Obscurin modulates the assembly and organization of sarcomeres and the sarcoplasmic reticulum

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ABSTRACT **Obscurin (~800 kDa) in striated muscle closely surrounds sarcomeres at the level of the M-band and Z-disk where, we hypothesize, it participates in the assembly of the contractile apparatus and membrane systems required for Ca^{2+} homeostasis. In this study, we used small inhibitory RNA (siRNA) technology to reduce the levels of obscurin in primary cultures of skeletal myotubes to study its role in myofibrillogenesis and the organization of the sarcoplasmic reticulum (SR). siRNA-treated myotubes showed a specific and dramatic reduction in the ~800 kDa form of obscurin by reverse transcription-polymerase chain reaction, immunoblotting, and immunofluorescence. M-bands and A-bands, but not Z-disks or I-bands, were disrupted when the synthesis of obscurin was inhibited. Small ankyrin 1, an integral protein of the network SR that binds to obscurin, also failed to align around developing sarcomeres in treated myotubes. Myosin and myomesin levels were significantly reduced in treated myotubes but α-actinin was not, suggesting that down-regulation of obscurin destabilizes proteins of the M-band and A-band but not of the Z-disk. Our findings suggest that obscurin is required for the assembly of the M-band and A-band and for the regular alignment of the network SR around the contractile apparatus.—Kontrogianni-Konstantopoulos, A., Catino, D. H., Strong, J. C., Sutter, S., Borisov, A. B., Pumplin, D. W., Russell, M. W., Bloch, R. J. Obscurin modulates the assembly and organization of sarcomeres and the sarcoplasmic reticulum. *FASEB J.* 20, 2102–2111 (2006)**

Key Words: obscurin · myosin · myomesin · titin · small ankyrin 1 · A-band · M-band

THE MYOPLASM OF ADULT striated muscle fibers contains several highly ordered components that are necessary for contraction. These include the myofibrils themselves and two associated systems of intracellular membranes: the transverse, or t-tubules, and the sarcoplasmic reticulum (SR).

The assembly of myofibrils is a complex process that depends on the coordinated integration of actin, myosin, and their associated proteins into sarcomeres (1, 2). The striking regularity of actin and myosin in sarcomeres cannot be explained simply by their ability to assemble independently into thin and thick filaments, respectively. Rather, it involves specific and dynamic interactions with other cytoskeletal components (2–5) that are still poorly understood. In one recent model of myofibrillogenesis, primitive Z-disks, termed "Z-bodies," composed of α-actinin interact with the N-terminus of titin, nebulin, and T-cap/telethonin to nucleate the polarized organization of actin filaments (6–8). Similarly, proteins of the M-band, including the COOH-terminal region of titin, myomesin, M-protein, and obscurin, are essential for the integration of myosin filaments into A-bands (5, 9–14). As myofibrils mature, they align parallel to each other, assume a diameter of 1–3 μm, and become organized as a series of sarcomeres with sharply delineated Z-lines, highly ordered I-bands consisting of ~1 μm-long thin filaments, and mature A-bands composed of ~1.6 μm-long, thick filaments that are centrally symmetrical and are bisected by M-bands.

As myofibrillogenesis proceeds, the t-tubules and SR, which together modulate cytosolic Ca^{2+} release and uptake during contraction and relaxation, assemble gradually and in close coordination with the sarcomeric cytoskeleton (15, 16). T-tubules are not associated with specific regions of the sarcomere until late in development, when they localize to the myoplasm surrounding the sites where the A and I bands overlap (the "A/I junction") in each sarcomere. Similarly, organized elements of the SR appear only at later stages of myofibrillogenesis, when the key features of sarcomeres are easily recognizable. At maturity, the SR of mammalian skeletal muscle contains three morphologically and functionally distinct, yet continuous, compartments: the terminal cisternae, the network SR, and the longitudinal SR (17). Like the t-tubules, these compartments

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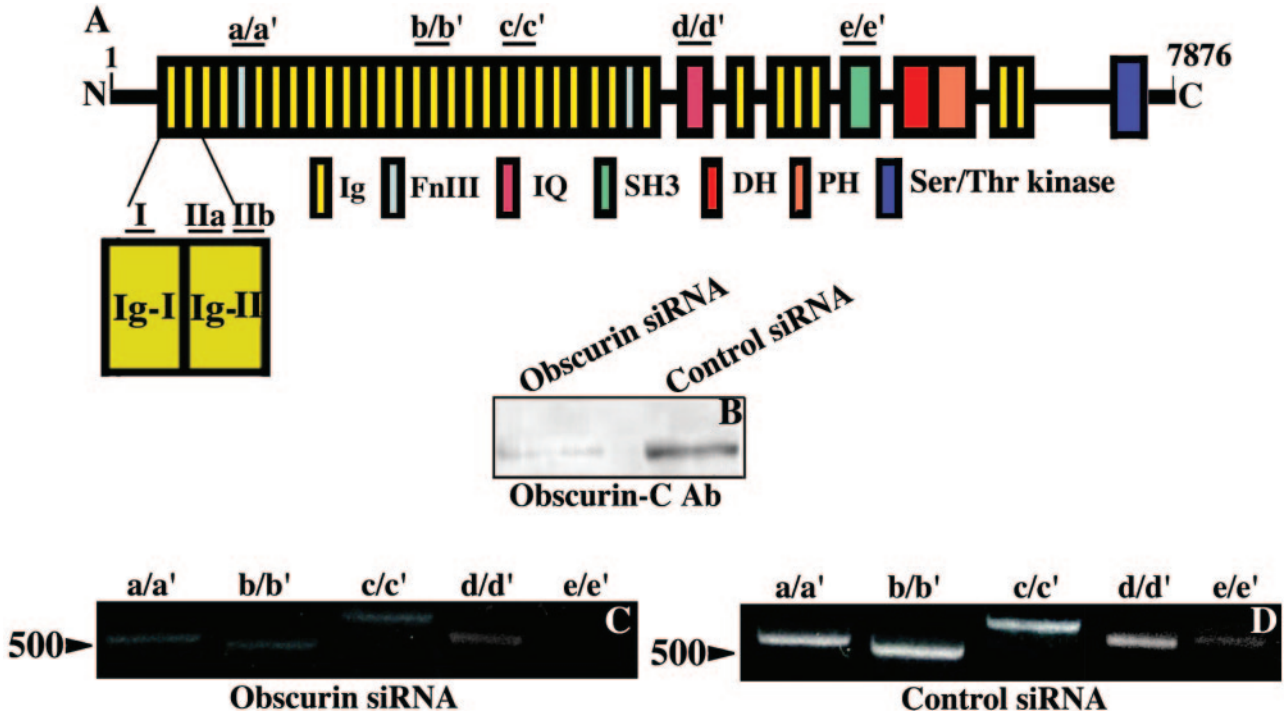

