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Andrei B. Borisov · Sarah B. Sutter
Aikaterini Kontrogianni-Konstantopoulos
Robert J. Bloch · Margaret V. Westfall
Mark W. Russell

Essential role of obscurin in cardiac myofibrillogenesis and hypertrophic response: evidence from small interfering RNA-mediated gene silencing

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Abstract Obscurin is a recently identified giant multidomain muscle protein (~800 kDa) whose structural and regulatory functions remain to be defined. The goal of this study was to examine the effect of obscurin gene silencing induced by RNA interference on the dynamics of myofibrillogenesis and hypertrophic response to phenylephrine in cultured rat cardiomyocytes. We found that that the adenoviral transfection of short interfering RNA (siRNA) constructs targeting the first coding exon of obscurin sequence resulted in progressive depletion of cellular obscurin. Confocal microscopy demonstrated that downregulation of obscurin expression led to the impaired assembly of new myofibrillar clusters and considerable aberrations of the normal structure of the contractile apparatus. While the establishment of the initial periodic pattern of α-actinin localization remained mainly unaffected in siRNA-transfected cells, obscurin depletion did cause the defective lateral alignment of myofibrillar bundles, leading to their abnormal bifurcation, dispersal and multiple branching. Bending of immature myofibrils, apparently associated with the loss of their rigidity, a modified titin pattern, the absence of well-formed A-bands in newly formed contractile structures as documented by a diffuse localization of sarcomeric myosin labeling, and an occasional irregular periodicity of sarcomere spacing were typical of obscurin siRNA-treated cells. These results suggest that obscurin is indispensable for spatial positioning of contractile proteins and for the structural integration and stabilization of myofibrils, especially at the stage of myosin filament incorporation and A-band assembly. This demonstrates a vital role for obscurin in myofibrillogenesis and hypertrophic growth.

Keywords Obscurin · Cardiac myocytes · Myofibrillogenesis · siRNA · α-Actinin · Myosin · Titin · Hypertrophy

A. B. Borisov (✉) · S. B. Sutter · M. W. Russell (✉)
Division of Pediatric Cardiology Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Room 8200, MSRB III, Ann Arbor, MI 48109, USA
E-mail: aborisov@umich.edu
E-mail: mruss@umich.edu
Tel.: +1-734-9368663
Fax: +1-734-6151386

A. Kontrogianni-Konstantopoulos · R. J. Bloch
Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

M. V. Westfall
Department of Molecular and Integrative Physiology and Department of Surgery, University of Michigan Medical School, Ann Arbor, MI 48109, USA

Introduction

The molecular mechanisms underlying the proper assembly and three-dimensional integration of sarcomeric filaments within nascent myofibrils during cardiac differentiation and hypertrophy are not completely understood. The studies performed over the last decade clearly demonstrated that loss-of-function mutations in structural muscle proteins result in the impairment of the contractile function and can lead to severe myocardial pathology including dilated and hypetrophic cardiomyopathy and congestive heart failure (Carlsson and Thornell 2001; Roberts and Sigwart 2001; Sehnert et al. 2002; Mohapatra et al. 2003; Bär et al. 2004; Stefanelli et al. 2004; Kasahara 2005; Van Driest et al. 2005).

Recently, a novel component of the sarcomere, a large multidomain ~800 kDa protein named obscurin, was identified in cardiac and skeletal muscle. Obscurin, localized mainly to M- and Z-lines in mature myofibrils, became the third known member of the family of giant muscle proteins that includes titin and nebulin/nebulette. Sequence analysis of the obscurin gene demonstrated that it encodes about 67 linked immunoglobulin domains and two fibronectin-3-like domains (Bang et al. 2001; Young et al. 2001; Russell et al. 2002). The structural organization of immunoglobulin

domains of obscurin resembles the elastic region of titin, and the fibronectin-3 domains share some similarity with the adhesive domains of several other contractile proteins (Young et al. 2001). Obscurin also contains Rho-guanine nucleotide exchange factor (Rho-GEF) and a calmodulin-binding IQ motif, which suggests its involvement in signal transduction in sarcomeres and regulation of the contractile function (Young et al. 2001; Bang et al. 2001; Russell et al. 2002). Earlier we found that the expression of obscurin is upregulated in vivo in hypertrophic murine hearts subjected to ascending aortic banding and that it is rapidly incorporated in newly forming myofibrils in vitro following stimulation of the hypertrophic response (Borisov et al. 2003). This protein appears to be the vertebrate homologue of the *unc-89* gene (Sutter et al. 2004) that has been demonstrated to be involved in the M-band assembly in *Caenorhabditis elegans* (Benian et al. 1996; Small et al. 2004).

It has been recently shown that mutations in the genes encoding other giant muscle proteins, titin and nebulin, have significant functional consequences and are associated with the development of cardiac and skeletal myopathies in humans (Hein and Schaper 2002; Itoh-Satoh et al. 2002; McElhinny et al. 2003; Anderson et al. 2004; Tskhovrebova and Trinick 2005). This suggests that the abnormal functional expression of these proteins has direct clinical implications and can explain the etiology and mechanisms of at least some types of muscle pathology.

The precise functional role of obscurin in muscle tissue is still poorly characterized. It remains unclear how important is obscurin for progression of myofibrillogenesis and for the stability of structural organization and integrity of myofibrils. Cell cultures of cardiac myocytes represent an established experimental model that has been successfully used in studies of normal, induced, and genetically modified patterns of sarcomeric protein expression (e.g., Eppenberger et al. 1994; Mitcheson et al. 1998; Person et al. 2000; Weikert et al. 2003). The heart cells isolated in vitro actively respond to a number of physiological and pharmacological stimuli including the hypertrophic ones, and the molecular mechanisms mediating the hypertrophic response are very similar in situ and cell culture (for literature see Hefti et al. 1997; Schaub et al. 1997, 1998; Komuro and Yazaki 2002; Opie 2004).

During the last several years, short interfering RNAs (siRNAs) became a new tool for the analysis of gene functions by selective and specific silencing of targeted homologous nucleotide sequences at the transcriptional level (reviewed in Chi et al. 2003; Montgomery 2004; Dorsett and Tuschl 2004; Campbell and Choy 2005; Huppi et al. 2005; Tomari and Zamore 2005). Transfection with replication-defective viral vectors has been successfully employed for efficient intracellular delivery of different siRNA constructs in several cell types including cardiomyocytes (Shen et al. 2003; Bantounas et al. 2004; Kasahara and Aoki 2005).

To determine the functional role of obscurin in the assembly and maintenance of the contractile apparatus, in this study we examined the structure of nascent and mature myofibrils and the progression of myofibrillogenesis induced by phenylephrine in cardiac myocytes following siRNA-mediated obscurin gene silencing in cell culture.

