The SH3 domain of UNC-89 (obscurin) interacts with paramyosin, a coiled-coil protein, in Caenorhabditis elegans muscle

Hiroshi Qadota, Emory University
Olga Mayans, University of Konstanz
Yohei Matsunaga, Emory University
Jonathan L. McMurry, Kennesaw State University
Kristy J. Wilson, Emory University
Grace E. Kwon, Emory University
Rachel Stanford, Emory University
Kevin Deehan, Emory University
Tina L. Tinley, Emory University
Verra M. Ngwa, Kennesaw State University

Only first 10 authors above; see publication for full author list.

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ABSTRACT UNC-89 is a giant polypeptide located at the sarcomeric M-line of Caenorhabditis elegans muscle. The human homologue is obscurin. To understand how UNC-89 is localized and functions, we have been identifying its binding partners. Screening a yeast two-hybrid library revealed that UNC-89 interacts with paramyosin. Paramyosin is an invertebrate-specific coiled-coil dimer protein that is homologous to the rod portion of myosin heavy chains and resides in thick filament cores. Minimally, this interaction requires UNC-89’s SH3 domain and residues 294–376 of paramyosin and has a $K_D$ of $\sim$1.1 $\mu$M. In unc-89 loss-of-function mutants that lack the SH3 domain, paramyosin is found in accumulations. When the SH3 domain is overexpressed, paramyosin is mislocalized. SH3 domains usually interact with a proline-rich consensus sequence, but the region of paramyosin that interacts with UNC-89’s SH3 is $\alpha$-helical and lacks prolines. Homology modeling of UNC-89’s SH3 suggests structural features that might be responsible for this interaction. The SH3-binding region of paramyosin contains a “skip residue,” which is likely to locally unwind the coiled-coil and perhaps contributes to the binding specificity.

INTRODUCTION To carry out their functions, most proteins interact with multiple other proteins in a time- and space-dependent manner. The quest to identify and understand the functions of interactors is most challenging when considering extraordinarily large polypeptides such as those found in striated muscle. The sarcomere, the unit of muscle contraction, consists of several hundred different proteins, among them being a number of giant polypeptides (>700,000 Da), which combine structural with signaling functions, and in some cases, provide muscle compliance through highly elastic regions. Among them are a number of giant polypeptides (Kontrogianni-Konstantopoulos et al., 2009). Much is known about the function of the largest of these polypeptides, vertebrate titin: its role in sarcomere assembly, muscle elasticity, protein turnover and quality control, and muscle-specific signaling pathways. Identification of titin’s binding partners has been one of the keys to understanding titin’s function. A nearly complete analysis identified >25 interacting proteins (Linke and Hamdani, 2014). Much less is known about the function and binding partners of the newest member of this family of proteins, UNC-89 of Caenorhabditis elegans and Drosophila, and its human homologue, obscurin (for a comparative overview, see Benian and Mayans, 2015). This type of protein was first discovered in the model genetic system C. elegans (Waterston et al., 1980; Benian et al., 1996). Loss-of-function unc-89 mutants have reduced locomotion, disorganized sarcomeres usually lacking M-lines, and disorganized myosin thick filaments (Waterston et al., 1980; Benian et al., 1999; Qadota et al., 2008a). unc-89 is a complex gene; through the use of three promoters and alternative splicing, at least eight major polypeptides are generated, ranging in size from 156,000 to 900,000 Da (Benian et al., 1996; Small et al., 2004; Ferrara et al., 2005). The largest of these
isofoms, UNC-89-B consists of 53 immunoglobulin (Ig) domains, two Fn3 domains, a triplet of SH3, DH, and PH domains near its N-terminus, and two protein kinase domains (PK1 and PK2) near its C-terminus. Antibodies generated to three different regions of UNC-89 localize the proteins to M-lines, structures in the middle of the sarcomere where thick filaments are cross-linked.

To learn how UNC-89 is localized and performs its functions, we are systematically identifying its binding partners. Our approach has been to screen a yeast two-hybrid library using as bait several-hundred-residue segments of UNC-89. We have obtained some significant biological insights. For example, near the N-terminus of UNC-89, Ig1-3 interacts with CPNA-1, a copine-domain-containing protein that is required not for the initial assembly of UNC-89 or myosin into sarcomeres but instead for their stable association once muscle activity begins in the embryo (Warner et al., 2013). Ig2-3 and Ig53-Fn2 interact with MEL-26, a substrate recognition protein for cullin 3 that acts as a scaffold for assembly of the ubiquitination machinery. Our studies (which include use of temperature-sensitive mutants) suggest that UNC-89 inhibits the MEL-26/CUL-3 complex from promoting the degradation of MEI-1 (katanin, which severs microtubules), and this is important for proper thick filament assembly or maintenance (Wilson et al., 2012b). A similar finding was made independently for mouse obscurin (UNC-89); degradation of small ankyrin 1.5 is dependent upon obscurin and is promoted by a cullin 3 substrate recognition protein, KCTD6 (Lange et al., 2012). Another intriguing result is that the two protein kinase domains of UNC-89 lining at its C-terminus interact with SCP-1, a CTD-type protein phosphatase (Qadota et al., 2008b). This was the first time that this class of phosphatase, well known to have roles in transcriptional regulation, was found to have a role in the sarcomere. Of interest, the phenotype of loss of function of unc-89 includes less ability to bend, and loss of function of scp-1 has greater ability to bend (Nahabedian et al., 2012). The idea that the protein kinase domains of UNC-89 interact with other enzymes and may act as signaling hubs was bolstered by finding that the Drosophila UNC-89 Kin1 kinase domain interacts with another kinase called Ball, and Ball is required for proper sarcomere assembly (Katzemich et al., 2015). We reported that both the PK1 region and the “interkinase region” interact with LIM-9 (Xiong et al., 2009), the closest nematoide homologue of human protein FHL (Lecroisey et al., 2013). lim-9(RNAi) shows disorganization of myosin thick filaments (Meissner et al., 2009). Similarly, obscurin has been reported to interact with FHL2 (Hu and Kontrogianni-Konstantopoulos, 2013). We also reported that the DH-PH region of UNC-89 activates RHO-1 (RhoA) specifically, and attenuated RNA interference for rho-1 results in disorganization of muscle thick filaments (Qadota et al., 2008a). A similar interaction between RhoA and obscurin was also demonstrated (Ford-Speelman et al., 2009). Early studies showed that the C-terminal region of some isoforms of vertebrate obscurin interact with the sarcoplasmic reticulum membrane proteins small ankyrin-1 isoform 5 (sAnk1.5) and ankyrin-2 (Ank2; Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003). The functional importance of this interaction is demonstrated by the obscurin-knockout mouse, in which longitudinal SR architecture is disrupted (Lange et al., 2009). The role of obscurin in linking the sarcomere to the SR may be conserved for UNC-89; UNC-89 is required for the proper organization of the ryanodine receptor and sarco/endo-sarcoplasmic reticulum Ca2+-ATPase, as well as for optimal calcium signaling (Spooner et al., 2012).

