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Obscurins: Unassuming Giants Enter the Spotlight

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Abstract

Discovered about a decade ago, obscurin (~720 kDa) is a member of a family of giant proteins expressed in striated muscle that are essential for normal muscle function. Much of what we understand about obscurin stems from its functions in cardiac and skeletal muscle. However, recent evidence has indicated that variants of obscurin (“obscurins”) are expressed in diverse cell types, where they contribute to distinct cellular processes. Dysfunction or abrogation of obscurins has also been implicated in the development of several pathological conditions, including cardiac hypertrophy and cancer. Herein, we present an overview of obscurins with an emphasis on novel findings that demonstrate their heretofore-unsuspected importance in cell signaling and disease progression.

Keywords

striated muscle; cardiac hypertrophy; breast cancer; *OBSCN*; obscurin; *UNC-89*.

Introduction

Named due to initial difficulties in characterization and detection (1), obscurin is now understood to be a family of proteins (“obscurins”) expressed from the single *OBSCN* gene, which in humans spans more than 170 kb on chromosome 1q42.13. The prototypical obscurin, obscurin A, is composed of 62 immunoglobulin (Ig) repeats interspersed with three fibronectin type-III (FNIII) domains and a calmodulin-binding IQ motif, followed by a src homology-3 domain, tandem Rho-guanine nucleotide exchange factor (RhoGEF) and pleckstrin homology (PH) domains, two additional Ig repeats, and a nonmodular COOH-terminal region of ~400 amino acids that contains consensus phosphorylation motifs for ERK kinases, yielding a total size of ~720 kDa (1) (Fig. 1). Another giant isoform, obscurin B (~870 kDa), is very similar to obscurin A, but lacks the nonmodular COOH-terminal region. Instead, it includes two Ser/Thr kinase domains with homology to myosin light chain kinases, referred to as SK2 and SK1, which are preceded by Ig, and Ig and FNIII domains, respectively (2) (Fig. 1). An alternative ribosomal entry site and start codon (3,4) allow the expression of smaller obscurin isoforms in humans, including a tandem kinase isoform that consists of partial SK2 and full length SK1, and a single kinase isoform that only contains SK1 (Fig. 1); recently, the activity of this alternate promoter was verified in mice (5). Expression differs among the obscurin isoforms; while giant obscurins A and B are

expressed in higher amounts in skeletal compared to cardiac muscles, the tandem and single kinase isoforms are largely present in cardiac muscle (2,4).

In addition to the aforementioned obscurin isoforms, new evidence indicates that many more variants can be generated from *OBSCN*'s 119 exons. Analysis of the *OBSCN* gene has revealed that most of its tandem Ig domains are encoded by individual exons that have complementary splice sites and preserve the reading frame, allowing for the modular assembly of isoforms containing all or select Ig domains (2,3). This is supported by immunoblots conducted with antibodies to epitopes spaced along the length of giant obscurins; immunoreactive bands of ~100 and ~150 kDa that contain both NH₂- and COOH-terminal epitopes were observed in rat skeletal muscles (6), while bands of ~110 and ~120 kDa that contain the RhoGEF and kinase domains, respectively, were detected in nuclear lysates prepared from epithelial cells (7). Furthermore, Bowman et al. (8) suggested that a unique isoform of obscurin containing a novel NH₂-terminus and the nonmodular COOH-terminus of obscurin A, but lacking the RhoGEF domain, is present at sarcomeric Z-disks. Collectively, the intricate alternative splicing that the *OBSCN* gene undergoes may represent a novel mechanism of generating specialized obscurin scaffolds that provide binding sites for select interacting partners.

Although this review focuses on the functions of mammalian obscurins, study of nonmammalian *OBSCN* orthologs has revealed their conservation across distant lineages (9). A *Caenorhabditis elegans* ortholog of *OBSCN*, *unc-89*, also produces multiple splice variants of the protein UNC-89 (10–12), for which many binding partners and functions have been determined (13–19). *Danio rerio* (zebrafish), which has two separate genes encoding the two largest obscurin isoforms, has been a useful model to study the effects of obscurin depletion by morpholino antisense technology (20–22). Furthermore, *Drosophila melanogaster* obscurin was recently characterized (23). In addition to its orthologs, *OBSCN* has two paralogs within mammalian genomes, striated preferentially expressed gene and obscurin-like-1, whose products may possess similar functional activities (24,25).