Materials and methods

Preparation of adenoviral-RNAi constructs

Short interfering RNA constructs were designed to inhibit the expression of the first coding exon of the rat obscurin gene (nucleotides 1–600 of the coding sequence). Target siRNA sequences were selected based on Tuschl's principles (Elbashir et al. 2002). BLASTn sequence homology searches [http://www.ncbi.nlm.nih.gov/BLAST/databases:non-redundant and EST] were used to confirm unique specificity of the RNAi constructs for the rat obscurin transcript. Potential target sequences that demonstrated greater than 70% sequence similarity with a rat cDNA transcript other than obscurin were excluded from further consideration. Three different siRNA target sites and one control sequence provided by Ambion, Inc., Austin, TX, USA were selected: ratObs5′BH143 (GTGGAGGCAGGAGCACGCT), ratObs5′BH373 (GACGCCACCTTCCGATGTC), ratObs5′BH416 (GCTGTGAGCTGGTCC AAAGA), and NEGCTL (ACTACCGTTGTTATA GGTG). The siRNA target sites were converted into oligonucleotide sequences per instructions supplied with the pSilencer^{TM} kit (Ambion Inc., Austin, TX, USA). For each template, 2 μg of the target and complementary oligonucleotides were heated at 95° for 5 min, then cooled to room temperature in annealing buffer (100 mM potassium acetate, 30 mM HEPES-potassium Hydroxide pH 7.4, 2 mM magnesium acetate). Duplex DNA was directionally cloned into the U6 promoter driven expression vector, pSilencer 2.0-U6 (Ambion Inc., Austin, TX, USA). The U6 promoter and RNAi construct were then subcloned into the *EcoRI* and *HindIII* sites of the adenoviral shuttle vector, pACpL + loxP-SSP. AdBH143, AdBH373, AdBH416 and AdNEGCTL were generated by Cre-lox recombination in vitro according to earlier published procedures (Aoki et al. 1999). Recombination occurred in a cell-free system consisting of only the viral and shuttle plasmids and Cre recombinase. Viral particles were produced in 293 cell line by transfection. After harvesting and lysing the infected cells, adenoviral lysates were sterilized by passage through 45 μm filters and titered using standard methods. The detailed characterization of the vectors and the technique of transfection were described earlier (Aoki et al. 1999; Westfall 2003). The effectiveness of transfection with the vector was virtually total, as evaluated by immunofluorescent labeling of the reporter

marker FLAG detected with anti-FLAG monoclonal antibody (Sigma Chemical, St. Louis, MO, USA).

Preparation of primary cultures and adenoviral transfection of adult rat cardiac myocytes

Primary cultures of cardiac myocytes have been isolated from adult female Fischer rats following enzymatic dissociation of perfused hearts with collagenase. We described earlier the detailed protocol of tissue dissociation (Westfall 2003). Cells were cultivated in 6-well plates containing 90% of F-12 medium and 10% of bovine fetal serum on 18 mm^2 coverslips in the CO_2 incubator (5% CO_2). Cell cultures were washed twice with serum-free F-12 media prior to transfection. Adenoviral transfection with the coding and non-coding (control) sequences was performed on day 2 following isolation in vitro in 400 µl of F-12 medium per 30 mm dish for 2 h at 37°C in a CO_2 incubator with gentle rocking every 15 min to ensure uniform delivery of the viral particles. At the end of 2 h, 2 ml of F-12 medium with 10% of fetal bovine serum was added to each dish, and 24 h after transfection, the media was removed and replaced with fresh media. Forty-eight hours after transfection, phenylephrine was added to the cell cultures at the final concentration of 35 mM.

Immunocytochemistry and confocal microscopy

The cells were fixed and processed for immunocytochemistry as described (Borisov et al. 1989). Monoclonal antibodies to the sarcomeric isoform of α-actinin in combination with polyclonal antibody to obscurin were used for double indirect immunofluorescent labeling of hypertrophic and control cultured cardiomyocytes. The properties and specificity of the monoclonal antibody to sarcomeric α-actinin, clone EA-53, were reported and tested earlier (Fridlianskaya et al. 1989; Borisov et al. 1989). This antibody is currently marketed by Sigma Chemical Co, St Louis, MO, USA. The polyclonal antibody to obscurin was developed in rabbits against carboxy-terminal region of obscurin molecule and its specificity has been examined in detail (Kontrogianni-Konstantopoulos et al. 2003). The monoclonal antibodies reacting to sarcomeric myosin heavy chains (MF-20) and the PEVK segment of titin (9D10) were obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA. The anti-titin antibody reacted to the epitope located in the I-bands near the I–A interface of sarcomeres (Trombitás et al. 1998). The secondary antibodies, FITC-conjugated anti-mouse IgG and TRITC-conjugated anti-rabbit IgG, were purchased from Sigma Chemical Co., St Louis, MO, USA. Immunostained samples have been examined in the Carl Zeiss LSM 510 confocal microscope.

Results

In control cultures, the cells started to resume contractile activity on days 3–5 following isolation in vitro, and this process typically has an asynchronous and protracted character. To activate sustained contractions and trigger synchronous onset of myofibrillogenesis, we used phenylephrine, an α-adrenergic agonist and a well-characterized stimulus for the hypertrophic response in isolated cardiac myocytes in culture (e.g., Perella et al. 1994; Lesniak et al. 1995; Aoki et al. 2000; Arya et al. 2004; Gan et al. 2005). In our earlier study, we found that the structural characteristics of myofibrillogenesis in cultures of rat cardiomyocytes were very similar in the presence/absence of phenylephrine, and that the addition of this agonist significantly accelerated and intensified this process (Borisov et al. 2003).

Addition of phenylephrine on day 4 stimulated rhythmic beating and cytoplasmic growth of myocytes, and by days 7–8 in culture the cells reestablished their contractile function. In order to examine the effects of obscurin depletion on new myofibril assembly and myofibrillar structure, we studied the intracellular distribution of sarcomeric α-actinin as a protein marker of mature and nascent myofibrils. Indirect immunofluorescent labeling for α-actinin has shown that following the period of structural remodeling, muscle cells treated with phenylephrine activated intense myofibrillogenesis. In the course of cultivation, the formation of myofibrils rapidly progressed from the central areas to the periphery of the sarcoplasm (Fig. 1a–f), which led to accumulation of contractile structures. Immunolabeling for obscurin has shown that during the assembly of myofibrils, this protein concentrated in the middle of the A-bands and in Z-lines of differentiating sarcomeres (Figs. 1a–c, 2a). After 11–16 days in culture, myocytes developed a highly organized contractile apparatus consisting of abundant well aligned bundles of myofibrils that occupied the whole volume of the sarcoplasm (Figs. 1d–f, 2c). Double immunolabeling clearly demonstrated that by that time a highly registered striated pattern of sarcomeric α-actinin in the Z-lines was interspersed with periodically localized foci of intensely labeled obscurin in the A-bands (Fig. 2c).

