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CSN-5, a Component of the COP9 Signalosome Complex, Regulates the Levels of UNC-96 and UNC-98, Two Components of M-lines in Caenorhabditis elegans Muscle

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In Caenorhabditis elegans two M-line proteins, UNC-98 and UNC-96, are involved in myofibril assembly and/or maintenance, especially myosin thick filaments. We found that CSN-5, a component of the COP9 signalosome complex, binds to UNC-98 and -96 using the yeast two-hybrid method. These interactions were confirmed by biochemical methods. The CSN-5 protein contains a Mov34 domain. Although one other COP9 signalosome component, CSN-6, also has a Mov34 domain, CSN-6 did not interact with UNC-98 or -96. Anti-CSN-5 antibody colocalized with paramyosin at A-bands in wild type and colocalized with abnormal accumulations of paramyosin found in unc-98, -96, and -15 (encodes paramyosin) mutants. Double knockdown of csn-5 and -6 could slightly suppress the unc-96 mutant phenotype. In the double knockdown of csn-5 and -6, the levels of UNC-98 protein were increased and the levels of UNC-96 protein levels were slightly reduced, suggesting that CSN-5 promotes the degradation of UNC-98 and that CSN-5 stabilizes UNC-96. In unc-15 and unc-96 mutants, CSN-5 protein was reduced, implying the existence of feed back regulation from myofibril proteins to CSN-5 protein levels. Taken together, we found that CSN-5 functions in muscle cells to regulate UNC-98 and -96, two M-line proteins.

INTRODUCTION

Caenorhabditis elegans is an excellent model system in which to study muscle because of its optical transparency and powerful genetic tools available (Waterston, 1988; Moerman and Fire, 1997; Moerman and Williams, 2006). The muscle used for locomotion is located in the body wall and consists of 95 spindle-shaped mononuclear cells arranged in interlocking pairs that run the length of the animal in four quadrants. The myofibrils are restricted to a narrow ~1.5-μm zone adjacent to the cell membrane along the outer side of the muscle cell. The thin filaments are attached to the dense bodies (Z-disk analogs), and the thick filaments are organized around M-lines. All the dense bodies and M-lines are anchored to the muscle cell membrane and extracellular matrix, which is attached to the hypodermis and cuticle. This allows the force of muscle contraction to be transmitted directly to the cuticle and allows movement of the whole animal. Thus, worm muscle M-lines and dense bodies serve the function of analogous structures in vertebrate muscle. But, in addition, because of their membrane anchorage and protein composition (see for example, Qadota et al., 2007), they are also similar to costameres of vertebrate muscle and focal adhesions of nonmuscle cells.

Over the last few years, multiple protein complexes have been found that link the muscle cell membrane to thick filaments at the M-line in C. elegans. The cytoplasmic tail of β-integrin is associated with a conserved four-protein complex that includes UNC-97 (PINCH in mammals; Mackinnon et al., 2002; Lin et al., 2003; Norman et al., 2007). UNC-97, in turn, has been found to interact with one or two additional proteins linked or bound to myosin in the thick filaments (Miller et al., 2006; Qadota et al., 2007). Among the myosin-interacting proteins are UNC-98 and -96. These proteins were first identified by isolation of their loss-of-function mutants (Zengel and Epstein, 1980). Mutants of each gene have a similar and characteristic phenotype: by polarized light microscopy, each shows a disorganization of myofibrils and birefringent needles at the ends of the muscle cells. Molecular cloning revealed that each of these genes encode fairly small polypeptides of 300–400 residues. UNC-98 contains four C2H2 Zn fingers (Merce et al., 2003), but UNC-96 has no recognizable domains (Merce et al., 2006). Antibodies to each localize them to the M-lines. There is genetic and biochemical evidence that UNC-98 and -96 interact with each other (Merce et al., 2006) and with paramyosin (Merce et al., 2006; Miller et al., 2008), an invertebrate-specific protein located in the cores of thick filaments, encoded by the gene unc-15.

