CPNA-1, a copine domain protein, is located at integrin adhesion sites and is required for myofilament stability in Caenorhabditis elegans

Adam Warner, University of British Columbia
Ge Xiong, Emory University
Hiroshi Qadota, Emory University
Teresa Rogalski, University of British Columbia
A. Wayne Vogl, University of British Columbia
Donald G. Moerman, University of British Columbia
Guy Benian, Emory University

Journal Title: Molecular Biology of the Cell
Volume: Volume 24, Number 5
Publisher: American Society for Cell Biology | 2013-03-01, Pages 601-616
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1091/mbc.E12-06-0478
Permanent URL: http://pid.emory.edu/ark:/25593/f3mzc

Final published version: http://www.molbiolcell.org/content/24/5/601

Copyright information:
© 2013 Warner et al. This article is distributed by The American Society for Cell Biology under license from the author(s). This is an Open Access work distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License (http://creativecommons.org/licenses/by-nc-sa/3.0/).

Accessed March 26, 2020 5:47 AM EDT
CPNA-1, a copine domain protein, is located at integrin adhesion sites and is required for myofilament stability in Caenorhabditis elegans

Adam Warner*a, Ge Xiongb, Hiroshi Qadotab, Teresa Rogalskia, A. Wayne Voglc, Donald G. Moerma, and Guy M. Benianb
*aDepartment of Zoology and bDepartment of Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC V6T 1Z4, Canada; cDepartment of Pathology, Emory University, Atlanta, GA 30322

ABSTRACT We identify cpna-1 (F31D5.3) as a novel essential muscle gene in the nematode Caenorhabditis elegans. Antibodies specific to copine domain protein atypical-1 (CPNA-1), as well as a yellow fluorescent protein translational fusion, are localized to integrin attachment sites (M-lines and dense bodies) in the body-wall muscle of C. elegans. CPNA-1 contains an N-terminal predicted transmembrane domain and a C-terminal copine domain and binds to the M-line/dense body protein PAT-6 (actopaxin) and the M-line proteins UNC-89 (obscurin), LIM-9 (FHL), SCPL-1 (SCP), and UNC-96. Proper CPNA-1 localization is dependent upon PAT-6 in embryonic and adult muscle. Nematodes lacking cpna-1 arrest elongation at the twofold stage of embryogenesis and display disruption of the myofilament lattice. The thick-filament component myosin heavy chain MYO-3 and the M-line component UNC-89 are initially localized properly in cpna-1–null embryos. However, in these embryos, when contraction begins, MYO-3 and UNC-89 become mislocalized into large foci and animals die. We propose that CPNA-1 acts as a linker between an integrin-associated protein, PAT-6, and membrane-distal components of integrin adhesion complexes in the muscle of C. elegans.

INTRODUCTION Sarcomeres—highly ordered assemblages of several hundred proteins—perform the work of muscle contraction. Despite ever-increasing knowledge of the components and functions of individual sarcomere proteins, we do not understand how sarcomeres are assembled during development or maintained during muscle contraction. The nematode Caenorhabditis elegans is an excellent model genetic system in which to study sarcomere assembly and maintenance (Waterston, 1988; Moerman and Fire, 1997; Moerman and Williams, 2006). In addition to being an excellent system in which to carry out mutational analysis in a whole organism through forward and reverse genetics, this organism offers several advantages for studying striated muscle. Two advantages of particular note are its optical transparency and its mode of reproduction. The transparency of the nematode allows for evaluation of sarcomere structure by polarized light (Epstein et al., 1974; Waterston et al., 1980; Zengel and Epstein, 1980) and visualization of green fluorescent protein (GFP)–tagged proteins (e.g., Hobert et al., 1999; Meissner et al., 2011). Because the nematode can propagate as a self-fertilizing hermaphrodite, many muscle mutants can be maintained even though these mutants are unable to mate.

Two major integrin adhesion complexes are responsible for building a functional sarcomere in C. elegans body-wall muscle: the dense body and the M-line. Prominent proteins in these complexes are α and β integrin (PAT-2, PAT-3; Gettner et al., 1995), kindlin (UNC-112; Rogalski et al., 2000), PINCH (UNC-97; Hobert et al., 1999), integrin-linked kinase (ILK, PAT-4; Mackinnon et al., 2002), and actopaxin (PAT-6; Lin et al., 2003). Another essential protein, vinculin (DEB-1), occurs only within dense bodies (Barstead and Waterston, 1991), and the giant protein UNC-89 occurs solely within M-lines (Benian et al., 1996). The Z-disk analogue in C. elegans body-wall muscle is the dense body, an array of proteins anchored by the PAT-2/PAT-3 integrin heterodimer to the muscle cell.
membrane, which is responsible for anchoring thin filaments composed mainly of actin. The M-line anchors myosin heavy chain–containing thick filaments and is structurally similar to a dense body but lacks the actin-anchoring proteins DEB-1 (vinculin) and ATN-1 (α-actinin; Francis and Waterston, 1991). Each of these integrid adhesion complexes is needed to transmit the force generated by muscle contraction into movement of the worm.

The aforementioned adhesion proteins are required in body-wall muscle in C. elegans to initiate sarcomere assembly and for muscle to function properly. In wild-type animals, at ~420–460 min into development, the embryo elongates to the 1.5-fold stage of embryogenesis and muscle contraction begins (Williams and Waterston, 1994). As the embryo elongates further to the point at which it is folded over on itself, called the twofold stage, movement within the eggshell increases (Williams and Waterston, 1994). In animals lacking one or more essential adhesion complex proteins, embryos arrest elongation at the twofold stage of embryonic development and are paralyzed (Williams and Waterston, 1994), a phenotype unique to defects in embryonic myofilament lattice assembly and contraction in C. elegans. Early work on β-integrin (PAT-3; Gittner et al., 1995) and kindlin (UNC-112; Rogalski et al., 2000) in C. elegans first implicated these proteins as key molecules in muscle assembly and maintenance. The worm has continued to be a valuable model organism for identification and study of genes required for early steps in muscle assembly (reviewed in Moerman and Williams, 2006). The similarity of dense body and M-line assembly to the formation of adhesion complexes present in tissue culture cells has been reviewed in Cox and Hardin (2004) and Moerman and Williams (2006).

In addition to the essential components for initiating sarcomere assembly, the sarcomere contains a number of extraordinarily large polypeptides (700,000 Da to 4 MDa) composed of multiple copies of immunoglobulin (Ig) and fibronectin type 3 (Fn3) domains, one or even two protein kinase domains, and, in some proteins, elastic regions. In general, these giants are considered to present multiple binding sites, help organize other proteins into the sarcomere, and participate in signaling (Kontrogianni-Kostantopoulou et al., 2009). One of these giant proteins is UNC-89, first identified in C. elegans (Waterston et al., 1980; Benian et al., 1996). The human homologue of UNC-89 is obscurin (Bang et al., 2001; Young et al., 2001; Kontrogianni-Kostantopoulou et al., 2009). In C. elegans UNC-89 is localized to the M-line (Benian et al., 1996; Small et al., 2004). Loss-of-function mutations in unc-89 result in worms that display reduced locomotion and disorganized myofibrils, especially at the A-band, and usually lack or display reduced M-lines (Waterston et al., 1980; Benian et al., 1999). Within the myofibril the myosin is disorganized in unc-89 mutants (Qadota et al., 2008a).