Here we report results of using the SH3-DH-PH-domain region of UNC-89 to screen a yeast two-hybrid library. We discovered that the SH3 domain interacts with UNC-15 (paramyosin), an invertebrate-specific thick filament protein with significant homology with myosin rods. We demonstrate that UNC-89’s SH3 domain interacts with an 82-residue segment of the mostly coiled-coil 873-residue-long paramyosin. unc-89 mutants lacking expression of giant isoforms that contain the SH3 domain show large aggregates of paramyosin. Overexpression of the SH3 domain results in mislocalization of paramyosin. Finally, we use homology modeling and bioinformatics analyses to explore the molecular features of the UNC-89 SH3 domain and its interaction with this α-helical segment of paramyosin. We speculate that although the SH3 domain of vertebrate obscurin is unlikely to interact with myosin, it may interact with another coiled-coil protein.

RESULTS
Identification of paramyosin as a binding partner of UNC-89
A segment of UNC-89 containing the SH3-DH-PH domains (Figure 1A) was used to screen a yeast two-hybrid library of C. elegans cDNAs. After screening of 442,000 colonies, 22 prey clones were found to be positive upon retransformation (Supplemental Table S2). Twenty of these clones represented portions of paramyosin. Paramyosin is an invertebrate-specific coiled-coil dimer that bears close resemblance to the rod domain of myosin (~40% identical in amino acid sequence) and is located in the cores of thick filaments (Cohen et al., 1971; Levine et al., 1976). Paramyosin is encoded by the unc-15 gene in C. elegans and is 873 residues long (Waterston et al., 1977; Kagawa et al., 1989). In C. elegans adult body-wall striated muscle, thick filaments are ~10 μm long and composed primarily of myosin heavy chain A (MHC A), myosin heavy chain B (MHC B), and paramyosin. Within the thick filament, these components are differentially localized. MHC A is located in the middle, whereas MHC B is in the outer or polar regions of the thick filament (Miller et al., 1983). MHC A and MHC B myosins and a portion of paramyosin are organized around a tubular core consisting of paramyosin and nematode-specific core proteins called filagenins in a specific geometry (Deitiker and Epstein, 1993; Epstein et al., 1995; Liu et al., 1998, 2000; Muller et al., 2001). As shown in Figure 1B, comparison of the spans of the prey clones indicates that the minimal portion of paramyosin required to interact with UNC-89 is 579 residues long (294–873).

As indicated in Figure 1C, various portions of the SH3-DH-PH region were used to further map the region of UNC-89 responsible for interaction with paramyosin by yeast two-hybrid assays. Results suggest that the SH3 domain is required for interaction with paramyosin. When a paramyosin clone containing residues 221–873 was used in two-hybrid assays against clones representing the entire largest UNC-89 isoform (isoform B), the only region with which UNC-89 was found to interact was the SH3-DH-PH region (unpublished data). This result further suggests the specificity of the interaction.

Minimally, the SH3 domain of UNC-89 interacts with UNC-15 (294–376)
To obtain additional and independent evidence for an UNC-89/paramyosin interaction, we used a biochemical assay with purified recombinant proteins. Paramyosin (221–873) was expressed as a maltose-binding protein (MBP) fusion, and portions of UNC-89− residues 1–500 containing the SH3-DH-PH region—were expressed as glutathione S-transferase (GST) fusions (Figure 2B). A far Western assay was conducted with MBP or MBP-paramyosin (221–873) on the blot and GST, GST-UNC-89 fusions in solution. As shown in Figure 2A, MBP-paramyosin, but not MBP, reacted with GST-a, GST-c, and GST-d but not GST-b. GST-b lacks the SH3 domain. We
concluded that UNC-89 can interact directly with paramyosin, and that the minimal region of UNC-89 that reacts with paramyosin is the SH3 domain (contained in GST-d, spanning UNC-89 (58–131)). We also noted that the apparent interaction was increased (shorter enhanced chemiluminescence [ECL] exposure time during the final step of the far Western assay) when the portion of UNC-89 that interacts with paramyosin is the SH3 domain. The indicated portions of SH3-DH-PH were used as bait to test for interaction with paramyosin (221–873) prey. +, growth, −, no growth of yeast on His− media.

Measurement of kinetics and affinity of binding
To further explore the interaction between the SH3 domain of UNC-89 (residues 58–131) and UNC-15 (221–873), we used biolayer interferometry (BLI; Abdiche et al., 2008; Concepcion et al., 2009). Much like surface plasmon resonance, BLI permits measurement of protein–protein interactions in real time. One protein, the ligand, is tethered to a fiber-optic sensor and then exposed to various concentrations of a second protein, the analyte, in solution. Binding is measured as a shift of the interference pattern of white light.
Parameters had low standard errors and tight 95% confidence intervals, indicating that fits are good; for example, the 95% confidence interval for $K_D$ is 0.96–1.14 μM.

**unc-89** mutants that fail to express **UNC-89** isoforms with the SH3 domain show aggregates of paramyosin

We reasoned that if **UNC-89** interacts with paramyosin in vivo, then in nematode mutants lacking the paramyosin-binding region of **UNC-89**, paramyosin should show an abnormal localization pattern. To examine this question, we determined the localization of paramyosin in two mutant alleles of **unc-89**: 1) **unc-89(su75)**, an **unc-89** mutant in which all the giant isoforms are missing (Small et al., 2004) and thus the paramyosin-binding SH3 domain is missing; and 2) **unc-89(tm752)**, an **unc-89** mutant in which all of the kinase-containing isoforms are missing but the giant isoforms containing the SH3 domain (UNC-89-A and -E) are present (Ferrara et al., 2005).