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Abbreviations used: ECL, enhanced chemiluminescence; HA, hemagglutinin; MBP, maltose-binding protein.
Interestingly, unc-96 mutants contain discrete accumulations of UNC-98 protein, and unc-98 mutants contain discrete accumulations of UNC-96 protein (Mercer et al., 2006). These accumulations very likely correspond to the birefringent needles visualized by polarized light. In addition, both unc-98 and -96 mutants contain discrete accumulations of paramyosin. Both UNC-96 and -98 have diffuse localization within muscle of a paramyosin (unc-15) null mutant and show colocalization with paramyosin paracrystals in a paramyosin missense mutant (Miller et al., 2008). By Western blot, in the absence of paramyosin, UNC-98 is diminished, whereas in paramyosin missense mutants, UNC-98 is increased. By yeast two-hybrid assay and ELISAs using purified proteins, UNC-98 interacts with paramyosin residues 31-693, whereas UNC-96 interacts with a separate region of paramyosin, residues 699-798. Paramyosin lacking the C-terminal UNC-96-binding region fails to localize throughout the A-bands. This data are compatible with a model we proposed in which UNC-98 and -96 may act as chaperones to promote the incorporation of paramyosin into thick filaments (Miller et al., 2008). To gain insight into the function of UNC-98 and -96, we have been identifying and learning the functions of their binding partners initially through a two-hybrid approach followed by confirmation by biochemical assays and interpretation using mutations and RNA interference (RNAi). Here we report that, unexpectedly, both UNC-98 and -96 interact with CSN-5, a component of the evolutionarily conserved COP9 signalosome, which has been implicated in a wide variety of biological functions usually linked to ubiquitin-mediated proteolysis. Our results are the first to implicate CSN-5 or the COP9 signalosome in myofibrillar organization or function.

MATERIALS AND METHODS

**Nematode Strains and Culture**

The following *C. elegans* strains were used in these studies: wild-type N2, unc-96 (e1067); unc-98 (e129), unc-15 (e1214), and unc-15 (e736). Nematodes were grown at 20°C on NGM agar plates with *Escherichia coli* sites of the vector pKS-HA8(Nhex2). After finding an error-free clone, the added XhoI site. This fragment was inserted between the EcoRV and SalI library were described in Miller et al. (2006).

**CSN-5 Functions in Sarcomeres**

CSN-5 is a key component of the COP9 signalosome, a protein complex required for the assembly and function of the ribosome. It is known to bind to various other proteins, including actin, to promote the incorporation of paramyosin into thick filaments. UNC-96 and -98, which are sarcomeric myosins, interact with CSN-5. This interaction is mediated by the C-terminal 202 residues of CSN-5 (aa 167-369), which are expressed and purified as a maltose-binding protein (MBP)-tagged fusion protein. To study the interaction between UNC-98 and CSN-5, we used anti-UNC-98 antibodies to affinity-purify the N-terminal residues of CSN-5 from various genes, and used Western blotting to compare the protein load per lane. Using these methods, we could compare the amount of actin or paramyosin present. We used quantities of extracts and dilutions of antibodies that would place us into the linear range of detection by enhanced chemiluminescence (ECL) and exposure to film. For detection of UNC-98, we used anti-UNC-98 affinity purified to the N-terminal residues 1-112 as described in Miller et al. (2006) at 1:200 dilution. Affinity-purified antibodies to UNC-96 (Mercer et al., 2006) and to CSN-5 were also used at 1:200 dilutions. Monoclonal antibodies to actin (clone C4; Chemicon International, Temecula, CA) were used at 1:12,000 dilution, and antibodies to paramyosin (5-23; Miller et al., 1983) were used at 1:2000 dilution. The quantitation of UNC-98 protein levels shown in Figure 8B was performed on a representative Western blot using Adobe Photoshop (San Jose, CA) and a method described in the following Web site: https://www.lukemiller.org/journal/2007/08/quantifying-Western-blots-without.html. For each lane, the UNC-98 level was normalized to that of actin. The relative amount of UNC-98 protein for each RNAi experiment, was expressed as follows: (experimental value/empty vector value) × 100.

For immunostaining, adult worms were fixed as described in Nonet et al. (1993). Anti-CSN-5 (see above) was used at 1:100 dilution, anti-a-actinin (monoclonal MH35; Francis and Waterston, 1985) at 1:200 dilution, anti-paramyosin (monoclonal 5-23; Miller et al., 1983) at 1:200 dilution, Secondary antibodies and confocal microscopy were as described in Qadota et al. (2007).

**HA Pulldown Assay**

The method for trying to determine whether yeast expressed HA-CSN-5 (full length) interacts with bacterially expressed MBP-UNC-96 (201-418) or MBP-UNC-98 (1-112) was performed essentially as described in Mercer et al. (2006).

**Far Western Assay**

A far Western assay for determining if bacterially expressed CSN-5-His interacts with bacterially expressed MBP-UNC-96 (201-418) or MBP-UNC-98 (1-112) was performed as described previously (Mercer et al., 2006).