We sought to identify novel proteins involved in sarcomere assembly. We used two approaches: one was an RNA interference (RNAi) screen for new paralyzed arrested at twofold (Pat) mutants (Meissner et al., 2009); the other was a yeast two-hybrid screen for new binding partners of UNC-89. Using these two approaches in parallel, we identified the same protein, copine domain protein atypical-1 (CPNA-1), which contains a copine domain.

RESULTS
F31D5.3/CPNA-1 is an “atypical” copine domain–containing protein

From existing Serial Analysis of Gene Expression libraries, we determined that cpna-1 is significantly enriched or specific to body-wall muscle (Meissner et al., 2009). From an RNAi screen of ~3300 genes known to be expressed in muscle, we identified cpna-1 in C. elegans as one of four new Pat mutants (gene model shown in Figure 1A). We examined the phenotype of an intragenic deletion, and likely null mutation, for cpna-1, gk266, provided by the C. elegans Gene Knockout Consortium. The phenotype of worms homozygous for the gk266 allele is Pat, identical to the RNAi phenotype.

CPNA-1 is most similar to homologues in other nematodes, but CPNA-1 is also similar to mammalian copines. An alignment of CPNA-1 with human copine V isoform CRA_d and a copine domain–containing protein (UniProt entry Q88U1J) in the mouse—its two closest homologues in those species (highest BLAST hits)—is shown in Figure 1B using Clustal W (Larkin et al., 2007). Copine domains (Pfam entry PF07002; InterPro entry IPR010734), also known as von Willebrand A–like or A domains, are ~180 residues long and have weak homology to the extracellular A domain of integrins. Within the copine domain, five key conserved residues (D x S x S . . . T . . . D ) constitute the metal ion–dependent adhesion site (MIDAS), which is found in integrins and copines and is believed to confer functional similarity to the protein upon association with metal ions (Lee et al., 1995; Whittaker and Hynes, 2002). Copine domains have an unknown function, although they are considered to be cytoplasmic and involved in protein–protein interactions (Tomsig and Creutz, 2002).

All previously characterized proteins containing copine domains also have two C2 domains, which are calcium-dependent phospholipid–binding motifs. Although CPNA-1 does not have predicted C2 domains, multiple online programs predict that CPNA-1 has a transmembrane domain. Each of the programs strongly predicts a transmembrane helix in the N-terminal region, with, for example, TMHMM2.0 (Sonnhammer et al., 1998; Krogh et al., 2001) predicting the region to be residues 23–45. (The closest mammalian homologues of CPNA-1 shown in Figure 1B do not contain predicted transmembrane regions.) Of interest, whereas CPNA-1 shares homology with copine domain–containing proteins in a diverse range of species, including humans, mice, Arabidopsis, and Dictyostelium, there do not appear to be any homologues in Drosophila. Our BLAST search of the C. elegans genome revealed that C. elegans has seven genes that encode proteins containing copine domains (Figure 2). When compared with CPNA-1, other copine domains in C. elegans share 28–55% identity. There is little homology outside of the copine domains, or C2 domains when present, among them. Of the seven genes, only nra-1 (Gottschalk et al., 2005) and gem-4 (Church and Lambie, 2003) encode proteins that also contain C2 domains. Thus we designated F31D5.3 as CPNA-1 and call it and the four other copine domain proteins that lack C2 domains “copine domain atypical,” with gene designations cpna-1 through cpna-5, respectively.

CPNA-1 localizes to dense body and M-line muscle integrid adhesion complexes and is required for maintenance of muscle stability

To determine the localization of CPNA-1 in nematode muscle, we raised antibodies to a non-copine–domain region of CPNA-1 (Figure 1A). These antibodies detected a single band on a Western blot using a protein lysate from C. elegans (Supplemental Figure S1). The size of this band, ~130 kDa, is very similar to the size of the predicted CPNA-1b isoform. Anti–CPNA-1 antibodies were used to visualize CPNA-1 localization in wild-type adult body-wall muscle. As shown in Figure 3A, CPNA-1 localizes to both M-lines and dense bodies, colocalizing with UNC-89 (M-lines), and with α-actinin (dense bodies). In cpna-1 (gk266) animals we do not observe any CPNA-1 staining, indicating the mutation is likely a null. UNC-112 (kindlin) is located at M-lines and dense bodies (Rogalski et al., 2000), and has been shown to interact with the cytoplasmic tail of PAT-3.
UNC-112, and therefore CPNA-1 is membrane proximal in both dense bodies and M-lines. In addition, we costained for CPNA-1 and one more membrane-proximal component, PAT-6 (actopaxin), which is a member of the conserved four-protein complex that associates with the cytoplasmic tail of PAT-3 (β-integrin; Lin et al., 2012). Thus UNC-112 can be considered as a marker for proteins that are located close to the muscle cell membrane. Anti-CPNA-1 antibodies were used to stain transgenic animals expressing GFP-tagged UNC-112. As shown in Figure 3A (third row), CPNA-1 colocalizes with the membrane proximal protein UNC-112, and therefore CPNA-1 is membrane proximal in both dense bodies and M-lines. In addition, we costained for CPNA-1 and one more membrane-proximal component, PAT-6 (actopaxin), which is a member of the conserved four-protein complex that associates with the cytoplasmic tail of PAT-3 (β-integrin; Qadota et al., 2012).
Figure 2: Copine family proteins in C. elegans. (A) The C. elegans genome has seven genes (1–7) that encode proteins containing copine domains. The protein names shown are based on gene names (e.g., CPNA-1) and sequence names (e.g., F31D5.3). Percentages refer to the percentage of identical amino acid residues in each copine domain compared with the copine domain of CPNA-1. The numbers in parentheses after the total number of amino acid residues in each protein denote the positions of copine domains. Note that only NRA-1 and GEM-4 are “typical,” in that they also contain C2 domains. Only CPNA-1 also has a predicted transmembrane domain. In addition, some isoforms of CPNA-1 and CPNA-2 are not shown. These isoforms, predicted on WormBase (CPNA-1c, CPNA-1d, CPNA-2b, and...
2003). As shown in Figure 3A (last row), CPNA-1 and PAT-6 colocalize to both dense bodies and M-lines.

As described previously, worms lacking cpna-1 due to either mutation or RNAi arrest elongation at the twofold stage of embryogenesis and die as abnormal L1 larvae. As shown in Figure 4A, whereas a wild-type worm hatches and progresses normally to the first larval stage, cpna-1(gk266) homozygotes (Figure 4B) arrest elongation during embryogenesis. In an attempt to rescue the embryonic lethal phenotype, we constructed a yellow fluorescent protein (YFP) translational fusion. The recombiner Fosmid fD1M1217 did not successfully rescue the lethal phenotype; however, YFP-tagged CPNA-1 localizes to dense bodies and M-lines (Figure 4C) in a pattern identical to that seen with our CPNA-1 immunostaining. Colocalization with PAT-3 staining (Figure 4D) is shown in Figure 3E. The localization pattern of CPNA-1::YFP is similar to that of PAT-3 in dense bodies and M-lines, and it is also seen more broadly around the dense bodies when compared with the localization pattern of PAT-3.