Figure 1. Reduced expression of obscurin by siRNA in primary cultures of skeletal myotubes. *A)* Schematic representation of obscurin (~800 kDa) showing its structural and signaling domains. Three sequences (I, IIa, and IIb) present in the first two Ig domains of obscurin were used to generate recombinant siRNA adenoviruses. *B)* Primary cultures of skeletal myotubes were infected with control or siRNA-IIa virus. After SDS-PAGE, lysates examined by immunoblotting for obscurin revealed significant reductions (~70%, $P<0.05$) in cells infected with obscurin siRNA virus compared with controls. *C, D)* RT-PCR using a series of primers designed to flank different parts of the obscurin sequence (shown in panel *A*: a/a′, b/b′, c/c′, d/d′, and e/e′) showed a decrease (~65%, $P<0.05$) in the levels of obscurin transcripts in myotubes treated with siRNA-IIa virus (*C*) *vs.* controls (*D*).

occupy specific locations around each sarcomere in skeletal myofibers: the network SR is positioned around M-bands and Z-disks whereas the terminal cisternae are located with the t-tubules around A/I junctions. Specific localization of the t-tubules and compartments of the SR requires a set of anchoring mechanisms that are strong enough to resist the strong shear forces that can be exerted on the membranes during the contractile cycle.

We have been studying the role of obscurin, a large (~800 kDa) protein of the titin superfamily, in organizing myofibrils and the SR (10, 11, 18–22). Like titin, obscurin is a multidomain protein composed of tandem adhesion modules and signaling domains (**Fig. 1*A***) (23). Specifically, its NH_2-terminal half is composed of 49 immunoglobulin (Ig) and 2 fibronectin III-like (FNIII) domains, and is followed by a complex region consisting of 4 additional Ig domains, flanked by nonmodular sequences, an IQ motif, and a conserved SH3 domain adjacent to Rho guanine nucleotide exchange factor and pleckstrin homology domains. Its COOH-terminal region consists of two additional Ig repeats, followed by a nonmodular region of 417 amino acids with several consensus phosphorylation motifs for ERK kinases.

Unlike titin, which is an integral component of sarcomeres, obscurin intimately surrounds myofibrils at the level of the Z-disk and M-band, where it is appropriately positioned to participate in their assembly and integration with elements of the SR (20). Consistent with this, obscurin binds to titin and sarcomeric myosin, and to small ankyrin 1 (sAnk1), an integral component of the network SR (11, 20, 23–25). During myofibrillogenesis in cultured neonatal rat cardiac and skeletal myotubes, obscurin associates primarily with developing M-bands and, at a later time and to a lesser extent, with Z-disks (18, 23, 26). Overexpression of its COOH-terminus in primary cultures of skeletal myotubes has a profound and specific effect on the organization of sarcomeric myosin, suggesting a role in the assembly of A-bands (11).

Here we use small inhibitory RNAs (siRNAs) to reduce the expression of obscurin and examine its role in developing skeletal myotubes. Our results show that siRNA targeted to the mRNA of the ~800 kDa form of obscurin reduces the levels of protein by ~70% and selectively inhibits the formation of M-bands and A-bands. They further suggest that the network SR also fails to differentiate and align with the contractile apparatus when obscurin levels are reduced. Our results are the first to demonstrate a key role for obscurin in the assembly and maintenance of sarcomeric A-bands and M-bands, and in the regular alignment of the network SR around developing sarcomeres.

MATERIALS AND METHODS

Cultures of skeletal myotubes

Primary cultures of rat myotubes were prepared as in ref. 27. Briefly, hind limb muscles from postnatal day 1 (P1) rats were dissociated enzymatically and suspended at 10^6 cells/ml in Dulbecco-Vogt-modified Eagle's medium (DMEM, Life Technologies, Inc., Carlsbad, CA, USA) containing 10% FBS (Life Technologies, Inc.). Aliquots (~0.5 ml) were applied to sterile glass coverslips and supplemented with 1 ml of the same medium the next day. Medium was replaced 48 h later with medium containing 2×10^{-5} M cytosine arabinoside (Sigma Chemicals, St. Louis, MO, USA) to kill dividing cells. Cultures were infected with adenoviral constructs 5 days after initial plating and analyzed 48 h later.

siRNA adenoviral constructs and infection of primary cultures

siRNA constructs were designed to inhibit the expression of the first coding exon of the rat obscurin gene (XM340807; nucleotides 1–600). Target siRNA sequences were selected based on Tuschl's principles (28). Three different siRNA target sites and one control sequence (Ambion Inc., Austin, TX, USA) were selected: I: 5′GTGGAGGCAGGAGCACGCT3′, IIa: 5′GACGCCACCTTCCGATGTC3′, IIb: 5′GCTGTGAGCTGGTCCAAAGA3′, and control: 5′ACTACCGTTGTTATAGGTG3′. The siRNA target sites were converted into oligonucleotide sequences as described in pSilencerTM kit (Ambion). Duplex DNA was directionally cloned into the pSilencer 2.0-U6 expression vector driven by the U6 promoter (Ambion). The U6 promoter and siRNA constructs were subcloned in the adenoviral shuttle vector, pACpL+loxP-SSP (http://www.med.umich.edu/vcore/pdf/pAC%20pL+loxP-SSP.pdf). Obscurin I, IIa, and IIb as well as control siRNA adenoviruses were produced, as described (10).

Cultures of rat myotubes were infected with 10^9 viral particles/ml of obscurin-I, obscurin-IIa, obscurin-IIb, or control siRNA viruses in 1 ml DMEM for 1 h at room temperature. Infected cells were supplemented with 1 ml of DMEM plus 20% FBS and 4×10^{-5} M cytosine arabinoside (Sigma Chemicals). After 48 h cultures were rinsed with PBS and fixed with 2% paraformaldehyde for 15 min at room temperature. Fixed cells were permeabilized with 0.1% Triton-X-100 for 10 min at room temperature, rinsed with PBS, and processed for immunolabeling and confocal imaging.

Experiments were repeated eight times and ~ 25 cells were analyzed from each.

Antibodies

Primary antibodies were rabbit antibodies to obscurin-C (3 μg/ml; ref. 20), titin-Z (to the first two Ig domains; 3 μg/ml), titin-M (to its M-band portion, 3 μg/ml; a gift of Dr. S. Labeit; ref. 29), and sAnk1 (3 μg/ml; ref. 30), and mouse antibodies to obscurin-N (to the two NH_2-terminal Ig domains; 3 μg/ml), myosin II (clone MY-32, diluted 1:500; Sigma), α-actinin (diluted 1:500, Sigma), and myomesin-B4 (diluted 1:100; a gift of Dr. J.-C. Perriard; ref. 31). Secondary antibodies were goat anti-mouse and goat anti-rabbit IgGs conjugated to $Alexa_{488}$ and $Alexa_{568}$, respectively, for immunofluorescence (Molecular Probes, Eugene, OR, USA; used at 1:200) or to alkaline phosphatase for immunoblotting (Jackson ImmunoResearch, West Grove, PA, USA; used at 1:2500).

