The Role of the UNC-82 Protein Kinase in Organizing Myosin Filaments in Striated Muscle of *Caenorhabditis elegans*

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ABSTRACT We study the mechanisms that guide the formation and maintenance of the highly ordered actin-myosin cytoskeleton in striated muscle. The *UNC-82* kinase of *Caenorhabditis elegans* is orthologous to mammalian kinases ARK5/NUAK1 and SNARK/NUAK2. *UNC-82* localizes to the M-line, and is required for proper organization of thick filaments, but its substrate and mechanism of action are unknown. Antibody staining of three mutants with missense mutations in the *UNC-82* catalytic domain revealed muscle structure that is less disorganized than in the null *unc-82(0)*, but contained distinctive ectopic accumulations not found in *unc-82(0)*. These accumulations contain paramyosin and myosin B, but lack myosin A and myosin A-associated proteins, as well as proteins of the integrin-associated complex. Fluorescently tagged missense mutant protein *UNC-82* E424K localized normally in wild type; however, in *unc-82(0)*, the tagged protein was found in the ectopic accumulations, which we also show to label with recently synthesized paramyosin. Recruitment of wild-type *UNC-82::GFP* to aggregates of differing protein composition in five muscle-affecting mutants revealed that colocalization of *UNC-82* and paramyosin does not require *UNC-96*, *UNC-98/ZnF*, *UNC-89/obscurin*, *CSN-5*, myosin A, or myosin B individually. Dosage effects in paramyosin mutants suggest that *UNC-82* acts as part of a complex, in which its stoichiometric relationship with paramyosin is critical. *UNC-82* dosage affects muscle organization in the absence of paramyosin, perhaps through myosin B. We present evidence that the interaction of *UNC-98/ZnF* with myosin A is independent of *UNC-82*, and that *UNC-82* acts upstream of *UNC-98/ZnF* in a pathway that organizes paramyosin during thick filament assembly.

KEYWORDS AMPK-related kinase; myosin; paramyosin; striated muscle; thick filament assembly

THE nematode *Caenorhabditis elegans* has been an excellent system for discovery and study of proteins important for proper muscle cell structure and function (reviewed in Waterston 1988; Moerman and Fire 1997; Moerman and Williams 2006; Gieseler *et al.* 2016). The contractile apparatus of striated muscle is a highly ordered array of interdigitated actin thin filaments and myosin thick filaments. The myosin filaments in *C. elegans* contain myosin A, myosin B, paramyosin, and the filagenins. The nematode thick filament has been modeled to consist of a series of concentric layers,

with an outer layer containing myosins A and B, an intermediate layer of paramyosin, and an inner layer or core of paramyosin and the filagenins (Epstein *et al.* 1985; Deitiker and Epstein 1993; Liu *et al.* 1998). Paramyosin is a “headless myosin,” which is homologous to the C-terminal three-fourths of the myosin coiled-coil rod (Kagawa *et al.* 1989). These three coiled-coil proteins segregate to three distinct compartments within the thick filament. Paramyosin, which has a more hydrophobic surface (Cohen *et al.* 1987), forms a core that runs the length of the filament. Paramyosin supports formation of the long thick filaments found in invertebrate muscle. The motor protein myosin assembles upon the surface of this paramyosin core, where its motor domain can interact with actin filaments to slide the two filament systems past each other, and accomplish contraction of the muscle cell. Myosin A is found in the central region of the thick filament, which is also the site where the thick filament attaches

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to the M-line. Myosin B, the major myosin isoform, is found in the filament arms that extend on either side of the M-line (Miller *et al.* 1983).

Contraction of the body-wall muscles, which are attached to the cuticular exoskeleton of the worm through the epidermis, causes the body bends that drive locomotion. In addition, contraction of the body-wall muscle is required for body elongation during embryogenesis. Embryonic body elongation is accomplished through constriction of circumferential actin filaments in the epidermis (Priess and Hirsh 1986), yet, in the absence of muscle contraction, body elongation arrests, and worms die as deformed L1 larvae with the *Pat* phenotype (paralyzed, arrested elongation at the two-fold stage) (Waterston 1989; Williams and Waterston 1994). Muscle and epidermal cells in *C. elegans* assemble and organize their cytoskeletons in response to contact with each other through integrin-mediated signaling at focal adhesion-like structures, which are present at the base of M-lines and dense bodies, the sites of thick filament and thin filament attachment, respectively (reviewed in Moerman and Williams 2006). Identification of many genes/proteins required in muscle occurred through forward genetics screens for *Unc* (uncoordinated) mutants, which have reduced muscle function and exhibit slow movement as adult worms, or the more severe *Pat* phenotype.

Mutations that eliminate myosin B, the major myosin (encoded by the gene *unc-54*), or paramyosin (encoded by the gene *unc-15*), cause disorganization of the contractile apparatus, resulting in an *Unc* phenotype (Epstein *et al.* 1974; Waterston *et al.* 1977). In contrast, mutations that eliminate the minor isoform myosin A (encoded by the *myo-3* gene) result in the *Pat* phenotype—an observation that revealed an essential role for myosin A in thick filament initiation (Waterston 1989). The region of myosin A necessary for the essential myosin A-specific function was mapped using chimeric myosins to two regions of the coiled-coil rod, consistent with a function in assembly of the thick filament (Hoppe and Waterston 1996).

The precise placement and structural regularity of thick filaments depend upon the action of many muscle proteins. The M-line, the site of thick filament attachment, comprises many structural and probable signaling proteins (reviewed in Qadota and Benian 2010; Gieseler *et al.* 2016). *UNC-89/obscurin* is a very large protein with many functional domains that is found at the M-line. Transmission electron microscopy (TEM) analysis revealed that *unc-89/obscurin* mutants lack M-lines, and have disorganized thick filaments (Waterston *et al.* 1980). The mutant alleles that lack the *UNC-89/obscurin* isoforms that contain the SH3 domain, which is proposed to link thick filaments to the M-line through an interaction with paramyosin, have distinctive paramyosin accumulations at the ends of muscle cells (Qadota *et al.* 2016). Mutations affecting the M-line-specific proteins *UNC-98* and *UNC-96* also cause moderate thick filament disorganization, coupled with distinctive paramyosin accumulations (Zengel and Epstein 1980). *UNC-98* is a 310 amino acid

protein that contains four C2H2 zinc fingers (*UNC-98/ZnF*). *UNC-96* is a novel protein (Mercer *et al.* 2006).

The body-wall muscle of *unc-82* mutant worms, which move more slowly than wild type in adulthood, exhibits abnormalities in myosin thick filament morphology and distribution when examined by polarized-light and electron microscopy (Waterston *et al.* 1980). Antibody staining of the putative null mutant *unc-82(e1323)* revealed that the thick filament components myosin A and paramyosin and the M-line protein *UNC-89/obscurin* were severely affected, and that these defects arose during body elongation of the embryo. A rescuing *UNC-82::GFP* fusion protein is detected at the M-line in body-wall muscle (Hoppe *et al.* 2010a). The protein product of *unc-82* is an AMPK-related kinase orthologous to human *ARK5/NUAK1* and *SNARK/NUAK2* (Hoppe *et al.* 2010a). Both orthologs are expressed in mammalian striated muscle (Lefebvre and Rosen 2005). Studies have implicated each of these genes individually in function of the contractile apparatus in mouse muscle. Muscle-specific knock-down of *ARK5/NUAK1* causes altered phosphorylation of contractile apparatus proteins, whereas disruption of *SNARK/NUAK2* causes an age-dependent reduction in muscle mass (Inazuka *et al.* 2012; Lessard *et al.* 2016). Double knockouts of these orthologs have not been studied in mammalian muscle; these mutants arrest with defects in embryonic morphogenesis (Ohmura *et al.* 2012). Interestingly, in human kidney cells, *ARK5* has been shown to regulate non-muscle myosin light chain phosphorylation (Zagórska *et al.* 2010). We propose that the *UNC-82* kinase associates stoichiometrically, directly or indirectly, with recently synthesized paramyosin to promote proper assembly of paramyosin into the thick filament. Our results also indicate that *UNC-82* affects one or more proteins of the contractile apparatus other than paramyosin, and suggest that one such protein is myosin B.

Materials and Methods

GFP and Red Fluorescent protein (RFP) fusion constructs

A previously isolated extrachromosomal array expressing wild-type *UNC-82::GFP* (*phEx22*; Hoppe *et al.* 2010a) was used in this study. The transgene was generated by recombination *in vivo* between two overlapping linear DNAs, a 17-kb genomic PCR fragment, and a linearized plasmid encoding the C-terminus of *UNC-82* fused to GFP, following the method of Yuan *et al.* (2000). The PCR fragment, amplified from cosmid *B0496*, begins within intron 5, 2.6 kb upstream of exon 6 (which contains the initiator methionine used in the majority of *unc-82* transcripts, P. E. Hoppe, unpublished data), and ends within intron 32. The plasmid contains genomic DNA spanning exons 29–35, with GFP inserted prior to the stop codon in exon 35. The numbering of the exons within the *unc-82* gene has changed from that in the original publication of this construct (Hoppe *et al.* 2010a), due to the discovery of minor transcripts that contain five additional 5' exons that were not included in the previously published gene structure.

In this study, full-length **UNC-82** E424K::GFP and **UNC-82** E424K::RFP fusion constructs were generated by the same method as described above for the wild-type GFP fusion, with the exception that genomic DNA from the *unc-82(e1220)* mutant was used as template to make the 17 kb PCR fragment using oligonucleotides GTCTCTGCTAAACAGCAATCG and GTTTGTGTACTTGTGTGTGTG, and LongAmp *Taq* DNA Polymerase (New England Biolabs, Beverly, MA). The GFP sequences in the plasmid-encoding exons 29–35 (Hoppe *et al.* 2010a) were removed by digestion with *AgeI* and *BsmI*, and replaced with a similarly digested 1240 bp fragment containing RFP, amplified from pKFMbRFP using Platinum Pfx DNA Polymerase (Invitrogen, Carlsbad, CA) and oligonucleotides ATTTTCAGGGCAGGG and GTTAGTAGAACTCAG. Prior to injection, plasmids were linearized with *HindIII*, extracted with phenol/chloroform, and ethanol precipitated.

