of the M-band. Obscurin B (~900 kDa) which has the N-terminal, Rho-GEF, and the C-terminal kinase-like domains, localizes at the level of the A/I junction. Additional isoforms, which lack one or more of these epitopes, are present at the Z-disk and Z/I junction.**

Keywords: Obscurin; I-band; Z-disk; M-line; Stretch

1. Introduction

Obscurin is a recently discovered giant (800–900 kDa) protein of striated muscle that has many Ig and several FnIII domains, as well as IQ, Src homology 3 (SH3), Rho guanine nucleotide exchange factor and pleckstrin homology (Rho-GEF/PH) domains, and can be alternatively spliced to produce smaller products or to include kinase-like domains that may function in signaling [1–6]. In longitudinal sections of adult skeletal muscle, obscurin concentrated at the level of the Z-disks and M-bands of sarcomeres [1,3,7–9], with one report suggesting it repositioned into the I-band in stretched samples [3] but the specific obscurin isoforms present at these sites were not identified. In cross-sections of myofibers, labeling for obscurin is in a reticular pattern, indicating that obscurin surrounds the contractile apparatus [9], a position that distinguishes it from the two other giant proteins of the sarcomere, titin and nebulin. Thus, products of the *obscurin* gene may have unique roles in myofibrillogenesis.

Understanding the subcellular distribution of obscurin is complicated by the presence of multiple alternatively spliced forms. Obscurin is present in skeletal muscle in an ~800 kDa form, now termed obscurin A [4], characterized by a C-terminal non-modular region that binds a small form of ankyrin in the network sarcoplasmic reticulum [7,9]. An additional variant, with a larger molecular mass than obscurin A, arises from the replacement, by alternative mRNA splicing, of the non-modular carboxy terminus with two serine/threonine kinase-like domains. Originally identified as the ''giant kinase'' isoform of obscurin [2,10,11] it has been more recently termed obscurin B (Fig. 1) [4]. Obscurin is also expressed as several smaller alternatively spliced variants [1,2,4,5,12]. Several alternatively spliced products have also been identified for obscurin's homologue in *C. elegans*, *UNC-89* [13].

We used antibodies to different regions of obscurin, the N-terminus, the C-terminus, the Rho-GEF domain, and the Ig domain N-terminal to the first kinase-like domain (Ob68) to characterize the subcellular location of some of the alternatively spliced forms of obscurin in skeletal muscle at resting length and after stretch (Fig. 1). We localized obscurin A and obscurin B to the M-bands. In addition, we found obscurin B near the A/I junction, and localized distinct obscurin domains, or combinations thereof, likely to be indicative of other alternatively spliced forms, to the Z-disk and at the Z/I junction. The presence of two and possibly more different forms of obscurin at distinct sarcomeric locations suggests that these proteins play several distinct roles in organizing and stabilizing the contractile apparatus and nearby structures.

2. Materials and methods

2.1. Antibodies

We used the reverse transcriptase polymerase chain reaction (rtPCR) to amplify the coding region of the Rho-GEF domain of obscurin from rat skeletal muscle poly A+ RNA (Clontech, Palo Alto, CA) using the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA). The primers were: reverse transcriptase, GCCACAGATCTGCTTCACCCA; forward, AGTGAATTCGTCATCCAGGAGTTGCTGAGTTC; reverse, ATCGGATCCCTAGCGCTGTGGCAGGGCAGA. This cDNA was cloned into the *Eco*RI/*Xho*I sites of pGEX-4T-1. Synthesis of the glutathione-*S*-transferase (GST)-Rho-GEF protein was induced (0.3 mM IPTG, 4 h). The protein was solubilized, purified and used to generate rabbit antibodies (Covance Research Products, Denver, PA), as described [14,15]. Antibodies were purified over affinity columns prepared with MBP-Rho-GEF. Immunodepletion experiments were performed by incubating 2 μg/ml of each antibody with 200 μg/ml of the corresponding antigen in PBS/BSA/NGS in a total volume of 1 mL (PBS containing 3% BSA, 10 mM NaN_3, 5% normal goat serum: Jackson ImmunoResearch Laboratories, West Grove, PA) for 16 h at 4 °C and using these pre-adsorbed antibodies for immunolabeling.