Indirect immunofluorescence was used to assess the localization of paramyosin and the two body-wall muscle cell myosin heavy chains MHC A and MHC B in wild type and **unc-89(su75)** and **unc-89(tm752)**. As shown in Figure 5A, **unc-89(su75)** has a disorganization of both MHC A and MHC B, whereas **unc-89(tm752)** has a disorganization of MHC A but normal organization of MHC B. Most importantly, however, large accumulations of paramyosin were found in **unc-89(su75)** but not in **unc-89(tm752)**. These accumulations of paramyosin were found in **unc-89(su75)** even in the muscle of L1-stage animals (Figure 5B). We conclude that when the $k_{on}$ (143 M$^{-1}$ s$^{-1}$). Parameters had low standard errors and tight 95% confidence intervals, indicating that fits are good; for example, the 95% confidence interval for $K_D$ is 0.96–1.14 μM.

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show accumulations of paramyosin. Therefore the interaction of UNC-89 with paramyosin is crucial for the organization (assembly or maintenance) of paramyosin into sarcomeres in body wall muscle.

To verify that mutations in unc-89(su75) and unc-89(r452) result in lack of expression of isoforms containing the SH3 domain and are not simply missing the epitopes (Ig3-6) for anti–UNC-89 antibodies.
unc-89 (su75)

UNC-98

UNC-15 (paramyosin)

UNC-98

UNC-15

CSN-5

UNC-15 (paramyosin)

CSN-5

FIGURE 7: Paramyosin accumulations in unc-89(su75) also contain UNC-98 and CSN-5. Each row shows images of several adult body wall muscle cells from unc-89(su75) coimmunostained with antibodies to paramyosin and either UNC-98 or CSN-5. Note that in the merged images, the white areas (overlap of magenta and green) show that the paramyosin accumulations also contain UNC-98 and CSN-5. Scale bar, 10 μm.

We wondered whether the paramyosin aggregates in unc-89(su75) might also contain these additional proteins. As shown in Figure 7, both UNC-98 and CSN-5 colocalize with paramyosin accumulations in unc-89(su75). Our interpretation is that when the paramyosin-binding portion of UNC-89 is missing, some paramyosin is not incorporated into thick filaments and instead accumulates, dragging with it its direct and indirect partners, UNC-96, UNC-98, and CSN-5.

Next we reasoned that since the proper localization of paramyosin depended on interaction with UNC-89 (Figure 6A), then perhaps the proper localization of UNC-89 would depend on paramyosin. unc-15(e73) and unc-15(e1215) are missense mutations of unc-15 (Gengyo-Ando and Kagawa, 1991) and contain multifa-

loss-of-function mutations in genes encoding two additional M-line components, UNC-98 and UNC-96, also result in abnormal accumulations of paramyosin (Mercer et al., 2006; Miller et al., 2008). UNC-98 is a 310-residue polypeptide without obvious human homologue and contains four C2H2 Zn-finger domains. These Zn-finger domains interact with UNC-97 (PINC), which is part of a conserved four-protein complex that interacts with the cytoplasmic tail of integrin (Mercer et al., 2003); the N-terminal 110 residues of UNC-98 interact with MHC A, which resides in the middle of the nema-

tode thick filament (Miller et al., 2006). UNC-96 is a 418-residue polypeptide without human homologue and contains no recognizable domains (Mercer et al., 2006). UNC-96 also interacts with MHC A (Qadota et al., 2007). UNC-98 and UNC-96 interact with each other, and each also interacts with paramyosin (Mercer et al., 2006; Miller et al., 2008). Paramyosin accumulations in unc-98 mutants contain UNC-96, and paramyosin accumulations in unc-96 contain UNC-98 (Mercer et al., 2006). Moreover, the para-

myosin accumulations of unc-98 and unc-96 mutants contain CSN-5, a highly conserved component of the COP9 signalosome, which is implicated in regulating ubiquitin-mediated proteolysis and is located at A-bands (Miller et al., 2009). CSN-5 interacts directly with UNC-98 and UNC-96. Paramyosin interacts directly with UNC-96 and UNC-98; UNC-96 interacts with UNC-98.

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heat shock promoter in adults with either hemagglutinin (HA) or enhanced green fluorescent protein (EGFP) N-terminal tags. After heat shock at 30°C for 2 h, we made protein lysates and were able to detect either tagged protein by Western blot. After repeating this heat shock procedure, we incubated the worms at 20°C (their normal growing temperature) for 24 h to allow for sarcomere assembly in the presence of the SH3 domain. After fixation and immunostaining with anti-paramyosin and either anti-HA or anti-EGFP, we found that paramyosin was normally localized, and we were not able to detect any HA-SH3 or EGFP-SH3 in the myofilament lattice. We suspected that HA-SH3 or EGFP-SH3 was not being incorporated into the sarcomere and consequently degraded. We next considered whether we might have a better result if we were able to direct the localization of the overexpressed SH3 domain by attaching it to a protein that localizes to the native UNC-89 site, that is, the M-line. We reasoned that a good candidate for this would be UNC-89 itself. We already knew that the small isoforms of UNC-89, UNC-89-C and UNC-89-D, which consist of partial PK1-interkinase-Ig-Fn-PK2, localize to M-lines (Small et al., 2004). We expressed portions of this region (with HA tags) in transgenic nematodes to determine the minimum portion that is required to localize to M-lines. We heat shocked at 30°C for 2 h and then fixed and stained with anti-HA. As summarized in Figure 9A, neither Fn-Ig-PK1 nor Ig-Fn-PK2 localized in the sarcomere. However, adding the C-terminal one-third of the interkinase region N-terminal of Ig-Fn-PK2 as the segment “1/3IK-Ig-Fn-PK2” did allow it to localize to both M-lines and dense bodies (Figure 9, A and B, bottom row). Although we have never seen localization of UNC-89 to dense bodies using antibodies developed to three different regions of UNC-89 (Small et al., 2004), we have encountered examples of other proteins that show additional sites of localization when examined using transgenic overexpression. Examples include UNC-98 (Mercer et al., 2003) and UNC-96 (Mercer et al., 2006). In the case of UNC-96, antibodies localize only to M-lines, but when overexpressed with a GFP tag, they localize to M-lines and dense bodies (Mercer et al., 2006).