Generation of transgenic lines

Transgenes expressing **UNC-82** fused to GFP or RFP were obtained by injecting a DNA cocktail containing 50 ng of PCR product, 125 ng of linearized plasmid, and 2 μ g of pRF4 plasmid containing the *rol-6* coinjection marker (Mello *et al.* 1991), brought up to 10 μ l with 10mM Tris, 1 mM EDTA, pH 8. Transgenic lines expressing *hsp::UNC-15::GFP* were obtained by injecting a 200 ng/ μ l DNA cocktail containing a 1:100 ratio of the plasmid containing full-length **UNC-15::GFP** under the control of a heat-shock promoter (described in Miller *et al.* 2008) to pRF4.

Anesthetizing hermaphrodites for crosses with rolling males

Transgenes in this study were followed in genetic crosses by the dominant rolling (**Rol**) phenotype conferred by the coinjection marker plasmid pRF4 *rol-6(su1006)* (Mello *et al.* 1991). It is advantageous to pass the transgene from the male because rolling cross progeny are easily distinguished from the hermaphrodites' self progeny. Because **Rol** males mate poorly, hermaphrodites were temporarily immobilized by treatment with M9 with 0.1% tricane and 0.01% tetramisole (TT) (Sigma, St. Louis, MO) (McCarter *et al.* 1999). Adult rolling males were picked to the bacterial lawn of a seeded plate. Adult hermaphrodites, about four per **Rol** male, were treated for 15–30 min in 0.1 ml TT in a sterile well of a three-well glass slide, and then picked to the unseeded area of the plate to allow the TT to seep into the agar. The hermaphrodites were then moved into the lawn, where they were straightened, and placed close to each other, either in rows or in clusters; males were then placed directly on top of a hermaphrodite. The following day, males were removed and hermaphrodites were transferred to fresh plates.

Crossing transgenes marked with *rol-6* into mutant backgrounds

To introduce a transgene into a single mutant background, rolling transgenic males were crossed to anesthetized homozygous mutant hermaphrodites (see previous), and the rolling

F1 heterozygous hermaphrodite progeny picked singly and allowed to self. The rolling F2 progeny were picked singly, and mutant homozygotes identified by movement and polarized light phenotypes of the F2 hermaphrodite and its F3 progeny. Double mutant worm strains were generated in the laboratory through similar crossing and classical genetic phenotype screening. All viable double mutant strains were verified by outcross to **N2** and reisolation of each single mutant. Due to synthetic lethal interaction between *unc-15* alleles and the **UNC-82::GFP** transgene *phEx22*, a chromosome containing the homozygous sterile mutation *gld-1(q485)*, which is closely linked to *unc-15*, was used to create balanced heterozygous *unc-15/gld-1(q485)* strains carrying the transgene *phEx22*.

Brood analysis

The lethality of the transgenic array expressing **UNC-82::GFP** in animals homozygous for an *unc-15* mutation was tested by examination of all progeny in two 1-day egg collections from a single balanced heterozygous hermaphrodite [*unc-15* +/+ *gld-1(q485)* I; *phEx22*]. One day after removal of the parent hermaphrodite, unhatched eggs were moved to a small area of the plate and monitored for an additional 2 days. Similarly, **Unc** progeny were grouped and monitored for progress to adulthood. All dead/arrested or **Unc** animals were mounted on slides to score for the presence of the **UNC-82::GFP** transgene using fluorescence microscopy. All moving transgenic progeny, identified by the *rol-6* marker, were picked singly for genotyping: animals homozygous wild type at the *unc-15* locus [+ *gld-1(q485)*/+ *gld-1(q485)* I; *phEx22*] are sterile with a tumorous germ line; *unc-15* heterozygotes [*unc-15* +/+ *gld-1(q485)* I; *phEx22*] segregate wild-type, **Unc**, and tumorous germ line offspring.

Temperature-enhanced phenotype assay

The following strains were used to screen for temperature-sensitive phenotypes by polarized light, anti-myosin A, and anti-paramyosin: **N2**, **CB1220** *unc-82(e1220)* IV, **PZ218** and **PZ219** *unc-82(gk205468)* IV, and **PZ220** and **PZ221** *unc-82(gk718459gk718460)* IV. Eggs were harvested from gravid adults grown at both 15° (5 days) and 25° (2 days) via bleaching methods described by Stiernagle (2006). Eggs were placed on unseeded plates at the appropriate temperature to hatch overnight. Hatched animals were washed off the plates with M9, placed on large seeded plates, and incubated at either 15° or 25°. When cultures reached the young adult stage, animals were collected and fixed using the Nonet fixation method (Nonet *et al.* 1993).

Microscopy

All worms were fixed using the Nonet method (Nonet *et al.* 1993), and incubated in primary antibodies overnight at room temperature. Mouse monoclonal antibodies 5/6 (1:500 dilution), 5/8 (1:500 dilution), 5/23 (1:200 dilution), and rabbit polyclonal antibody EU131 (1:200) were used for immunofluorescence localization of myosin A, myosin B, paramyosin, and **UNC-98**, respectively (Miller *et al.* 1983;

Mercer *et al.* 2003). Rabbit and mouse anti-GFP antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA) were used at 1:250 dilution. Alexa Fluor 488- and 594-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories were used at 1:400 dilutions. Worms were incubated in secondary antibodies for 2 hr. For staining of UNC-89 the mouse monoclonal MH42 (1:200) and rabbit polyclonal EU30 (1:200) were used (Benian *et al.* 1996). For staining of UNC-95, UNC-97, CPNA-1, PAT-6, UNC-112, and CSN-5 the following antibodies were used: Benian-13 (1:100), Benian-16 (1:100), anti-CPNA (1:100), anti-PAT-6 (1:100), anti-UNC-112 (1:100), anti-CSN-5 (1:100) (Benian *et al.* 1996; Hikita *et al.* 2005; Miller *et al.* 2006, 2009; Qadota *et al.* 2007; Warner *et al.* 2013). The polarized light microscopy procedures of Mackenzie *et al.* (1978) were followed. All images were acquired in ImagePro 6.0 (Rockville, MD), using a Retiga Exi Fast cooling mono12-bit Qimaging camera mounted to a Leica DM5500 microscope equipped with a polarizing lens. All images were processed using Adobe Photoshop CS6 software (San Jose, CA).

Nematode strains

Unless otherwise noted, worm strains were maintained at 20° on nematode growth medium (NGM) plates, seeded with the uracil mutant OP50 strain of *Escherichia coli* (Brenner 1974).

Strains obtained from Guy Benian or The Caenorhabditis Genetics Center (CGC) Strains obtained from Guy Benian or the CGC are as follows: Bristol N2 [wild type]; CB1220 [*unc-82(e1220)* IV]; CB1323 [*unc-82(e1323)* IV]; CB73 [*unc-15(e73)* I]; GB246 [*unc-98(sf19)* X]; HE75 [*unc-89(su75)* I]; and CB1214 [*unc-15(e1214)* I].

Transgenic strains carrying fluorescently tagged wild-type UNC-82: Transgenic strains carrying fluorescently tagged wild-type UNC-82 are as follows: PZ278 [+; *phEx22* (UNC-82::GFP)]; PZ238 [*unc-82(e1323)*; *phEx22*]; PZ236 [*unc-98(sf19)* X; *phEx22*]; PZ237 [*unc-89(su75)* I; *phEx22*]; PZ240 [*unc-82(e1323)* IV; *unc-98(sf19)* X; *phEx22*]; PZ249 [*unc-89(su75)* I; *unc-82(e1323)* IV; *phEx22*]; PZ259 [*unc-15(e1214)* +/+ *gld-1(q485)* I; *phEx22*]; PZ279 [*unc-96(sf18)* X; *phEx22*]; PZ290 [*unc-15(e73)* +/+ *gld-1(q485)* I; *phEx22*]; and PZ289 [*unc-15(su228)* +/+ *gld-1(q485)* I; *phEx22*].

Transgenic strains carrying fluorescently tagged E424K UNC-82: Transgenic strains carrying fluorescently tagged E424K UNC-82 are as follows: PZ169 [+; *phEx67* (E424K UNC-82::GFP)]; PZ171 [*unc-82(e1323)* IV; *phEx67*]; and PZ176 [*unc-82(e1323)* IV; *phEx68* (E424K UNC-82::RFP)].

Transgenic strains carrying heat-shock-inducible paramyosin::GFP: Transgenic strains carrying heat-shock-inducible paramyosin::GFP are as follows: PZ182 [+; *phEx70* (UNC-15::GFP)]; PZ187 [*unc-82(e1323)* IV; *phEx70*]; and PZ190 [*unc-82(e1220)* IV; *phEx70*].