We also used antibodies to: α-actinin (mouse IgG EA53, 1:400 dilution, Sigma Chemical Co., St. Louis, MO); mouse 9D10 to the PEVK region of titin (supernatant fraction, used at 1:1 dilution, Developmental Studies Hybridoma Bank, Iowa City, IA); the N-terminal (Z-disk region) epitopes of titin (titin-Z, 3 μg/ml; [12]); the N-terminal [12] and C-terminal [9] regions of obscurin A; and the Ob68-serine-threonine

*Corresponding author. Fax: +1 410 706 8341.
E-mail address: rbloch@umaryland.edu (R.J. Bloch).

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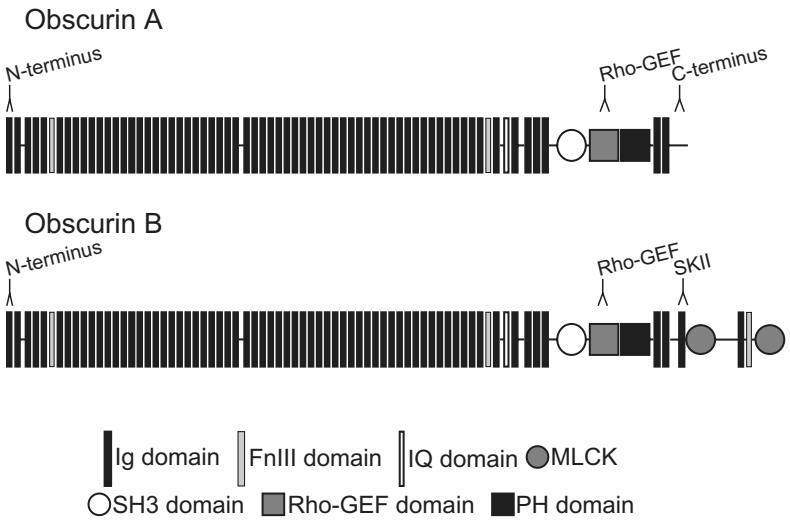

Isoform	Recognized by antibodies to:
Obscurin A	N-terminus, Rho-GEF, C-terminus
Obscurin B	N-terminus, Rho-GEF, SKII

Fig. 1. Obscurin isoforms. Antibodies were prepared to distinct regions of obscurin: the N-terminal Ig domain, and the Rho-GEF domain, the non-modular C-terminus of obscurin A and to Ob68, adjacent to the first kinase-like domain (SKII) in the giant kinase isoform, obscurin B.

kinase region of obscurin B [16]. All antibodies to titin and obscurin were generated in rabbits, except to the N-terminal domain of obscurin and the 9D10 antibody to titin, which were created in mice.

Goat antibodies to mouse and rabbit IgGs, linked to Alexa-488 or Alexa-568 (Molecular Probes, Eugene, OR), were diluted 1:200.

2.2. Immunoblotting

Homogenates of adult rat muscle EDL were prepared [17] and supplemented with 50 U/mL benzonase (Novagen, Madison, WI). Aliquots (50 μg) were processed for SDS–PAGE and immunoblotting [5].

2.3. Stretched muscle

Extensor digitorum longus (EDL) muscles, removed from adult rats with proximal and distal tendons intact, were placed in Krebs solution equilibrated with 95% O_2, and 5% CO_2, pH 7.2. Silk threads (#4) were tied around the tendons and muscles were extended to L_o, the resting tension length in vitro from the proximal to the distal myotendinous junction [18,19], or to distances ranging from 1.3 to 1.7 L_o. (Only smaller bundles of fibers could be stretched to 1.7 L_o, due to the passive resistance of the connective tissue in intact muscle). The muscles were fixed immediately in 2% paraformaldehyde in PBS for 20 min, snap frozen, and cryosectioned [9].