**Generation of Anti-CSN-5 Antibodies**

The C-terminal 202 residues of CSN-5 (aa 167-369) were expressed and purified in E. coli as an MBP fusion protein. To do this primers, GACTGCTGCTGCGCTGCTGACGGTGCAGTGCAGTGCGCCTCAGACCGAC with a deleted BamHI site and the 3′ primer AGTCTGCTGCGCTGCTGACGGTGCAGTGCAGTGCGCCTCAGACCGAC with an added XhoI site. The fragment was excised, cloned into pMAL-KK1 using the same restriction sites, and used for protein expression as described in Mercer et al. (2006). The resulting MBP-CSN-5 (167-369) was shipped to Spring Valley Laboratories (Woodbine, MD) for generation of rabbit polyclonal antibodies. After removal of most of the anti-MBP antibodies by immunoprecipitation with MBP-UNC-96 (201-418) (Mercer et al., 2006), anti-CSN-5 antibodies were affinity-purified against MBP-CSN-5 as described previously (Mercer et al., 2003).

**Western Blots and Immunofluorescence Localization of Proteins**

We used the procedure of Hannak et al. (2002) to prepare total protein lysates from wild-type, unc-96, -98, and -15 mutant worms and from rfi3(pk1426) RNAi hypersensitive worms (Simmer et al., 2002) subjected to RNAi by feeding with empty vector, or for csn-5, -6, or both csn-5 and -6 (see below). When comparing wild type and mutants, or empty vector and RNAi for various genes, we loaded approximately equal total amounts of protein extract estimated by finding volumes of extracts that would give equal intensity of banding after Coomasie staining. To assure that equal total protein was loaded per lane, using Western blotting, we compared the amount of actin or paramyosin present. We used quantities of extracts and dilutions of antibodies that would place us into the linear range of detection by enhanced chemiluminescence (ECL) and exposure to film. For detection of UNC-98, we used anti-UNC-98 affinity purified to the N-terminal residues 1-112 as described in Miller et al. (2006) at 1:200 dilution. Affinity-purified antibodies to UNC-96 (Mercer et al., 2006) and to CSN-5 were also used at 1:200 dilutions. Monoclonal antibodies to actin (clone C4; Chemicon International, Temecula, CA) were used at 1:12,000 dilution, and antibodies to paramyosin (5-23; Miller et al., 1983) were used at 1:2000 dilution. The quantitation of UNC-98 protein levels shown in Figure 8B was performed on a representative Western blot using Adobe Photoshop (San Jose, CA) and a method described in the following Web site: https://www.lukemiller.org/journal/2007/08/quantifying-Western-blots-without.html. For each lane, the UNC-98 level was normalized to that of actin. The relative amount of UNC-98 protein for each RNAi experiment, was expressed as follows: (experimental value/empty vector value) × 100.

For immunostaining, adult worms were fixed as described in Nonet et al. (1993). Anti-CSN-5 (see above) was used at 1:100 dilution, anti-a-actinin (monoclonal MH35; Francis and Waterston, 1985) at 1:200 dilution, anti-paramyosin (monoclonal 5-23; Miller et al., 1983) at 1:200 dilution, Secondary antibodies and confocal microscopy were as described in Qadota et al. (2007).
RNAi for csn-5 and -6

RNAi was performed by a feeding method (Simmer et al., 2002). The following plasmids were used: pPD129.36-csn-5 for csn-5, pPD129.36-csn-6 for csn-6, and pPD129.36-csn-5/-6 for csn-5 and -6 double RNAi. To construct pPD129.36-csn-5, the XhoI fragment of 4c-4-2 (originally isolated from yeast two-hybrid screening with UNC-98 N-terminus as bait (Miller et al., 2006) containing full-length csn-5 cDNA) was cloned into the Xhol site of pPD129.36 (Timmons et al., 2001). pPD129.36-csn-6 was made by insertion of PCR-amplified csn-6 full-length cDNA into pPD129.36 between BamHI and Xhol sites. For making the pPD129.36-csn-5/-6 plasmid, the XhoI fragment of 4c-4-2 was cloned into the Xhol site of pPD129.36-csn-6.

For immunostaining of RNAi worms, the following method was used. Feeding bacteria containing the RNAi plasmids noted above were cultured in liquid culture and induced using a final concentration of 0.4 mM IPTG for 4 h. Liquid culture of induced cells was spotted onto NGM plates containing ampicillin (50 µg/ml) and tetracycline (15 µg/ml). Worms were picked onto 25 6-cm RNAi plates (each containing three spots of induced bacteria) and incubated at 20°C overnight to eliminate RNAi nonaffected eggs. The next day, the worms were transferred from the five 6-cm plates to five 10-cm plates (each containing 11 spots of induced bacteria) and incubated at 20°C for 8 h for these animals to lay F1 generation eggs. After 8 h, the 10 parental worms were removed from each of the 10-cm plates. After 3-d incubation at 20°C, the F1 generation attained young adulthood, a good stage for immunostaining using the method of Nonet al. (1993).