The vast size of the giant obscurins is necessitated by one of their functions in striated muscle cells, that is, acting as molecular “rulers.” Along with titin (~3–4 MDa) and nebulin (~600–800 kDa), obscurins may serve to define the dimensions of the developing sarcomere. It was in this context that obscurin was originally discovered as an interacting partner of titin in a yeast-two-hybrid screen (1). Herein, we will provide an overview of the functions of obscurins in striated muscles, discuss recent evidence regarding their roles in non-muscle tissues, and conclude with their involvement in different diseases. Readers interested in a detailed description of the role of giant obscurins in myofibrillogenesis are directed to a recent comprehensive review (26).

Obscurins in Striated Muscles

Subcellular Distribution

The striated appearance of cardiac and skeletal muscle is due to the regular arrangement of actin and myosin filaments and their associated proteins into contractile units, termed sarcomeres (Fig. 2A). These incorporate into bundles of myofibrils that comprise the muscle cell (myofiber). Sarcomeres are defined by protein-dense structures, termed Z-disks, which contain structural and signaling molecules and serve as anchoring sites for actin thin filaments, which occupy I-bands. The central region of sarcomeres contains bundles of myosin thick filaments, organized into A-bands, which are bisected by M-bands that are devoid of myosin heads. Upon release of calcium ions from the sarcoplasmic reticulum (SR), which intimately surrounds each sarcomere, the heads of the myosin filaments hydrolyze adenosine triphosphate to “pull” on the interdigitating actin filaments. This causes

shortening of the sarcomere and hence contraction of myofibers; reuptake of Ca^{2+} to the SR leads to relaxation until a new cycle starts (27).

The subcellular distribution of obscurins has been studied extensively in cardiac and skeletal muscle of mouse and rat, and to a lesser extent in human. This has been primarily accomplished through confocal optics and immunofluorescence or immuno-electron microscopy, using antibodies raised to epitopes spanning the entire length of giant obscurins. Obscurins containing both A and B epitopes concentrate at M-bands and Z-disks in fully differentiated rodent (6,8) and human (28) skeletal and cardiac muscles (28–30). In addition to their sarcomeric localization, obscurins are also present at the nuclei and sarcolemma of striated muscle cells and at a specialized domain of the sarcolemma, the intercalated disc, in cardiocytes (31) (Fig. 2A). During differentiation, however, obscurins' subcellular distribution is variable. In skeletal myoblasts, obscurins assemble into primordial M-bands, prior to the organization of myosin into A-bands, while they incorporate into Z-disks after those have matured (32). In contrast, obscurins appear at Z-disks early during myofibrillogenesis in cardiomyocytes, but later translocate to M-bands (1). Whether this dynamic localization during cardiac development is due to the presence of multiple splice variants, or simply an artifact of epitope accessibility, remains to be determined.

In contrast to titin and nebulin, which are oriented longitudinally along the long axis of sarcomeres, specifying their length, obscurins exhibit a reticular distribution, presumably defining their diameter (6,29). This unique topography allows obscurins to provide binding sites for diverse binding partners located in distinct subcellular compartments (26).

Interacting Partners

Obscurin was originally discovered in the course of a yeast-two-hybrid screen of a human cardiac library for binding partners to a fragment of titin that is localized to the peripheral Z-disk in striated muscles (1). Specifically, obscurin domains Ig58/59 interact with titin Ig domains Z9/Z10, anchoring obscurin to the Z-disk. The Ig58/59 region of obscurin also binds to a novel isoform of titin that resides at the I-band, novex-3 (33). Because muscle extension results in stretching of the novex-3:obscurin complex, the authors proposed that it might be involved in strain-induced signaling during muscle development and cardiac disease (33).