Overexpression of UNC-89’s SH3 domain results in mislocalization of paramyosin

We heat-shock expressed an artificial small UNC-89 molecule consisting of SH3-1/3IK-Ig-Fn-PK2 to direct SH3 to the M-line (again, we used UNC-89 residues 1–151, which contain the SH3 domain and flanking sequences.) As shown in Figure 9C, this chimeric protein also localizes to M-lines and dense bodies (after a 2-h heat shock and immediate fixation). However, when this chimeric protein was expressed with a 2-h heat shock followed by 24 h at 20°C, we did not detect a change in the localization of paramyosin (Supplemental Figure S3). In fact, after this protocol, we could not detect the chimeric protein by Western blot (Supplemental Figure S3A). Suspecting that if the protein was expressed it might be unstable, we heat shocked at 30°C for 2 h and then moved the worms to 20°C and made protein lysates from them immediately and at 2, 4, 6, 8, and 24 h later. As shown by Western blot (Supplemental Figure S3B), the protein levels decreased with time after heat shock and were undetectable at 24 h. Therefore we changed our heat shock protocol to a 30°C exposure continuously for 24 h. With this procedure, we found that both HA-1/3IK-Ig-Fn-PK2 and HA-SH3-1/3IK-Ig-Fn-PK2 were localized to M-lines and dense bodies and, in some muscle cells, just M-lines (Figure 10A, top). Transgenic lines expressing each of these chimeric proteins were immunostained with anti-paramyosin and anti-UNC-95 (a protein that localizes to the bases of both M-lines and dense bodies). As shown in Figure 10A, middle, neither heat shock on wild-type animals (“no array”) nor overexpression of HA-1/3IK-Ig-Fn-PK2 in transgenic animals affects the normal localization of paramyosin to A-bands. However, overexpression of HA-SH3-1/3IK-Ig-Fn-PK2 in transgenic animals results in mislocalization of paramyosin to M-lines and dense bodies. As shown in Figure 10B at higher magnification, overexpressed HA-SH3-1/3IK-Ig-Fn-PK2 clearly results in paramyosin being abnormally localized to M-lines (arrows) and dense bodies (arrowheads). The effect of overexpressing HA-SH3-1/3IK-Ig-Fn-PK2 is specific: the localization of UNC-95 is unaffected (Figure 10A, bottom, rightmost column), as are the localizations of the thick filament components MHC A, MHC B, and twitchin (Supplemental Figure S4). Therefore this experiment provides additional evidence that there is specific interaction between UNC-89’s SH3 domain with paramyosin in vivo.

Bioinformatics insight into the interaction between the SH3 domain of UNC-89 and paramyosin

The interaction of UNC-89’s SH3 domain with paramyosin was unexpected, given that SH3 domains typically interact with proline-rich sequences. Paramyosin is almost entirely α-helical, with only three proline residues, all in the nonhelical, 30-residue-long N-terminus, which is not required for binding. The SH3 fold consists of a five-stranded β-barrel comprising three prominent loops (termed RT, n-Src, and distal loops) and a β12-helix turn (Figure 10A, top). The

**FIGURE 8:** Paramyosin accumulations in unc-89(su75) and in two paramyosin missense mutants. Paramyosin paracrystals in unc-15(e73) and unc-15(e1215) show only partial colocalization with UNC-89. Each row contains images of the same portion of body wall muscle from the indicated nematode strains costained with anti-UNC-89 (green) and anti-UNC-15 (magenta); colocalization is indicated by white. A wider field view of the anti-paramyosin staining of these mutants is shown in Supplemental Figure S2. Scale bar, 10 μm.
peptide-binding groove is located between the RT loop and the n-Src loop/310-turn. The groove is mostly hydrophobic and consists of three shallow pockets defined by conserved aromatic residues (Figure 11A, gray). The peptide ligand is typically proline rich, commonly containing a PxxP motif in extended, left-handed polyproline-2 conformation. Generally, proline residues in the peptide ligand are immediately preceded by hydrophobic residues forming two ΦP pairs in register (ΦPΦP). Each ΦP dipeptide is accommodated in a hydrophobic pocket of the SH3. The third pocket includes arginine that protrudes from the binding groove. A BLAST analysis revealed that lysine is also found in this position across sequences from vertebrates, insects, and nematodes, although nematodes often contain a serine instead (asparagine is only rarely observed). The “specificity” glutamate in loop RT (blue in Figure 11A) is identifiable only in the fly, possibly having shifted positions in the human and worm domains. This variability in the PxxP binding groove might point to a nonconsensus ligand sequence for this site or, potentially, a lack of binding functionality.

FIGURE 9: A small portion of UNC-89, even with an added SH3 domain, can localize to sarcomeres. (A) Top, schematic of domains within the C-terminal portion of UNC-89. Below, portions tested with HA tags in transgenic animals for localization to the sarcomere. HA-1/3IK-Ig-Fn-PK2 localized to M-lines and dense bodies. (B) Localization of transgenically expressed portions of UNC-89. Each row shows co-staining with anti-HA to localize the fragment and anti-UNC-95 to mark M-lines and dense bodies. As shown, HA-Ig-Fn-PK2 does not localize, but HA-1/3IK-Ig-Fn-PK2 does. (C) Localization of transgenically expressed chimeric protein HA-SH3-1/3IK-Ig-Fn-PK2. Note that inclusion of the SH3 domain still permits 1/3IK-Ig-Fn-PK2 to localize to M-lines and dense bodies. Arrows, M-lines; arrowheads, dense bodies. Scale bar, 10 μm.
The sequences of the rod portion of myosin heavy chains and paramyosin share very high conservation and both consist of heptad repeats that induce their folding into coiled-coils. Simultaneously, the sequences have a longer, 28-residue repeat (38 copies) that results in charged residues being displayed on the surface of the coiled-coil in a periodic manner. This charge distribution directs the staggered parallel assembly of myosin rods into a thick filament (McLachlan and Karn, 1982; Kagawa et al., 1989). There are four widely spaced “skip residues” in the rod portion of myosin, which introduce a discontinuity in the phasing of the heptads that is believed to result in the deformation of the coiled-coil. Each skip is located at the end of a 28-residue repeat following position c of a heptad. The crystal structure of regions of human cardiac myosin containing each of the skip residues confirmed that the skips result in local relaxation of the coiled-coil (Taylor et al., 2015). The biological significance of each skip residue was tested by introducing tagged myosin into cardiomyocytes in which each of the skip residues had been deleted; deletion of skip 3 resulted in misincorporation by the presence of myosin aggregates; deletion of skip 4 resulted in lack of antiparallel assembly of myosin rods in the bare zone. Of interest, deletion of skip 1 or skip 2 had no obvious phenotype, and these were speculated to play other, as-yet-unidentified sarcomeric roles (Taylor et al., 2015). The SH3-binding segment of paramyosin residues 294–376 shares high similarity (64%) with the region of myosin MYH7 containing skip 2 (Figure 11D). The glutamate residue that causes this skip in MYH7 (E1385) is also present in UNC-15 (E312; Figure 11D). Our assignment of E312 as skip 2 in UNC-15 is in agreement with an earlier analysis (Kagawa et al., 1989). It is conceivable that UNC-89’s SH3 might specifically recognize the quaternary structure of UNC-15 at this locus due to the unwinding distortion that this skip introduces in its coiled-coil.