Missense *unc-82* mutations from the Million Mutation Project (MMP): The MMP created 2000 mutagenized *C. elegans* strains, each of which carries many unique mutations, and determined the genomic DNA sequence of these strains (Thompson *et al.* 2013). From this collection, 22 lines carrying a missense mutation in *unc-82* were examined in Donald Moerman's laboratory using polarized light microscopy, with the help of Teresa Rogalski. Two of these strains, VC40608 [*unc-82(gk718459gk718460)*] and VC40253 [*unc-82(gk537592)*], have abnormal muscle structure, and both have a missense mutation within the UNC-82 kinase domain; VC40608 contains a second *unc-82* missense mutation outside the kinase domain. The strains VC40041 [*unc-82(gk205469)*] and VC40253 [*unc-82(gk537592)*] have a missense mutation in the kinase domain but have normal muscle structure. The remaining 18 strains examined, which carry *unc-82* missense mutations outside the kinase domain, have normal muscle structure: VC20602 [*unc-82(gk350480)*], VC20766 [*unc-82(gk396209)*], VC40085 [*unc-82(gk455403)*], VC20628 [*unc-82(gk361893)*], VC30173 [*unc-82(gk430314)*], VC20724 [*unc-82(gk384013)*], VC30166 [*unc-82(gk428568)*], VC20233 [*unc-82(gk205474)*], VC40473 [*unc-82(gk654885)*], VC20013 [*unc-82(gk205476)*], VC40884 [*unc-82(gk864500)*], VC20779 [*unc-82(gk400373)*], VC20418 [*unc-82(gk205477)*], VC20013 [*unc-82(gk205482)*], VC20720 [*unc-82(gk382630)*], VC40847 [*unc-82(gk844906)*], VC20144 [*unc-82(gk205484)*], and VC30024 [*unc-82(gk405473)*].

Outcrossing of MMP lines: To remove many background mutations, and to randomize the background of remaining mutations, wild-type males were mated to each MMP line, and the resulting F1 males crossed again to wild-type hermaphrodites. Single F2 hermaphrodites from this cross were picked, and the F3 progeny examined by polarized light microscopy to identify wells segregating the *unc-82* mutation. Individual *Unc* animals were picked to establish 2× outcrossed lines. The process was repeated, and two independent 4× outcrossed lines isolated for each MMP allele: PZ218 and PZ219 [*unc-82(gk205468)* IV], and PZ220 and PZ221 [*unc-82(gk718459gk718460)* IV]. The mutational changes reported for these MMP alleles were confirmed in the outcrossed lines by DNA sequencing.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All strains and reagents generated in this study are available upon request.

Results

Myosin and paramyosin distribution in *unc-82* mutants

Adults homozygous for either of the *unc-82* mutant alleles *e1323* or *e1220* exhibit defects in thick filament and M-line organization when examined using polarized light and electron

microscopy. In both mutants, TEM revealed abnormal, large-diameter filaments, which, based on analysis of double mutants of *unc-82* with myosin B and paramyosin null mutations, were likely composed of paramyosin (Waterston *et al.* 1980). Subsequent molecular analyses found that *e1323* is a presumptive null allele [*unc-82(0)*] containing a premature stop codon (Q403stop) within the kinase domain; in Q403stop homozygotes localization of myosin A, paramyosin, and the large M-line protein UNC-89/obscurin in adult muscle was severely disrupted, with defects appearing during body elongation stages of embryogenesis (Hoppe *et al.* 2010a). In contrast, the *e1220* allele was identified as a missense mutation E424K [*unc-82* E424K] that affects a highly conserved residue in the kinase catalytic domain, and, therefore, likely produces a full-length but catalytically impaired protein product. The predicted UNC-82 protein contains >1000 amino acids that are C-terminal to the kinase domain, but these sequences are poorly conserved among species, have no recognizable conserved domain, and their importance to UNC-82 function is not known (Hoppe *et al.* 2010a). It was therefore of interest to examine the cellular phenotype of the E424K missense mutant, and compare it to that of the null mutant.

In wild type, paramyosin and myosins A and B appear in stripes that are localized within the birefringent A bands, which mark the position of the myosin-containing thick filaments in striated body-wall muscle cells (Figure 1, A–F). Myosin A is restricted to the center of the thick filament, and therefore myosin A stain appears in thin stripes that coincide with the position of the M-line, whereas myosin B appears more broadly in the A-band (see *Introduction*, *cf.* Figure 1, C and E). In the presumptive *unc-82* null Q403stop mutant, the staining for paramyosin, myosin A or myosin B coincides with amorphous birefringent patches that are distributed throughout the muscle cell (Figure 1, G–L), suggesting that these patches are disorganized thick filament accumulations that contain all three proteins. In *unc-82* E424K mutants, the overall disruption of muscle cell structure appears less severe than in the null (Figure 1, M–R; Waterston *et al.* 1980). The polarized light phenotype of *unc-82* E424K shows more uniformly distributed birefringence throughout the body of the cell, and includes more areas that contain discernible striations than found in Q403stop animals. However, most E424K cells also contain small brightly birefringent accumulations at the ends of the spindle-shaped cells; these stain brightly for paramyosin (Figure 1, M and N). Paramyosin is weakly detected elsewhere in the body of these muscle cells. In contrast, myosins A and B are strongly detected throughout the disorganized contractile apparatus. The birefringent accumulations at the ends of muscle cells coincide with a portion of the myosin B stain in the cell. However, the accumulations at the ends of the muscle cell do not stain positive for myosin A. These results suggest that the presence of a full-length, catalytically impaired UNC-82 protein leads to formation of distinctive abnormal structures at the ends of muscle cells. The birefringence of the

structures indicates that they contain organized molecular assemblages. The intensity of the antibody stain suggests that a major component of the assemblages is paramyosin, with some myosin B also present.

The *unc-82(e1220)* E424K substitution introduces a charge change (negative to positive) in a highly conserved residue of the catalytic loop (Figure 2, A and B). An alanine substitution at this position in a yeast kinase reduced catalytic activity to 1.7% of wild type (Gibbs and Zoller 1991). In structural studies of a transition state mimic, the homologous, negatively charged, glutamic acid residue E170 of cAMP-dependent kinase (protein kinase A, PKA) forms a salt bridge with a positively charged arginine in the peptide substrate (Madhusudan *et al.* 2002). The lysine substitution in UNC-82 E424K changes this to an unfavorable interaction between two positive charges, which likely adversely affects binding of the catalytic loop with the substrate, delaying, or preventing, transfer of the phosphate group. The distinctive accumulations in this mutant might therefore be the result of disruption of this specific step in catalysis.

To determine whether other alterations in the UNC-82 kinase domain would result in similar distinctive paramyosin-containing accumulations, we used GExplore (Hutter *et al.* 2009) to identify strains generated by the MMP (Thompson *et al.* 2013) that contained novel *unc-82* mutations. Two of the four MMP strains that contained missense mutations within the kinase domain (VC20479, G456R, VC40608, and S442P S969F; see *Materials and Methods*) had a defective polarized light muscle phenotype. Both alleles are recessive and failed to complement *unc-82(0)*, confirming that the novel *unc-82* mutations in the MMP strains were responsible for the observed muscle defects. Both strains contain a substitution within the activation segment (Figure 2, A and B), a region that regulates substrate access to the catalytic cleft in a phosphorylation-dependent manner (reviewed in Johnson and Lewis 2001; Huse and Kuriyan 2002). The strain VC40608 contains a second missense mutation in the *unc-82* gene that is located outside the kinase domain (S969F, Figure 2A). Because the residues outside the UNC-82 kinase domain are poorly conserved (Hoppe *et al.* 2010a) it is unlikely that the second missense mutation is responsible for the observed muscle phenotype. Consistent with this proposal, examination of 18 other MMP strains that contain an *unc-82* missense mutation outside the kinase domain (see *Materials and Methods*) found that all have a normal polarized light muscle phenotype.

The two MMP strains that have abnormal muscle, and a mutation in the activation segment, were outcrossed four times to wild type (see *Materials and Methods*), and the reported mutational changes in *unc-82* confirmed by DNA sequencing. Synchronized populations of the outcrossed novel mutants along with wild-type and E424K worms were grown at 15 and 25° (see *Materials and Methods*), and young adult animals examined using polarized light microscopy and antibody staining for paramyosin or myosin A. At 15 and 25°, most muscle cells in E424K worms contain birefringent

Figure 1 Thick filament components paramyosin, myosin A, and myosin B have distinct subcellular distribution patterns in the *unc-82* null compared to the E424K missense mutant. (A–F) In wild type, antibody staining of thick filament components appears in organized lines of signal. Paramyosin (PM) antibody stain [arrow in (A)] coincides with the birefringent A-bands visible by polarized light (PO) microscopy [arrow in (B)]. Asterisks (A, B) mark the ends of a single spindle-shaped muscle cell. Myosin A (MyoA) staining [arrow in (C)] is found at the center of the A band [arrow in (D)]. Myosin B (MyoB) staining [arrow in (E)] is localized more broadly in the A-band [arrow in (F)]. (G–L) Antibody stains in *unc-82(0)* [P296 *unc-82(e1323)*] mutants reveal amorphous patches of paramyosin [rectangles in (G)], myosin A [rectangle in (I)], and myosin B [rectangles in (K)] that coincide with the birefringent amorphous patches present in the polarized light micrographs [rectangles in (H), (J), and (L)]. (M–R) Antibody staining of paramyosin in *unc-82* E424K [CB1220 *unc-82(e1220)*] worms shows that paramyosin accumulates at

the ends of muscle cells in distinctive compact aggregates [arrowheads in (M)], which are also birefringent [arrowheads in (N)]. Myosin A staining in E424K worms is localized to the disorganized A-bands [broad arrow in (O) and (P)], and is not detected in the distinctive birefringent accumulations at the ends of muscle cells [arrowheads in (O) and (P)]. Myosin B stain in E424K is localized to the disorganized A-bands [broad arrow in (Q) and (R)], and the distinctive birefringent accumulations [arrowheads in (Q) and (R)]. Bar, 20 μ m.

accumulations that stain positive for paramyosin, but not myosin A (Figure 2, C–F), similar to the results obtained at 20° (Figure 1, M–P). The G456R mutant had markedly disrupted patterns of myosin A and paramyosin staining at 15 and 25°, and contained some birefringent accumulations at muscle cell ends at both temperatures, but not in the majority of cells (Figure 2, G–J; data not shown). Where present, the accumulations contained paramyosin but not myosin A. The S442P S969F mutant is temperature sensitive, having a near wild-type phenotype at 15° (Figure 2, K–N), but resembling the E424K mutant at 25°, with paramyosin accumulations, which do not stain for myosin A, present in all cells (Figure 2, O and R). Even at 25°, the muscle cells of the S442P S969F mutant have some striations discernible by polarized light, and myosin A stain in much of the cell body, and therefore this mutant appears less severe than the other activation segment mutant, G456R. The presence of distinctive paramyosin accumulations in these activation segment missense mutants indicates they are not specific to the E424K lesion. Because the E424K mutant has a high penetrance of paramyosin accumulations at all temperatures, it was chosen for fluorescent tagging experiments to elucidate the interaction of *UNC-82* with paramyosin.