Obscurins also bind the region of titin that extends into the M-band; in particular, the Ig1 domain of obscurin interacts with the M10 domain of titin (34). Interestingly, atomic force microscopy demonstrated a relatively low unbinding force (~ 30 pN) for the titin-M10:obscurin-Ig1 complex (35). The weak titin-M10:obscurin-Ig1 coupling at the M-band is also part of a ternary complex with myomesin, a protein that is involved in anchoring thick filaments to the M-band, which directly binds to the Ig3 domain of obscurin (34). Upon introduction of exogenous obscurin Ig1 or Ig3, which contain binding sites for titin and myomesin, respectively, to neonatal rat cardiomyocytes, endogenous obscurins are displaced from the M-band and remain diffuse in the cytoplasm (34). This dominant negative effect emphasizes the importance of titin and myomesin in targeting and stabilizing obscurins to the M-band.

The NH_2 -terminal region of obscurins also binds to a novel variant of myosin-binding protein-C slow (sMyBP-C variant 1) in skeletal muscle (36). The obscurins' second Ig domain, Ig2, directly interacts with the COOH-terminal Ig domain, C10, of sMyBP-C variant 1. The presence of 26 novel amino acids following the C10 domain of variant 1 considerably strengthen the interaction between obscurins and sMyBP-C variant-1. Overexpression of obscurin Ig2 disrupts the formation of M-bands and the assembly of

myosin filaments into A-bands, underlining the essential role of obscurins' NH₂-terminus to the assembly and stabilization of thick filaments.

In addition to playing key roles in sarcomeric organization, obscurins also contribute to Ca²⁺ signaling. Although alterations were observed in the localization and expression level of SR proteins in *OBSCN* knockout mice (37), no changes in calcium release or reuptake were reported (5). However, upon characterization of obscurin A, it was discovered that its IQ motif interacts with the calcium sensor calmodulin in a calcium-independent manner (1). Therefore, it is conceivable that the IQ-calmodulin interaction may play a role in coupling calcium signaling to other obscurin functions, such as guanine nucleotide exchange in the nearby RhoGEF domain, a possibility that merits further investigation.

Obscurins' RhoGEF domain can specifically induce exchange of GDP for GTP in the small GTPases RhoA (38) and TC10 [also known as RhoQ; (39)], leading to activation of their downstream effectors, but not in Rac1 or Cdc42 (37,38). This specificity is observed in *C. elegans* UNC-89 as well (14), with conservation indicative that obscurin RhoGEFs' activation of RhoA and TC10 is necessary for the proper function of striated muscles. Indeed, RhoA activity plays an important role in normal myofibril growth and during pathologic hypertrophy (40), consistent with the up-regulation of RhoGEF-containing obscurins during normal myofibrillogenesis (41) and stress-induced cardiac remodeling (42).

RhoGEF domains are often coupled to PH domains, which regulate their activity and membrane recruitment (43). Although functional studies of obscurins' PH motif have not been conducted, the structure of the UNC-89 PH domain suggests that it is likely not involved in binding phosphoinositides, a canonical function of PH domains (13). However, as UNC-89 and obscurin PH domains share only 23% identity, it is possible that they have different properties and functions, and may influence novel activities of adjacent signaling domains. Consistent with this, a unique function of the obscurin RhoGEF domain is its interaction with Ran-Binding Protein-9 (RanBP9) (44), a scaffolding protein that binds to the nuclear import-export regulators, RanGTPases. RanBP9 is present at M-bands and Z-disks, and when overexpressed in cultured skeletal myotubes inhibits the incorporation of the NH₂-terminus of titin to developing Z-disks. Interestingly, both RanBP9 and the RhoGEF domain of obscurins bind directly to the NH₂-terminus of titin, suggesting that a ternary complex of titin, obscurin, and RanBP9 may be important for Z-disk assembly.