Generation of P1 myotube lysates and immunoblotting

Protein lysate was prepared from infected myotubes as described (11), and ~50 μg from each sample were solubilized in 2 × SDS Laemmli buffer either at 42°C for 30 min (for obscurin) (20) or at 90°C for 5 min (for myosin, myomesin, α-actinin, and sAnk1). Samples fractionated by SDS-PAGE were transferred to nitrocellulose, processed (11), and probed with primary antibodies at 100 ng/ml (or other concentrations; see above). These experiments were repeated at least four times and yielded consistent results.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from cultures of rat myotubes, infected with control siRNA viruses or with siRNA viruses targeting obscurin, with Trizol as reagent (Invitrogen Corporation, Carlsbad, CA, USA). Aliquots containing ~5 μg of RNA were reverse transcribed using the Superscript First Strand Synthesis System for RT-polymerase chain reaction (RT-PCR) (Invitrogen) following the manufacturer's instructions. Polymerase chain reaction (PCR) amplification yielded cDNAs encoding fragments of the rat obscurin gene (accession number: XM_340807), with the following sets of oligonucleotide primers: a: 5′GCACCCAGGAGGCCACCCCTGTACCCT3′ (sense) and a′: 5′GTGGTCCGAGGGGCTCT GGGGCTG3′ (antisense), b: 5′GAGTCCCAAACTCCACAGAGGCCT3′ (sense) and b′: 5′CTTTCCTCTGTAGTTCCCCGAGTG3′ (antisense), c: 5′ACTGTGGAAGCTCGGGAGGTG ACA3′ (sense) and c′: 5′CAAGAGTTTAGTCCCTGTGAGCCT3′ (antisense), d: 5′CCTGCACAGCCCAGCCTGCCTCCC3′ (sense) and d′: 5′CTGCTGCTTCATCTCCTTCCT CAC3′ (antisense) and e: 5′GCCAAAGAAGAGGAGCAGGAGAGA3′ (sense) and e′: 5′AGGAGATAGCTTGAGCCTCTTGTC3′ (antisense).

cDNAs encoding sequences of different isoforms of muscle myosin were also assessed (32). A sense oligonucleotide primer, A: 5′ GAAGGCCAAGAAGGCCATC 3′, annealed to a highly conserved region shared by all four isoforms of the heavy chain of muscle myosin (MHC) expressed in adult rat skeletal muscle. A slightly modified primer A, termed A′: 5′ GAAGGCCAA*R*AA*R*GCCAT*Y*A 3′ was used for the embryonic (E) and neonatal (N) myosin isoforms. Primers A and A′ were used in combination with antisense oligonucleotide primers designed to anneal to highly specific sequences for each MHC gene, present in their 3′ untranslated regions. These included MHC-IIa: 5′ TCTACAGCATCAGAGCTGCC 3′, MHC-IIb: 5′ GTGTGATTTCTTCTGTCACC 3′, MHC-IIx: 5′ GGTCACTTTCCTGCTTTGGA 3′, MHC-I: 5′ GGTCTCAGGGCTTCACAGGC 3′, MHC-E: 5′ CCCTCACCAAGAGGACATGC 3′ and MHC-N: 5′ GCGGCCTCCTCAAGATGCGT 3′. In parallel experiments, cDNAs encoding segments of α-actinin-3 (AF450248) and myomesin-2 (XM_240481) from rat skeletal muscle (accession numbers: AF450248 and XM_240481, respectively) were amplified using primers: 5′GTTATGCAGCCCGAGGGT3′ (sense) and 5′CAGCGGAAAACAGCACCA3′ (antisense) and 5′ATGTCCCTGGTTGTAGTG3′ (sense) and 5′GGTGTGGGACCTTAACCT3′ (antisense), respectively. All PCR products were analyzed by electrophoresis in 1% agarose gels and their authenticity was verified by sequencing.

Immunofluorescence staining and confocal microscopy

Fixed, permeabilized cultures were immunolabeled with primary antibodies and analyzed with a Zeiss 410 confocal microscope (Carl Zeiss, Inc, Tarrytown, NY, USA) equipped with a 63×, numerical apertune (NA) 1.4 objective, with pinholes set at 18, as in ref. 11.

Electron microscopy

Myotube cultures, prepared on glass coverslips and infected with viruses encoding either control siRNA or siRNA targeting obscurin, were fixed with 2% paraformaldehyde for 15 min at room temperature, washed with PBS, and incubated in 100 mM glycine for 10 min at room temperature. Samples were permeabilized with 0.5 mg/ml saponin in PBS, 1%BSA (PBS/BSA/Saponin) for 1 h at room temperature, immunolabeled with obscurin-N or obscurin-C antibodies, washed in PBS/BSA/Saponin, counterstained with goat anti-mouse-Alexa$_{488}$ or goat anti-rabbit-Alexa$_{568}$ (Molecular Probes), and mounted onto slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). The locations of infected cells that showed reduced staining for obscurin were marked with a diamond stylus. Coverslips were removed and samples were fixed overnight in 0.2M cacodylate buffer, 2% glutaraldehyde, 5 mg/ml tannic acid. After washing in 0.2M cacodylate buffer, cultures were postfixed in 50 mM acetate buffer, 1% osmium tetroxide, *en bloc* stained with 1% uranyl acetate, dehydrated, and embedded in araldite-embed 812 (Electron Microscopy Sciences, Fort Washington, PA, USA). After hardening of the resin, glass coverslips were separated from the cells with hydrofluoric acid. For cross sections, samples were oriented under a dissecting microscope to allow sectioning of myotubes in a direction perpendicular both to the substrate and to the long axis of filament bundles. Sections were cut at a thickness of ~60–80 nm with an LKB MT5000 microtome (LKB-Pharmacia, Bromma, Sweden) and picked up on 200 mesh copper grids. They were stained again with uranyl acetate, followed by lead citrate, and examined under a Philips 201 electron microscope (Philips, Eindhoven, The Netherlands) at a magnification of 30,000. Pictures were taken on Kodak 4489 film and digitally scanned at 720 dpi.