2.4. Fluorescent immunolabeling and confocal microscopy

Frozen longitudinal sections were incubated in PBS containing 3% BSA and 5% non-immune goat serum (PBS/BSA/NGS) followed by primary antibodies in PBS/BSA/NGS overnight at 4 °C [9]. Samples were washed, incubated with species-specific secondary antibodies in PBS/BSA/NGS, mounted in Vectashield, and observed with a Zeiss 410 confocal laser scanning microscope (Carl Zeiss, Inc., Tarrytown, NY).

3. Results

3.1. Localization of obscurin epitopes in skeletal muscle at resting sarcomere lengths

We used immunofluorescence to identify sites in resting muscle fibers labeled by antibodies to different epitopes of obscurins A and B. The specificity of the C-terminal and SKII antibodies have been previously described [5,9,16]. In addition, we performed immunodepletion experiments demonstrating that immunoreactivity was eliminated by preadsorbtion with the immunogen (Fig. 2A, D, G, J, insets). Antibodies to the N-terminus primarily recognize structures at the level of the M-band, with additional but faint labeling near the Z-disk (Fig. 2A), as determined by double immunofluorescence with antibodies to epitopes of titin found at the Z-disk (Fig. 2B and C). Double immunofluorescence experiments with antibodies to α-actinin (Fig. 2E and F) indicate that the Rho-GEF domain of obscurin is primarily found in structures at the level of the M-band (Fig. 2D). As previously reported, antibodies to the C-terminal region of obscurin A and to the Ob68-serine-threonine kinase region of obscurin B (Fig. 2G and J) recognize structures at both the Z-disk and M-band, as determined by double immunofluorescence experiments with antibodies to α-actinin (Fig. 2H, I; K, L) [9,16]. These results suggest that both obscurin A and the epitopes within Ob68 and its adjacent kinase-like domain, associated with the B isoform, are present at the level of the M-band, though the latter may be present at lower levels. Furthermore, an isoform of obscurin or a closely related protein that contains epitopes of the non-modular C-terminal domain of obscurin A is likely to be present at the level of the Z-disk. However, we do not discount the possibility that Rho-GEF and N-terminal epitopes are not as accessible at the Z-disk and this is an additional site of localization of obscurin A.

3.2. Localization of obscurin epitopes in skeletal muscle at stretched sarcomere lengths

Epitopes of obscurin that fail to label at particular sarcomeric locations may be inaccessible. To increase accessibility,