For the preparation of Western blot lysates or total RNA (see below), the following method was used. Worms were picked onto 25 6-cm RNAI plates (each containing three spots of induced bacteria) at 20°C for 8 h. These plates were incubated at 20°C overnight to eliminate RNAI nonaffected eggs. The next day, the 10 worms were transferred from the 25 6-cm plates to 25 10-cm plates (each containing 11 spots of induced bacteria) and incubated at 20°C for 8 h for these animals to lay F1 generation eggs. After 8 h, the 10 parental worms were removed from each of the 10-cm plates. After 3-d incubation at 20°C, the F1 generation attained young adulthood, and the worms were collected from the plates by washing them off with M9 buffer.

Real-Time PCR Assays

Total RNA from approximately 100–200 µl pellets of F1 worms (described above) that had been fed bacteria containing either csn-5 RNAi or empty vector was extracted using Trizol (Invitrogen, Carlsbad, CA) using the manufacturer's procedure and quantitated by UV absorbance. First-strand cDNAs were synthesized with random decamers using a RETROscript kit (Applied Biosystems, Foster City, CA). The following primers were used to amplify ~120-base pair cDNAs from unc-96 and ges-1: Q982F: GTTGGATGCACGTGATCAAACAGG, Q982R: GAGTGTTCATGAAGTTGAATGTGAG, ges-1F: GGAATCCAGAACAGG, Q982R: GAGTGTTCATGAAGTTGAATGTGAG, ges-1R: CGTGAAATCCAGAACA-GAAGTC. Standard PCR using these primers and first-strand cDNAs revealed contamination of the RNA with genomic DNA. Therefore, the RNA was treated with RNase-free DNAse (TURBO DNA-free from Applied Biosystems), the first-strand cDNAs were made again, and agarose gel analysis of trial PCRcs revealed production of the single bands of expected size without genomic DNA contamination. Real-time PCR was performed in 50-µl volumes using SYBR-green detection in a 96-well plate on an iCycler machine (Bio-Rad, Hercules, CA). Three independent reactions were performed in duplicate from each of two independently generated first-strand cDNAs for each sample (empty vector or csn-5 RNAi). The PCR reactions were initiated at 95°C for 1 min followed by 40 cycles of, 15 s at 95°C, 60 s at 60°C, and 30 s at 68°C using Platinum Taq DNA polymerase (Invitrogen). The following equation, adapted from Maeda et al., 2007, was used: % Δ in unc-98 mRNA = 2Δ C of unc-98 mRNA − Δ C of empty vector × 100, in which C of csn-5 (RNAi) = (average Ct value of unc-98 mRNA − average Ct value of ges-1 mRNA), and Ct of empty vector = (average Ct value of unc-98 mRNA − average Ct value of ges-1 mRNA).

Polarized Light Microscopy

Polarized light microscopy was performed as described previously (Mercer et al., 2006).

RESULTS

CSN-5 Interacts with UNC-98 and -96

As reported previously (Miller et al., 2006), when we used the N-terminal 112 residues of UNC-98 as bait to screen a yeast two-hybrid library of C elegans cDNAs, preys representing 18 unique proteins were recovered. Among them were 10 clones representing full-length cDNA for csn-5 (Supplemental Data in Miller et al., 2006). Because of the similarity in mutant phenotype of unc-98 and -96, their genetic interaction, their interaction as proteins, and their colocalization at the M-line (Mercer et al., 2006), a similar screen was conducted with UNC-96. A bait representing the C-terminal half of UNC-96 yielded two independent clones for CSN-5. CSN-5 is the C elegans ortholog of subunit 5 of the COP9 signalosome (CSN) complex conserved in plants and animals and functions in controlling protein degradation, either in stabilizing some proteins, or in promoting the degradation of other proteins (for reviews see Cope and Deshaies, 2003; Schwechheimer, 2004). In C elegans, the COP9 signalosome contains five proteins with PCI domains and two proteins, CSN-5 and CSN-6, with Mov34 (also known as MPN) domains (Luke-Glaser et al., 2007). As shown in Figure 1, by two-hybrid analysis, interaction of the C-terminal half of UNC-96 or the N-terminal 112 residues of UNC-98 require full-length CSN-5. Neither residues 1-165, containing the Mov34 domain, nor residues 166-369 of CSN-98 alone are sufficient. In addition, the other Mov34 domain containing protein in C elegans, CSN-6, failed to interact with either UNC-96 or -98 (Figure 1).