RESULTS

We used siRNA to reduce the levels of obscurin in primary cultures of rat myotubes to study the role of obscurin in myofibrillogenesis and in the formation of the SR. Adenoviral constructs that expressed three different siRNAs designed to target sequences within the first (Fig. 1*A*, siRNA-I) or the second (Fig. 1*A*, siRNA-IIa and siRNA-IIb) Ig domains of the rat obscurin gene were used to infect myotubes. As a control, we infected primary cultures with adenovirus carrying a scrambled siRNA sequence. Infections were performed on 5-day-old cultures and assayed 2 days later. Here we present the results with siRNA-IIa, but we made similar observations with siRNA-I and -IIb.

Immunoblots showed that primary myotubes infected with siRNA-IIa expressed significantly lower levels of the ~800 kDa form of obscurin than controls (Fig. 1*B*). Quantitation of the immunoreactive bands with Metamorph Imaging Software showed a decrease of ~70% ($P<0.05$) in the amount of obscurin expressed in cultures treated with siRNA-IIa virus compared with the scrambled control. RT-PCR analysis confirmed that the levels of transcripts encoding the ~800 kDa form of obscurin were reduced. These experiments used five different sets of primers designed to anneal to sequences flanking either the first FnIII domain (a/a′), internal Ig repeats (b/b′ and c/c′), the IQ motif (d/d′), or the SH3 domain (e/e′) of the ~800 kDa protein (Fig. 1*A*). There was a significant decrease in all RT-PCR products (~65%, $P<0.05$) in cultures infected with the obscurin siRNA virus (Fig. 1*C*) compared with controls (Fig. 1*D*).

Immunolabeling of rat myotubes 2 days after infection with viruses expressing siRNA-IIa showed that ~65% of the cells were severely depleted of obscurin, which failed to accumulate into M-bands and Z-disks (**Fig. 2*A*, *B***, asterisk). Remarkably, these cells also showed significantly reduced labeling for sarcomeric myosin (Fig. 2*A′*, *B′*, asterisk). In the other ~35% of infected myotubes, obscurin was also decreased compared with controls but not as dramatically (Fig. 2*B, C*). In these cells, obscurin was present either along striated fibrils near the periphery (Fig. 2*B*, arrow), punctate throughout the myoplasm (Fig. 2*B*), or in aggregates

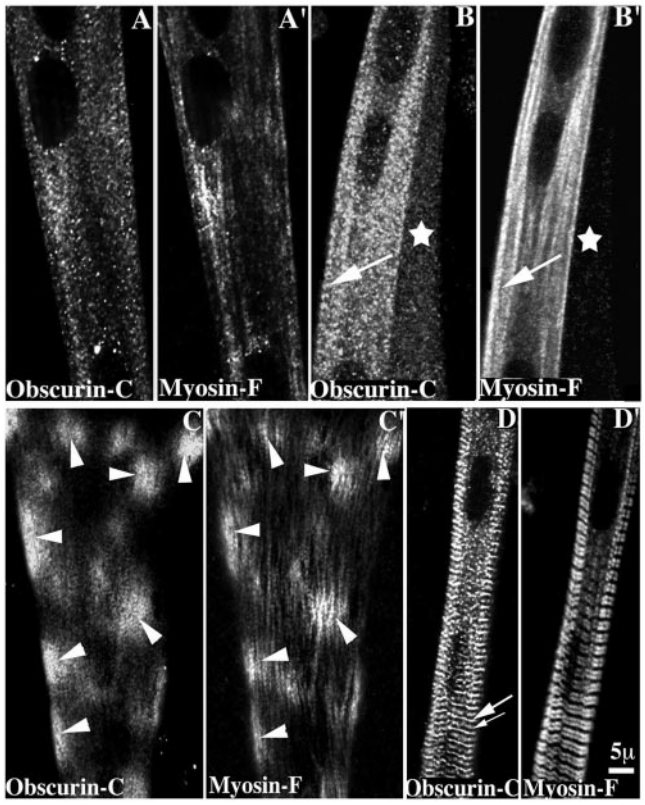

Figure 2. Failure of myosin to assemble into A-bands in myotubes with reduced levels of obscurin. Myotubes were treated with obscurin-IIa (*A–C′*) or control siRNA virus (*D, D′*). Two days later, cultures were fixed and labeled for obscurin and sarcomeric myosin. *A–C′)* The apparent levels and distribution of endogenous obscurin were dramatically altered in ~ 65% of myotubes treated with siRNA-IIa virus (*A, B*, asterisk). These cells were also devoid of sarcomeric myosin (*A′*, *B′*, asterisk). In the other ~35% of cells, obscurin labeling was significantly reduced and the protein was detected either at residual striated fibrils (*B*, arrow), punctate structures (*B*), or aggregates (*C*, arrowheads). Myosin also significantly diminished in these cells; it concentrated in the same structures as obscurin (*A′*, *B′*, arrow, and *C′*, arrowheads). *D, D′)* In cells infected with control virus, obscurin assumed its typical organization at M-bands (*D*, big arrow) and Z-disks (*D*, small arrow) and myosin occupied A-bands (*D′*).

that exhibited occasional periodicity (Fig. 2*C*, arrowheads). As in more severely affected cells, sarcomeric myosin also failed to assemble into A-bands in these myotubes and instead tended to concentrate in the same structures as obscurin (Fig. 2*B′*, arrow and *C′*, arrowheads). By contrast, ~95% of myotubes infected with control virus showed a regular organization of obscurin at the level of the M-band and Z-disk (Fig. 2*D*, big and small arrows, respectively), with sarcomeric myosin expressed at high levels and assembled into A-bands (Fig. 2*D′*).

As obscurin is an early component of the M-band that is present even before A-bands assemble (10, 26), we examined the effects of siRNA-IIa on two other M-band markers: myomesin and COOH-terminal epitopes of titin (**Fig. 3**). In ~65% of myotubes infected with the virus encoding siRNA targeting obscurin, the amounts of both myomesin and COOH-terminal epitopes of titin detected by immunofluorescence were dramatically reduced. The normally strong, linear labeling of M-bands was replaced by hazy staining of the myoplasm, with occasional accumulations in short fibrils (Fig. 3*A, A′* and *D, D′*, respectively). In another ~35% of infected cells, myomesin and the COOH terminus of titin were detected in the same punctate or filamentous structures as the residual obscurin (Fig. 3*B, B′* and *E, E′*, arrows, respectively). Notably, in ~95% of myotubes infected with virus encoding control siRNA, both myomesin and the COOH-terminal epitopes of titin were present at M-bands, which appeared to be normal (Fig. 3*C, C′* and *F, F′*, respectively).