DISCUSSION

Our results demonstrate that paramyosin is a new binding partner for the giant muscle polypeptide UNC-89 in C. elegans muscle. The UNC-89/paramyosin interaction was initially discovered by screening a yeast two-hybrid library with the SH3-DH-PH region of UNC-89 as bait. In vitro binding assays using purified recombinant proteins showed that minimally this interaction requires the SH3 domain (residues 58–131) of UNC-89 and residues 294–376 of paramyosin (Figure 12A). Biolayer interferometry showed that the two protein segments bind with $K_D = 1.1 \mu M$ with a relatively slow off-rate. Evidence that the UNC-89/paramyosin interaction occurs in vivo includes both mutant and transgenic analysis.
Paramyosin forms accumulations or aggregates in unc-89 mutants that lack expression of the large SH3 domain–containing isoforms; these paramyosin aggregates are not found in normal muscle or unc-89 mutants that do express the SH3 domain–containing isoforms. Paramyosin accumulations can be found in as early as L1 larvae. These results suggest that the interaction of UNC-89 with paramyosin is important during sarcomere assembly (during the L1 stage) and perhaps during sarcomere maintenance via turnover, as there is no new sarcomere assembly during the adult stage (Mackenzie et al., 1978).

Paramyosin accumulations in unc-89 mutants were also shown to contain a trio of interacting proteins found previously in paramyosin accumulations in unc-98 and unc-96 mutants: UNC-96 and UNC-98, two nematode-specific M-line proteins, and CSN-5, a highly conserved component of COP9 signalosomes, which regulates ubiquitin-mediated proteolysis. After identifying a minimal region of UNC-89 that can localize to M-lines and dense bodies when expressed in transgenic animals, we used it to direct the localization of the SH3 domain to these structures. This chimeric artificial protein (SH3-minimal localizing fragment) is able to direct paramyosin to M-lines and dense bodies, providing further evidence that the SH3 domain of UNC-89 can interact with paramyosin in vivo. The interaction of UNC-89’s SH3 domain with paramyosin was surprising, given that SH3 domains usually interact with proline-rich sequences that are absent in coiled-coil proteins like paramyosin. To provide some insight into how UNC-89’s SH3 domain binds to paramyosin, we made a homology model of UNC-89’s SH3 domain from both C. elegans and Drosophila. The models suggested a somewhat degenerated PxxP binding groove, which may suggest lack of binding ability to this type of sequence, as well as conservation of exposed surfaces of strands β2 and β3, which may be significant for binding properties of UNC-89’s SH3 domain.

**FIGURE 11:** Predicted molecular features of UNC-89-SH3 and UNC-15(294-376). (A) Sequence alignment of SH3 domains of known structure most similar to UNC-89-SH3. PDB codes are given; 1v1c is from human obscurin. Hydrophobic residues (gray) and negatively charged residue (blue) classically found in the PxxP binding groove are highlighted. SH3, known to bind helical peptides in their convex surface, are in red; 1m5z is Pex13p-SH3, and 1ng2 is p47phox-SH3. Conserved groups across UNC-89-SH3 from human, nematode, and fly are boxed. Conserved residues that are potential mediators of interactions at the convex face are in yellow. (B) Two perpendicular views of the crystal structures of Pex13p-SH3 (1m5z; blue) and p47phox-SH3 (1ng2; orange). Pex13p-SH3 is in complex with a canonical PxxP-containing peptide from Pex14p (red); the binding site of the helical peptide from Pex15p is magenta (strand β2). p47phox-SH3 contains the SH3-surrounding inhibitory sequence. (C) Left, NMR structure of human obscurin’s SH3 (PDB 1V1C) displaying residues in the PxxP binding region. Right, 3D models of the SH3 domains from UNC-89 from C. elegans (blue) and D. melanogaster (green) superimposed on human obscurin’s SH3. The conserved charged residues in the convex region are shown. (D) Alignment of skip-2–containing sequences from UNC-15 and human cardiac myosin hMYH7 (E312 is marked and sequence identity highlighted). (E) A 3D model of UNC-15 showing the skip 2 residue, E312.
paramyosin, as this region has been shown to bind α-helical motifs in Pex13p SH3 and p47phox. We also found that the region of paramyosin that binds to UNC-89’s SH3 contains a skip residue and has strong homology (and conservation of the skip-causing glutamate) to skip 2 of human cardiac myosin MYH7, which was shown recently by crystallography (Taylor et al., 2015) to result in relaxation of the coiled-coil. We speculate that UNC-89’s SH3 domain might recognize and bind to the surface structure created by the unwinding distortion of the coiled-coil.

Of interest, the novel function of UNC-89’s SH3 domain is a second example in which UNC-89 likely uses “classical” domains for “nonclassical” functions. PH domains usually bind to acid phospholipids and function in membrane targeting. However, the PH domain of UNC-89 does not bind to inositol-1,4,5-triphosphate, and its NMR structure shows that it has a closed conformation, and its presumed phospholipid-binding pocket is lined with negative charges, which would make it incapable of binding to acidic phospholipids (Blomberg et al., 2000). Although Blomberg et al. (2000) speculated that UNC-89’s PH domain might alternatively function in protein–protein interactions, such a role has not yet been found by experiment.

The optical biosensing experiments indicated fast on-rate and a slow off-rate between UNC-89’s SH3 and the segment of paramyosin. This is compatible with the robust signals we obtained using far Western assays. The measured $K_D = 1.1 \mu M$ of the interaction is also compatible with reports of the binding of the myosin rod or paramyosin with several other proteins. For example, the mammalian protein myosin-binding protein C binds through its C-terminal Ig domain to myosin, and, when measured for binding by cosedimentation to light meromyosin (LMM) filaments, had $K_D = 3.5 \mu M$ (Flashman et al., 2007). We reported that for C. elegans muscle, the N-terminal portion of the M-line protein UNC-98 binds to purified myosin by enzyme-linked immunosorbent assay (ELISA) with $K_D = 0.30 \mu M$ (Miller et al., 2006). In contrast, we also reported that full-length UNC-98 and UNC-96 bind to fragments of paramyosin by ELISA with higher affinity—56 and 49 nM, respectively (Miller et al., 2008).