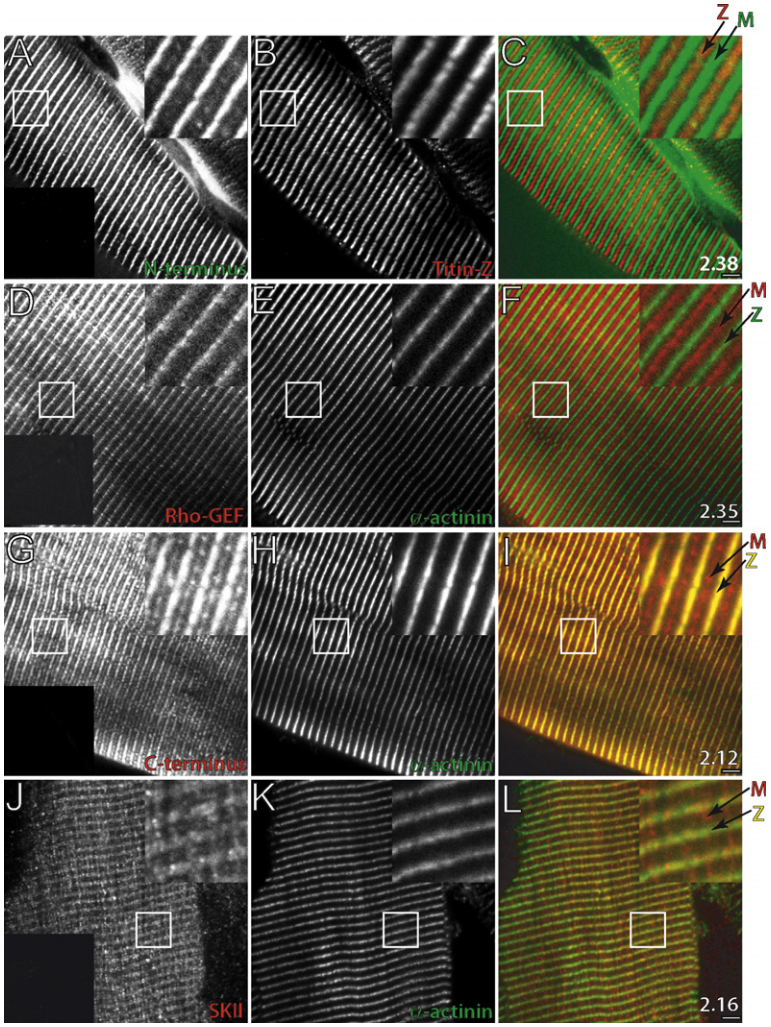

Fig. 2. Localization of obscurin isoforms in muscle at resting length. (A–F) Double immufluorescence experiments with antibodies to Z-disk epitopes of titin (B, C, red) and α-actinin (E, F, green) to mark Z-disks, and antibodies to the N-terminus (A, C, green) and Rho-GEF domain of obscurin (D, F, red) show that the latter primarily label at the level of the M-band. (G–L) Similarly, with the two C-terminal antibodies, to the non-modular C-terminal domain (G, I, red) and the SKII antibody to Ob68 (J, L, red). Both label primarily at the level of the Z-disk and M-band, identified by comparison with α-actinin (H, I; K, L green). Immunodepletion experiments are shown in the lower left corners of the larger panels (A, D, G, J). Average sarcomere lengths are indicated. Bars: 5 μm.

we stretched EDL muscles to 130–170% of their resting length (1.3–1.7 L_o) before immunolabeling with the same anti-obscurin antibodies. Antibodies to the N-terminal and Rho-GEF domains labeled stretched samples at the level of the M-band, and also at the level of the I-band (Fig. 3A, D, G, J; compare with labeling by antibodies to Z-disk epitopes of titin, Fig. 3B, E, and α-actinin, Fig. 3H and K). Labeling at the I-band was near the A/I junction, which also labeled with antibodies to the PEVK region of titin (supplemental figure 1).

Antibodies to the non-modular C-terminus labeled both the M-band and Z-disk in stretched samples, similar to unstretched muscle (Fig. 3M–O). Further increases in sarcomere length to ~3.6 μm did not further alter their labeling pattern, although a slight splitting could be detected at the level of the Z-disk (Fig. 3P), resulting in a decrease in overlap with α-actinin (Fig. 3Q and R).

As in unstretched muscle, the SKII antibody that detects obscurin B labeled the M-band and the periphery of the Z-disk, near the Z/I junction, in stretched muscle. SKII antibodies also labeled structures further into the I-band in stretched muscle, as described above with antibodies to the N-terminal and Rho-GEF domains (Fig. 3S–X).

3.3. Relative protein levels of obscurin A and B

Using antibodies to the Rho-GEF domain, C-terminus and N-terminus, we performed western blots on muscle extracts of adult rat EDL. As expected, obscurin B has a greater molecular mass than obscurin A and is recognized by antibodies to the Rho-GEF domain and the N-terminus, but not the C-terminus. In addition, obscurin B appears to be present at significantly lower levels (Fig. 4).

4. Discussion

Products of the *obscurin* gene play an important role in myofibrillogenesis, especially in the assembly of the M-band [16,17,20], but the functions of the various, alternatively spliced gene products in this process are still unclear [1,2]. Our results suggest that the two giant forms of obscurin, termed A and B,