Contrary to myofibrillar proteins of the A-band and M-band, which were severely disrupted in the absence of obscurin, two markers of the Z-disk, α-actinin and the NH_2-terminal region of titin, were not significantly affected by treatment of myotubes with either control

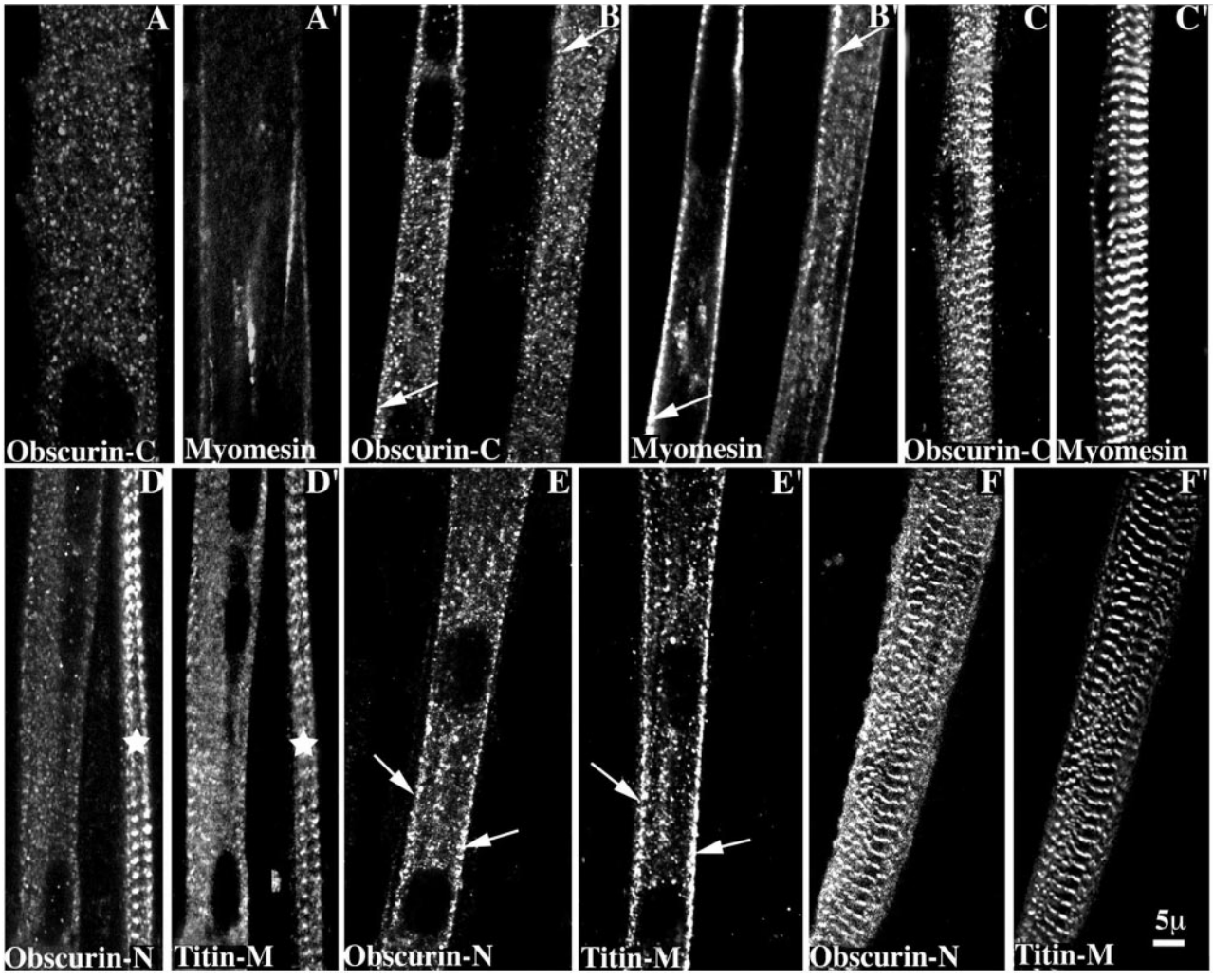

Figure 3. Disorganized M-bands in myotubes with reduced levels of obscurin. Procedures were as in Fig. 2. *A–B′* and *D–E′*. Down-regulation of obscurin with specific siRNA (*A, D*) reduced immunolabeling for myomesin (*A′*) and epitopes of titin located at the M-band (*D′*). In the subset of treated myotubes in which obscurin was still detectable, it was either concentrated along striated fibrils close to the periphery of the cell (*B, E*, arrows) or showed a punctate cytoplasmic distribution (*B, E*). Like obscurin, myomesin (*B′*) and epitopes of titin located at the M-band (*E′*) were detected either along fibrils with occasional periodicity (*B′, E′*, arrows) or in puncta. *C–C′* and *F–F′)* Obscurin (*C, F*), myomesin (*C′*), and COOH-terminal epitopes of titin (*F′*) assumed their typical striated organization in myotubes infected with control virus. Components of the M-band were regularly organized in uninfected cells (*D–D′*, asterisks).

virus or virus targeting obscurin. Indeed, the distribution of α-actinin at Z-disks was apparently normal whether the expression of obscurin was inhibited or not (**Fig. 4*A, A′, D, D′***, respectively). The same was true for the NH_2-terminal region of titin in most (~60%) myotubes depleted of obscurin and in ~95% of myotubes infected with control virus (Fig. 4*B, B′* and *E, E′*, respectively). The remaining ~40% of cells treated with siRNA-IIa showed extensive disorganization of the N-terminus of titin (Fig. 4*C, C′*, arrow).

We also examined the subcellular distribution of sAnk1, an integral component of the network SR that directly binds to the COOH-terminus of obscurin and localizes around M-bands and Z-disks of the contractile apparatus (20, 24). The organization of sAnk1 was severely disrupted in myotubes infected with siRNA-IIa virus and instead concentrated in longitudinally oriented structures throughout the myoplasm (**Fig. 5*A, A′***). In controls, sAnk1 was regularly organized at the level of the M-band, where it coincided with obscurin (Fig. 5*B, B′*).

To determine whether the reduced immunolabeling of myosin and myomesin in cells treated with the obscurin siRNA virus was due to their specific loss, we collected homogenates of P1 myotubes infected with either control or siRNA-IIa virus and analyzed them by SDS-PAGE, followed by immunoblotting (**Fig. 6*A*, *B***). Immunoreactive bands of ~220 and ~150 kDa that correspond to sarcomeric myosin and myomesin were detected in extracts of cells infected with control siRNA virus, but were severely reduced in extracts of cells infected with the siRNA virus targeting obscurin ($P<0.05$). By contrast, immunoblots of the same extracts for α-actinin and sAnk1 showed no significant differences ($P>0.05$; Fig. 6*C, D*), consistent with immunofluorescence data (Figs. 4, 5). These results suggest that siRNA targeted to obscurin selectively reduces the levels not only of obscurin, but also of myosin and myomesin, two proteins with which it closely associates in the middle of the sarcomere.