Based on gradual solubilization of purified thick filaments with buffers of increasing salt concentrations (Epstein et al., 1985), the model for the nematode thick filament is that it is a series of concentric layers—an outer layer consisting of myosins MHC A and B, an intermediate layer of paramyosin, and an inner layer of paramyosin and the filagenins (Deitkiker and Epstein, 1993; Liu et al., 1998; Figure 12B). Analysis of electron microscope (EM) images of isolated cores indicated that the core consists of seven subfilaments of paramyosin coupled to form a tube by the filagenins (Epstein et al., 1995). In vertebrate muscles, the M-line is observable by EM to be a structure in which the shafts of thick filaments are cross-linked at their surfaces by a series of struts and additional filaments (Knappes and Carlsen, 1968; Luther and Squire, 1978). Presumably, similar cross-linking of thick filaments occurs in C. elegans. UNC-89 is localized exclusively to the M-line, from its base, where it is anchored at the muscle cell membrane, continuing throughout the depth of the myofilament lattice (Warner et al., 2013; Reed, Hoppe, and Benian, unpublished data). Because paramyosin seems buried under an outer layer of the myosins, it is difficult to understand how interaction of UNC-89 with paramyosin can occur at the M-line. Both myosin rods and paramyosin dimers have alternating zones of positive and negative charges along their surface that support their assembly into filaments primarily by electrostatic interactions (McLachlan and Karn, 1982; Kagawa et al., 1989). It is possible that the distortions introduced by skip residues in the helical rod bundles of paramyosin and myosin are such that these proteins do not form tight smooth layers but that bulges and/or crevices exist in their surface topography. These discontinuities would permit access of UNC-89’s SH3 domain to the otherwise internal paramyosin layer (Figure 12C). Not mutually exclusive with this possibility is that during sarcromere assembly, paramyosin and UNC-89 associate before they are incorporated into thick filaments and M-lines. Consistent with this idea, during embryonic muscle development, paramyosin can be detected in 420-min embryos (Epstein et al., 1993), during which time
thick filament precursors are being assembled, and this is the same time at which staining with MH42, the monoclonal later shown to react with UNC-89 (Benian et al., 1996), is first detected (Hresko et al., 1994). Although additional sarcomers are not being added in adult muscle, presumably there is removal of damaged proteins and their replacement with newly synthesized ones. One way to interpret the paramyosin accumulations found in unc-89(su75) and unc-89(r452) is that in the absence of UNC-89, which is capable of binding to paramyosin, paramyosin forms abnormal aggregates, and this reflects a paramyosin pool that was otherwise destined to form replacement thick filaments.

A related question is that, if UNC-89 interacts with paramyosin that is distributed throughout the long axis of thick filaments, how is UNC-89 restricted to M-lines? The answer likely arises from other binding partners of UNC-89 that emanate from the cell membrane base of the M-lines, which begins with the transmembrane protein integrin. The cytoplasmic tail of integrin exists in a complex with UNC-112 (kindlin), PAT-4 (ILK), PAT-6 (α-parvin), and UNC-97 (PINCH; Qadota and Benian, 2010). UNC-97 interacts with LIM-9 (FHL), which in turn interacts with UNC-89 via its Ig52-Fn1-PK1 and 1/3 interkinase region (Xiong et al., 2009). PAT-6 (ε-parvin) interacts with CPNA-1, which in turn interacts with UNC-89 via its Ig1-3 domains (Warner et al., 2013).

Given that Drosophila thick filaments also contain paramyosin, we suggest that Drosophila UNC-89 (Small et al., 2004; Katzemich et al., 2015) might also associate with paramyosin through its SH3 domain. Although vertebrate muscle does not contain paramyosin, perhaps it is possible for the SH3 domain of vertebrate obscurin to interact with myosin. An obscurin–myosin interaction seems unlikely, however, for the following reasons. In obscurin-A isoforms (Fukuzawa et al., 2005), there are up to 55 Ig and Fn3 domains separating the M-band targeting N-terminal Ig1-3 (via titin–myomesin interactions; Fukuzawa et al., 2008) from the obscurin SH3 domain, and the C-terminus of obscurin-A interacts with small ankyrin 1 at the sarcoplasmic reticulum (Bagnato et al., 2003; Kontrogianni-Kostantinopoulou et al., 2003). Myosin and the SH3 are therefore separated by ~200 nm, and a direct interaction seems unlikely. Nevertheless, it is possible that obscurin’s SH3 domain interacts with another coiled-coiled protein.

### MATERIALS AND METHODS

#### Screening of yeast two-hybrid library

Yeast two-hybrid screening of the C. elegans cDNA library RB2 was performed as described (Miller et al., 2006) with some modifications. We first tried to screen the library with this previous protocol using the bait plasmid pGBDU-UNC-89 SH3-DH-PH (plasmid described in Warner et al., 2013). However, after shuffling off the bait plasmid, the yeast host strain harvesting only prey plasmids did not grow well in selective media, meaning that isolation of prey plasmids from yeast cells was practically impossible. We changed the bait plasmid to pGBKTKT-UNC-89 SH3-DH-PH, a bait plasmid with kanamycin resistance as the bacterial marker (pGBKTK7; Takara Clontech, Mountain View, CA). To make pGBKTK7-UNC-89 SH3-DH-PH, we cloned the Ncol/Xho fragment insert from pGBDU-UNC-89 SH3-DH-PH into Ncol/Sal-digested pGBKTK7. Because of the kanamycin selection for the bait, it was not necessary to remove the bait plasmid from the yeast cells of the two-hybrid positive clones in order to grow and isolate the ampicillin-resistant prey plasmids.

#### Domain mapping by yeast two-hybrid assay

Twenty prey clones containing various UNC-15 regions were originally isolated from cDNA library screening. UNC-89 bait fragments lacking SH3, DH, or PH domain were amplified by two-step PCR using GK1-8 primers (Supplemental Table S1) and confirmed by DNA sequencing. Using those prey and bait clones, we detected interaction by our standard yeast two-hybrid method (Miller et al., 2006).