To identify the functions of obscurin in cardiac muscle, its expression was inhibited using adenoviral introduction of obscurin-specific siRNA constructs. We found that the transfection with anti-obscurin siRNA led to its gradual cellular depletion, considerably impaired the hypertrophic response of cardiomyocytes and changed the structure of their contractile system. Adenoviral delivery of anti-obscurin siRNA did not visibly affect the contractile activity and myofibrillar morphology during the first several days following transfection. However, the rate and intensity of cell contractions decreased at later stages. By day 9, the rate of contractions in transfected cultures was approximately 60–70% of that noted in control cells. Double

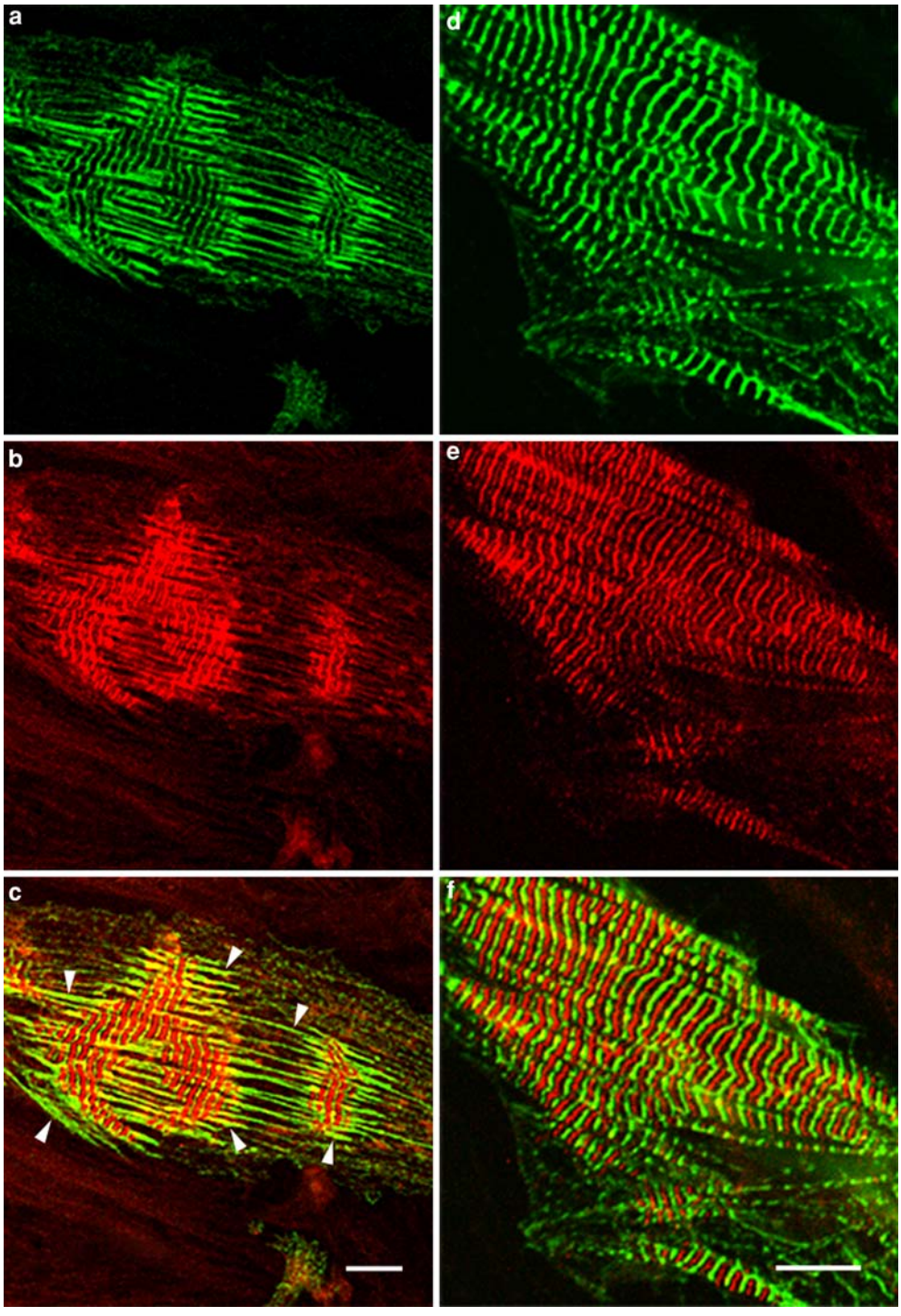

Fig. 1 Localization of obscurin in cardiac myocytes during myofibrillogenesis in control cultures. Double immunolabeling of obscurin (*red fluorescence*) and sarcomeric α-actinin (*green fluorescence*) after 6 days (**a**–**c**) and 11 days (**d**–**f**) in vitro. **a** and **d**—localization of α–actinin, **b** and **e**—localization of obscurin, **c** and **f**—merged images showing the localization of α-actinin and obscurin in the cells shown **a** and **d**, respectively. Immunoreactivity for obscurin is clearly seen in the M-bands and to a much lesser extent, in the Z-lines of progressively differentiating myofibrils. The *arrowheads* in **c** show the non-striated precursors of myofibrils intensely immunopositive for sarcomeric α-actinin. *Bars* 10 μm in **c** for **a–c** and in **f** for **d–f**

immunolabeling for obscurin and sarcomeric α-actinin showed that obscurin began to diminish in the contractile structures by days 5–6 following transfection (Fig. 2b). The intensity of labeling for obscurin was considerably lower in the transfected myocytes compared to the control cultures (Fig. 2b, d and a, c, respectively). In transfected cells, an increasing number of striated myofibrils were noted to be intensely positive for α-actinin but immunonegative or weakly immunopositive for obscurin (Fig. 2b). One can suggest two possible interpretations of this fact: either the process of newly formed Z-band differentiation during assembly of myofibrils can successfully progress to a certain stage without obscurin, or the Z-bands of pre-existing myofibrils are able to retain their striated aligned morphology, at least transiently, after the depletion of the intracellular obscurin pool.

Starting days 8–10 following siRNA exposure, we observed the presence of morphologically unusual, flattened muscle cells with poorly developed cytoplasmic