It is clear that CPNA-1 is located at dense bodies and M-lines, but its position vis-à-vis the plasma membrane needed to be clarified. We took two approaches to address this question. We did a confocal Z-series beginning at the plasma membrane and extending into the cell at 0.5-μm increments (see Supplemental Figure S2). We see that CPNA-1 is close to the plasma membrane, at the same level as the ECM protein UNC-52 (perlecan) and the membrane-proximal proteins UNC-112 and PAT-6, but also extends more deeply into the cell than these proteins. However, CPNA-1 does not extend as deeply into the cell as the M-line protein UNC-89 (obscurin).

We also examined transmission electron microscopy (TEM) sections of CPNA-1::YFP animals using colloidal gold–labeled anti-YFP antibodies to determine whether the protein is close to the plasma membrane. In the tested animals, YFP sequence is fused to CPNA-1 at the C-terminal end of the protein, whereas the N-terminal of the protein is predicted to be transmembrane, so we did not expect to see colloidal gold within the plasma membrane even if CPNA-1 does traverse the membrane. We focused on dense bodies rather than M-lines because dense bodies are more visible in TEM images. In worm sections, CPNA-1 labeled with colloidal gold is visible within and surrounding the dense body (Figure 3B, middle) at much higher levels than the random localization of gold particles in control images (Figure 3B, right). We compared the number of gold particles observed within a dense body or directly adjacent to the visible portion of dense bodies in control sections and experimental sections. Control sections had an average of 6 gold particles per dense body, whereas sections with colloidal gold–labeled CPNA-1 had an average of 24.25 gold particles per dense body. CPNA-1 is observed in dense bodies both distal and proximal to the cell membrane (Figure 3B, middle), supporting the idea that some CPNA-1 molecules localize close to or within the basal membrane of body-wall muscle cells at integrin adhesion sites.

CPNA-1 acts to maintain sarcomere integrity

We examined the localization pattern of essential body-wall muscle proteins in the cpna-1–mutant background using immunostaining for each protein tested. For each of PAT-3 (B-integrin; Figure 4G), DEB-1 (vinculin; Figure 4I), PAT-4 (ILK; Figure 4K), and PAT-6 (actopaxin; Figure 4M), each protein was localized at integrin adhesion complexes in muscle quadrants, albeit with some minor organization when compared with wild-type embryos. These results are typical when staining embryos for proteins that act earlier in sarcomere assembly than the protein under study (Williams and Waterston, 1994; Norman et al., 2007). These observations indicate that CPNA-1 is not necessary for the earliest steps in sarcomere initiation.

In cpna-1–null embryos, UNC-89 (obscurin) is properly localized to adhesion sites before the 1.5-fold stage (Figure 4O), but in later stages (1.75- and 2-fold), UNC-89 is found in abnormal accumulations (Figure 4Q). Similarly, in cpna-1–null embryos, MYO-3 is properly organized into discrete sarcomeres before the 1.5-fold stage (Figure 4S), but in later stages (1.75- and 2-fold), MYO-3 is found in abnormal accumulations (Figure 4U). These observations suggest that CPNA-1 is essential to maintain the integrity of both UNC-89 and MYO-3 at muscle integrin adhesion sites, and in cpna-1–null animals, elongation arrests due to either the mislocalization of MYO-3 or other factors.

Using anti-CPNA-1 antibodies, we carried out the reciprocal experiment and examined the localization of CPNA-1 in the mutant background of a number of Pat mutants. In pat-3–arrested (Figure 5B) and pat-4–arrested (Figure 5C) embryos, CPNA-1 is mislocalized, appearing in bright fluorescent patches within the muscle cells (arrows). In pat-6 embryos (Figure 5, D and E), CPNA-1 shows some variability in its localization, ranging from severe mislocalization (Figure 5D), similar to that seen in the pat-3 and pat-4 backgrounds, to proper polarization in the membrane but disorganized (Figure 5E). In contrast, CPNA-1 is localized normally in myo-3–arrested embryos (Figure 5F), closely resembling the localization of CPNA-1 in wild-type embryos (Figure 5A). These cumulative results place cpna-1 between pat-6 and myo-3 in the M-line/dense body assembly pathway.

CPNA-1 interacts with UNC-89 and several other muscle integrin adhesion complex proteins

In an effort to identify new binding partners for UNC-89, we used a portion of UNC-89 comprising Ig domains 1–5 (Ig1–5) as bait to screen a yeast two-hybrid library of C. elegans cDNAs (Figure 6A). Two positive preys representing CPNA-1 were recovered. The smallest prey clone (residues 825–1058) contains essentially just the copine domain, suggesting that this region of CPNA-1 is minimally required for interaction with UNC-89. CPNA-1 was used in yeast two-hybrid assays as both bait and prey to test for interaction with an additional 16 portions of UNC-89. As indicated in Figure 6B, CPNA-1 (825–1058) interacts only with Ig1–5. Given our understanding of the regulation of the unc-89 gene (Ferrara et al., 2005), CPNA-1 is predicted to interact with the large UNC-89 isoforms (A, B, E, and F) but not the small isoforms (C and D). When tested by two-hybrid assays against copine domains from each of the seven copine domain–containing proteins, UNC-89 Ig1–5 interacted only with the copine domains.
To identify additional CPNA-1 binding partners, we used CPNA-1 to conduct a yeast two-hybrid screen of a collection of 23 known components of dense bodies and M-lines (Supplemental Table S1). As indicated in Figure 6, D–H, we identified four additional CPNA-1–interacting proteins: SCPL-1 (a CTD-type protein phosphatase; Qadota et al., 2008b), LIM-9 (FHL; Qadota et al., 2007), PAT-6 (actopaxin; Lin et al., 2003), and UNC-96 (Mercer et al., 2006). The minimal portions required for interaction with CPNA-1 were also determined. Although only the copine domain of CPNA-1 is required to interact with UNC-96, nearly full-length CPNA-1 is required for interaction with SCPL-1, LIM-9, and PAT-6. These interactions were confirmed using in vitro binding experiments with purified proteins (Supplemental Figure S4). Thus CPNA-1 has both dense body and M-line binding partners.

We demonstrated that the copine domain of CPNA-1 can interact with UNC-89 Ig1–5 and with UNC-96 (201–418). We used in vitro mutagenesis to test whether CPNA-1 has distinct binding sites for these proteins. Alignment of copine domains from the C. elegans proteins reveals 12 invariant amino acid positions (shaded green in Figure 2B), which are also conserved in the PFAM consensus sequence for all copine proteins. We mutated three of the 12 conserved residues (asterisks in Figure 2B): G922 was changed to V, which has a larger side chain and may cause a local conformational change; Y985 to A to alter the side chain; or F, which eliminates a possible phosphorylation site or eliminates hydrogen bonding or even ionic bonding independent of phosphorylation. Similarly, S1015 to A to eliminate a possible phosphorylation site. As shown in Figure 7A, the G922V mutation eliminates only the interaction with UNC-89, and the S1015A mutation eliminates only the interaction with UNC-96, suggesting that the copine domain of CPNA-1 might have distinct binding sites for these two M-line proteins.