#### Domain mapping by far Western assay

Segments of paramyosin spanning residues 221–873, 221–446, 294–446, 294–376, and 365–446 were expressed as an MBP fusion protein in Escherichia coli. cDNAs encoding UNC-15–corresponding regions were PCR amplified using primers GK10-19 (Supplemental Table S1), and its sequence was confirmed to be error-free. Plasmids expressing MBP with UNC-15 (1–693, 1–446, 437–709, 699–873) were described previously (Miller et al., 2008). Portions of UNC-89 (1–500, 1–70/125–500, 1–151, 58–131) were cloned into pGEX-KK-1 for expression of the corresponding GST fusion proteins, beginning with cDNA PCR fragments generated using primers (GK1, 2, 5, 6, 9, 20, 21) indicated in Supplemental Table S1. MBP, GST, and fusion proteins were purified as described previously (Benian et al., 1993; Mercer et al., 2006). Supplemental Figure S5 shows SDS–PAGE of 2 μg each of the 15 expressed recombinant proteins. Far Western assays were conducted as follows: 2 μg of MBP and 4 μg of MBP-UNC-15 (221–873) or 2 μg of MBP-UNC-15 (1–693, 1–446, 437–709, 699–873, 221–446, 294–446, 221–373, 294–376, 365–446) were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and blocked overnight at room temperature in 5% milk and Tris-buffered saline/Tween 20 (TBS-T). Portions of this blot containing MBP and MBP-UNC-15 were separately incubated with 1 μg/ml GST or GST-UNC-15 (1–500, 1–70/125–500, 1–151, 58–131) at room temperature for 1 h, washed multiple times in TBS-T, reacted with anti-GST conjugated to horseradish peroxidase (at 1:3000 dilution; A7340; Sigma-Aldrich, St. Louis, MO), washed, and then reactions visualized by enhanced chemiluminescence (ECL; Pierce, Thermo Fisher Scientific, Waltham, MA).

#### Biolayer interferometry

BLI experiments were performed using a FortéBio (Menlo Park, CA) Octet QK using anti-GST sensors. Assays were done in 96-well plates at 25°C. We used 200-μl volumes in each well. Ligand GST-SH3 (UNC-89 (58–131)) was loaded onto sensors for 600 s and followed by baseline measurements in binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol) for 300 s. Association was measured by dipping sensors into solutions of analyte protein and was followed by moving sensors to buffer only to monitor dissociation. For qualitative experiments, all analyte concentrations were 7.2 μM. A serial dilution of MBP-UNC-15 (294–376) was made for full characterization by our standard yeast two-hybrid method (Miller et al., 2006).

#### C. elegans strains

Standard growth conditions for C. elegans were used (Brenner, 1974). Wild-type nematodes were the N2 (Bristol) strain. Strain HE75 unc-89(su75) was provided by Henry Epstein (University of Texas Medical Branch at Galveston, Galveston, TX). unc-89(tm752) was obtained from Shohei Mitani (Tokyo Women’s Medical University School of Medicine, Tokyo, Japan). CB1460 unc-89(e1460) was obtained from Robert Waterston (University of Washington, Seattle, WA). Strain TR625 unc-89(r452) was obtained from Phil Anderson (University of Wisconsin, Madison, WI). These unc-89 mutants were outcrossed to wild type three or four times. The unc-15 alleles e73 and e1215, and N2 (Bristol) were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN).
Immunolocalization in adult body-wall muscle

Adult and L1 larva nematodes were fixed and immunostained according to the method described by Nonet et al. (1993) and described in further detail by Wilson et al. (2012a). The following primary antibodies were used at 1:200 dilution: anti-UNC-89 (rabbit polyclonal EU30; Benian et al., 1996), anti-paraphysin (mouse monoclonal 5-23; Miller et al., 1983), anti–MHC A (mouse monoclonal 5-6; Miller et al., 1983), anti–MHC B (mouse monoclonal 5-8; Miller et al., 1983), anti–UNC-95 (rabbit polyclonal Benian-13; Qadota et al., 2007), anti-twitchin (rabbit polyclonal I II II; Benian et al., 1996), and anti-HA (mouse monoclonal; H3663; Sigma-Aldrich). The following antibodies were used at 1:100 dilution: anti–UNC-98 (rabbit polyclonal EU131; Mercer et al., 2003), and anti–CSN-5 (rabbit polyclonal; Miller et al., 2009). Secondary antibodies, also used at 1:200 dilution, included anti-rabbit Alexa 488 (Invitrogen, Thermo Fisher Scientific) and anti-mouse Alexa 594 (Invitrogen). Images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope and an Apochromat ×63/1.4 numerical aperture oil immersion objective in ×2.5 zoom mode. The color balances of the images were adjusted by using Photoshop (Adobe, San Jose, CA).

Western blots

We used the procedure of Hannak et al. (2002) to prepare total protein lysates from nematodes. Western blots to detect the large isoforms of UNC-89 were carried out on wild type and the unc-89 alleles su75, r452, e1460, and tm752. Briefly, equal amounts of total protein from these strains were separated by 4% polyacrylamide–SDS Laemmli gels, transferred to nitrocellulose membrane, and reacted with affinity-purified rabbit EU30 anti–UNC-89 antibody (generated at Ig3-6) at 1:500 dilution. As a loading control, equal amounts of total protein from these strains were separated by 10% SDS–PAGE, transferred to nitrocellulose membrane, and reacted with the mouse monoclonal MH4 antibody at 1:100, which detects an intermediate filament protein expressed in the hypodermis (Francis and Waterston, 1985). To detect expression of HA-SH3-1/3IK-Ig-Fn-PK2, Hannak extracts were separated by 10% SDS–PAGE, transferred to membrane, and reacted with mouse monoclonal to HA (H3663; Sigma-Aldrich) at 1:200 dilution.

Determination of mutation sites in unc-89(su75) and unc-89(r452)

Genomic DNA from each strain was prepared and provided to the Emory Integrated Genomics Core (EIGC) for illumina sequencing. The Genomic Services Laboratory at the HudsonAlpha Institute for Biotechnology (Huntsville, AL) performed quality control and constructed standard illumina sequencing libraries following the manufacturer’s instructions. Sequencing was performed on an Illumina HiSeq version 3 with 100-base pair paired-end reads. Raw data were returned to the EIGC for bioinformatics analysis. Raw reads were mapped against the C. elegans reference genome (WS190/cce6) with the PEMapper software package. PEMapper maps short reads to a reference genome by first decomposing those reads into k-mers (16-mers in this case) and then performing a hash-based “rough mapping” of each read. After roughly mapping the read, the fine-scale position of the read was determined by a local Smith–Waterman alignment. Genotypes were determined using PECaller. Intuitively, we envision the six channels of data (number of A, C, G, T, deletion, and insertion reads) as being multinomially sampled, with some probability of drawing a read from each of the channels, but the probability varies from experiment to experiment and is drawn from a Dirichlet distribution. PECaller combines data across samples in order to identify the genotype with the highest likelihood at each sequenced base. Both PEMapper and PECaller are part of a custom software package developed at Emory University for mapping and identifying variant sites from illumina raw sequencing data (D. J. Cutler and M. E. Zwick, personal communication). Our analysis then focused on the ~60-kb unc-89 gene. PECaller identified a single homoygous mutation in the coding sequence in each sequenced mutant strain.