The most studied obscurin ligand is a splice variant of the *ANK1* gene, small ankyrin-1 (sAnk1, also known as Ank1.5), an integral component of the network SR. Two groups discovered this interaction independently (29,45), and while both found that sAnk1 bound to the nonmodular COOH-terminus of obscurin A, discrete sites were identified. Bagnato et al. narrowed the sAnk1-binding site to residues 6,236–6,260 (NP_443075.3) (44) that has a predicted random coil structure and a dissociation constant of 384 nM (46), while Kontogianni-Konstantopoulos et al. identified residues 6,316–6,345, with a ~28% predicted α -helical structure and a dissociation constant of ~130 nM (29,46). Both binding sites rely on electrostatic interactions (47,48), while the higher affinity of the more COOH-terminal site (amino acids 6,316–6,345) is also supported by hydrophobic interactions (49).

Characterization of the obscurin-binding site on sAnk1 revealed two regions that can mediate binding; the first, residues 57–89 (AAH61219.1), is conserved across ankyrin isoforms, while the second, residues 90–122, is unique to sAnk1 (47) and another *ANK1* splice variant, ank1.9, which also binds to the COOH-terminus of obscurin A (50). Recently, two homologous sites have been found in a novel splice variant of ankyrin B, encoded by the *ANK2* gene, which also binds to the nonmodular COOH-terminus of obscurin A and

serves to recruit protein phosphatase 2A to the M-band (51). The identification of sAnk1 (and related isoforms) as a binding partner of obscurin A was particularly significant because it represented the first direct molecular link between the contractile sarcomere and the SR membrane, illuminating a possible mechanism for the alignment and anchoring of the SR around the developing myofibril. Indeed, in the absence of obscurins, sAnk1 remains associated with the SR membrane, although in a disorganized manner, and the SR fails to assemble around the contractile apparatus (52). Although no studies have been conducted on the interaction of UNC-89 and SR-localized ankyrin isoforms, in UNC-89 mutant nematodes, which no longer express the largest UNC-89 isoforms, SR proteins are disorganized and calcium signaling is compromised (17).

The binding of obscurins to sAnk1 appears to mediate another novel biological process. Through the adaptor protein, potassium channel tetramerization domain containing-6 (KCTD6), the ubiquitin E3 ligase cullin-3 binds and ubiquitylates sAnk1, targeting it for degradation (37). However, a subset of the sAnk1 lysines subject to ubiquitylation are located within the obscurin-binding domain, and therefore are inaccessible when sAnk1 is bound to obscurin; this is consistent with the observation of lower sAnk1 levels in obscurin-null mice (5,37). Reinforcing the emerging importance of obscurin in mediating protein turnover is the recent observation of UNC-89s' interaction with maternal effect lethal-26 (MEL-26) (18). Upon binding to either the Ig2-Ig3 or Ig53-FnIII-2 regions of UNC-89, MEL-26 recruits cullin-3 and promotes degradation of the microtubule-severing protein katanin. Although the nature of the obscurins' interaction with cullin-3 is quite different in these two cases, the idea that obscurins may assemble protein degradation complexes is intriguing.

Increasingly, the kinase-containing isoforms of obscurins are understood to play distinct roles in muscle, particularly in the heart (2,4). Qadota et al. demonstrated that small-CTD-phosphatase-like-1 (SCPL-1) binds to both kinase domains of UNC-89 (15). In addition, Xiong et al. showed that the first kinase domain of UNC-89 forms a ternary complex with the four and one-half LIM protein-9 (LIM-9) and SCPL-1 (16). This complex is localized to the M-band and, through bridging proteins, links UNC-89 to costameric integrins, and thus the extracellular matrix (ECM). The complex of LIM-9 and SCPL-1 also interacts with UNC-89 Ig domains 1–5 through copine domain atypical-1 (CPNA-1), providing an additional link between UNC-89 at the M-band and integrin adhesion complexes (19).