Based on our mutant analysis (Figures 4 and 5), PAT-6 is required for assembly of CPNA-1 into integrin adhesion sites, and CPNA-1 is required for the stable association of UNC-89 with integrin adhesion sites. As such, we might expect to obtain evidence that PAT-6, CPNA-1, and UNC-89 form a ternary structure. To test this idea, we performed a yeast three-hybrid assay. UNC-89 Ig1–5 as bait was coexpressed with hemagglutinin (HA)-tagged CPNA-1 (173–1107), or empty vector as control, and PAT-6 as prey. As shown in Figure 7B, the interaction of UNC-89 Ig1–5 with PAT-6 depends on the presence of CPNA-1. This result is consistent with the existence of a ternary domain from CPNA-1 (Figure 2). This is a further indication of the specificity of the UNC-89 to CPNA-1 interaction. Deletion derivatives of Ig1–5 were used in two-hybrid assays against CPNA-1. As shown in Figure 6C, the minimal region necessary and sufficient to interact with CPNA-1 is Ig1–3. Two biochemical approaches were used to verify the interaction of UNC-89 with CPNA-1 (Supplemental Figure S3).
complex containing PAT-6, most of CPNA-1, and a small portion of UNC-89.

The role of CPNA-1 in postembryonic body-wall muscle
Previsouly we used RNAi on postembryonic-stage animals to inves-
tigate whether CPNA-1 was required in body-wall muscle (Meissner
et al., 2009). In cpna-1 (RNAi)-treated adult animals, the arrays of
myofilaments in body-wall muscle are highly disorganized, as visual-
ized by polarized light microscopy.

PAT-6 is the earliest protein in the M-line/dense body assembly
pathway that interacts with CPNA-1. Our mutant analysis indicates
that CPNA-1 is downstream of PAT-6 in embryonic muscle. These
results led us to explore the relationship between PAT-6 and CPNA-1
in adult muscle. We used RNAI to knock down pat-6, beginning at
the L1 larval stage, in order to avoid the embryonic requirement of
pat-6. In muscle cells with nearly undetectable PAT-6, CPNA-1 was
mislocalized, being found in abnormal accumulations (arrowhead)
or at the edges of the muscle cell (arrows; Figure 8).

We next did the same experiment with UNC-89, SCPL-1, UNC-
96, and LIM-9. None of these four proteins is required for embryonic
muscle development, but when deficient, they have phenotypes in
adult muscle (Waterston et al., 1980; Mercer et al., 2006; Meissner
et al., 2009; Nahabedian et al., 2011). As shown in Figure 9, the lo-
calization of CPNA-1 at M-lines (indicated by arrows) is unaffect-
ed by the absence or reduced levels of UNC-89, SCPL-1, UNC-96, or
LIM-9 in L4 or early- or late-stage adults. These results are consistent
with these proteins being downstream of CPNA-1 in the M-line as-
sembly pathway in postembryonic muscle.

DISCUSSION
A model for CPNA-1 in maintenance of integrin adhesion
complexes
Our studies identified CPNA-1 as a new component of integrin ad-
hesion complexes, based on four criteria. First, CPNA-1 is localized
to M-lines and dense bodies, similar to integrin and integrin-asso-
ciated proteins (Moerman and Williams, 2006). Second, loss of
function of CPNA-1 leads to a Pat embryonic lethal phenotype.
This terminal phenotype can result from loss of function of genes
encoding integrin and integrin-associated proteins. Third, whereas
MYO-3 and UNC-89 are initially localized in cpna-1–null embryos,
as the embryo proceeds to the twofold stage, both proteins be-
come mislocalized and accumulate at the edges of muscle cells.
This demonstrates that CPNA-1 is required for maintaining the
adjacent to the dense body, observed by the green localization
pattern around the dense body; stronger M-line staining by PAT-3
(red) overshadows that of CPNA-1 (arrowhead). Compared to
wild-type staining (F, H, J, L), when cpna-1 embryos are
immunostained with antisera for PAT-3 (G), DEB-1 (I), PAT-4 (K), and
PAT-6 (M), only slight disorganization is seen and each of the four
proteins is still localized to the appropriate integrin adhesion sites
(arrowheads). In pre–1.5-fold embryos, UNC-89 is able to localize to
M-lines in both wild-type embryos (N) and cpna-1–null embryos (O),
but after the 1.5-fold stage, wild-type embryos (P) show normal
UNC-89 localization, whereas cpna-1–null embryos show UNC-89
mislocalized into large foci within muscle cells (Q; arrows). Similarly,
MYO-3 is able to organize into nascent thick filaments in both
pre–1.5-fold wild-type (R) and cpna-1–null (S) embryos, but after the
1.5-fold stage, wild-type embryos (T) show normal MYO-3 localization
to nascent thick filaments, whereas in cpna-1–null embryos, MYO-3 is
disorganized and mislocalized into large accumulations within the
muscle cells (U; arrows). Bars, (A and B) 20 μm, (C-U) 2 μm.
structural stability of thick-filament attachments. Finally, CPNA-1 binds directly to PAT-6, one of the integrin-associated proteins. We suggest that the molecular function of CPNA-1 is to act as a linker between integrin-associated proteins near the muscle cell membrane and proteins that are found deeper inside the muscle cell (Figure 10). In striated muscle of C. elegans, both the M-lines and dense bodies are integrin adhesion complexes containing both shared and specific protein components. At the base of each M-line and dense body, associated with the cytoplasmic tail of β-integrin, is a complex of four conserved proteins, including UNC-112 (kinderlin), PAT-4 (ILK), UNC-97 (PINCH), and PAT-6 (actopaxin). Lin et al. (2003) showed that PAT-4 (ILK) interacts with the C-terminal CH domain of PAT-6 (actopaxin). Here, we show that the N-terminal portion of PAT-6, including the first CH domain, interacts with CPNA-1. CPNA-1, in turn, interacts with UNC-89 (obscurin), LIM-9 (FHL), SCPL-1 (SCP), and UNC-96 at M-lines (Figure 10). We speculate that there are as-yet-unidentified dense body–specific proteins that interact with CPNA-1.

CPNA-1 is not required for the initial assembly of sarcomeric components, but embryonic muscle lacking CPNA-1 becomes disorganized after muscle contraction begins. CPNA-1 binds to PAT-6, UNC-89, and other integrin adhesion complex proteins to possibly hold together the integrin adhesion complex. In this manner, MYO-3 (myosin) and other membrane-distal components of the sarcomere remain in their proper locations, and the sarcomere is stable. In the absence of CPNA-1, the integrin adhesion complex would not be maintained or repaired during the stress of contraction, and the sarcomere would fall apart. Work by Tomsig et al. (2003) might support this proposed mode of action. The copine domain of CPNA-1 contains key residues of a MIDAS (Figure 1B), and Tomsig et al. (2003) demonstrated that these sites are important for human copines to recruit binding partners in a calcium-dependent manner. Given that muscle contraction is a calcium-regulated process, one could infer that CPNA-1 might be involved in the recruitment of binding partners during contraction and consequently plays a role in maintaining sarcomere integrity. An alternative possibility is that CPNA-1 maintains stability by recycling damaged sarcomere components or acts by recruiting new proteins to the integrin adhesion complexes. In this regard, one could speculate that the predicted transmembrane domain of CPNA-1 inserts into membrane vesicles and is involved in moving damaged or newly synthesized proteins from or to muscle integrin adhesion complexes.