Mutant *UNC-82* E424K colocalizes with abnormal paramyosin accumulations

To investigate the relationship between the E424K protein and the formation of the paramyosin-containing accumula-

tions, transgenes expressing fluorescently tagged *UNC-82* E424K were generated (see *Materials and Methods*), and examined in wild-type and *unc-82* mutant backgrounds. When expressed in the *unc-82* null mutant, the tagged E424K *UNC-82* proteins reproduced the polarized light phenotype of the chromosomal E424K *unc-82(e1220)* allele, exhibiting brightly birefringent accumulations at the ends of muscle cells (Figure 3). Fluorescence microscopy of live animals revealed that the RFP-tagged E424K signal was concentrated in these birefringent accumulations (Figure 3, A and B). The E424K::GFP signal was also found in distinctive accumulations in the *unc-82* null background (Figure 3C), indicating that the behavior was independent of the fluorescent tag. In a wild-type background, the E424K::GFP signal appeared at the M-line (Figure 3D), the pattern typical of the wild-type *UNC-82::GFP* fusion (Hoppe *et al.* 2010a), indicating that the presence of wild-type *UNC-82* protein allowed normal localization of the GFP-tagged mutant protein. Similarly, wild-type *UNC-82::GFP* expressed from the extrachromosomal transgene *phEx22* rescued the muscle phenotype of the genomic *unc-82(e1220)* allele, which contains the E424K substitution (data not shown). The significance of the nuclear *UNC-82::GFP* signal (Figure 3D) is not known, but is seen with both wild-type *UNC-82::GFP* and E424K::GFP. Interestingly, nuclear localization has also been reported for the zinc-finger-containing M-line protein *UNC-98/ZnF* (Mercer *et al.* 2003). The mammalian *UNC-82* orthologs exhibit nuclear localization under conditions of stress (Kuga *et al.* 2008; Hou *et al.* 2011).

Figure 2 Kinase domain missense mutants vary in prevalence of birefringent paramyosin-containing accumulations at ends of muscle cells. (A) The long rectangle represents the predicted 1817-amino-acid UNC-82 protein made from a hypothetical transcript that contains all 35 exons. The dark shading indicates the kinase domain. The positions of the nonsense and missense mutations used in this study are shown, giving the allele number and the resulting alteration in the protein sequence. The text below the rectangle shows the amino acid sequence of the portion of the kinase domain that contains four of the mutations; affected residues are marked with a bar above the letter. The residues comprising the catalytic loop and activation segment (underlined), as well as the threonine in the activation segment that is phosphorylated in the active form of the enzyme (asterisk) were assigned by homology. (B) A Clustal X alignment of protein kinase sequences in the portion of the catalytic domain that is affected in *unc-82* mutants shows the high conservation of affected residues (highlighted positions) in the catalytic loop and activation segment [underlined as in (A)], and the position of the threonine residue (red T) that is phosphorylated in the activated kinase. ARK5/NUAK1 and SNARK/NUAK2 are the human (Hs) orthologs of UNC-82. Homology to the human cAMP-dependent protein kinase (HsPKAc) sequence was used to define the residues within the catalytic loop and activation segment. The last three sequences are human, worm (Ce), and yeast (Sc) representatives from the closely related AMPK family. Paramyosin and myosin A staining in E424K (C–F) and G456R [PZ219 *unc-82(gk205468)*] worms (G–J) grown at 25° reveals dis-

tinctive birefringent accumulations that contain paramyosin but not myosin A at the ends of muscle cells (arrowheads) in both strains, but they are present in fewer cells in G456R. (K–N) Paramyosin and myosin A staining of the temperature-sensitive mutant S442P S969F [PZ221 *unc-82(gk718456 gk718460)*] grown at 15° shows a near wild-type phenotype (K–N), while most cells in animals grown at 25° exhibit distinctive birefringent accumulations of paramyosin that do not stain for myosin A at the ends of muscle cells [arrowheads in (O)–(R)]. Bar, 20 μ m.

The colocalization of paramyosin and the mutant UNC-82 E424K protein in the distinctive accumulations suggests that the two proteins interact physically during assembly of the thick filament, and that loss of UNC-82 catalytic activity leads to aggregation of both proteins. If so, wild-type UNC-82 may have chaperone-like activity that promotes proper entry of soluble paramyosin into the thick filament. To test whether UNC-82 influences the localization of recently synthesized paramyosin, an unstable transgenic array in which expression of paramyosin::GFP is driven by a heat shock promoter was generated, and crossed into wild type and *unc-82* mutants. Transgenic young adult animals were heat-shocked at 30° for 5 hr, and fluorescent images taken within 15 min of

treatment. In wild type, the paramyosin::GFP fusion protein appeared in stripes at or near the M-line (Figure 3E; Miller *et al.* 2008). In the *unc-82(0)* background, paramyosin::GFP was seen in a patchy distribution throughout the muscle cell (Figure 3F). In the *unc-82* E424K mutant, some GFP signal was detected in longitudinal stripes that may be abnormal M-lines, while bright signal was seen in distinctive accumulations at the ends of muscle cells (Figure 3G). These observations are consistent with the hypothesis that newly synthesized paramyosin physically associates with UNC-82, and that this complex localizes to the M-line in wild type, but in distinctive accumulations at the ends of muscle cells in the UNC-82 E424K mutant.

Figure 3 UNC-82 protein containing the E424K substitution localizes to the distinctive paramyosin accumulations, which also label with recently synthesized paramyosin. (A, B) Live worms expressing the mutant UNC-82 E424K::RFP protein in the *unc-82(0)* background [PZ176 *unc-82(e1323); phEx68*] contain birefringent aggregates at the ends of muscle cells [arrow in (A)], similar to those present in the strain homozygous for the genomic missense E424K mutation *unc-82(e1220)*. UNC-82 E424K::RFP colocalizes with the distinctive birefringent aggregates [arrow in (B)]. The intestine (i) contains round autofluorescent granules. (C) The UNC-82 E424K protein tagged with GFP accumulates in distinctive aggregates [arrow in (C)] in an *unc-82* null mutant background [PZ171 *unc-82(e1323); phEx67*]. (D) The signal from the same UNC-82 E424K::GFP transgene in a wild-type background [PZ169 +; *phEx67*] is present at the M-line [broad arrow in (D)], and in the nucleus [chevron in (D)]. (E–G) Paramyosin::GFP expressed following heat shock goes to the M-line [broad arrow in (E)] in wild-type worms [PZ182 +; *phEx70*], is disorganized [rectangle in (F)] in *unc-82* null mutants [PZ187 *unc-82(e1323); phEx70*], and joins distinctive accumulations at the ends of muscle cells [arrow in (G)] in the E424K mutant [PZ190 *unc-82(e1220); phEx70*]. Bar, 20 μ m.

Overexpression of UNC-82 in paramyosin mutants

To test the hypothesis that wild-type UNC-82 associates with paramyosin to promote proper assembly, we examined the subcellular localization of wild-type UNC-82::GFP in the paramyosin missense mutant *unc-15(e73)*. This mutant contains large needle-like birefringent paramyosin-containing aggregates that are thought to form due to increased affinity of paramyosin for itself (Gengyo-Ando and Kagawa 1991). The paramyosin *e73* mutation is semidominant: heterozygotes exhibit slow movement and smaller birefringent aggregates, whereas *e73* homozygotes move very poorly and have prominent aggregates (Waterston *et al.* 1977). If UNC-82 binds to paramyosin to promote entry into the thick filament, we might expect expression of UNC-82::GFP in the *e73* mu-

tant to reduce the size of the aggregates and improve motility. Increased expression of myosin A has been shown to dramatically suppress the *e73* phenotype by increasing incorporation of mutant paramyosin into normal thick filaments (Riddle and Brenner 1978; Otsuka 1986; Hoppe and Waterston 2000). An extrachromosomal array expressing wild-type UNC-82::GFP (*phEx22*), and carrying the coinjection marker *rol-6*, was crossed to *e73* homozygotes. Repeated attempts to obtain a strain that carried the transgene in a homozygous *e73* background were not successful. The transgene, which restores muscle structure in *unc-82(e1323)* mutant worms (Hoppe *et al.* 2010a), appeared to increase the severity of the movement defect in *e73* heterozygotes.