Recent work in our laboratory has revealed that both the more NH₂-terminal (SK2) and COOH-terminal (SK1) kinase domains of obscurin B are active and capable of autophosphorylation. Importantly, N-cadherin is a substrate of SK2, while Na⁺/K⁺-ATPase is a binding partner of SK1 (31). Furthermore, a small isoform of obscurin containing only SK1 is localized extracellularly in cardiocytes and can undergo *N*-glycosylation (31). The exofacial localization of at least one obscurin isoform raises the possibility that obscurins may interact with and/or modify the ECM.

Embryonic Development

The effects of obscurins' depletion on developing zebrafish embryos have been observed using morpholino antisense technology. Knockdown of obscurin A specifically results in defects in the development of striated muscles, including ventricular hypoplasia and decreased heart rate, failure of skeletal myofibrils to assemble into larger functional units, and SR disorganization (20). Somite boundaries were less well-defined in the absence of obscurin A (20,22), suggesting that it may link myofibrils to the ECM. However, as expression of the kinase-containing isoform(s) was unaffected by the morpholino treatment, the proposed ECM interaction is distinct from that involving the kinase domains (see "*Interacting Partners*," above). In particular, knockdown of obscurin A resulted in deficient

integrin clustering, causing disorganization of the fibronectin matrix (22). Importantly, the severe abnormalities observed upon depletion of obscurin A, including the irregular organization of adherens junctions that affected both muscle and retina development, could be rescued by expression of a “mini-obscurin” consisting of the RhoGEF and ankyrin-binding regions of obscurins (21). This implicates obscurin-stimulated small GTPase function in tissue differentiation.

The effects of obscurin/UNC-89 knockdown or mutation in *D. melanogaster* and *C. elegans* also result in altered organization of striated muscle (23,53), although the effects of depletion on other tissues have not been investigated. In contrast to the robust phenotypes observed in obscurin A/UNC-89-depleted zebra-fish, flies, and nematodes, *OBSCN* knockout mice (5) do not show any developmental or structural abnormalities, despite alterations in the organization of sAnk1 and morphological changes in the SR (37). Nevertheless, additional studies need to be conducted in light of the presence of multiple obscurin isoforms and their diverse roles in distinct cell processes.

Obscurins in Non-Muscle Tissues

There has been little work examining the functions of obscurins in organs other than striated muscles. Obscurin transcripts have been observed at low levels in other tissues, however, including brain (54), liver, kidney, and pancreas (2). In zebra-fish, normal brain and retina express obscurins at the transcript and protein level, and morpholino-induced silencing of the expression of obscurins caused defects in retina differentiation and therefore eye development (20,21).

Our group recently described that giant obscurin proteins are expressed abundantly in breast, skin, and colon epithelial cell lines, where they localize to the plasma membrane and cell-cell contacts, cytosolic puncta that codistribute with the Golgi apparatus, and the nucleus (7) (Fig. 2B). Notably, the nucleus contains at least two unique forms of obscurin, not found in the cytoplasm, raising the possibility that they may impact cell cycle progression, DNA replication, or transcription; these exciting prospects merit further investigation.

We have recently begun to characterize the expression of obscurins in rodent non-muscle tissues and have shown that giant obscurins as well as smaller, yet uncharacterized forms, are present in the brain, kidney, liver, lung, spleen, and skin, where they exhibit membrane, cytosolic, and nuclear localization (our unpublished observations). Given the wide expression of obscurins in different organs and tissues and their diverse subcellular distributions, it is not surprising that they have been directly linked to an array of pathologies.

Obscurins in Disease

Genomic linkage analysis has revealed the presence of a G > A transition of nucleotide 13,031 of *OBSCN*, resulting in a mis-sense mutation within the protein (55). This Arg4344Gln mutation has been directly linked to the development of hypertrophic cardiomyopathy in humans. Interestingly, the Arg4344Gln substitution falls within Ig58, abrogating obscurins' binding to titin. Consistent with this, *in vitro* studies have demonstrated that mutant obscurins fail to incorporate into Z-disks (55).