**New classes of copine domain–containing proteins**

Copines are evolutionarily conserved proteins found in plants and animals and have been analyzed genetically in Arabidopsis (Hua et al., 2001; Jambunathan et al., 2001), Dictyostelium (Damer et al., 2007), and C. elegans (Church and Lambie, 2003; Gottschalk et al., 2005). Copines were first identified as Ca++–dependent phospholipid-binding proteins in Parmeum (Creutz et al., 1998), contain two C2 domains, and are usually believed to be involved in membrane trafficking. CPNA-1 is the first characterized copine domain–containing protein that lacks C2 domains. The Pfam web site (http://pfam.sanger.ac.uk/), as of April 2011, notes 200 sequences from diverse organisms that have a copine domain plus two C2 domains, 25 with copine domains plus a single C2 domain, and 168 containing only a copine domain. This copine domain–only category includes an uncharacterized putative protein from the mouse (UniProt entry Q8BJJ1 MOUSE; 346 amino acids) and isofrom CRA_d of copine V from humans (UniProt entry Q7Z6C8 HUMAN; 301 amino acids), as shown aligned with CPNA-1 in Figure 1B. These are the two closest homologues to CPNA-1 in those species.

Among the copine domain–containing proteins in the C. elegans proteome, both typical and atypical, only CPNA-1 contains a predicted transmembrane domain. In fact, our in silico analysis has not revealed any human copine proteins that have a predicted transmembrane domain. Although both confocal (Figure 3A and Supplementary Figure S2) and electron microscopic (Figure 3B) imaging reveal some fraction of CPNA-1 at or near the muscle cell membrane at the base of dense bodies and M-lines, whether this predicted transmembrane domain inserts into this or any other membrane will require additional experiments. Note that our domain mapping (Figure 6F) indicates that the predicted transmembrane domain of CPNA-1 is not required for its association with PAT-6. Thus CPNA-1 might have two ways to localize to integrin adhesion complexes—binding to the integrin-associated protein PAT-6, and direct insertion of its transmembrane domain into the muscle cell membrane.

**FIGURE 5:** Immunolocalization of CPNA-1 in the null background of other Pat genes. In late-stage, wild-type embryos, CPNA-1 is organized into bands of dense bodies and M-lines in the muscle cell quadrants (A; arrowheads). In null pat-3 embryos, however, CPNA-1 is mislocalized into large aggregates within body-wall muscle cells (B; arrows). Similarly, large accumulations of CPNA-1 are also seen in null embryos for pat-4 (C) and pat-6 (D; arrows), although in some pat-6 embryos, CPNA-1 mislocalized to a lesser extent than for pat-3 and pat-4 embryos (E; arrow). In arrested twofold embryos lacking MYO-3, however, CPNA-1 is organized properly into bands of dense bodies and M-lines (F), as it is in wild-type embryos (A; arrowheads). Bar, 2 μm.
FIGURE 6: CPNA-1 interacts with M-line proteins UNC-89, PAT-6, LIM-9, SCPL-1b, and UNC-96. (A) Schematic representation of domains within the largest isoform of UNC-89, and indication that Ig1-5 was used as bait to screen a yeast two-hybrid library. Two positive preys representing CPNA-1 were recovered, as indicated. CPNA-1, isoform b, has a predicted transmembrane domain (TM) and a copine domain (copine). (B) When CPNA-1 was used to test for interaction with the other 16 clones that fully cover UNC-89, interaction was only found with Ig1–5. (C) Domain mapping of UNC-89 Ig1–5 shows that Ig1–3 are minimally required for interaction with CPNA-1 (173–1107). (D–H) Proteins found to interact with CPNA-1 by screening a collection of known M-line and dense body proteins using the two-hybrid method. (D–G) Depiction of the results of domain mapping to determine the minimal region (indicated as a blue bar) of each protein required for interaction with the indicated regions of CPNA-1. (H) Summary of the results showing protein domains of each protein and which regions are involved in the interactions.
We identified CPNA-1 as an interacting partner for the giant poly-

We identified and characterized CPNA-1, a copine domain–containing protein required in C. elegans body-wall muscle during embryogenesis specifically for maintaining the structural integrity of the sarcomere. CPNA-1 is localized to integrin adhesion sites in muscle tissue and might act as a linker between membrane-proximal attachment proteins and those deeper within muscle cells. To date, this is the only copine domain–containing protein implicated in muscle maintenance/stability. Given that there are copine homologues in a diverse range of species including humans, further study on copines in other species is needed to determine whether this muscle function of copine domain containing proteins is evolutionarily conserved.

MATERIALS AND METHODS

Strains used

N2 (Bristol) is the primary wild-type strain used for C. elegans research, and standard growth conditions were used (Brenner, 1974). VC516 cpna-1(gk266)/mIs14[mls14 dpy-10(e128)] II contains a deletion in cpna-1, and VC209 lim-9(gk106) I and VC349 lim-9(gk210) I carry mutations in the gene lim-9. Each was provided by the International C. elegans Gene Knockout Consortium (Vancouver, Canada). DM7439 ex217 was created by transforming wild-type C. elegans with the YFP-recombineered fosmid fDM1217, and the strain DM5151 cpna-1(gk266)/+; ex217 was created by crossing the gk266 allele from strain VC516. Strain WB201 pat-4(st587) III; ex(pat-4::GFP/pat-3::YFP) was provided by Ben Williams (University of Illinois, Urbana, IL). Strains RW3600 pat-3(st564)/c01 dpy-19(e1259) glp-1(q339) III and RW3568 pat-6(st561)/dpy-

21 interacting proteins. The targets fell into several functional categories, including regulators of protein phosphorylation, transcription, or ubiquitination, calcium-binding proteins, and cytoskeletal or structural proteins. A majority of the interactors (14 of 21) contained predicted coiled-coil domains. CPNA-1 interactors are also regulators of phosphorylation (UNC-89 has two kinase domains and SCPL-1 is a phosphatase) and cytoskeletal proteins (PAT-6, LIM-9, and UNC-96). None of our interactors, however, contains predicted coiled-coil regions, and none of our proteins is a homologue of any proteins found by these authors. This is a further indication that the copine domain can interact with proteins of diverse functions.

Summary

Alternatively, the predicted transmembrane domain might not be involved in localization to the muscle cell membrane but might instead be required in other membrane compartments, for example, vesicles involved in repair or replacement of sarcomeric proteins (see earlier discussion).

Copine domains as protein-interacting modules

We identified CPNA-1 as an interacting partner for the giant polypeptide UNC-89, as well as for other M-line and dense body proteins. This interaction with UNC-89 is specific in two ways: 1) Only Ig domains 1–3, and not any of the other 50 Ig domains or any other segment of UNC-89, interact. 2) Only the copine domain of CPNA-1, and none of the copine domains from the six other copine domain-containing proteins in C. elegans, interacts with UNC-89.