To better analyze the interaction of increased UNC-82 expression with the paramyosin *e73* mutation, the UNC-82::GFP (*phEx22*) transgene was crossed into a heterozygous line in which *e73* is balanced by a closely linked *gld-1* mutation on the opposite chromosome [PZ290 *unc-15(e73) +/+ gld-1(q485) I; phEx22*]. Examination of transgenic progeny revealed that overexpression of UNC-82 increased severity of the polarized light muscle phenotype in *e73* heterozygotes, and resulted in lethality or developmental arrest in *e73* homozygotes (Figure 4). Muscle cells in transgenic heterozygotes contained larger birefringent aggregates than heterozygotes without the transgene, and UNC-82::GFP localized to these aggregates (Figure 4, F–H). Wild-type animals carrying the transgene did not contain abnormal birefringent aggregates, although small accumulations of GFP signal were seen outside the contractile apparatus (Figure 4, A and B). Enhancement of aggregate formation by overexpression of UNC-82 was also observed in animals heterozygous for another missense paramyosin mutation, *su228* [PZ289 *unc-15(su228) +/+ gld-1(q485) I; phEx22*]. The *su228* allele contains an arginine to cysteine substitution near the paramyosin C-terminus (Gengyo-Ando and Kagawa 1991). Whereas *unc-15(su228)/+* heterozygotes rarely contain birefringent aggregates at the ends of cells, transgenic heterozygotes contained prominent aggregates, most of which colocalized with GFP signal (Figure 4, I–K). Therefore, rather than promoting incorporation of mutant paramyosin into normal filaments, increased levels of UNC-82 led to larger aberrant accumulations that contain UNC-82. Interestingly, transgenic animals heterozygous for the paramyosin null mutation [PZ259 *unc-15(e1214) +/+ gld-1(q485) I; phEx22*] also had a small number of birefringent accumulations at the end of the cells, whereas nontransgenic heterozygotes do not (Figure 4, C–E). However, in these animals, no GFP signal was detected on the accumulations. Therefore, in addition to increasing severity of defects in animals heterozygous for paramyosin missense mutants, increased UNC-82 expression also leads to birefringent accumulations in cells where levels of wild-type paramyosin have simply been reduced. The protein composition of the accumulations in the latter case is not known, but the accumulations do not colocalize with UNC-82 signal.

Figure 4 Overexpression of UNC-82 increases the severity of phenotypes in paramyosin mutant heterozygotes and homozygotes. (A, B) Polarized light microscopy of a live wild-type animal expressing UNC-82::GFP [PZ278+; *phEx22*] reveals wild-type muscle (compare with Figure 1F) with defined A bands and no ectopic aggregates. Fluorescence microscopy of this animal shows UNC-82::GFP localized to the M-line [broad arrow in (B)] and in small round ectopic accumulations [thin arrow in (B)]. The intestine, with larger autofluorescent granules, is labeled (i). (C–E) Muscle cells in *unc-15(0)*/+ heterozygotes have near-normal muscle with decreased birefringence at muscle cell ends [arrowhead in (C)]. Expression of UNC-82::GFP in *unc-15(0)*/+ results in brightly birefringent accumulations at the ends of some cells; these accumulations do not strongly label with UNC-82::GFP [arrowheads in (D) and (E)]. (F–H) Animals heterozygous for the semidominant missense paramyosin mutant *e73* have bright birefringent accumulations at the ends of muscle cells (F). The accumulations are larger in *e73* heterozygotes that carry the UNC-82::GFP transgene [brackets in (F) and (G)], and UNC-82::GFP colocalizes with the birefringent structures [arrowheads in (G) and (H)]. (I–K) Animals heterozygous for the paramyosin missense mutant *unc-15(su228)* occasionally have small birefringent accumulations at muscle cell ends (I). The accumulations are enhanced in size and prevalence in an *su228* heterozygote expressing UNC-82::GFP [brackets in (H) and (K)]. UNC-82::GFP colocalizes with the birefringent structures. (L, M) In early larval stages, UNC-82::GFP expressed in wild-type worms is visible in the muscle in stripes, and in some accumulations [arrows in (L)]. The corresponding DIC image shows the normal unkinked hatched larval phenotype (M). (N, O) This arrested transgenic *unc-15(0)* homozygote shows unorganized UNC-82::GFP signal, and a kinked body phenotype. (P, Q) The head portion of a later-stage arrested *e73* larva shows bright defined accumulations of UNC-82::GFP. (R, S) This arrested *su228* transgenic homozygote has disorganized UNC-82::GFP signal with areas of apparent muscle detachment, which may account for the kinked body phenotype [chevron in (R) and (S)]. Bar, 5 μm.

For all three *unc-15* alleles, expression of UNC-82::GFP in mutant homozygotes produced lethality or developmental arrest, as determined by brood analysis of transgenic balanced heterozygotes (see *Materials and Methods*) (Table 1). All control nontransgenic heterozygous parent hermaphrodites produced viable *Unc* progeny (*unc-15* homozygotes) in numbers that were close to the expected 25% proportion based on a Mendelian genotypic ratio of 1:2:1. A small percentage of progeny from nontransgenic *unc-15(e1214)*/+ and *unc-15(su228)*/+ hermaphrodites were scored as dead/arrested (3.6 and 1.8%): all of these were elongated embryos that failed to hatch. In contrast, transgenic hermaphrodites carrying the UNC-82::GFP array had a decrease in the number of viable *Unc* offspring with a concomitant increase in the dead/arrested class. Most died as unhatched elongated larvae. Of those that arrested at later developmen-

tal stages, the majority had abnormal body elongation, and/or kinked morphology, consistent with muscle contraction and attachment defects (see *Introduction*). A few transgenic *unc-15* homozygotes survived to adulthood. Most were mosaic animals in which UNC-82::GFP was expressed in a subset of the animal's muscle cells.

The UNC-82::GFP signal in arrested larvae homozygous for either missense allele appeared in bright, defined accumulations (Figure 4, P–R) like those found in the transgenic heterozygous adults (Figure 4, H and K). In contrast the UNC-82::GFP signal in arrested *unc-15(0)* larvae was more diffuse (Figure 4N). Surviving *unc-15(0)* adults that expressed UNC-82::GFP in a subset of muscle cells allowed examination of cell phenotype by polarized light microscopy. In these mosaics (Figure 5, A and B), the cells that expressed UNC-82::GFP had irregularly shaped accumulations of GFP

Table 1 Overexpression of UNC-82 increases the severity of paramyosin mutant phenotypes

Genotype of Parent	Phenotype or Genotype of Progeny			
	Unc	+/+	<i>unc-15/+</i>	Dead/Arrested
<i>e1214/+</i>	25 (18.1%)	30 (21.7%)	78 (56.5%)	4 (3.6%) ^a
<i>e1214/+; phEx22</i>	10 mosaic (5.3%)	42 (22.2%)	89 (47.1%)	48 (25.4%) ^{a,b}
<i>e73/+</i>	32 (19.2%)	59 (35.5%)	75 (45.2%)	0 (0%)
<i>e73/+; phEx22</i>	4 mosaic (2.9%)	30 (21.6%)	66 (47.5%)	39 (28.1%) ^{a,b}
<i>su228/+</i>	48 (29.4%)	37 (22.7%)	78 (47.9%)	3 (1.8%) ^a
<i>su228/+; phEx22</i>	1 (1%)	28 (29.8%)	53 (56.4%)	14 (14.7%) ^{b,c}
	1 mosaic (1%)			

Each of the three *unc-15* alleles tested was maintained in a balanced strain, *unc-15 +/+ gld-1*, with or without the *phEx22* UNC-82::GFP extrachromosomal array. The genotype of the parent hermaphrodite of a given brood is given in the first column. The phenotypes and *unc-15* genotypes scored in each brood are listed in the first row. Each subsequent row contains counts of a brood from a single hermaphrodite, with the percentage of the total progeny from that brood given in parentheses. In control (nontransgenic) broods, all progeny were counted and scored. In broods from transgenic hermaphrodites, only transgenic progeny were included in the analysis (see *Materials and Methods*). The recessive *gld-1(q485)* mutation is closely linked to *unc-15* (0.2 map units). Progeny homozygous for *gld-1*, which are sterile with a tumorous germ line, were scored as the *unc-15* genotype of +/+. The dead/arrested class includes any animals that did not survive to adulthood. Mosaic animals were those that did not have the unstable transgene expressing UNC-82::GFP in all muscle cells.

^a Arrested worms elongated in the egg shell but failed to hatch.

^b Worms hatched but arrested in larval stages, many were kinked or folded.

^c Eggs that did not develop to the twofold stage of body elongation were not counted.

signal at the ends of cells, with little GFP signal in the cell body. The GFP-positive cells contained large round birefringent structures that are not typically found in the paramyosin null mutant; these structures were not strongly GFP-positive (Figure 5, A and B). Because paramyosin is absent, these structures may arise from aggregation of one or more other muscle proteins during filament assembly, or the later collapse of the contractile apparatus. The observation that transgenic *unc-15(0)/+* hermaphrodites (Figure 4D) contain birefringent accumulations that are not GFP positive in an otherwise well-ordered cell is consistent with the aggregation model. These results suggest that the stoichiometric relationship between paramyosin and UNC-82 is critical for proper muscle cell structure, and that the imbalance affects additional proteins other than paramyosin and UNC-82.

Interaction between accumulated paramyosin and UNC-82 does not require UNC-96, UNC-98/ZnF, UNC-89/obscurin, myosin A, or myosin B

The recruitment of UNC-82::GFP to the abnormal aggregates in the paramyosin missense mutant *e73* (Figure 4, G and H) is consistent with a physical interaction between UNC-82 and paramyosin. However, because the *e73* aggregates also stain positive for myosin B, and perhaps for myosin A and UNC-89/obscurin (Epstein *et al.* 1987; Gengyo-Ando and Kagawa 1991; Qadota *et al.* 2016), the interaction between UNC-82 and paramyosin might be indirect. We therefore obtained other mutant strains that have ectopic paramyosin accumulations of differing composition. Paramyosin aggregates that do not stain for either myosin A or B are found in *unc-96* mutants (where aggregates also lack UNC-96), in *unc-98* mutants (where aggregates also lack UNC-98/ZnF), and in *unc-89(su75)* (where aggregates also lack UNC-89/obscurin). Interestingly, like the accumulations in *unc-82* missense mutants, the paramyosin accumulations in *unc-96*, *unc-98/ZnF*,

and *unc-89(su75)*/obscurin mutants reside at the ends of the muscle cells (Mercer *et al.* 2006; Miller *et al.* 2008; Qadota *et al.* 2016). The extrachromosomal transgene *phEx22* expressing UNC-82::GFP was crossed into these mutant lines. All three strains were viable and fertile, and, in all three, UNC-82::GFP colocalized with the ectopic birefringent structures (Figure 5, C–H).