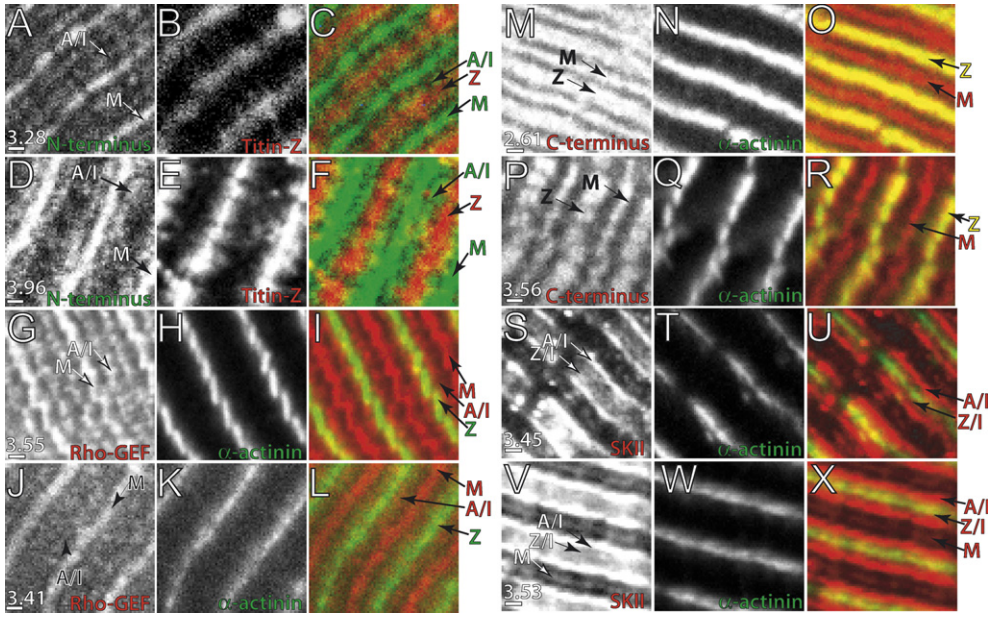

Fig. 3. Localization of obscurin isoforms in stretched muscle. As in Fig. 2, but muscle was stretched to 1.3–1.7 L_o before immunolabeling. (A–F) In addition to M-bands, antibodies to the N-terminus of obscurin label the I-band near the A/I junction (A, C, D, F; green; compared to titin at the Z-disk: B, C, E, F; red). (G–L) Antibodies to the Rho-GEF domain also react with structures at both the M-band and A/I junction (G, I, J, L; red; compared to α-actinin H, I, K, L; green). (M–R) The non-modular C-terminus of obscurin is at the level of the Z-disk and M-band (M, O; red; compared to α-actinin: N, O; green) at ~1.3 L_o. Greater stretching to ~1.7 L_o produces splitting of the C-terminal antibody labeling of the Z-disk (P, R; red; compared to α-actinin: Q, R; green). (S–X) Antibodies to Ob68 label at the level of the M-band and at two locations within the I-band, near the A/I junction, and at the Z/I interface (S, U, V, X; red, compared to α-actinin: T, U, W, X; green). Average sarcomere lengths are indicated. Bars: 1 μm.

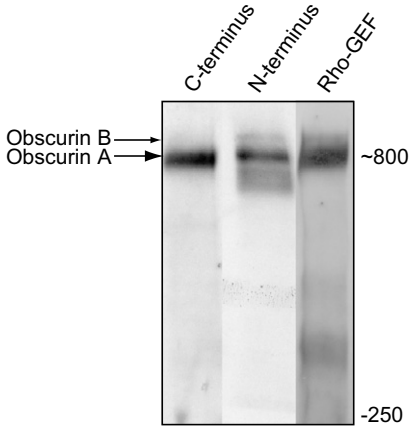

Fig. 4. Immunoblotting of obscurins A and B. In immunoblots of extracts of adult quadriceps, antibodies to the Rho-GEF domain of obscurin and the N-terminal Ig domain recognize the 800 kDa isoform (large arrow), likely to be obscurin A, and a larger isoform that is present at much lower levels (small arrow), likely to be obscurin B. Antibodies to the C-terminus of obscurin A recognize the obscurin A isoform, but not the obscurin B isoform. Molecular masses are indicated.

are selectively enriched at different sites in skeletal muscle, and that smaller alternatively spliced forms—or post-translationally modified proteins—may be associated with the Z-disk and nearby regions of the I-band. These results are the first to document the differential distribution of different forms of obscurin in mammalian striated muscle.