Previous studies demonstrated that the copine domain is a protein–protein interacting domain (Hua et al., 2001; Yang et al., 2006; Li et al., 2010). Tomsig et al. (2003) used the copine domains of three human copine proteins (copines I, II, and IV) to screen a mouse embryo two-hybrid library and identified FIGURE 7: Point mutations in the copine domain of CPNA-1 affects its binding to UNC-89 and UNC-96; evidence for a ternary complex containing PAT-6 and portions of CPNA-1 and UNC-89. (A) The copine domain of CPNA-1 has distinct binding sites for UNC-89 and UNC-96. Three of the 12 conserved residues of copine domains (indicated with asterisks in Figure 2B) were changed: G922 was changed to V; Y985 was changed to A and F, respectively; S1015 was changed to A. The G922V mutation eliminates binding to UNC-96 but not UNC-89. Similarly, the S1015A mutation eliminates the binding to UNC-96 but not UNC-89. (B) UNC-89 Ig1-5 as bait was coexpressed with HA-tagged CPNA-1 (residues 173–1107; or empty vector as control) and PAT-6 (full length) as prey. +, growth on –Ade plates; –, no growth on –Ade plates. Right, the copine domain–containing protein required in other membrane compartments, for example, vesicles involved in repair or replacement of sarcomeric proteins (see earlier discussion).
pat-6 (RNAi)

FIGURE 8: In adult muscle, PAT-6 is required for localization of CPNA-1. RNAi was used to knock down pat-6 beginning at the L1 larval stage, and the resulting adults were immunostained for PAT-6 and CPNA-1. Top and bottom, portions of body-wall muscle from two such animals. In muscle cells in which PAT-6 was knocked down, CPNA-1 is found in abnormal accumulations (arrowhead) or mislocalized to the edge of the muscle cell near the muscle cell membrane (arrows). Bar, 10 μm.

Screening of yeast two-hybrid library

Two-hybrid screening was performed as described in Miller et al. (2006). The first screen of the RB2 library of C. elegans random primed cDNAs (kindly provided by Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK) used the bait plasmid pGBDU-UNC-89 GX34, which contains coding sequence for UNC-89 Ig1–5. To make the Ig1–5 bait, an insert generated by PCR using primers GX3 with added BamHI site and GX4 with added XhoI site was ligated into pGBDU-C1 (see Supplemental Table S2 for primer sequences). From a screen of 819,500 colonies, the following prey clones were isolated: 35 preys representing VIG-1, 1 prey from MEL-26 (residues 25–343), and 1 prey from CPNA-1 (called GX9-109, residues 173–1107). A second screen of the library used a bait plasmid that contains coding sequence for UNC-89 Ig2–5. This bait was generated by insertion of a PCR fragment using primers GX2/5 with added BamHI site and GX4 with added XhoI site was ligated into pGBDU-C1 (see Supplemental Table S2 for primer sequences). From a screen of 1,206,000 colonies, the following prey clones were isolated: 4 preys from VIG-1, 1 prey from UNC-54 (residues 1–602), 1 prey from KETN-1 (residues 4394–4889), and 1 prey from CPNA-1 (called GX9-109, residues 173–1107). A second screen of the library used a bait plasmid that contains coding sequence for UNC-89 Ig3–5. This bait was generated by insertion of a PCR fragment using primers GX3/4 with added BamHI site and GX4. From a screen of 1,020,000 colonies, the following prey clones were isolated: 4 preys from VIG-1, 1 prey from UNC-54 (residues 1–602), 1 prey from MEL-26 (residues 25–343), and 1 prey from CPNA-1 (called B15-101, residues 825–1058). Interaction with VIG-1 was not pursued because it is part of a RISC complex (Caudy et al., 2003) and thus we believed that the resulting fragment did not make biological sense. We did not pursue interaction with UNC-54 (myosin heavy chain B), which is located in the polar regions of the A-band (Miller et al., 1983) rather than at the M-line where UNC-89 is located. Similarly, interaction with KETN-1 was not pursued, as KETN-1 is located in the I-band/dense body region of the sarcomere (Ono et al., 2006). The interaction of UNC-89 and MEL-26 is described in Wilson et al. (2012).

Construction of two-hybrid clones covering all of UNC-89-B and its screening with CPNA-1

Seventeen segments (Figure 6B), covering all of UNC-89-B, were cloned into two-hybrid bait and prey vectors. Three of these segments were described previously: Fn1-Ig52-PK1 and Ig53-Fn2-PK2 (Qadota et al., 2008b) and interkinase (Xiong et al., 2009). For the other segments, corresponding cDNA fragments of UNC-89-B were amplified by using PCR with primers listed in Supplemental Table S2 and cloned into pBluescript. After confirming that the DNA sequence of each fragment was error-free, we cloned each fragment into pGBDU or pGAD yeast two-hybrid plasmids. To screen for interaction with CPNA-1, first, PJ69-4A yeast host strains carrying each fragment of UNC-89-B in pGBDU were prepared, and then each strain was transformed with CPNA-1 prey clone B15-101 (residues 825–1058). The yeast two-hybrid assay was performed by scoring growth on media lacking histidine or adenine.

Screening of yeast two-hybrid bookshelf of known M-line and dense body proteins

CPNA-1 was used as both bait and prey to screen a collection (“bookshelf”) of 23 known components of nematode M-lines and dense bodies (Supplemental Table S1). Both CPNA-1 (825–1058) and CPNA-1 (173–1107) were used.

Domain mapping of UNC-89 Ig1–5, SCPL-1, LIM-9, PAT-6, and UNC-96

To map the region of UNC-89 Ig1–5 minimally required for interaction with CPNA-1, we generated deletion derivatives of Ig1–5 by PCR using primers listed in Supplemental Tables S2 and S3. After cloning into pBluescript and identifying clones lacking PCR induced errors, we cloned the fragments into pGBDU and then tested them by two-hybrid assay against GX9-109 library prey clone CPNA-1 (173–1107). Domain mapping experiments reported in Figure 6, D–H, used constructs described previously: SCPL-1 (Qadota et al., 2008b), LIM-9 (Qadota et al., 2007), PAT-6 (Lin et al., 2003), and UNC-96 (Mercer et al., 2006; Qadota et al., 2007). Derivatives of SCPL-1, LIM-9, and PAT-6 in pGAD were tested against CPNA-1 (173–1107) in pGBDU. Derivatives of UNC-96 in pGBDU were tested against CPNA-1 (825–1058) in pACT.

Testing for interaction of UNC-89 Ig1–5 against copine domains of other C. elegans proteins

To test the specificity of interaction between UNC-89 Ig1–5 and the copine domain of CPNA-1, we generated the copine domains of other copine domain–containing proteins by PCR using the primers listed in Supplemental Table S3. The 5′ primers have added BamHI or Smal sites, and the 3′ primers have added XhoI sites. The products were ligated into pBluescript, sequenced, and then cloned into pGBDU and pGAD vectors. Yeast two-hybrid assays were performed by using pGAD copine domain clones against pGBDU UNC-89 Ig
A. Warner et al. Molecular Biology of the Cell

fragments were cloned into pGAD and tested against pGBDU UNC-89 Ig1–5 and pGBDU UNC-96 (201–418) yeast strains. Mutant fragments were also cloned into pGBDU and tested against pGAD UNC-89 Ig1–5 and pGAD UNC-96 (201–418).