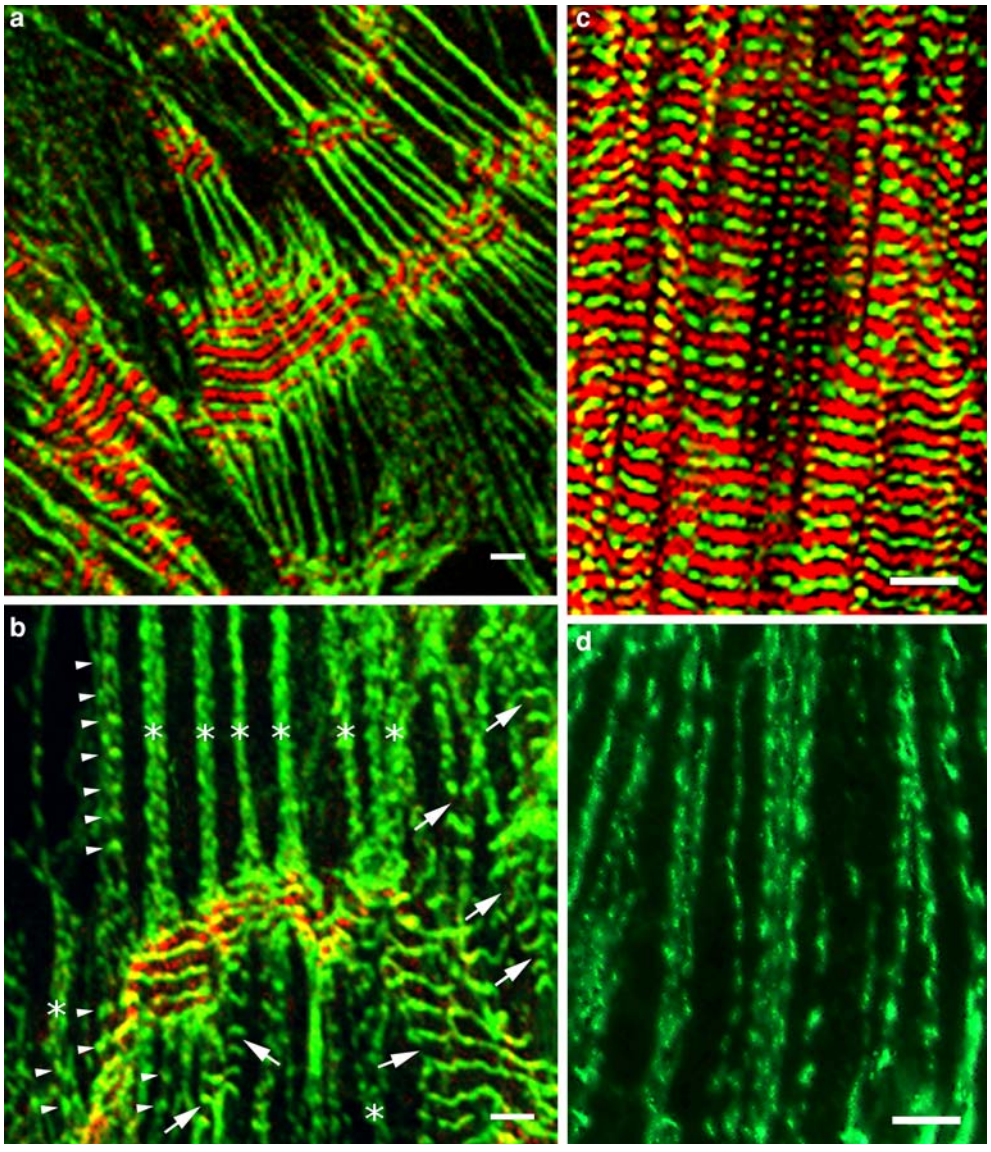

Fig. 2 Double immunolabeling of obscurin (*red fluorescence*) and sarcomeric α-actinin (*green fluorescence*) during myofibrillogenesis in control cardiac muscle cells (**a, c**) and obscurin siRNA-transfected cells (**b, d**) on day 9 (**a**, **b**) and day 16 (**c**, **d**) in culture. **a** Intense incorporation of obscurin in the areas of M-bands within the A-bands of newly formed sarcomeres and intense immunoreactivity for α-actinin in non-striated precursors of myofibrils in control cells. **b** Assembly of myofibrils in obscurin siRNA-transfected cardiomyocytes. Note residual immunoreactivity for obscurin in several sarcomeres and its complete absence in many others (*arrows*). The *arrowheads* in **b** show the development of striated periodic pattern of nascent Z-lines. The *asterisks mark* the areas of loose and misaligned distribution of sarcomeric α-actinin in premyofibrillar structures in the transfected cells. **c** Abundant presence of obscurin in the M-lines and regular organization of α-actinin in Z-lines in well-developed and aligned myofibrils in control cells. **d** Lack of lateral alignment and registered organization of nascent myofibrils observed in the peripheral sarcoplasm of obscurin-depleted cells. Note the presence of narrow striated bundles and a variable thickness and periodicity of α-actinin-containing bodies in immature myofibrils. Double immunolabeling for obscurin and sarcomeric α-actinin demonstrates the suppression of obscurin expression following siRNA transfection (**b**, **d**). *Bars* 3 μm (**a**, **b**), 4 μm (**c**, **d**)

processes. Immunofluorescent labeling of such cells for sarcomeric α-actinin demonstrated that this morphology was associated with the impairment of registered alignment of the contractile structures following obscurin siRNA transfection (Fig. 3a–c). By days 12–16, the majority of transfected cells showed a weak immunoreactivity for obscurin or did not express this protein at immunocytochemically detectable levels in the peripheral areas of the sarcoplasm despite a very clear and intense immunopositivity for sarcomeric α-actinin (Fig. 3b, c), although some residual immunolabeling occasionally persisted in the central myofibrillar clusters. We found that α-actinin-immunopositive structures in the transfected cells formed multiple thin intricately branching bundles (Fig. 3). The development of brush-like structures consisting of multiple, dispersed, immature, thin myofibrils located on the tips of larger myofibrillar bundles (Fig. 3a, b) shows that the block of