We next used RT-PCR to learn whether the down-regulation of obscurin by siRNA affects sarcomeric myosin and myomesin by reducing the amounts of their mRNAs. For these experiments we used primers that specifically amplified sequences in mRNAs encoding myomesin and different muscle myosin forms, including fast IIa, IIb, and IIx, slow I, embryonic and neonatal. P1 myotubes infected with siRNA-IIa (Fig. 6*E*) or control virus (Fig. 6*F*) expressed similar amounts of myomesin and of the fast IIb, embryonic, and neonatal myosins ($P>0.05$). Sequences encoding the other forms of skeletal myosin were not detected in experimental or control samples (Fig. 6*E* and *F*, respectively). Products of the RT-PCR reaction with oligonucleotide primers for the isoform of α-actinin present in skeletal muscle were similarly unchanged (Fig. 6*E, F*). These results suggest that siRNA viruses that target the expression of obscurin reduce the levels of myosin and myomesin without affecting the transcription or stability of their mRNAs. Thus, the reduced levels of these proteins must be a consequence of the reduced expression of obscurin, which alters their ability to assemble into A-bands and M-bands.

We also used electron microscopy (EM) to examine myotubes treated with siRNA targeting obscurin in order to learn whether the ultrastructural features of the altered A-bands and M-bands in these cells were consistent with their decreased stability. In electron

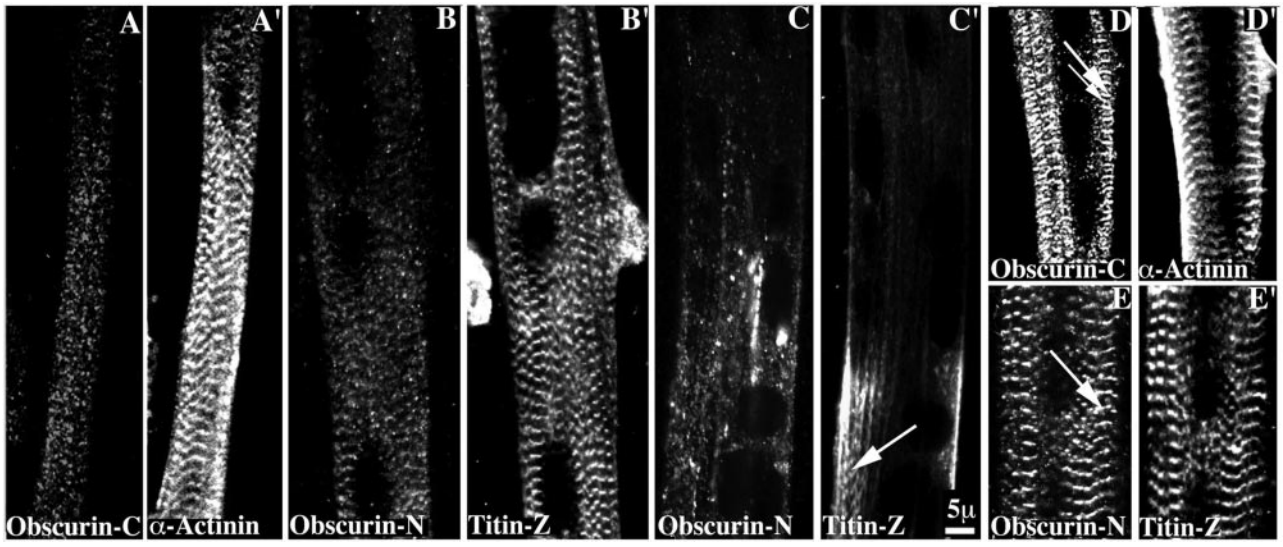

Figure 4. Reduced levels of obscurin have no significant effect on components of the Z-disk. The procedures were as in Fig. 2. *A–A′*. Myotubes infected with siRNA-IIa virus showed reduced labeling for obscurin (*A*) but regular organization of α-actinin at Z-disks (*A′*). *B–B′* and *C–C′)* Similarly, a significant percentage of cells (~60%) infected with virus encoding siRNA to obscurin showed reduced levels of obscurin (*B*) but strong periodic labeling of the N terminus of titin at Z-disks (*B′*). Another ~40% of treated cells (*C*) had reduced staining for NH_2-terminal epitopes of titin at Z-disks, and instead showed fibrillar labeling with occasional periodicity (*C′*, arrow). *D–D′* and *E–E′)* Cells infected with control virus showed obscurin not only at M-bands (*D, E,* big arrows), but also at Z-disks (*D*, small arrow), where it colocalized with α-actinin (*D′*) and titin epitopes (*E′*).

micrographs, myotubes treated with siRNA-IIa, which were severely depleted of obscurin, resembled empty "myosacs" with no discernible structures (not shown). Electron micrographs of longitudinal sections of myotubes treated with siRNA-IIa that were only moderately depleted of obscurin (**Fig. 7*A***) revealed the total absence of M-bands and the presence of irregular A-bands in regions of the myoplasm that contained Z-disks, which appeared normal. Residual myosin filaments extended uniformly along the entire length of assembled sarcomeres, between neighboring Z-disks, rendering both A- and I-bands indistinct (Fig. 7*A*). Consistent with longitudinal views, ultrastructural views of cross sections of such myotubes showed that myosin filaments were less frequent and more poorly organized in myotubes lacking obscurin (Fig. 7*C*). By contrast, myotubes infected with control siRNA virus showed regularly organized sarcomeres in longitudinal and cross sections (Fig. 7*B, D*). We further analyzed images of cross sections with Fast Fourier transforms (FFT). The transform from a control myotube (Fig. 7*D*, inset) showed the typical pattern of myosin filaments equally spaced in a hexagonal array (i.e., six dots surrounding a central core). The transform from a myotube depleted of obscurin (Fig. 7*C*, inset) also had densities corresponding to myosin filaments, but the pattern was distorted, reflecting the decreased order in the myosin array. In agreement with this, quantitative measurements of the distances between myosin filaments in the two samples showed that the variance of the distances in myotubes treated with siRNA-IIa virus was significantly higher (s^2=7.41 nm, n=4) than in controls (s^2=5.46 nm, n=5; P = 0.014, Mann-Whitney U test), although the means were approximately the same—39 nm and 40 nm, respectively.