Demonstration of interactions using purified proteins
To verify the interaction of CPNA-1 with UNC-89 Ig1–3, we pulled CPNA-1 out of a worm lysate using glutathione S-transferase (GST)–Ig1-3. To generate GST–Ig1-3, we excised the insert from pGBDU-Ig1-3 with BamHI and BgIII and cloned it into the BamHI site of pGEX-KK1. After identifying a clone with the proper orientation, we prepared a GST fusion protein. Preparation of a C. elegans lysate 1–5 yeast strain and pGBDU copine domain clones against the pGAD UNC-89 Ig1–5 clone, respectively. The results of both assays are consistent and are shown in Figure 2.

Testing for interaction of UNC-89 Ig1–5 and UNC-96 (201–418) against single–amino acid mutants of the copine domain of CPNA-1
To test whether the conserved amino acid residues within the copine domain of CPNA-1 are important for the binding with other proteins, we generated four point mutants using the primers listed in Supplemental Table S3 and used a method involving two rounds of PCR similar to that described in Qadota et al. (2012). Mutant fragments were cloned into pGAD and tested against pGBDU UNC-89 Ig1–5 and pGBDU UNC-96 (201–418) yeast strains. Mutant fragments were also cloned into pGBDU and tested against pGAD UNC-89 Ig1–5 and pGAD UNC-96 (201–418).

Demonstration of interactions using purified proteins
To verify the interaction of CPNA-1 with UNC-89 Ig1–3, we pulled CPNA-1 out of a worm lysate using glutathione S-transferase (GST)–Ig1-3. To generate GST–Ig1-3, we excised the insert from pGBDU-Ig1-3 with BamHI and BgIII and cloned it into the BamHI site of pGEX-KK1. After identifying a clone with the proper orientation, we prepared a GST fusion protein. Preparation of a C. elegans lysate

FIGURE 9: Analysis of mutants places the M-line proteins UNC-96, LIM-9, and SCPL-1 downstream of CPNA-1 in late larval or adult muscle. The indicated loss-of-function mutants or RNAi animals were coimmunostained with anti–CPNA-1 and anti–α-actinin. The localization of CPNA-1 at M-lines (indicated by yellow arrows) is unaffected by the absence or reduced levels of UNC-89, SCPL-1, UNC-96, or LIM-9. Note that the unc-89–mutant allele su75, which lacks all large UNC-89 isoforms, lacks CPNA-1–binding sites. Bar, 10 μm.
essentially as described in Mercer et al. (2003). PAT-6 (actopaxin) and the protein lysates from wild-type animals and from worms that had undergone pat-6(RNAi) by L1 feeding (Miller et al., 2006). Approximately 50–100 µg of total protein was separated by 10% SDS–PAGE, transferred to a blot, incubated with 6His–Ig1-3, and then detected with anti-His and anti-rabbit horseradish peroxidase (HRP). To express His–CPNA-1 (173–1107), pGBDU-CPNA-1 (173–1107) was digested with EcoRI (site in the vector) and SaII (in the insert), and this fragment, encoding the N-terminal two-thirds of CPNA-1 (173–1107) was ligation into pET28a to create clone a. The C-terminal one third of CPNA-1 (173–1107) was run on SDS–PAGE, transferred to a blot, incubated with either MBP–SCPL-1 (phosphatase domain; Qadota et al., 2008b), MBP–PAT-6 (full length; Lin et al., 2003), or MBP–LIM-9 (LIM domains; Qadota et al., 2007), and then detected with anti–MBP–HRP. Procedures for growth of yeast for expressing HA-tagged proteins, growth of bacteria, and purification of GST, MBP-, and His-tagged proteins were described previously (Mercer et al., 2006; Qadota et al., 2008a).

**Generation of antibodies to CPNA-1**

Residues 176–385 of CPNA-1b were expressed and purified in Escherichia coli as a GST fusion protein. To do this, primers tag-149-1 and tag-149-2 with added EcoR1 and XhoI sites were used to create a PCR fragment using cDNA GX9-109 as template. This fragment was cloned into pBluescript, and a clone without PCR-induced errors was used for excising the insert. This insert was ligated into pGEX-KK1 using the same enzyme sites. Using methods described in Mercer et al. (2006), we expressed GST–CPNA-1 (176–385), purified it, and shipped it to Spring Valley Laboratories (Woodbine, MD) for generation of rabbit polyclonal antibodies. Most of the anti-GST antibodies were removed by immunoprecipitation using GST, and anti–CPNA-1 was affinity purified using Affi-Gel conjugated with GST–CPNA-1 (176–385), as described previously (Mercer et al., 2003). These antibodies detect a band of expected size on Western blot (Supplemental Figure S1) and react to wild-type but not cpna-1–null embryos on immunostaining.

**Western blots**

The procedure of Hannak et al. (2002) was used to prepare total protein lysates from wild-type animals and from worms that had undergone pat-6(RNAi) by L1 feeding (Miller et al., 2006). Approximately 50–100 µg of total protein was separated by 10% SDS–PAGE, transferred to nitrocellulose membrane, and reacted with affinity purified and OP50 E. coli absorbant anti–CPNA-1 at 1:100 or 1:500 dilution, or affinity-purified and OP50 E. coli absorbant anti–PAT-6 at 1:200 dilution, followed by reaction with appropriate HRP-conjugated secondary antibodies and visualization using enhanced chemiluminescence (cat. no. 32106; Pierce, Rockford, IL).

**Immunolocalization in adult body-wall muscle**

Worms were fixed by the Nonet method (Nonet et al., 1993), with the exception of Figure 3A (third row), in which strain DMS115 (UNC-112::GFP), which was fixed using the constant-spring method (Benian et al., 1996). Primary antibodies were used at the following dilutions: anti–CPNA-1 at 1:100, anti–UNC-89 (monoclonal MH42; Benian et al., 1996) at 1:200, anti-α-actinin (MH35; Francis and Waterston, 1991) at 1:200, anti–myosin heavy chain A (MHCA A; 5–6;
Miller et al., 1983) at 1:200, and anti–PAT-6 at 1:100. For anti–CPNA-1, the secondary was anti-rabbit conjugated to Alexa 488 (the one exception was when DMS5115 was stained; then we used anti-rabbit Cy3); for the monoclonals (MH35, MH42, and 5-6) the secondary was anti-mouse-Alexa 594; and for anti–PAT-6, we used anti–rat-Alexa 594. Images were captured at room temperature with a Zeiss confocal system (LSM510; Zeiss, Jena, Germany) equipped with an Axiovert 100M microscope using an Apochromat 63x/1.4 oil objective in 2.5x zoom mode. The color balances of the images were adjusted with Photoshop (Adobe, San Jose, CA).

RNAi
RNAi of pat-6 was performed by feeding wild-type strain N2 worms bacteria expressing double-stranded RNA beginning at the L1 larval stage and continuing until the animals reached adulthood. Adults were then fixed and immunostained (Miller et al., 2006).