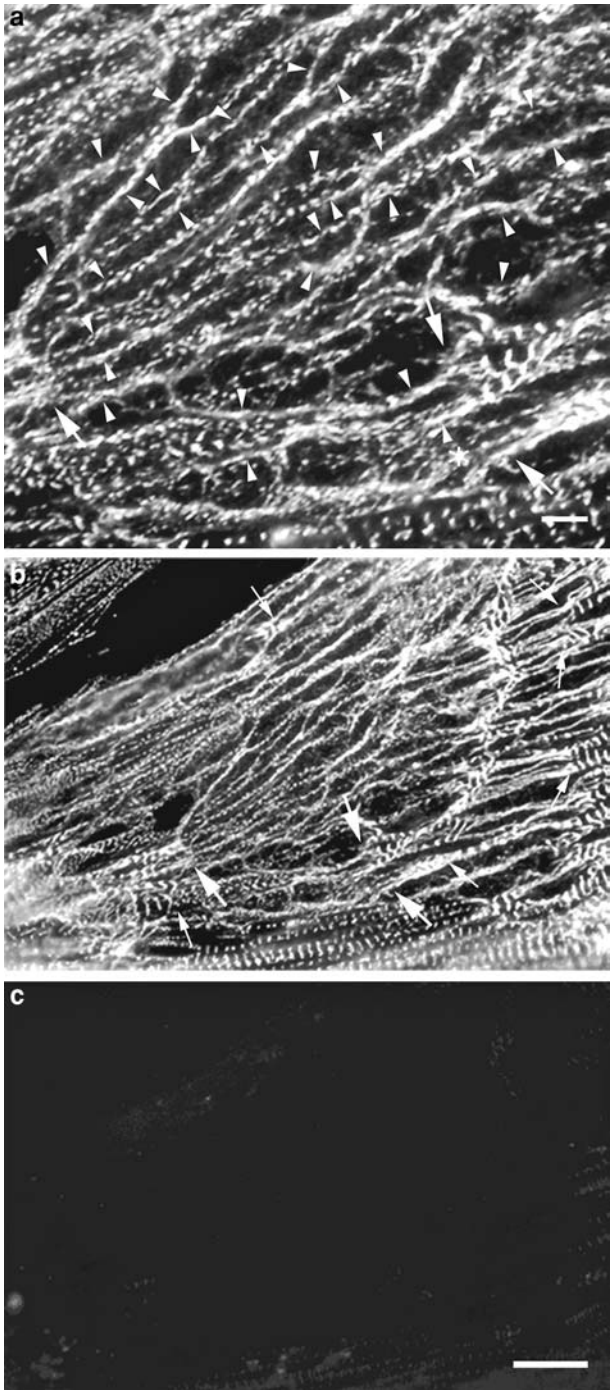

Fig. 3 Aberrant assembly and alignment of myofibrils in cardiac myocytes following transfection with obscurin siRNA. **a** Multiple branching of myofibrils and their inability to undergo lateral fusions visualized by immunolabeling for sarcomeric α-actinin. Numerous thin cross-striated bundles form brush-like structures on the tips of persisting myofibrils (*arrows*). The pairs of *arrowheads* facing each other show bifurcating thin branches of myofibrils. **b** A view of the same cell at a lower magnification. The *large arrows* are in the same positions as **a**. The *small arrows* show the sites of transition from the striated to branching pattern of α-actinin distribution. Note that misaligned immature myofibrils and their structural precursors occupy large areas of the sarcoplasm. **c** immunofluorescent labeling for obscurin in the field presented in **b** shows the suppression of its expression. Eleven days in culture (compared to the control cell at the same time point shown in Fig. 1e). *Bars* 4 μm (**a**), 10 μm (**b**, **c**)

obscurin expression affects lateral alignment and fusion of sarcomeric bundles and impairs further registered organization of myofibrils. An interesting characteristic of these structures is their repeated bifurcations and dichotomic branching (Figs. 3a, b, 4a). This type of localization is dramatically different from the uninterrupted cross-striated α-actinin distribution in control cells illustrated in Figs. 1f, 2a, c.

Irregularities of sarcomere spacing and a loose, widened, or irregular localization of α-actinin bodies in premyofibrils (Figs. 2d, 3a, 4a–c) show that obscurin may also be involved in the control of intermediate stages of myofibrillogenesis, specifically in the positioning and lateral binding of individual myofibrils into the continuous pattern typical of differentiated cardiac muscle (Fig. 1d–f, 2a). At the same time, the presence of striated contractile structures in the cells with a significantly depleted obscurin pool (Figs. 3, 4) shows that the initial stages of myofibril assembly, that include the establishment of linear longitudinal periodic localization of Z-bodies and nascent Z-lines, are not highly dependent on the expression of this protein. Interestingly enough, many cells retained the cross-striated pattern of α-actinin localization 14–18 days following transfection, but most of them failed to bind and integrate the myofibrillar bundles into larger structural units and formed clearly visible gaps between these structures (Figs. 3, 4). The analysis of the samples at a higher magnification showed that both single myofibrils and their clusters were indeed spatially separated (the inserts in Figs. 4a, b). This effect was more pronounced in the areas of newly formed myofibrils, whereas the Z-lines retained a significantly higher level of integrated organization in the myofibrillar clusters located in the central sarcoplasm.

It is important to note that the transfected cultures exhibited a considerably heterogeneous morphology of the contractile apparatus and intensity of myofibrillogenesis in individual cells. This heterogeneity is likely due to differential effects of obscurin depletion at each phase of the remodeling process. The structural modifications of the contractile apparatus observed in anti-obscurin siRNA transfected cells are shown in Fig. 4. In summary, these changes include a thin, atrophic appearance of myofibrillar bundles, a looser distribution of α-actinin, and a failure to assemble mature contractile structures, as evidenced by more or less generalized myofibrillar misalignment and disorganization (Figs. 2, 3, 4). Local disarrays of α-actinin-containing thin bundles or their breakdown into dotted, closely spaced patterns (Fig. 4c) was a characteristic of one more cell type that developed in cultures by 12–18 days following the transfection. This morphological variety was practically absent among non-transfected myocytes and in the cultures transfected with control non-coding siRNA constructs.

Since obscurin has been reported to interact with titin, another giant, muscle-specific protein (Bang et al. 2001; Sanger and Sanger 2001; Young et al. 2001), it was