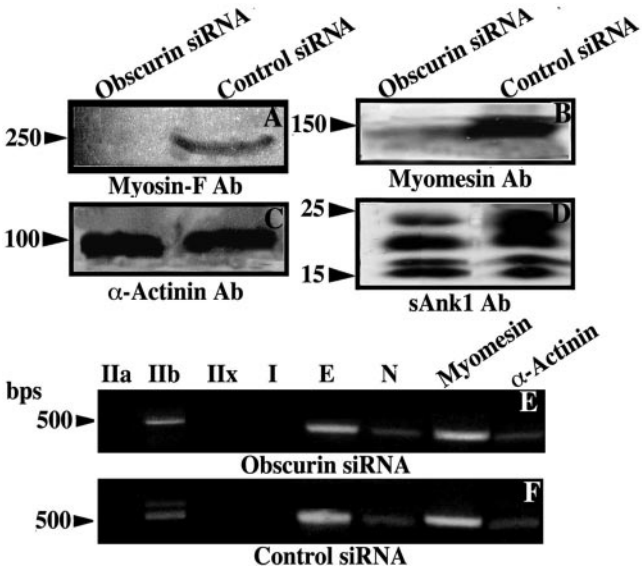

Figure 6. Reduced levels of myosin and myomesin, but not α-actinin and sAnk1, in myotubes with reduced levels of obscurin. *A–D)* Immunoblot of lysates prepared from myotubes in which obscurin levels were reduced by siRNA showed a dramatic decrease in the amount of sarcomeric myosin (*A*) and myomesin (*B*) compared with lysates from cells infected with control siRNA virus. No significant difference in the levels of α-actinin (*C*) and sAnk1 (*D*) was observed between experimental and control samples. *E–F)* RT-PCR analysis with a set of primers designed to amplify sequences of different isoforms of muscle myosin [fast: IIa, IIb, IIx, slow: I, embryonic (E) and neonatal (N)], myomesin, and α-actinin showed no significant changes in the levels of their mRNAs in myotubes with reduced levels of obscurin (*E*) compared with controls (*F*).

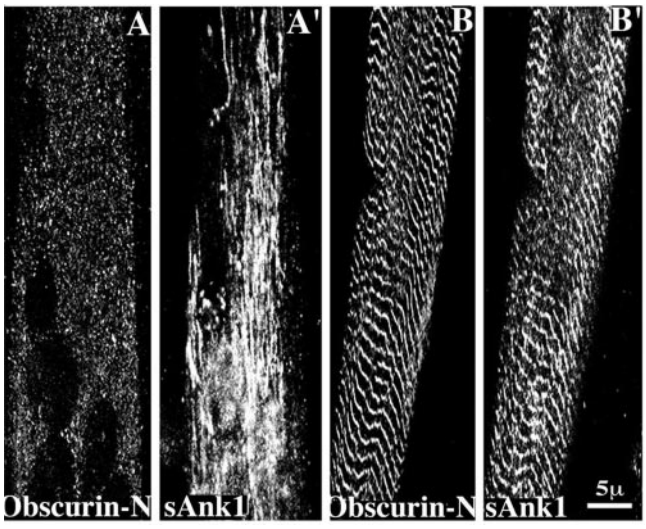

Figure 5. Failure of sAnk1 to assemble in transverse structures in myotubes with reduced levels of obscurin. Procedures were as in Fig. 2. *A–A′).* Myotubes treated with siRNA-IIa virus failed to organize sAnk1 in transverse structures (*A′*) when the levels of obscurin (*A*) were reduced. *B–B′).* Most cells infected with control siRNA virus showed sAnk1 organized transversely around M-bands (*B′*), where it codistributed with obscurin (*B*). Control (*B′*) and experimental (*A′*) levels of sAnk1 did not seem significantly altered, however.

DISCUSSION

Myofibrillogenesis is a complex process that depends on the coordinated assembly and integration of actin and myosin filaments and their associated proteins into regular arrays that support contraction (1, 5, 7, 33–38). In this study we used small inhibitory RNA (siRNA) to inhibit the synthesis of the giant sarcomeric protein, obscurin (~800 kDa), with the aim of determining its roles in myofibrillogenesis and the organization of the sarcoplasmic reticulum (SR). Our earlier results suggested an important role for obscurin in the formation of myosin-rich A-bands (11). Here, we report that reduced levels of obscurin inhibit the formation and stability not only of A-bands, but also of M-bands, and the proper organization of the SR around developing sarcomeres. These effects are specific, as siRNA targeting the ~800 kDa form of obscurin has no obvious effect on the assembly of Z-disks and I-bands or on the levels of expression of their major components, α-actinin and actin, respectively. Our results are the first to demonstrate a central role for obscurin, or any protein