OBSCN dysregulation may also contribute to symptoms associated with the development of myopathy. For instance, upon treatment of mouse myotubes with siRNA to dystrophin to mimic the molecular effects of Duchenne muscular dystrophy, the obscurin transcripts are reduced to half of control levels (56). In contrast, obscurin transcripts are upregulated during cardiac sarcomeric remodeling (42,57,58).

Recently, a number of solid tumors have been shown to possess mutant obscurins (59,60). The first modest evidence that mutant obscurins may be associated with tumor formation and progression came with the observation that the *OBSCN* gene is bisected within the first intron by a chromosomal translocation associated with the childhood kidney disease, Wilms' tumor (61). Although the possible contributions of the disrupted *OBSCN* gene were not pursued at the time, recent data suggesting that *OBSCN* mutations may contribute to the formation of several cancers underscores the importance of the Wilms' tumor connection.

During large-scale sequencing efforts aiming to determine consensus coding sequences that were mutated in breast and colorectal cancers, *OBSCN* was identified as one of only two genes (the other being *TP53*) which, when mutated, could drive the formation of both tumor types (59). In a follow-up study, *OBSCN* mutations were also detected in glioblastoma and melanoma (60). In addition, the relative expression levels of *OBSCN* and *PRUNE2* transcripts were sufficient to differentiate between two phenotypically similar cancers, leiomyosarcoma and gastrointestinal stromal tumors (62). This accumulated evidence led us to investigate the role of obscurins in cancer. We found that giant and smaller isoforms of obscurin are dramatically reduced in breast, skin, and colon cancer cells relative to their nonmalignant counterparts (7). Furthermore, RNAi-mediated knockdown of obscurins in nontumorigenic breast epithelial cells was sufficient to reduce apoptosis by 70% upon exposure to a DNA-damaging agent (7). Given the wide expression of obscurins in epithelial cells (our unpublished work), and their loss from different cancer epithelial cell lines, it seems likely that obscurins may have important roles in the formation and progression of different types of cancer.

In a testament to the diversity of cellular processes in which obscurins participate, a recent analysis of the genetics of aspirin exacerbated respiratory disease in Korean patients demonstrated that *OBSCN* single-nucleotide polymorphisms (SNP) may contribute to aspirin sensitivity in asthmatics (63). The authors postulated that altered SR architecture occurs in airway smooth muscle cells bearing variant *OBSCN* alleles, resulting in defective calcium signaling and broncho-constriction upon aspirin ingestion. Although the *OBSCN* SNPs have not been experimentally shown to affect SR structure or calcium flux, it is likely that linkage disequilibrium and transcriptomic analyses will continue to implicate the dysfunction of obscurins in various pathologies and syndromes. Therefore, it is imperative that these disease-associated obscurin variants be molecularly and functionally characterized, potentially with the end goal of developing novel, obscurin-targeting therapeutics.

Conclusions

Despite early evidence that obscurins are expressed in muscle exclusively, where they play key structural and regulatory roles, accumulating evidence indicates that they are expressed in an array of tissues, where they have distinct topographies and ligands, and are dysfunctional in multiple pathological conditions. In just the last 5 years, obscurins have been implicated in cancer formation, found to interact with the ubiquitin proteasome pathway, and suggested as targets for drug development (64). No longer "obscure," the obscurins have emerged as critical regulators of normal tissue function. Without question, the future will yield further exciting discoveries about obscurins as they continue to become more widely studied.

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FIG 1.

Domain architecture of the obscurin isoforms, illustrating their motifs and ligands. Binding partners and interaction sites identified in mammalian obscurins and *C. elegans* UNC-89 are shown in regular and italicized font, respectively.

FIG 2.

Localization of obscurins in muscle and epithelial cells. (A) In muscles, obscurins are found at sarcomeric M-bands (red), Z-disks (dark green), and I-bands (yellow). They are also localized to the sarcolemma (purple), the intercalated disk (blue), nuclei (light green), and the extracellular space (orange). (B) Obscurins in epithelial cells are found at the plasma membrane (purple), sites of cell–cell contact (blue), the Golgi apparatus (yellow), and within nuclei (light green).