Generation and characterization of antibodies to PAT-6
Residues 1–99 of PAT-6 were expressed in E. coli and purified as a GST fusion protein. Primers PAT-6-1 and PAT-6-99 with added BamHI and Xhol sites were used to amplify a fragment from the full-length clone of pat-6 in the two-hybrid bait vector. The resulting fragment was cloned directly into BamHI- and Xhol-cut pGEX-KK1. After identification of a clone with an error-free insert, the clone was used to produce GST-PAT-6(1-99). This protein was shipped to Spring Valley Laboratories for generation of polyclonal antibodies in rats. Affinity purification was performed using Affigel conjugated to GST-PAT-6(1-99). Western blot extracts, as described earlier, were prepared from adults that had been subjected to RNAi by feeding beginning at the L1 stage, either with bacteria containing the empty vector or pat-6 cDNA sequence, and subjected to Western blot analysis using anti–PAT-6 (see Supplemental Figure S5). Wild-type adult muscle was costained with anti–CPNA-1 and anti–PAT-6 and imaged as described.

Immunostaining of embryonic muscle
Antibody staining of C. elegans embryos was carried out using a method modified from Albertson (1984). Adult worms were washed off plates and incubated for 5 min in embryo preparation solution containing 75% distilled H2O, 20% sodium hypochlorite, and 5% 10 M NaOH to dissolve the adult worm cuticles. After spinning down the suspension, the remaining embryos and worm carcasses were incubated for another 2 min in the embryo preparation solution, followed by three washes in M9 buffer. The remaining embryos were then either suspended in a 4% sucrose and 1 mM EDTA, pH 7.4, solution or left to incubate for 6 h to obtain later-stage embryos by resuspension. The egg suspension was transferred to glass slides coated with 2 mg/ml poly-l-lysine, covered by a rectangular glass coverslip, and frozen on an aluminum slab at ~80°C overnight. The coverslips were removed from the slides using a razor blade, and slides were transferred to ~20°C acetone for 4 min, with subsequent 1-min intervals of 75% acetone at 20°C, 50% acetone, 25% acetone, and Tween-TBS for 2 min. Embryos were double stained with anti–CPNA-1 together with antibodies to the various integrin adhesion complex proteins indicated in Figure 4. The double staining allowed identification of cpna-1/−null embryos among the progeny of cpna-1+/− parental worms. Primary antibodies solutions were added to the slides for 5 h and included anti–CPNA-1 (rabbit) at 1:100 together with anti–PAT-3 (mouse MH25; Francis and Waterston, 1985) at 1:500, anti–DEB-1 (vinculin; mouse MH24; Francis and Waterston, 1985) at 1:250, anti–PAT-6 (rat) at 1:40, or anti MYO-3 (mouse DMS-6; Miller et al., 1983) at 1:250 dilutions. It was not possible to double stain with anti–CPNA-1 and anti–PAT-4, as both are rabbit antibodies. However, none of the embryos from cpna-1/−/ parents showed mislocalization of PAT-4 (anti–PAT-4 used at 1:40 dilution). After incubation, slides were washed for 1 h in TBS-Tween and then removed and coated with secondary antibodies for 3 h: anti-rabbit Alexa 488 (Invitrogen, Carlsbad, CA) for PAT-4 and CPNA-1 antibodies, anti-mouse Alexa 594 (Invitrogen) for MH25, MH24, and DMS-5, and anti-rat Alexa 594 (Invitrogen) for anti–PAT-6. After a 1 h wash in TBS-Tween, mounting solution was added (20 mM Tris, pH 8.0, 0.2 M DABCO, and 90% glycerol) and slides were sealed with nail polish.

Generation of a translational YFP fusion for cpna-1
A C-terminal YFP translational fusion construct was created for cpna-1 using the fosmid recombineering method described in Tursun et al. (2009). The recombinered fosmid was named fDM1217, and a solution containing 94 ng/μl pRF4 rol-6(su1006dm) and 6 ng/μl fDM1217 was injected into the gonad of adult N2 animals using conventional methods as described in Mello et al. (1991).

Electron microscopy
For noncolloidal gold electron microscopy (EM), fixation and imaging was carried out identically to the procedure outlined in Warner et al. (2011). For colloidal gold EM, CPNA-1::YFP worms were transferred from agar plates and immersed in fixative. Under a dissecting microscope, worms were cut in half using a scalpel and collected in a vial. The fixation protocol used was a two-step method described in detail elsewhere (Vaid et al., 2007). Worms were embedded in Lowicryl, sectioned, placed on Formvar/carbon-coated grids, phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA)/0.5% Tween 20/1:20 normal goat serum (NGS) was aliquoted onto Parafilm in 8-μl drops, and grids were placed face down onto drops for 5 min. Primary chicken anti-GFP antibody (13970; Abcam, Cambridge, MA; also reacts with YFP) was diluted in PBS/0.1% BSA/0.5% Tween 20/1% NGS at a 1:1000 dilution, and 8 μl per grid (grids placed face down on drops) was used for an overnight incubation. Grids were then washed three times with PBS/0.1% BSA/0.05% Tween 20 (grids placed face down on drops). The secondary rabbit polyclonal anti chicken IgY antibody conjugated with 10 nm colloidal gold (39612; Abcam) was diluted 1:200 in PBS/0.1% BSA/0.05% Tween 20/5% fetal bovine serum (FBS), with the solution diluted 1:20 prior to antibody addition. The secondary antibody solution was aliquoted into 8 μl drops, and grids were placed face down for 2 h. Controls were incubated solely with either secondary antibody without primary, or chicken IgY (119138; Abcam) followed by secondary antibody incubation. Grids were then washed twice with PBS using hanging drops, then fixed for 10 min with 1% glutaraldehyde in PBS. Grids were then washed twice with distilled water using hanging drops, incubated on 8 μl drops of uranyl acetate for 3 min, then washed three times with distilled water, dried, and stored in a desiccator prior to imaging. Imaging was carried out using a Tecnai G2 Spirit electron microscope (FEI, Hillsboro, OR) operated at 120 kV. Quantification of gold particle localization was carried out by counting the number of gold particles in a region of 100 nm centered about a dense body. Four control sections and four colloidal gold-labeled CPNA-1 sections were utilized.

Yeast three-hybrid assay
The yeast three-hybrid assay was done essentially as described in Lin et al. (2003).
Sequence analysis
BLAST, using CPNA-1 as query against the C. elegans translated genome, was used to identify the complete set of copine containing proteins in this organism, as well as those in other organisms. PFAM was used to identify the copine domain boundaries and to identify other possible domains (e.g., C2 domains). The multisquence alignment of copine domains from C. elegans was performed using www .genome.jp/tools/clustalw/. To predict possible transmembrane helices in the copine family, we used the following programs: TMHMM Server v. 2.0 (www.cbs.dtu.dk/services/TMHMM-2.0), PRED-TMR (http://athina.bioc.uoa.gr/PRED-TMR/input.html), and HMMTop (www.enzim.hu/hmmtop/server/hmmtop.cgi).

ACKNOWLEDGMENTS

We thank Kim M. Gernert (Emory University, Atlanta, GA) for help in predicting the transmembrane domain of CPNA-1. We also thank Pamela Hoppe (Western Michigan University, Kalamazoo, MI) for her help editing the manuscript. G.M.B. thanks the Department of Pathology, Emory University, for current support, and the National Institutes of Health for previous support (Grants AR051466 and AR052133). D.G.M. thanks the Canadian Institute for Health Research and the National Science and Engineering Research Council of Canada for current support. D.G.M. is a Canadian Institute for Advanced Research Fellow.

REFERENCES