To verify the interactions identified by two-hybrid assay, two different biochemical assays were used with purified CSN-5, UNC-98, and -96 proteins. Total protein extracts were prepared from yeast expressing HA-tagged CSN-5, immunoprecipitated with beads coated with antibodies to HA, washed, and incubated with purified, bacterially expressed MBP-UNC-98 (1-112), MBP-UNC-96 (201-418), or MBP, and then washed, and the bound proteins were eluted. As shown by the immunoblots in Figure 2A (top), each reaction contained equal amounts of purified HA-CSN-5, but only MBP-UNC-98 was copelleted. This verifies interaction between purified CSN-5 and purified UNC-98. In the second assay, far Western blot analysis was used. Bacterially expressed CSN-5 with a C-terminal 6His tag was purified, separated on a gel, and blotted, and then the blot was incubated with either MBP-UNC-98 (1-112), MBP-UNC-96 (201-418), or MBP. As shown in Figure 2B, reaction
with antibodies to MBP revealed an interaction of CSN-5 with UNC-96. This verifies interaction between purified CSN-5 and purified UNC-96.

**Anti-CSN-5 Localizes to Muscle A-Bands**

As one way to determine if these interactions occur in vivo, we wanted to determine if and where CSN-5 is located in the sarcomere. We raised rabbit polyclonal antibodies to a region of CSN-5 outside the Mov34 domain to avoid possible cross-reaction with CSN-6 (Figure 3A). After affinity purification, these antibodies primarily react with a protein of 40 kDa, the size expected from the CSN-5 sequence. Molecular-weight standards are shown at the right. (C) Anti-CSN-5 localize to A-bands in body wall muscle. Anti-CSN-5 and anti-α-actinin were coincubated with wild-type C. elegans, and the muscle was imaged by confocal immunofluorescence microscopy. The images show a portion of one body wall muscle cell. α-Actinin is a marker for dense bodies. Bar, 10 μm.

**CSN-5 Colocalizes with Abnormal Accumulations of Paramyosin in unc-98, -96, and -15 Mutant Muscle**

The hallmark of the unc-96 or -98 mutant body wall muscle is the presence birefringent “needles” at the ends of the muscle cells as observed by polarized light microscopy (Zengel and Epstein, 1980; Mercer et al., 2003, 2006). These
needles likely contain accumulations of paramyosin in either unc-96 or -98 mutants, UNC-98 protein in unc-96 mutants, and UNC-96 protein in unc-98 mutants (Mercer et al., 2006; Miller et al., 2008). Thus, given the interaction between CSN-5 and UNC-96 and -98, we wanted to determine if CSN-5 was also present in the needles or paramyosin accumulations. When the anti-CSN-5 antibodies were used to stain unc-96 and -98 mutants, CSN-5 was found at both A-bands and in accumulations at the ends of the muscle cells, colocalizing with paramyosin (Figure 4). Additionally, we examined the localization of CSN-5 in an unc-15 missense mutant, e1215, that contains paramyosin paracrystals. As shown in Figure 5, CSN-5 and paramyosin colocalize, including in the paramyosin paracrystals. Similarly, we had shown that these paramyosin paracrystals in unc-15 (e1215) also contain UNC-98 and -96 (Miller et al., 2008). Thus, in three conditions (unc-96, -98, and -15 mutants) in which abnormal accumulations or paracrystals of paramyosin are formed, CSN-5 is associated with the abnormal paramyosin.

**RNAi Knockdown of csn-5 Increases the Level of UNC-98 Protein**

Because CSN-5 localizes to the A-band, we wondered whether A-band organization would be disrupted when csn-5 was reduced in activity. In addition, because CSN-5 and -6 each have a Mov34 domain and thus might have redundancy in function, we wondered whether A-band organization would be disrupted when csn-6 was reduced in activity. Because intragenic deletions that are presumably null for csn-5 are sterile (our unpublished observations), such strains would be inconvenient for protein localization studies. In addition, Smith et al. (2002) reported that RNAi for csn-5 results in sterility with small gonads and lack of oocytes. Nevertheless, we could feed large numbers of wild-type worms bacteria producing csn-5 double-strand RNA (dsRNA) and examine the muscle structure of the F1 adults. This was conducted for csn-5, csn-6 and a double csn-5 csn-6 RNAi construct. As shown in Figure 6, these treatments resulted in no defects in A-band organization, either in the localization of paramyosin, which is located in the polar

![Figure 4](image1.png)

**Figure 4.** Immunofluorescence localization of CSN-5 and paramyosin in wild-type and in unc-96 (sf18) and unc-98 (sf19) mutant muscle. In wild-type muscle, CSN-5 localizes to A-bands, colocalizing with paramyosin. In both unc-96 and -98, some CSN-5 is found in A-bands, but much of it is found in needle-like accumulations containing paramyosin at the ends of muscle cells. Bar, 10 μm.