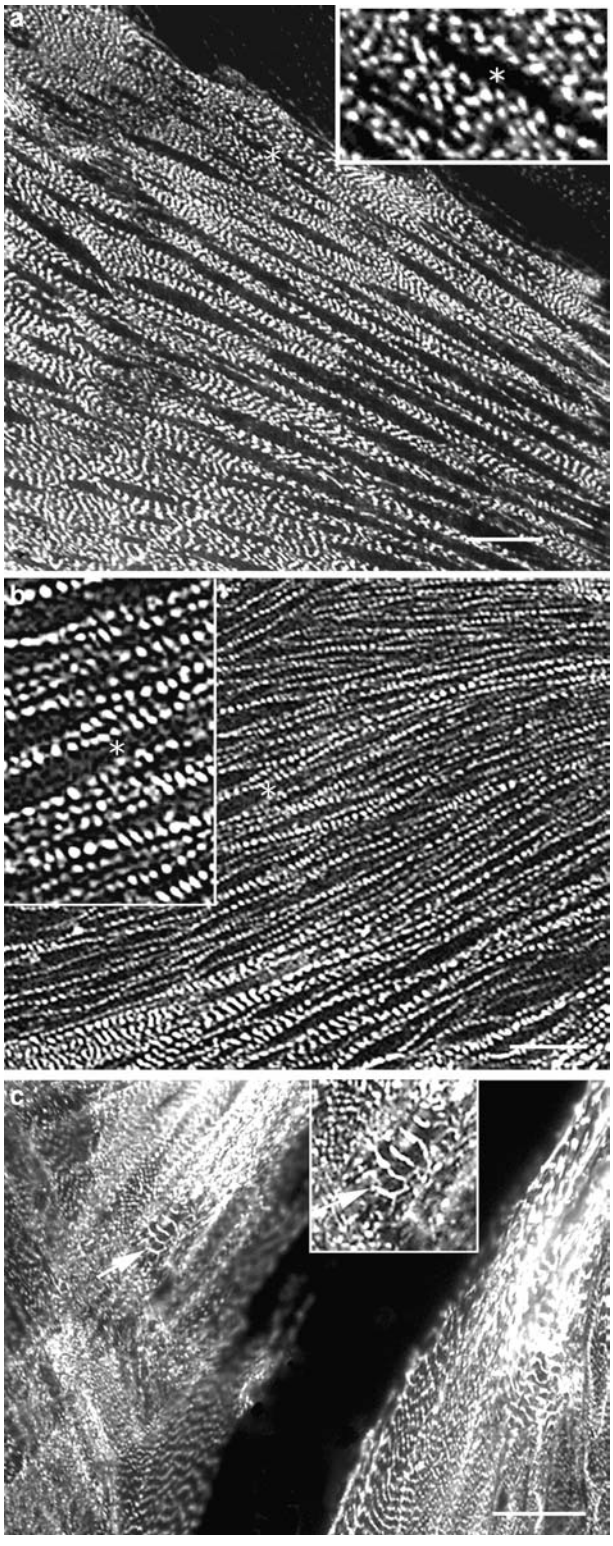

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Fig. 4 Structural modifications of the contractile apparatus immunolabeled for sarcomeric α-actinin in cardiac myocytes following exposure to anti-obscurin siRNA. **a, b** Division of myofibrils into separate bundles of different sizes. Note parallel rows of poorly aligned and underdeveloped branching myofibrils. The periodic pattern of α-actinin localization is generally well preserved. The *insets* show the areas of longitudinal separation and misalignment of individual thin myofibrillar bundles. The *asterisks* in **a** and **b** mark the same sites in the figures and in the *insets*, respectively. **c** Dispersion of myofibrils into thin bundles and tiny Z-bodies immunopositive for α-actinin. The *arrow* indicates the area shown at a higher magnification in the *insert*. Note the presence of five sarcomeres that retained intact normal spacing and striated morphology surrounded by multiple narrow-spaced small Z-bodies some of which are arranged in a punctate dotted linear pattern. Myofibrils with normal periodicity of striations are seen at the *bottom* of figure **c**. Day 16 (**a, b**) and day 11 in culture(**c**). *Bars* 18 μm

important to determine how depletion of obscurin affected the distribution of titin. The immunolocalization pattern of titin in relation to α-actinin and obscurin during myofibrillogenesis in this model have been illustrated and discussed in our recent publication (see Fig. 4a–d in Borisov et al. 2004). In that study, obscurin was demonstrated to incorporate into the middle of the A-bands of nascent myofibrils rather early and become clearly visible soon after the formation of Z-lines at the time when titin also acquired the cross-striated pattern (Borisov et al. 2004). We found that siRNA-mediated downregulation of obscurin expression resulted in significant changes in the topographical distribution of titin (Fig. 5). Unlike control cells (Fig. 5a), the sarcomeric organization of titin in siRNA-transfected cells was less developed (Fig. 5b). The area occupied by mature cross-striated pattern of titin localization in individual obscurin siRNA-transfected cells varied considerably, but in general was about a half that observed in control myocytes. This shows that either the blockage of normal interactions of titin and obscurin impairs the progression of myofibrillogenesis, or the depletion of obscurin affects the interactions of other sarcomeric proteins resulting in misalignment of titin as a secondary phenomenon.

Given obscurin's localization to the middle of the A-bands, we explored the possibility of its involvement in the organization of thick (myosin) filaments within the sarcomere. To that end, we examined the effect of siRNA-mediated obscurin downregulation on the localization of sarcomeric myosin, the major protein of the A-bands. Immunofluorescent labeling has shown that the normal regular cross-striated pattern of sarcomeric myosin typical of control cells (Fig. 6a, b) was considerably perturbed in obscurin-depleted myocytes (Fig. 6c–f). In the transfected cultures, the areas occupied by well-organized cross-striated myosin filaments were considerably diminished. In some myocytes, myosin was diffusely distributed in the sarcoplasm (Fig. 6c, e), and such cells were practically absent in the control cultures. Diffuse and patchy distribution of myosin occurred both in the central and peripheral areas (Fig. 6c, d). While the magnitude of these changes varied among individual siRNA-transfected cells and in some cells the elements of cross-striation persisted through the post-transfection period, they were not noted in control cells which typically displayed organized patterns of myosin labeling in sarcomeric A-bands that extended from the

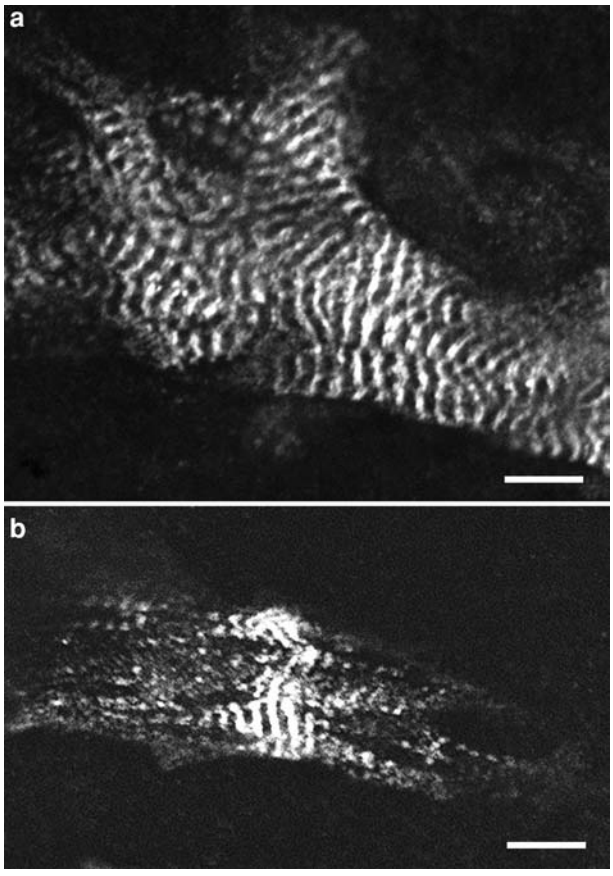

Fig. 5 Immunofluorescent labeling of titin in control (**a**) and obscurin siRNA-transfected (**b**) cardiac muscle cells. Note cross-striated distribution of titin in a control cell. Downregulation of obscurin expression induces the development of diffuse and granular patterns of titin localization in the peripheral areas of the sarcoplasm. Eleven days in culture (**a, b**). *Bar* 6 μm

center to the cell periphery (Fig. 6b). This suggests that obscurin is involved in the registered incorporation and/or alignment of myosin within nascent and mature myofibrils and that downregulation of its expression blocks the assembly of new mature contractile structures following induction of the hypertrophic response.

Discussion

The results of this study demonstrate that obscurin appears to be indispensable for the advanced stages of sarcomere assembly and probably for lateral alignment of myofibrils into larger structural units during differentiation and hypertrophic growth of cardiac myocytes. Earlier we suggested that the pattern of obscurin localization in the middle of A-bands during myofibril formation, its high molecular weight and multidomain structure make this protein a very suitable candidate for the role of the functional integrator of myosin filaments and structural stabilizer of the whole sarcomere (Borisov et al. 2003, 2004; Kontrogianni-Konstantopoulos et al. 2004). The data presented above completely support and extend this hypothesis.