Taken together, these data indicate that recruitment of UNC-82 to paramyosin aggregates does not require UNC-96, UNC-98/ZnF, or myosins A or B individually. The three transgenic mutant strains were examined to determine if overexpression of UNC-82 altered the muscle phenotype, as it did in the three paramyosin mutants (Figure 4). Expression of UNC-82::GFP in the *unc-98(sf19)/ZnF* mutant animals caused no obvious difference in movement, or in the morphology of paramyosin aggregates compared to those of the *unc-98/ZnF* mutant alone (data not shown). The expression of UNC-82::GFP in the *unc-89/obscurin* and *unc-96(sf18)* mutants, which move more slowly than *unc-98/ZnF* mutants, appeared to increase the severity of the movement defects. Because of the unstable nature of the transgene, and the abnormal rolling locomotion caused by the *rol-6* marker, quantitative assessment of the decrease in locomotion was not attempted.

Analysis of double mutants of *unc-82* with other paramyosin-affecting mutations

The detrimental effect of increasing UNC-82 expression in the paramyosin missense mutant *e73* is consistent with previous reports that the double mutant containing *e73* and *unc-82(0)* moved better than the *e73* single mutant, and that the appearance of the paramyosin aggregates was altered in doubles with either the *unc-82* null or the missense allele E424K (Waterston *et al.* 1980; Brown and Riddle 1985). We therefore reisolated the double mutant strains and examined the

Figure 5 UNC-82::GFP localization is affected in mutants that have abnormal paramyosin distribution. (A, B) An adult escaper homozygous for the paramyosin null mutation, and carrying the unstable UNC-82::GFP transgene is a mosaic. Cells lacking paramyosin and expressing UNC-82::GFP contain large birefringent aggregates [arrow in (A)] that do not coincide with bright GFP signal [arrow in (B)]. Bright GFP signal is found in abnormal accumulations at the ends of cells [ovals in (B)] where some birefringence is also detected [ovals in (A)]. Cells that do not express UNC-82::GFP [bracket in (B)] show the uniform, diffuse birefringent signal typical of *e1214* null mutant animals [bracket in (A)]. (C–H) UNC-82::GFP signal colocalizes with the birefringent paramyosin-containing aggregates at the ends of muscle cells in live adults from strains homozygous for a mutation affecting an M-line protein: UNC-89/obscurin [PZ237 *unc-89(su75)* I; *phEx22*] (C–D); UNC-98/ZnF [PZ236 *unc-98(sf19)* X; *phEx22*] (E and F); UNC-96 [PZ279 *unc-96(sf18)* X; *phEx22*] (G, H). Bar, 20 μ m

muscle phenotype by polarized light microscopy. The double mutant *e73*; *unc-82* E424K [PZ267 *unc-15(e73)* I; *unc-82-e1220* IV] contained birefringent accumulations at the ends of cells that more closely resembled those found in the *unc-82* mutant, rather than the longer defined needles characteristic of *e73* (Figure 6, A–F). Similarly, the *e73*; *unc-82(0)* double [PZ263 *unc-15(e73)* I; *unc-82(e1323)* IV] more closely resembled *unc-82(0)* alone, containing patchy disorganized birefringence and none of the large needle-like accumulations characteristic of *e73* (Figure 6B). The observation that the *e73*; *unc-82(0)* double mutant has improved movement, rather than an additive more severe phenotype, suggests that the two genes act in a single pathway to organize paramyosin, and that UNC-82 protein promotes paramyosin aggregate formation in the *e73* mutant.

To test the interaction of *unc-82* with mutations in other genes that result in formation of prominent paramyosin aggregates, double mutant strains of *unc-82* with *unc-89/obscurin* and *unc-98/ZnF* were obtained by genetic crossing (see *Materials and Methods*). The double of *unc-82(0)* with *unc-89(su75)/obscurin* is sterile, and has a muscle cell phenotype unlike that of either single mutant. The muscle phenotype of the double mutant was studied by constructing a double mutant strain in which *unc-82* is rescued by an unstable transgene expressing UNC-82::GFP [PZ249 *unc-89(su75)* I; *unc-82(e1323)* IV; *phEx22*], and examining progeny that had lost the transgenic array. Muscle cells in these worms lacking UNC-82::GFP had little birefringent signal

outside of large round accumulations in the middle of the cell (Figure 6H). To examine the sterile phenotype, single nonrolling adults were picked from this strain. Some of these animals were likely mosaics that had lost the UNC-82::GFP transgene in the epidermis (where the *rol-6* marker is expressed), but retained the transgene in other cells. Most animals (7 out of 12) produced no eggs. The gonad was often grossly abnormal or absent, and, in a few, a round clear internal “bubble” of unknown origin was present in the posterior of the animal. The maximum number of progeny produced by a single adult was 29, and the median number was four. The few progeny that were produced had variable defects, including severe *Unc*, and dumpy and folded morphologies. None of the progeny were fertile. The double of *unc-89/obscurin* and *unc-82* E424K [PZ245 *unc-89(su75)* I; *unc-82(e1220)* IV] is viable, but has reduced body size, decreased motility, and lower fecundity. The muscle showed an additive polarized light phenotype with patchy birefringence in the cell body, and bright birefringent accumulations at the ends of cells (Figure 6I).

In contrast, the doubles of either the *unc-82* null or E424K missense mutation with the presumptive null allele *unc-98(sf19)/ZnF* were viable and fertile, and had polarized light muscle phenotypes similar to those of the *unc-82* mutations alone (Figure 6, J–L). The distinctive paramyosin accumulations characteristic of *unc-98/ZnF* were absent in the double with the *unc-82(0)* as determined by polarized light microscopy (Figure 6K) and antibody staining (Figure 7, A–C).

Figure 6 Distinct polarized light muscle phenotypes are observed in double mutants of *unc-82* with other mutations that produce paramyosin aggregates. (A) Wild-type body-wall muscle contains highly regular longitudinal birefringent A-bands (broad arrow). (B) Worms homozygous for the *unc-82(0)* mutation contain irregular amorphous patches of birefringent material (rectangle). (C) The missense *unc-82* mutant E424K has brightly birefringent accumulations at the ends of cells (arrow), and, compared to the null mutant, a more uniform distribution of disorganized birefringence in the rest of the cell. (D) The paramyosin missense mutant e73 contains large birefringent needles (arrow), and has reduced, unpatterned birefringence in the rest of the cell. (E) Double mutants homozygous for the missense paramyosin, and *unc-82* null mutations [PZ263 *unc-15(e73)* I; *unc-82(e1323)* IV], exhibit patchy birefringence and no needles (rectangle), resembling *unc-82(0)* [cf. (B)], but are thinner in body width. (F) Double mutants homozygous for the missense paramyosin and *unc-82* E424K mutations [PZ267 *unc-15(e73)* I; *unc-82(e1220)* IV] have reduced overall birefringence with little organization (broad arrow), and bright accumulations, which lack the definition of the e73 needles, at the ends of cells [arrow in (F)]. (G) The *unc-89/obscurin* mutant exhibits bright accumulations at the ends of cells (thin arrow), and reduced, poorly patterned signal in the rest of the cell (broad arrow). (H) Cells in an *unc-89; unc-82(0)* double mutant that segregated from a strain in which *unc-82* is rescued with an unstable transgene [PZ249 *unc-89(su75)* I; *unc-82(e1323)* IV; *phEx22*] exhibit an enhanced phenotype, with large round brightly birefringent accumulations (arrowhead), and little signal in the rest of the cell (broad arrow). (I) The double mutant of *unc-89/obscurin* with *unc-82* E424K [PZ245 *unc-89(su75)* I; *unc-82(e1220)* IV] is more severe than either mutant alone, with bright accumulations at ends of cells (arrow) and patchy birefringence (rectangle) in the rest of the cell. (J) The *unc-98(sf19)/ZnF* mutant [GB246] has bright accumulations at the ends of cells (thin arrow), with faint but relatively well organized A bands (broad arrow) in the rest of the cell. (K) The double mutant of *unc-98/ZnF* and *unc-82(0)* [PZ240 *unc-82(e1323)* IV; *unc-98(sf19)* X] has amorphous patches of birefringence (rectangle), and no bright accumulations, resembling *unc-82(0)* mutants [compare to (B)]. (L) Double mutants carrying *unc-98/ZnF* and *unc-82* E424K [PZ250 *unc-82(e1220)* IV; *unc-98(sf19)* X] have bright accumulations at end of cells (small arrow) and disorganized signal in the rest of the cell (broad arrow), resembling the *unc-82* E424K single [cf. (C)]. Bar, 20 μ m.

Brightly birefringent accumulations are found in both single mutants *unc-82* E424K and *unc-98(0)/ZnF*. The double mutant exhibited bright accumulations as well as the more disorganized contractile apparatus characteristic of the *unc-82* missense mutant (Figure 6L). Antibody staining of this double revealed accumulations that contain paramyosin and myosin B (Figure 7, D–F), a property of accumulations in the E424K single mutant, but unlike those in the *unc-98/ZnF* single, which do not stain for myosin B. The presence of “*unc-82-like*” accumulations in the double, along with the more severe disorganization of the contractile apparatus in *unc-82(0)* compared to *unc-98(0)/ZnF*, suggests that *unc-82* may act upstream of *unc-98/ZnF* in a single pathway that organizes paramyosin, or that *unc-82* acts in a pathway that

affects a larger portion of the paramyosin pool than the *unc-98/ZnF* pathway.