![Figure 5](image2.png)

**Figure 5.** CSN-5 colocalizes with paramyosin in a paramyosin missense mutant. The paramyosin missense mutant, unc-15 (e1215), has paramyosin paracrystals within its body wall muscle cells. These accumulations of paramyosin also contain CSN-5. Bar, 10 μm.

![Figure 6](image3.png)

**Figure 6.** Knockdown of csn-5, -6, or both together has no effect on the localization of two thick filament components, MHC A and paramyosin. Wild-type worms were subjected to RNAi by feeding using either the empty vector or with vectors containing the indicated genes. F1 adults were fixed and immunostained with either anti-MHC A or anti-paramyosin. RNAi with the empty vector resulted in worms with the same staining pattern as unfed wild-type worms. Bar, 10 μm.
Loss-of-Function Mutations in unc-96 and -15 Result in Diminished Levels of CSN-5 Protein

We also wanted to determine whether the levels of CSN-5 might be affected by the levels of UNC-96, -98, or the level or state of paramyosin. As shown in Figure 9A, and as shown previously (Miller et al., 2008), the levels of paramyosin are similar in wild-type, unc-96, and -98 loss-of-function mutants. However, in unc-96 mutants (but not unc-98 mutants), the level of CSN-5 is significantly diminished. As shown in Figure 9B, the level of CSN-5 was examined in three mutant alleles of unc-15 (paramyosin): in the null allele, e1214, and in the missense alleles e1215 and e73, each of which form paramyosin paracrystals. In the absence of paramyosin (e1214), CSN-5 is significantly diminished. (Similarly, in the absence of paramyosin, UNC-98 is diminished; Miller et al., 2008.) In the presence of abnormal paramyosin, e73 and to some extent e1215, moderate decreases in the levels of CSN-5 were also observed. (In contrast, in the presence of these abnormal paramyosins, UNC-98 levels were increased; Miller et al., 2008).

DISCUSSION

In summary, we have identified an interaction between the highly conserved COP9 signalosome component CSN-5, implicated in control of protein levels, with two known components of nematode sarcomeric M-lines: UNC-98 and -96. CSN-5 was found to colocalize with paramyosin in wild-type muscle and to colocalize with abnormal aggregates of paramyosin in unc-96 and unc-98 loss-of-function mutants and in paramyosin (unc-15) missense mutants. RNAi knockdown of csn-5 together with the similar protein csn-6 did not result in a defect in myofibrillar structure, but it did result in a mild improvement in the muscle structure of unc-96 mutant muscle.

We have obtained insight into the meaning of direct interaction between CSN-5 and UNC-98 and -96 from the Western blot experiments (Figure 8). In csn-5 (RNAi) and csn-5 csn-6 double RNAi animals, the amount of UNC-98 levels as loading controls and the results are depicted in Figure 8B. As compared with animals fed bacteria carrying the empty RNAi vector, csn-5 (RNAi) and csn-5 (RNAi) csn-6 (RNAi), resulted in 2.1- and 2.7-fold higher levels of UNC-98 protein, respectively. Because nematode CSN-5 has been shown by antibody staining to reside in both cytoplasm and nuclei of most cell types (Smith et al., 2002; Pintard et al., 2003), we wondered if csn-5 knockdown might affect unc-98 transcription. This was investigated by performing quantitative real-time RT-PCR for unc-98 mRNA in worms fed RNAi empty vector versus csn-5 (RNAi). We normalized the unc-98 mRNA levels to those of ges-1 mRNA, which encodes a carboxylesterase expressed specifically in the gut (Aamodt et al., 1991). As shown in Figure 8C, knockdown of csn-5 resulted in a 1.3-fold increase in unc-98 mRNA. Because the increase in UNC-98 protein is greater than that in unc-98 mRNA (2.1- vs. 1.3-fold), the increase of unc-98 mRNA might not be the main reason for the increase in UNC-98 protein caused by csn-5 knockdown. Our results are at least consistent with a normal role of CSN-5 in promoting the degradation of UNC-98 protein (see Discussion). Finally, as shown in Figure 8A, the effects on protein levels of UNC-96 were not so obvious although there was a mild decrease of UNC-96 when csn-5, -6, or especially both csn-5 and csn-6 were knocked down. Thus, this result would be consistent with a normal role of CSN-5 in promoting the stability of UNC-96.
was increased (2.1- and 2.7-fold, respectively). From the quantitative RT-PCR experiment, csn-5 (RNAi) resulted in a 1.3-fold increase in unc-98 mRNA. However, because the increase in UNC-98 protein was 2.1-fold, we propose that the increase of unc-98 mRNA is not the main reason for the increase in UNC-98 protein. Based on the interaction between UNC-98 protein and CSN-5 protein that we have demonstrated here, the known affect of the COP9 signalosome on ubiquitin-mediated proteolysis, and the lack of detectable CSN-5 protein in muscle cell nuclei, we suggest, that at least in \textit{C. elegans} muscle, one normal role of CSN-5 protein is to promote the degradation of UNC-98 protein. In contrast, knockdown of csn-6, or csn-6 together with csn-5 resulted in a mild decrease in UNC-98, implying that the normal function of CSN-5 is to stabilize UNC-98. Our interpretation that the normal function of CSN-5 is to promote degradation of UNC-98 is similar to most studies on the COP9 signalosome implicating a role for promoting degradation of proteins via ubiquitin-mediated proteolysis. This function for CSN-5 has also been seen in \textit{C. elegans}, for