We previously studied the localization of obscurin with antibodies to the non-modular C-terminus of obscurin A and determined it to be at the level of Z- and M-lines in structures that surround the contractile apparatus, where it may link the sarcoplasmic reticulum to the contractile apparatus through binding to small ankyrin 1 [7,9]. We subsequently created antibodies to the N-terminal Ig, Rho-GEF, and Ob68 regions (Ob68 is adjacent to the first of two kinase-like domains of obscurin) and localized their epitopes in skeletal muscle fibers of resting and stretched lengths [3,5]. Obscurin A, as well as an epitope associated with obscurin B, are present, at the M-band, as previously reported [1,7,9,16]. We find that obscurin B is also present at the I-band near the A/I junction. We suggest further that the isoform of obscurin present at the Z-disk that reacts with antibodies to the non-modular C-terminus is a unique isoform which appears well after the formation of the Z-disk [11]. Our data suggest that this isoform may contain a novel N-terminus and lack the Rho-GEF domain, although we cannot now rule out the possibility that the non-modular C-terminus is more accessible to antibodies, compared to other regions of the molecule associated with the Z-disk. The SKII antibody, to domains of obscurin B, labeled a form of the molecule in the I-band near the periphery of the Z-disk. We also observed this isoform to be recognized by antibodies to the second kinase-like domain (link 7, data not shown). This is likely to represent one of the smaller kinase-containing isoforms of obscurin, some of which are known to be expressed at low levels in skeletal muscle, but more studies of these splice forms will be needed before they can be characterized and localized in the myoplasm [2]. Whether this isoform may account for the labeling of the I-band by antibodies to Ob58/Ob59/Ob60 (formerly referred to as I48/I49/I50), as previously described by Bang et al. [3], remains to be determined. A summary of these isoforms and their sarcomeric locations is summarized in Fig. 5.

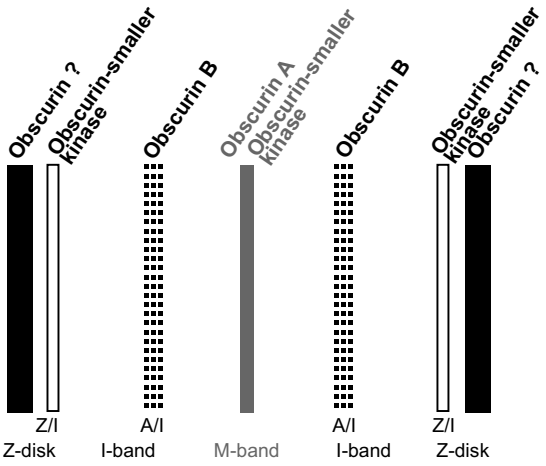

Fig. 5. Schematic representation of localization of distinct obscurin isoforms. The sarcomeric locations of each of the isoforms of obscurin we have identified are indicated. An additional isoform that has not been well characterized is likely to be located at the level of the Z-disk. See text for discussion.

The discovery of new subcellular locations for different forms of obscurin suggests that these proteins may have additional roles in myofibrillogenesis and muscle architecture that remain to be discovered, in addition to their involvement in the assembly of M- and A-bands or in the sarcomeric alignment of the SR [8,11,12,16,17]. The molecules of obscurin present at the A/I junction are near or adjacent to the PEVK region of titin, which serves as the principle elastic component of the titin filament [21]. Thus, the form of obscurin present at this site (obscurin B), may function to transduce stretch signals from this region of titin. Experiments by Borisov et al. [10] indicate that both obscurin Rho-GEF (obscurin A and B) and kinase-encoding (obscurin B) transcripts are upregulated after induction of hypertrophy, consistent with a role of obscurin isoforms A and B in these cellular processes.

Our results suggest further that other proteins or protein epitopes in the I-band are 'hidden' and thus unable to react with antibodies, and that stretching muscle may expose them, perhaps by reducing local protein densities or altering local conformations. Our findings may also indicate that some proteins originally identified as being at the Z-disk may actually associate with nearby regions of the I-band [22]. Immunofluorescence studies of stretched muscle can therefore provide a more complete characterization of the location of sarcomeric proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.03.011.

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