Localization of other M-line proteins in *unc-82* mutants

UNC-98/ZnF has been shown to bind to paramyosin, and has been proposed to promote the proper assembly of paramyosin into thick filaments (Miller *et al.* 2008). It was therefore of interest to examine the localization of *UNC-98/ZnF* in *unc-82* mutants. The absence of *UNC-82* did not prevent association of *UNC-98/ZnF* with the disorganized contractile apparatus (Figure 7, G–I), where its distribution partially overlapped that of paramyosin. In the *unc-82* E424K mutant, *UNC-98/ZnF* was again detected in the contractile apparatus where the signal partially overlapped that of anti-paramyosin.

Figure 7 Protein composition of muscle cell aggregates investigated by immunostaining. (A–C) Animals homozygous for presumptive null mutations in both *unc-82* and *unc-98/ZnF* resemble the *unc-82* single, exhibiting patchy paramyosin and myosin B stain with no distinct accumulations at muscle cell ends. (D–F) The double mutant of *unc-98/ZnF* null with the missense *unc-82* mutation, resembles the *unc-82* E424K single, having little paramyosin stain in the contractile apparatus and bright accumulations, which also stain for myosin B, at the ends of cells (P–R). The paramyosin and myosin B stains overlap in the accumulations, but are not completely coincident (inset). (G–I) UNC-98/ZnF colocalizes with paramyosin stain in some areas of the muscle cells in *unc-82(0)*. (J–U) Ectopic accumulations of paramyosin in *unc-82* E424K do not stain with UNC-98/ZnF, CSN-5, or UNC-89/obscurin, but are positive for myosin B. (V–X) In the absence of paramyosin, myosin A and UNC-98/ZnF stains overlap in areas of the disorganized contractile apparatus. (Y–Zz) Ectopic filamentous balls of myosin A recruit UNC-98/ZnF in strain RW3880, which expresses myosin A with an altered C-terminus (2SerAlaΔ25; Hoppe *et al.* 2010b). Bar, 20 μ m.

However, UNC-98/ZnF signal was not detected in the ectopic paramyosin accumulations (Figure 7, J–L), which contain the mutant UNC-82 kinase and myosin B, but not myosin A (Figure 1, O–R, Figure 3, A–C, and Figure 7, P–R).

The absence of UNC-98/ZnF from the paramyosin-containing accumulations in UNC-82 E424K mutants suggests that the association of UNC-98/ZnF with paramyosin requires UNC-82 activity *in vivo*.

The disparity between the paramyosin and UNC-98/ZnF staining patterns prompted us to examine the *in vivo* interaction of UNC-98/ZnF and myosin A, which have been shown to physically interact (Miller *et al.* 2006). We found that UNC-98/ZnF and myosin A colocalize in *unc-15(0)* animals, which do not contain paramyosin (Figure 7, V–X), and in

animals that express a mutant myosin A that forms ectopic accumulations (Figure 7, Y–Zz). Both myosin A and myosin B contain a C-terminal nonhelical tailpiece that is homologous to the N-terminal nonhelical headpiece of paramyosin, which is phosphorylated by an endogenous kinase (Kagawa *et al.* 1989; Schrieffer and Waterston 1989). The myosin A nonhelical tailpiece is 29 amino acids long, and contains three copies of the proposed phosphorylation motif S₁S₂A that was identified in the paramyosin headpiece (Hoppe *et al.* 2010b, Schrieffer and Waterston 1989). The mutant myosin construct is missing the C-terminal 25 amino acids, and the serine residues within the four remaining residues of the tailpiece (which contain one motif) are changed to alanine. The mutant myosin assembles outside the contractile

apparatus, suggesting that this construct has a loss of negative control of myosin assembly that normally occurs through phosphorylation. The ectopic myosin A accumulations appear to be balls of jumbled filaments and do not stain with anti-paramyosin (Hoppe *et al.* 2010b). Therefore, our results suggest that **UNC-98/ZnF** associates with myosin A in the absence of paramyosin, and that the C-terminal 29 amino acids of myosin A are not required to bind **UNC-98/ZnF** *in vivo*. In yeast two-hybrid experiments, the C-terminal 200 amino acids of myosin A were found to be sufficient for binding **UNC-98/ZnF**, and removal of the last 32 amino acids abolished this binding (Miller *et al.* 2006). Interestingly, the additional three amino acids removed in the yeast two-hybrid experiment, but present in our mutant myosin construct, are within the four amino acids at the C-terminus of the coiled-coil rod that are essential for viability when tested in double mutants lacking endogenous myosin A and B (Hoppe *et al.* 2003).

The failure of **UNC-98/ZnF** to associate with the paramyosin aggregates in **unc-82 E424K** led us to examine the localization pattern of other M-line proteins in this mutant. The paramyosin aggregates in **unc-96** and **unc-98/ZnF** mutants (Miller *et al.* 2009) and the **unc-89(su75)/obscurin** mutant (Qadota *et al.* 2016) stain positive for **CSN-5**, a component of the conserved COP9 signalosome complex. **CSN-5** interacts with both **UNC-96** and with **UNC-98/ZnF**, and likely promotes the degradation of **UNC-98/ZnF**. Antibody staining experiments did not detect **CSN-5** on the paramyosin/myosin B accumulations in **UNC-82 E424K** (Figure 7, M and O). Experiments using antibodies against **UNC-89/obscurin**, **UNC-97**, **UNC-95**, **CPNA-1**, **PAT-6**, and **UNC-112** (see *Materials and Methods*) indicated that none of these antibodies stain cells in a pattern that resembles the distinctive accumulations at the ends of cells in **unc-82 E424K** (Figure 8). **UNC-89/obscurin**, which has been shown to interact with paramyosin (Qadota *et al.* 2016), does appear in bright irregular patches in **E424K** (Figure 8G), as it does in the **unc-82(0)** (Hoppe *et al.* 2010a). A double stain with paramyosin found that, although paramyosin and **UNC-89/obscurin** stain showed some overlap, the distinctive paramyosin accumulations at ends of cells did not stain for **UNC-89/obscurin** (Figure 7, S–U). Interestingly, in the plane of the cell containing the paramyosin accumulations, the **UNC-89/obscurin** stain was moderately disorganized (Figure 7T). But, in focal planes that were more distal from the integrin complex, which is located in the plasma membrane adjacent to the epidermis, the **UNC-89/obscurin** stain included large irregular patches (Figure 8G). Therefore, the only proteins detected in the distinctive accumulations formed in the **unc-82 E424K** kinase domain missense mutant are the mutant **UNC-82** protein itself, paramyosin, and myosin B.

Discussion

Loss-of-function of the AMPK-related kinase **UNC-82** results in defects in thick filament morphology and organization in

striated muscle of *C. elegans* (see *Introduction*). In this study, we present evidence that the major thick filament components paramyosin, myosin A, and myosin B are not equally affected by alterations in the catalytic activity of **UNC-82**. Our analysis of three different **unc-82** missense mutants revealed distinctive ectopic accumulations at the ends of cells, which contain paramyosin but not myosin A. Notably, such accumulations are not found in the null (Figure 1 and Figure 2). Accumulations in at least one of these mutants (**E424K**) also contain myosin B (Figure 1) and the mutant **UNC-82** protein itself (Figure 3). We propose that **UNC-82** is required in an early step in paramyosin, and probably myosin B, assembly that occurs prior to the association of paramyosin with **UNC-98/ZnF**, myosin A, or several other M-line proteins (Figure 9).

The **UNC-82** missense mutations used in this study fall within the kinase domain, either in the catalytic loop, which performs the transfer of phosphate from ATP to peptide substrate, or the activation segment, which regulates catalytic activity of the domain in response to phosphorylation (Figure 2). Our results suggest that at least two of these alleles (**E424K** and **S442P S969F**), both of which exhibit a high incidence of ectopic paramyosin accumulations, can generate stable, but presumably catalytically impaired, protein products. The GFP-tagged **E424K** protein accumulates, and localizes normally to the M-line in wild-type cells, and is strongly detected in the birefringent accumulations at the ends of muscle cells in the **unc-82(0)** background (Figure 3). Because the mutant **UNC-82 E424K::GFP** protein is present in these accumulations, and the accumulations are not present in the **unc-82** null, we propose that these accumulations represent a complex of the catalytically impaired **UNC-82** protein with its substrate in a stalled intermediate. The protein produced by the second mutant, **S442P S969F**, is clearly stable at the permissive temperature, given the normal muscle structure in the homozygote (Figure 2). At the restrictive temperature, we suggest that the **S442P** change may destabilize the fold adopted by the activation segment upon phosphorylation, impeding substrate access to the catalytic cleft.

The third mutant, **G456R** (which also affects the activation segment), contains some accumulations, but not in all muscle cells. Given our model that catalytically impaired **UNC-82** protein produces the distinctive accumulations, one possible explanation for fewer accumulations in the **G456R** mutant is lower levels of mutant **UNC-82** protein. Alternatively, the **G456R** mutant protein might have a decreased tendency to associate with paramyosin, or another component of the aggregates. The **G456R** substitution affects a highly conserved glycine, and places the large, positively charged, arginine side chain within three residues of the threonine that is phosphorylated in the active form of the enzyme (Figure 2). The change might impair phosphorylation of the threonine, which would decrease the pool of activated **UNC-82**, and potentially alter the stability of the mutant **UNC-82**, or its interactions with other muscle proteins. Similar effects have been demonstrated for unphosphorylated PKA (Iyer *et al.* 2005).

Figure 8 Other M-line proteins are not found on abnormal accumulations in the missense mutant *unc-82* E424K. Immunofluorescence images of stained wild type (A–F) and *unc-82* missense mutant E424K (G–L) adults show varying degrees of disorganization of the different M-line components in the *unc-82* mutant. However, none of the proteins is detected in distinctive accumulations at muscle cell ends (arrowheads). Bar, 20 μ m.