α-Actinin, a well-characterized marker of myofibrillogenesis, is one of the earliest proteins incorporated into the precursors of myofibrils during development of the contractile system (Du et al. 2003; Wang et al. 2005). According to a current concept of myofibril assembly (e.g., Du et al. 2003), thick (myosin) filament integration into the nascent sarcomeres occurs at later stages of myofibrillogenesis. Myosin begins to become aligned along actin after the formation of a developed scaffold of the I–Z–I complexes consisting of I-bands attached to the nascent Z-lines (for discussion see Borisov 1991; Holtzer et al. 1997; van der Ven et al. 1999). While this process was thought to be guided by titin (Gregorio et al. 1999; Tskhovrebova and Trinick 2003; Miller et al. 2004; Wang et al. 2005), our data suggest that at this stage, obscurin may play an important role in organization of the contractile apparatus. This makes more plausible the hypothesis that obscurin acts as a flexible linker between titin and other cellular structures (Young et al. 2001). The findings that this protein binds small ankyrin-1 and mediates structural interaction between the myofibrils and the sarcoplasmic reticulum in skeletal muscle (Bagnato et al. 2003; Kontrogianni-Konstantopoulos et al. 2003) further prove its involvement in the integrating function. Preservation of the striated and dotted patterns of α-actinin localization in the obscurin-depleted cells shows that the lack of obscurin is not critical during the early stages of myofibrillogenesis.

Our current results provide evidence that the process of myosin filament incorporation is considerably more dependent on the presence of obscurin, than are the initial stages of myofibril assembly. The bundling and lateral alignment of myofibrils in register at terminal stages of cardiac and skeletal myogenesis is traditionally attributed to desmin, the major muscle-specific intermediate filament protein (reviewed in Borisov 1991; Paulin and Li 2004). However, mice lacking desmin did not show considerable abnormalities of myofibril structure during muscle development (e.g., Capetanaki et al. 1997; Shah et al. 2001; Paulin and Li 2004). In our previous studies, we found that the initial stages of lateral alignment of new myofibrils during differentiation, remodeling, and hypertrophic growth of rat cardiac myocytes were coincident with obscurin localization to the sarcomere and occurred when desmin was still localized diffusely rather than concentrated at Z-lines (Borisov et al. 2003, 2004). This suggests that obscurin may be involved in this process earlier than desmin. Taking into account the sarcomeric localization of obscurin at M- and Z-lines, these observations do not exclude the possibility of interaction of these two proteins in the final stages of myofibril assembly.

Interestingly, the effects of obscurin depletion on myofibrillogenesis in our experiments to some extent are similar to those observed in a titin-deficient myogenic cell line (van der Ven et al. 2000) and in rat cardiac myocytes in which titin expression was inhibited by

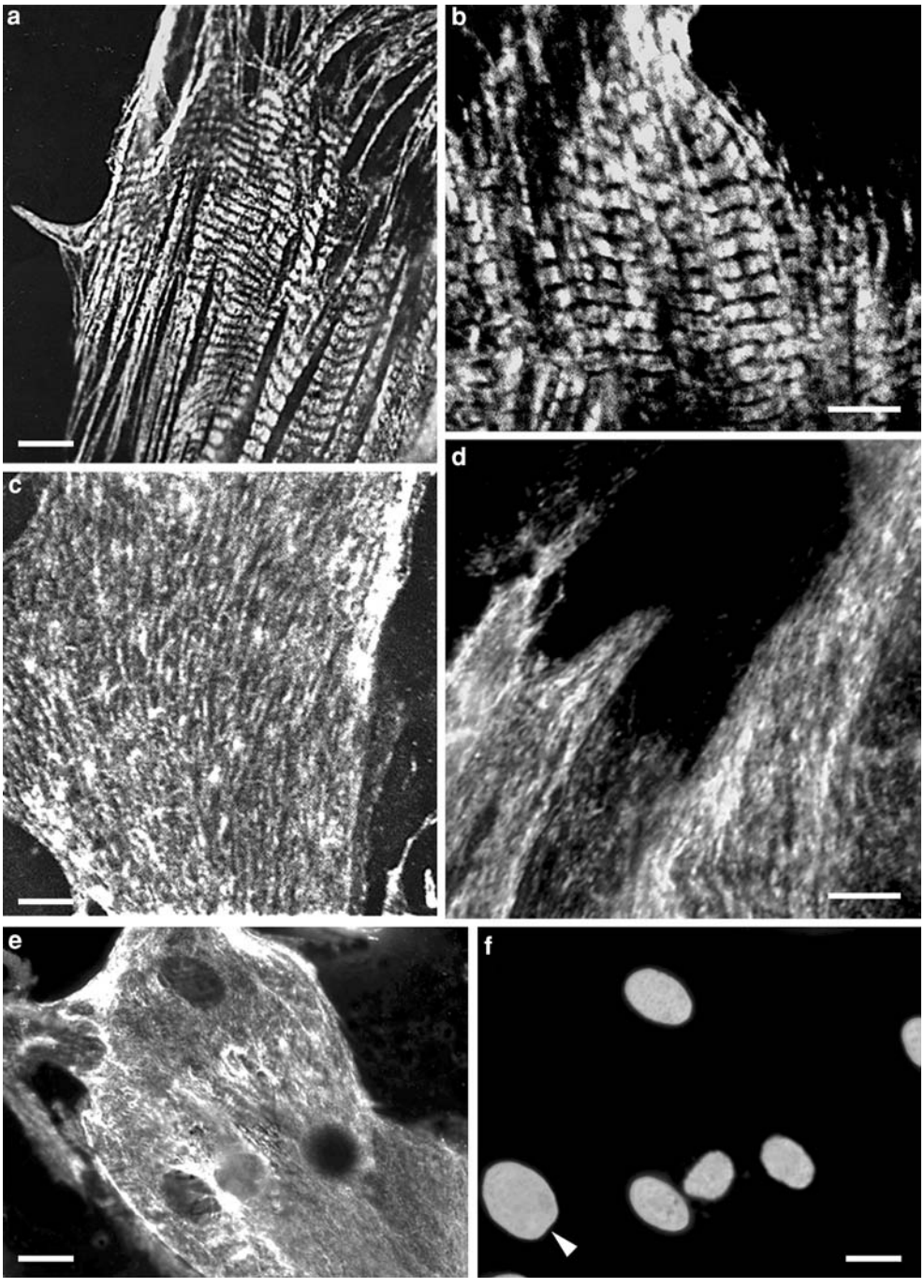

Fig. 6 Immunofluorescent localization of sarcomeric myosin in control (**a**, **b**) and anti-obscurin siRNA-transfected cells (**c**, **d**, **e**). Note the striated pattern of myosin distribution typical of the A-bands of normal myofibrils compared to the diffuse localization of myosin and the presence of scattered small spots and aggregates of myosin filaments similar in size to the size of A-bands in anti-obscurin siRNA-transfected cultures. **b**, **d** Immunolabeling for sarcomeric myosin in the peripheral cytoplasmic processes. **a**, **c** Immunolabeling for sarcomeric myosin in the central areas of the cytoplasm shown at a lower magnification. **e** A general view of a group of anti-obscurin siRNA-transfected muscle cells immunolabeled for sarcomeric myosin. **f** Labeling for obscurin and staining of nuclear DNA with bisbenzimide in the same field shows downregulation of obscurin expression following the transfection. The *arrowhead* in **f** shows the localization of the nucleus of a non-muscle cell immunonegative for sarcomeric myosin. **a**, **c** 16 days in culture, **b**, **d** 14 days in culture, **e**, **f** 11 days in culture. *Bars 6* μm (**a**–**d**), 14 μm (**e**, **f**)