Figure 8. RNAi for csn-5 results in an increase in the levels of UNC-98 protein. (A) Extracts containing equal amounts of total Laemmli-soluble proteins from F1 progeny of animals fed either the RNAi by feeding empty vector or vectors containing csn-5 or -6 or both csn-5 and -6, were separated on a gel, blotted, and reacted with antibodies to the indicated proteins. As expected, the level of CSN-5 is reduced in csn-5 (RNAi) or in csn-5 (RNAi) csn-6 (RNAi). Unexpectedly, CSN-5 is also reduced in csn-6 (RNAi). Note that when csn-5 was knocked down, the level of UNC-98 was increased. The levels of actin and paramyosin served as loading controls. (B) Quantitation of UNC-98 protein levels under different conditions. For each lane, the UNC-98 level was normalized to that of actin. As compared with empty RNAi vector, csn-5 (RNAi) resulted in a 2.1-fold increase, and csn-5 csn-6 double RNAi resulted in a 2.7-fold increase in UNC-98 protein. (C) Quantitative real-time PCR of unc-98 mRNA with and without RNAi for csn-5. mRNA levels of unc-98 upon csn-5 (RNAi) were calculated relative to the empty vector, which was arbitrarily set at a value of 100. As compared with empty vector, csn-5 (RNAi) resulted in a 1.3-fold increase in unc-98 mRNA levels. The graphs depict means from six experiments, and the error bars depict standard deviations.

Figure 9. The effects of mutations in unc-96, -98, and -15 on the levels of CSN-5. Extracts containing equal amounts of total Laemmli-soluble proteins from wild-type (N2), loss-of-function alleles for unc-96 (sf18) and unc-98 (sf19), and the unc-15 null (e1214) and two unc-15 missense mutations (e1215 and e73) were separated on a gel, blotted, and reacted with antibodies to the indicated proteins. (A) The level of CSN-5 protein is diminished in the unc-96 mutant. (Paramyosin, the level of which was shown previously not to be affected in unc-96 or -98 and actin, serve as loading controls.) (B) The level of CSN-5 protein is significantly decreased in the paramyosin null (e1214), and moderately decreased in the two paramyosin missense mutants (especially e73).
example, in promoting degradation of MEI-1, a katanin-like protein (Pintard et al., 2003). Our results implicating a function of CSN-5 in stabilizing UNC-96, are unusual, but not unprecedented: for example, in C. elegans, GLH-1, a germ-line RNA helicase found in oocyte P granules is also stabilized by CSN-5 (Orsborn et al., 2007).

It is notable that the effects of CSN-5 and -6 are different on UNC-98 and -96. Whereas csn-5 (RNAi) results in elevation of UNC-98 levels, it has no effect on UNC-96. In contrast, csn-6 (RNAi) results in no change in UNC-98 and reduced levels of UNC-96. Several studies in Schizosaccharomyces pombe and in Drosophila have shown that loss-of-function or knockdown of one CSN subunit does not always result in the same phenotype as loss-of-function or knockdown of other individual subunits, suggesting that some components may function independently from the COP9 complex (Mundt et al., 2002; Oron et al., 2002). Although both CSN-5 and -6 share the Mov34 domain, outside this domain there is no similarity, and thus different functions might be expected. On the other hand, it is somewhat surprising that knockdowns of either csn-5 or -6 have different phenotypes because C. elegans CSN-5 and -6 have been shown to interact by a two-hybrid assay (Pintard et al., 2003), and a reconstituted human COP9 complex has been shown by a mass spectrometry approach to contain two subcomplexes, one of which has an interaction between CsN5 and CsN6 (Sharon et al., 2009). Furthermore, we do not know whether the other six components of the COP9 signalosome are involved in regulating UNC-98 and -96 levels.