Construction and expression of UNC-89 fragments in transgenic nematodes

HA-tagged UNC-89 fragments were expressed under the control of two different types of heat shock promoters (pPD49.78 and pPD49.83). UNC-89 1/3IK-Ig-Fn-PK2, UNC-89 SH3-1/3IK-Ig-Fn-PK2, and UNC-89 SH3 were amplified by PCR using GK22-26 (Supplemental Table S1) and cloned into the HA-tagged vector, pKS-HA(Nhex2), and their DNA sequences were confirmed. UNC-89 SH3 was also cloned into pEGFP-C1 vector. Nhel-digested fragments of HA-UNC-89 Fn-Ig-PK1 (Qadota et al., 2008b), HA-UNC-89 Ig-Fn-PK2 (Qadota et al., 2008b), HA-UNC-89 1/3IK-Ig-Fn-PK2, HA-UNC-89 SH3-1/3IK-Ig-Fn-PK2, and EGFP-UNC-89 SH3 were cloned into Nhel-digested pPD49.78 and pPD49.83, resulting in pPD49.78/83-HA-UNC-89 Fn-Ig-PK1, pPD49.78/83-HA-UNC-89 Ig-Fn-PK2, pPD49.78/83-HA-UNC-89 1/3IK-Ig-PK2, pPD49.78/83-HA-UNC-89 SH3-1/3IK-Ig-Fn-PK2, pPD49.78/83-HA-UNC-89 SH3, and pPD49.78/83-EGFP-UNC-89 SH3. pPD49.78/83-HA-UNC-89 (Fn-Ig-PK1, Ig-Fn-PK2, 1/3IK-Ig-Fn-PK2, SH3-1/3IK-Ig-Fn-PK2, SH3) were mixed with pTG96 (SUR-5::NLS::GFP) as a transformation marker (Yochem et al., 1998) and injected into wild-type N2 worms. pPD49.78/83-EGFP-UNC-89 SH3 were mixed with pRF4 (rol-6 dominant) as a transformation marker (Mello et al., 1991) and injected into wild-type N2 worms. Transgenic lines with extrachromosomal arrays containing pPD49.78/83-HA-UNC-89 Fn-Ig-PK1, HA-UNC-89 Ig-Fn-PK2 (called sfEx56), HA-UNC-89 1/3IK-Ig-Fn-PK2 (called sfEx52), HA-UNC-89 SH3-1/3IK-Ig-Fn-PK2 (called sfEx51), HA-UNC-89 SH3, and pTG96 were established by picking up GFP-positive worms using a GFP dissection microscope. Transgenic lines with extrachromosomal arrays containing pPD49.78/83-EGFP-UNC-89 SH3 and pRF4 were established by picking up ROL worms.

Integration of extrachromosomal arrays

The extrachromosomal arrays containing pPD49.78/83-HA-UNC-89 (1/3IK-Ig-Fn-PK2 and SH3-1/3IK-Ig-Fn-PK2) and pTG96 were integrated into the genome by ultraviolet irradiation (Mitani, 1995) with some modifications (P. Barrett, personal communication). The resulting integrated nematode lines are called sfIs11, for HA-UNC-89 SH3-1/3IK-Ig-Fn-PK2 and sfIs13, for HA-UNC-89 1/3IK-Ig-Fn-PK2.

Overexpression experiments

To examine the localization of HA-tagged UNC-89 fragments (Figure 8), expression of the HA-tagged UNC-89 proteins was induced by incubation of the transgenic worms at 30°C for 2 h (heat shock). Heat-shocked transgenic worms were fixed using the method described previously (Nonet et al., 1993) and stained by anti-GFP to verify the existence of extrachromosomal array (at 1:200 dilution; Life Technologies, Thermo Fisher Scientific; also used at 1:200 dilution, included anti-rabbit Alexa 488 (Invitrogen, Thermo Fisher Scientific) and anti-mouse Alexa 594 (Invitrogen). Images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope and an Apochromat ×63/1.4 numerical aperture oil immersion objective in ×2.5 zoom mode. The color balances of the images were adjusted by using Photoshop (Adobe, San Jose, CA).

Molecular Biology of the Cell
worms were exposed at 30°C for 24 h. Worms were fixed using the method described previously (Nonet et al., 1993) and stained by anti-paramyosin (to examine the localization of paramyosin), anti-UNC-95 (to identify dense bodies and M-lines in muscle cells), anti-HA (to determine the localization of HA-tagged UNC-89 fragments), and anti-MHC A, anti-MHC B, and anti-twitchin (to determine any effects on these thick filament proteins). We prepared worm lysates from transgenic worms with or without heat shock and examined the expression of HA-tagged UNC-89 proteins by Western blot, reacting with anti-HA (1:200 dilution; H3663; Sigma-Aldrich).

Sequence analysis and homology modeling of SH3 domains and paramyosin

Various 3D models of the SH3 domains of UNC-89 from C. elegans and D. melanogaster were calculated using the Phyre2 (Kelley et al., 2015) and ModWeb v.r166 (https://modbase.compbio.ucsf.edu/modweb) servers, as well as a local installation of MODELLER v9.15 (https://salilab.org/modeller/). The latter used as template various combinations of SH3 domain structures (listed in Figure 11A), with sequence similarity being identified and evaluated using BLAST (http://blast.ncbi.nlm.nih.gov/) against the Protein Data Bank (www.pdb.org). Visual inspection of 3D models was in PyMOL v1.7.4 (www.pymol.org), which revealed good consensus in the modeling of the β-barrel but typical variability in the loop regions. We calculated 3D models of UNC-15(294–376) using Phyre2 with PDB ID 4XA3 as template, as well as by manual mutagenesis in silico in PyMOL.

General sequence searches and alignments of SH3 domains of UNC-89/obscurin from vertebrates, insects, and nematodes were carried out independently per each taxon using BLAST. Other sequence alignments and comparisons used T-coffee (www.tcoffee.org/).

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