antisense oligonucleotides (Person et al. 2000). In both models, suppression of titin expression was associated with significant structural disturbance of the contractile apparatus and a failure to incorporate myosin filaments in the nascent sarcomeres. Defective myofibrillogenesis has also been reported in differentiating cultures of mouse skeletal myotubes following expression of titin with a targeted deletion of its terminal end (Miller et al. 2003). The authors found that downregulation of titin and/or disruption of the titin kinase domain affected both proper obscurin localization and M- and Z-line organization. Combined with our data, these results suggest a cooperative interaction between titin and obscurin in the assembly and stabilization of the myosin filament arrays. The heterogeneity of responses of individual cells to obscurin depletion may depend on the intrinsic differences in their levels of differentiation, and a similar heterogeneous response has also been observed in titin-depleted cells (Person et al. 2000).

Titin has been demonstrated to directly bind myosin and to anchor the thick filaments to Z-lines and M-lines as an elastic spring along the distance of a

half-sarcomere (Houmeida et al. 1995; Bennett and Gautel 1996; Tskhovrebova and Trinick 2003). While a direct interaction between obscurin and myosin has not yet been identified, the preliminary data suggest the possibility of such interactions (Kontrogianni-Konstantopoulos et al. 2004). Furthermore, obscurin and myosin can be co-immunoprecipitated using antibodies to either one, indicating that the two directly interact or are both part of a tightly bound protein complex (Knotrogianni-Konstantopoulos et al. 2004). Given the apparent capacity of obscurin to bind titin (Young et al. 2001; Bang et al. 2001), it is likely that these two giant proteins are expressed and function in a coordinate manner, providing a precisely structured three-dimensional scaffold, or template, for proper myofilament incorporation. These observations agree well with the data presented here and provide further evidence that obscurin may interact with both myosin and titin directly or indirectly. The fact that overexpression of the ankyrin-binding obscurin domain in cultures of skeletal myotubes has a specific and profound effect on the organization of sarcomeric myosin (Kontrogianni-Konstantopoulos et al. 2004) suggests that ankyrin may mediate the interactions between obscurin and myosin. Myomesin and other myosin-binding M-line-associated proteins may also be involved in this process. The sarcomeric M-band is thought to provide a link between the thick filaments and the elastic filament system (Agarkova et al. 2003; Lange et al. 2005). Myomesin, one of the major M-band protein components, consists mainly of immunoglobulin-like and fibronectin-3 domains, and its N-terminal and central parts harbor binding sites for myosin, titin, and muscle myosin kinase (Lange et al. 2005; Schoenauer et al. 2005).

It is essential that both titin and obscurin also contain multiple immunoglobulin repeats and fibronectin-3-like domains (Bang et al. 2001; Young et al. 2001; Tskhovrebova and Trinick 2004). This is another indicator of the possible similarity of their functional specialization as adhesive hubs and rulers controlling spatial protein interactions during myofibrillogenesis. The similarity between the structural changes induced by the reduced expression of titin and obscurin indicate that these proteins may perform synergistic but distinct functions during myofibril assembly. It remains to be determined whether the local misalignments of titin and myosin are the direct result of obscurin-downregulation as their primary binding and integrating partner, or this structural shift simply reflects the block of advanced stages of myofibril assembly affected by additional interprotein interactions and control mechanisms. Whether the diffuse localization of sarcomeric myosin in newly formed sarcomeres in obscurin-depleted cells is explained by the defective targeting/incorporation of the thick filaments, by their defective cross-linking, or by a simple superimposition of the layers of misaligned thin myofibrils is of special interest. Further studies are required to identify the precise molecular events related to the role of obscurin in the morphogenesis of the M-lines, A-bands and Z-lines.

The presence of the regulatory Rho-GEF and calmodulin-binding motifs in the obscurin molecules shows that the functions of this protein are not limited to a merely structural role and strongly suggests direct its involvement in signal transduction. Of special interest are the reports that Rho protein family is implicated in the control of cytoskeletal remodeling and cardiac muscle gene transcription (Finkel 1999; Clerk and Sugden 2000; Burridge and Wennerberg 2004). In addition, Wei et al. (2002) reported that the inhibition of Rho-family GTPases disrupted cardiac morphogenesis and suppressed cardiomyocyte proliferation in murine embryos. Thus, it is possible that the effect of obscurin downregulation on the structure of the contractile apparatus is mediated by inactivation of its signaling domains.

Obscurin-like giant muscle proteins were apparently developed and evolved in common phylogenetic ancestors of invertebrate and vertebrate animals. It was reported that several motifs within mammalian obscurin gene have a high sequence homology to the nematode *Caenorhabditis elegans* muscle-specific gene *unc-89* (for discussion, see Sutter et al. 2004). Despite a lower molecular weight of *unc-89* (732 kDa), the general similarity of its primary structure to the structure of obscurin and its localization in the middle of A-bands of myofibrils suggests that these proteins are evolutionary orthologs. This is also supported by the fact that the nematodes carrying spontaneous mutations of *unc-89* demonstrate significant disruption of the contractile system due to aberrant myofibril assembly in the body wall muscles (Benian et al. 1996). Despite a normal number of myosin filaments, the deletion mutants showed severely disorganized A bands and disrupted M-lines. Recent identification of the *unc-89* isoforms containing myosin light chain kinase (MLCK)-like domains (Sutter et al. 2004; Small et al. 2004) similar to those found among the isoforms of obscurin (Russell et al. 2002) provides new evidence of functional and phylogenetic homology of these two genes and their involvement in signal transduction. As the only identified homolog of nematode *unc-89* in vertebrate animals, obscurin appears to play a similar functional role in the processes of assembly, adaptive plasticity, and structural maintenance of the contractile system in mammalian muscle cells.

It is becoming increasingly apparent that the mutations associated with the loss of function of orthologous proteins can cause similar types of pathological conditions in different taxonomic groups of animals (O'Brian et al. 2004). This fact has important implications for the potential involvement of obscurin in various types of human muscle pathology. Taken together, these findings suggest that obscurin plays a vital role in the assembly, integration and control of functional activity of the contractile apparatus.

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