It has been reported that csn-5 mRNA is expressed in both somatic and germline tissues, and is in fact, enhanced in the germline (Smith et al., 2002). Two groups (Smith et al., 2002 and Pintard et al., 2003), using specific anti-CSN-5 antibodies, have shown that CSN-5 protein is expressed in each cell of the adult and embryo, where it is localized in the cytoplasm and also in most nuclei. Our results are the first to indicate that CSN-5 is localized to body wall muscle A-bands, and indeed the first time that any COP9 complex member has been localized to sarcomeres in any animal. Interestingly, we did not observe nuclear localization of anti-CSN-5 in body wall muscle cells.

Moreover, our results indicate that there is very high expression of csn-5 in body wall muscle because loss-of-function mutations in unc-96 or -15, genes that are primarily expressed in body wall muscle, resulted in very large decreases in the total amount of CSN-5 extracted from whole nematodes. This is surprising given that body wall muscle comprises only 95% of a total of 959 somatic cells of the worm. This high level of CSN-5 in body wall muscle is another indication that CSN-5 has an important role to play in muscle. This is an additional role for CSN-5 at least in C. elegans, in which it had been reported previously to be essential for oocyte development (Smith et al., 2002; Pintard et al., 2003).

Moreover, although the COP9 signalosome has been shown in many organisms to regulate a myriad of cellular processes, ranging from cell cycle and signal transduction to transcription (Cope and Deshaies, 2003; Schwechheimer, 2004), our data are the first to show that COP9 has a role in myofibril organization and/or assembly.

Our findings also imply a novel way to regulate CSN-5: In either unc-96 loss-of-function or unc-15 (paramyosin) null mutants there was a substantial decrease in levels of CSN-5. This suggests that normally UNC-96 or paramyosin, by some unknown mechanism(s), indirectly promotes the expression or stabilization of CSN-5. This could occur by binding to UNC-96 or may be occurring at the transcriptional or translational levels. The possibility of transcriptional control is suggested by the large decrease in CSN-5 protein observed and that several of the proteins that UNC-96 interact with directly or indirectly are found in both myofibrils and nuclei (e.g., UNC-98, Mercer et al., 2003; UNC-97, Hobert et al., 1999). The stabilization of CSN-5 by paramyosin is more difficult to explain and may not occur through direct interaction of CSN-5 and paramyosin. By two-hybrid assays, full-length CSN-5 bait was tested for interaction with full-length and various deletion derivatives of paramyosin as preys. No interactions were revealed (data not shown). One possibility, however, is suggested by our previous finding that in the absence of paramyosin, the level of UNC-98 protein is reduced (Miller et al., 2008); thus, less UNC-98 is available to translocate into the nucleus and stimulate transcription of target genes such as possibly, csn-5.

In our previous studies, we had shown that the birefringent needles in unc-96 and -98 mutants contain abnormal aggregates of paramyosin residing outside the thick filaments (Mercer et al., 2006; Miller et al., 2008). These needles also likely contain UNC-98 in unc-96 mutants and UNC-96 in unc-98 mutants (Mercer et al., 2006). We can now add a fourth protein, CSN-5, by virtue of its interaction with either UNC-98 or -96 and its presence in accumulations of paramyosin in unc-98 and -96 mutants, as also being a component of these needles. Previously, we showed that in the absence of paramyosin, there was a decrease in UNC-98, and in the presence of abnormally aggregated paramyosin there was an increase in UNC-98. We had postulated that the dependence of UNC-98 and possibly UNC-96 levels on the state of paramyosin might be due to a chaperone function for UNC-98 or -96 to prevent aggregation of paramyosin and therefore to promote the incorporation of paramyosin into thick filaments. We can extend our model to include the regulation of UNC-98 and -96 levels (albeit in opposite directions) by CSN-5 (Figure 10).

![Figure 10. CSN-5 cooperates with UNC-98 and -96 to promote assembly of paramyosin into thick filaments. UNC-98 and -96 are two components of the nematode M-line and interact with each other. Paramyosin, a major component of thick filaments, is likely to exist in two pools: paramyosin incorporated into thick filaments and free paramyosin created by new synthesis or by normal turnover of thick filaments. Previous results supported a model in which UNC-98 and -96 act as chaperones to prevent abnormal aggregation of free paramyosin and thus promote its incorporation into thick filaments. In this study we show that CSN-5, a component of the COP9 signalosome, interacts with both UNC-98 and -96; that CSN-5 promotes the degradation of UNC-98 (arrow), that a combination of CSN-5 and -6 (shown by other studies to interact with each other) stabilize UNC-96 (converging arrows), and that UNC-96 and free paramyosin indirectly or through a positive feedback loop act to increase the levels of CSN-5 (dotted arrows). Overall CSN-5 and -6 act together with UNC-98 and -96 to promote the incorporation of paramyosin into thick filaments.](image-url)
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