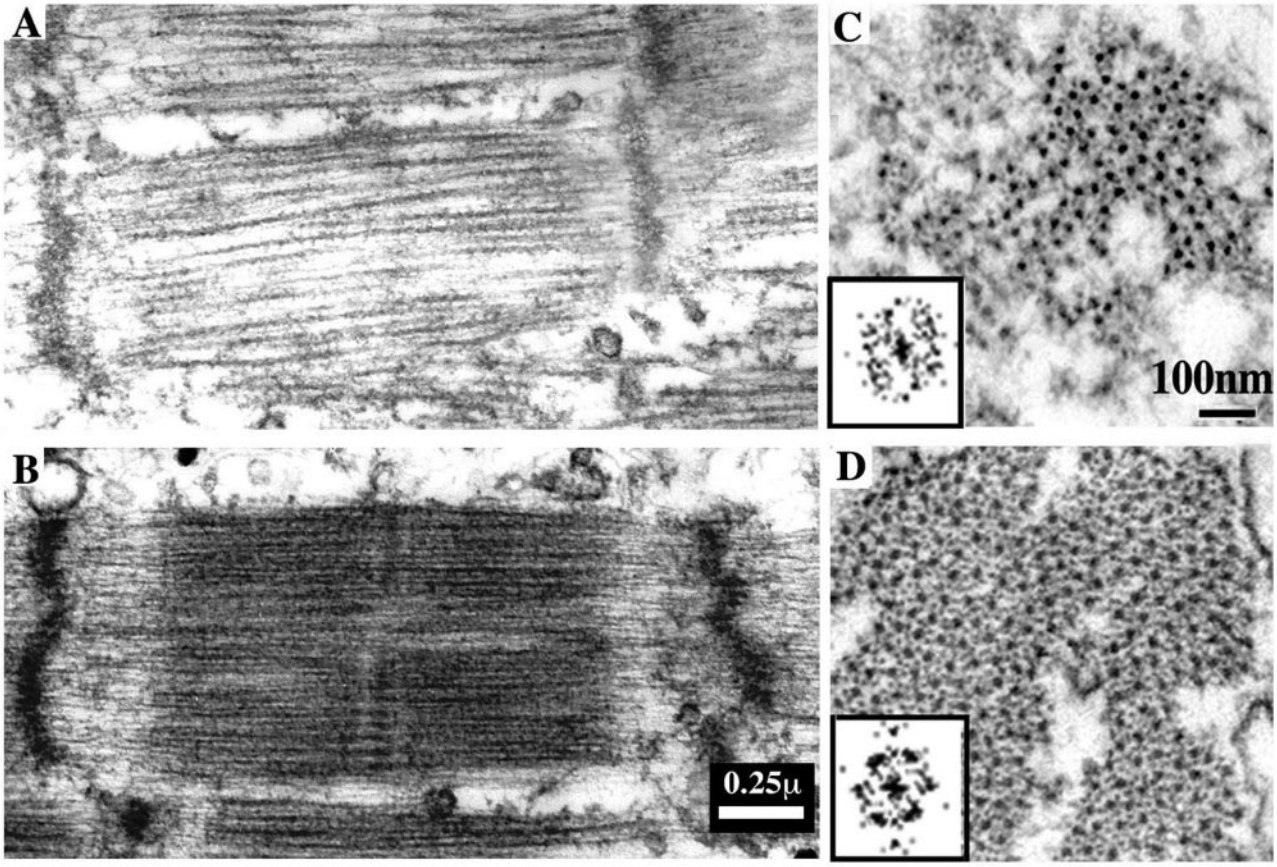

Figure 7. Disruption of A-bands at the ultrastructural level in myotubes with reduced levels of obscurin. *A, B)* After processing for EM, longitudinal sections of myotubes infected with siRNA-IIa virus (*A*) showed sarcomeres with disorganized A-bands devoid of M-lines. Myosin filaments in these cells ran uniformly along the entire length of assembled sarcomeres, making I-bands less distinct. By contrast, myotubes infected with control siRNA virus (*B*) showed regularly organized A- and M-bands. *C, D)* Ultrastructural examination of cross sections of myotubes infected with siRNA-IIa virus (*C*) revealed that the number of myosin filaments was diminished and that their organization was less regular than in controls (*D*). This was confirmed by FFT analysis, which showed that the normally hexagonal arrays of myosin filaments in control cells were distorted in cells treated with siRNA-IIa (see insets).

for that matter, in the assembly and maintenance of sarcomeric A-bands and M-bands, and in the regular alignment of the network SR membranes around the middle of the sarcomere. Thus, like titin and nebulin (39, 40), obscurin may function as a "molecular template" for the assembly of the contractile apparatus and closely associated structures.

Early in myofibrillogenesis, obscurin assembles with other M-band proteins, including myomesin, M-protein, and the COOH terminus of titin to form primordial M-bands (26). This occurs well before myosin filaments assemble into A-bands, suggesting a possible role for obscurin in A-band formation (10, 11, 26). Consistent with this, formation of A-bands is specifically inhibited by overexpression of the COOH-terminal region of obscurin (11). Our present study further supports this possibility and indicates that obscurin is essential not only for the assembly of thick filaments into A-bands, but also for the formation or maintenance of the M-band lattice. These results are also consistent with the role of UNC-89, obscurin's orthologue in *C. elegans* (41, 42), as mutants lacking UNC-89 have normal numbers of thick filaments but severely disrupted A-bands devoid of M-lines.

At the time of viral infection in our cell system, M-bands have already formed, but sarcomeric myosin is present only in primordial A-bands and has not yet assembled into regular A-bands (11, 26). Thus, the reduced expression of obscurin appears to inhibit the maturation of A-bands and impair the stability of M-bands. This is consistent with the early localization of obscurin at M-bands of differentiating muscle cells, where it may stabilize a core complex of titin and myomesin at the middle of each sarcomere, on which thick filaments later assemble (18). Remarkably, the absence of these structures, when obscurin levels are reduced, has no significant effect on the assembly of two other key sarcomeric elements—Z-disks and I-bands—perhaps because obscurin's association with these structures occurs only late in the process of myofibrillogenesis. Although the NH_2-terminus of titin was disorganized in ~40% of treated cells, this was likely an indirect effect of its failure to anchor to the M-band. This agrees with reports that disruption of the

COOH-terminus of titin in cultured skeletal myoblasts by gene targeting affected its organization at the Z-line (43), and that electroporation of an antititin antibody (Ab) targeted to its M-band disrupted its assembly at Z-disks as well as M-bands (12).

In contrast to the reduction of obscurin in cells treated with targeted siRNA, which directly correlates with the reduction in its mRNA, the reduction of myosin and myomesin appears to be due to translational or posttranslational regulatory processes. Although obscurin may contribute to the rate of synthesis or turnover of sarcomeric proteins—for example, through its signaling domains—the loss of myosin and myomesin in treated myotubes is more likely due to increased degradation linked to their failure to incorporate into sarcomeres. This is consistent with our previous report of a mild diminution of myosin levels in myotubes in which the COOH-terminal region of obscurin was overexpressed (11). Notably, the effects of reducing the overall levels of obscurin in developing myotubes are greater than the effects of overexpressing the COOH-terminal region of the molecule, which inhibits the assembly of A-bands but has no effect on M-bands, Z-disks, or I-bands (11). Our current results suggest that domains of obscurin in addition to those at the COOH-terminus are important for the assembly or stability of M-bands and are likely to be required for the subsequent assembly of A-bands.

Unlike myosin and myomesin, which are lost when obscurin levels are reduced, sAnk1 remains stably associated with intracellular membranes, consistent with its identity as an integral protein of the SR. Remarkably, however, the SR fails to organize around the contractile elements when obscurin is depleted. This supports our hypothesis that interactions between obscurin at the periphery of the M-band and proteins in the SR, such as sAnk1, organize the network SR in its stereotypical pattern around the middle of each M-band of the sarcomere.

Our studies show that down-regulation of the ~800 kDa form of obscurin results in the failure of two important structures to form A-bands and their central M-bands, and the network compartment of the SR. Thus, our findings are the first to demonstrate a role for obscurin in assembling and stabilizing M-bands and A-bands and in linking the contractile apparatus to the network SR in vertebrate skeletal muscle. Since multiple obscurin isoforms are produced by alternative splicing (21, 23), there may be isoforms that are not targeted by our siRNA constructs. Certainly, the expression of the small kinase isoforms of obscurin-MLCK, which have a distinct translation initiation site (21), would not be directly affected by these siRNAs. However, they do appear to target many, if not all, of the isoforms derived from the ~25 kb mRNA transcript, as indicated by the diminished abundance of all of the RT-PCR products tested in this study (Fig. 1*C*, *D*). Still, given the potential for obscurin and obscurin-MLCK isoforms to remain unaffected by our siRNA constructs, as well as the limitations of our model, we cannot rule out the possibility that some forms of obscurin may play additional roles in myofibrillogenesis, including roles at the Z-disk, in the lateral association of myofibrils, or in the links between superficial myofibrils and the sarcolemma. Further studies are required to determine the additional functions of obscurin and its isoforms in sarcomerogenesis, and more generally in the physiology of striated muscle. FJ

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