The proposal that **UNC-82** interacts with paramyosin early in the assembly pathway is supported by results from three types of experiments. First, recently synthesized paramyosin::GFP colocalizes with both wild-type and a mutant form of **UNC-82**. In wild type, paramyosin::GFP driven by a brief heat shock is detected at the M-line (Figure 3; Miller *et al.* 2008), the site of **UNC-82**::GFP localization. This initial localization of paramyosin at the M-line may represent a staging area for thick filament components prior to incorporation into growing thick filaments. In the E424K *unc-82*(e1220) genomic mutant, newly made paramyosin associates with distinctive birefringent structures at the ends of cells (Figure 3F), which we have shown stain positive for both paramyosin and myosin B (Figure 1, M, N, Q, and R and Figure 7, P–R). Similar birefringent structures containing **UNC-82** E424K::GFP are formed when the mutant protein is expressed in the *unc-82*(0) (Figure 3). This is consistent with our model that the accumulations represent a stalled paramyosin/**UNC-82** complex that normally forms as an intermediate, prior to incorporation of paramyosin into the thick filament.

The composition of the E424K aggregates provides a second line of evidence that this putative intermediate is formed prior to paramyosin association with other proteins. The accumulations in *unc-82* E424K mutants contain paramyosin, **UNC-82** E424K mutant protein, and myosin B, but not proteins of the myosin A-associated complex (myosin A, **UNC-98/ZnF**) (Figure 1 and Figure 7), proteins of the integrin-associated complex (**UNC-112**, **UNC-98/ZnF**, **CPNA-1**, **PAT-6**, or **CSN-5**) (Figure 7 and Figure 8), or the giant M-line protein **UNC-89/obscurin** (Figure 7). Conversely, we found that wild-type **UNC-82**::GFP colocalized with ectopic birefringent aggregates resulting from mutation of four other genes that disrupt thick filament assembly or organization (Figure 4 and Figure 5). In addition to paramyosin, these aggregates all contain additional proteins (see *Results*) that are not found in **UNC-82** E424K aggregates. These observations suggest that arrest of the assembly process in these

other mutants occurs at a later step than in **UNC-82** E424K mutants.

Finally, the genetic interactions between *unc-82* and other paramyosin-affecting mutations also support its early action in a paramyosin assembly pathway. The phenotype of double mutants of *unc-82* alleles with *unc-98*(sf19)/ZnF is similar to the phenotype of the *unc-82* mutation alone (Figure 6, J–L and Figure 7, A and B). The lack of an additive increase in severity argues that the two proteins may act in a common linear pathway. The aggregates present in the *unc-98*/ZnF mutant contain **UNC-82** (Figure 5, E and F); these aggregates do not form in the absence of **UNC-82** in the *unc-82*(0); *unc-98*/ZnF double mutant (Figure 6K and Figure 7, A–C). Further, the accumulations typical of *unc-82* E424K mutants (accumulations that contain myosin B) are present in the double with *unc-98*/ZnF (Figure 7, D–F). These observations are consistent with **UNC-82** acting upstream of **UNC-98/ZnF**, and **UNC-82** protein playing a role in aggregate formation in *unc-98*/ZnF mutants.

In contrast, the double mutants of *unc-82* alleles with a mutation in *unc-89*/obscurin show a more severe phenotype than either mutation alone (Figure 6, G–I). The *unc-89*(su75) allele eliminates all large isoforms of **UNC-89/obscurin**, which contain the SH3 domain. The SH3 domain is proposed to crosslink thick filaments at the M-line through an interaction with paramyosin (Qadota *et al.* 2016). The double mutant combination of *unc-89*(su75)/obscurin and *unc-82*(0) is sterile, and muscle cells have grossly abnormal structure, with birefringent signal appearing in large round accumulations (Figure 6H). This additive interaction suggests that the two proteins function independently, with **UNC-82** promoting paramyosin assembly into the thick filament, and **UNC-89/obscurin** mediating filament attachment at the M-line.

Birefringent, and therefore ordered, assemblages of paramyosin form in a variety of mutant backgrounds, and also when wild-type paramyosin is overexpressed from transgenes (Hoppe *et al.* 2010b). The more hydrophobic surface of

Figure 9 Model for the order of UNC-82 protein interactions during early steps of thick filament assembly. UNC-82 first associates with newly made paramyosin and myosin B. UNC-82 kinase activity promotes the subsequent association of this complex with a complex of UNC-98-myosin A during filament and body elongation.

paramyosin compared to myosin (Cohen *et al.* 1987) may be responsible for the apparent propensity to form aggregates. Aggregates may form in a given mutant because the affected protein has a direct role in paramyosin organization. However, because increased dosage of normal paramyosin can lead to aggregate formation, aggregates may form due to a block in a separate step in thick filament assembly that secondarily leads to high levels of cytosolic paramyosin. The colocalization of UNC-82 with all paramyosin-containing aggregates examined (Figure 3 and Figure 5, C–H) suggests a close interaction of UNC-82 with paramyosin *in vivo*. Consistent with this hypothesis, the localization pattern of UNC-82::GFP is highly abnormal in the paramyosin null mutant (Figure 5, A and B).

Considering the roles of phosphorylation in regulating the assembly of smooth and nonmuscle myosin heavy chains (Castellani and Cohen 1987; Murakami *et al.* 1988; Kelley and Adelstein 1990; Rovner *et al.* 2002), it is tempting to speculate that UNC-82 exerts its effects by phosphorylating paramyosin to regulate its assembly. Several observations are consistent with this model. The level of paramyosin and myosin phosphorylation in purified *C. elegans* thick filaments has been correlated with its state of assembly, and solubilized myosin and paramyosin phosphorylation by a copurifying kinase was demonstrated (Dey *et al.* 1992). In worm extracts, paramyosin is phosphorylated by an endogenous kinase on serine residues within the N-terminal nonhelical headpiece (Schrieffer and Waterston 1989). Point mutation of the headpiece, or the homologous C-terminal tailpiece residues in myosin A, leads to formation of abnormal aggregates in muscle cells (Hoppe *et al.* 2010b). Unpublished data cited in

Schrieffer and Waterston (1989) indicates that the more acidic forms of paramyosin are absent in *unc-82* mutants, consistent with decreased phosphorylation of the protein. This reported effect on paramyosin phosphorylation might occur because paramyosin is an UNC-82 substrate, or through an indirect mechanism. The mammalian UNC-82 ortholog ARK5/NUAK1 increases myosin light chain phosphorylation in human embryonic kidney cells by phosphorylating and inactivating a myosin phosphatase (Zagórska *et al.* 2010).

However, the dosage effects observed in our experiments are not consistent with a simple model in which UNC-82 phosphorylates newly synthesized paramyosin, either to prevent aggregation, or to directly promote its assembly into a growing thick filament. The effect of increased expression of wild-type UNC-82 in paramyosin missense mutants was to increase, rather than decrease, aggregate formation in heterozygotes (Figure 4, F–K), and to cause synthetic lethality in homozygotes (Figure 4, P–S). Increased UNC-82 levels also caused lethality and aggregate formation in the absence of paramyosin (Figure 5, A and B). These data indicate that a stoichiometric relationship of UNC-82 and paramyosin is critical for normal muscle structure, and suggest that UNC-82 has both catalytic and structural roles during thick filament assembly.

The synthetic lethality and formation of novel birefringent structures in the muscles cells of paramyosin null animals with increased UNC-82::GFP expression implicates UNC-82 in organizing additional components of the contractile apparatus (Figure 5, A and B). The composition of the birefringent aggregates formed in these cells was not determined. Given that the paramyosin accumulations in UNC-82 E424K mutants

and paramyosin *e73* missense mutants contain myosin B (Figure 1, Q and R; Epstein *et al.* 1987; Gengyo-Ando and Kagawa 1991), and that the paramyosin accumulations in other mutants do not (Mercer *et al.* 2006; Miller *et al.* 2008; Qadota *et al.* 2016), these aggregates may be assemblages of myosin B. Removal of myosin B in a paramyosin mutant background is lethal (Waterston 1988). Induction of myosin B aggregation in the paramyosin null may mimic that double mutant phenotype. The possibility that *UNC-82* is involved in the organization of myosin B and paramyosin (Figure 9), which compose the thick filament arms (see *Introduction*), is consistent with defects in *unc-82(0)* mutant muscle cells becoming apparent during body elongation stages of embryogenesis, when muscle cells and thick filaments are increasing in length.

Myosin A is not found associated with the *UNC-82*-containing aggregates (Figure 1, O and P). The opposite effects of increasing myosin A dosage in a paramyosin *e73* background, which suppresses (Riddle and Brenner 1978; Otsuka 1986; Hoppe and Waterston 2000), and increasing *UNC-82* dosage, which enhances (Figure 4, E–H, P, and Q), also suggest that *UNC-82* and myosin A do not act as part of the same complex in organizing paramyosin. We have found that *UNC-98*/ZnF colocalizes with myosin A in structures that do not contain paramyosin (Figure 7, V–Zz). We propose that *UNC-82* catalytic activity is required in a processing step that precedes the association of paramyosin with *UNC-98* and myosin A (Figure 9).

There are two mammalian orthologs of *UNC-82*, ARK5/NUAK1 and SNARK/NUAK2 (Hoppe *et al.* 2010a); both are required in mouse muscle. Phosphoproteome analysis of a muscle-specific knock-out of ARK5/NUAK1 revealed changes in phosphorylation levels of proteins in the actin-myosin cytoskeleton, including myosin (Inazuka *et al.* 2012). Reduction of SNARK/NUAK2 resulted in an age-dependent loss of muscle mass (Lessard *et al.* 2016). These observations suggest potential conserved roles for *UNC-82*/ARK5/SNARK in the contractile apparatus across diverse animal lineages.

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