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Kettin, the large actin-binding protein with multiple immunoglobulin domains, is essential for sarcomeric actin assembly and larval development in *Caenorhabditis elegans*

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Abstract

Among many essential genes in the nematode *Caenorhabditis elegans*, *let-330* is located on the left arm of Chromosome V and was identified as the largest target of a mutagen in this region. However, the *let-330* gene has not been characterized at the molecular level. Here, we report that two sequenced *let-330* alleles are nonsense mutations of *ketn-1*, a previously characterized gene encoding kettin. Kettin is a large actin-binding protein of 472 kDa with 31 immunoglobulin domains and is expressed in muscle cells in *C. elegans*. *let-330/ketn-1* mutants are homozygous lethal at the first larval stage with mild defects in body elongation. These mutants have severe defects in sarcomeric actin and myosin assembly in striated muscle. However, α-actinin and vinculin, which are components of the dense bodies anchoring actin to the membranes, were not significantly disorganized by *let-330/ketn-1* mutation. Kettin localizes to embryonic myofibrils before α-actinin is expressed, and α-actinin deficiency does not affect kettin localization in larval muscle. Depletion of vinculin minimally affects kettin localization but significantly reduces colocalization of actin with kettin in embryonic muscle cells. These results indicate that kettin is an essential protein for sarcomeric assembly of actin filaments in muscle cells.

Graphical Abstract

Kettin is a large actin-binding protein with more than 30 immunoglobulin domains. Genetic studies in the nematode *Caenorhabditis elegans* reveal that *ketn-1*, the gene encoding kettin, is allelic to *let-330* and essential for muscle sarcomere assembly and larval development. *ketn-1* mutations cause severe disorganization of sarcomeric actin and myosin in striated muscle.

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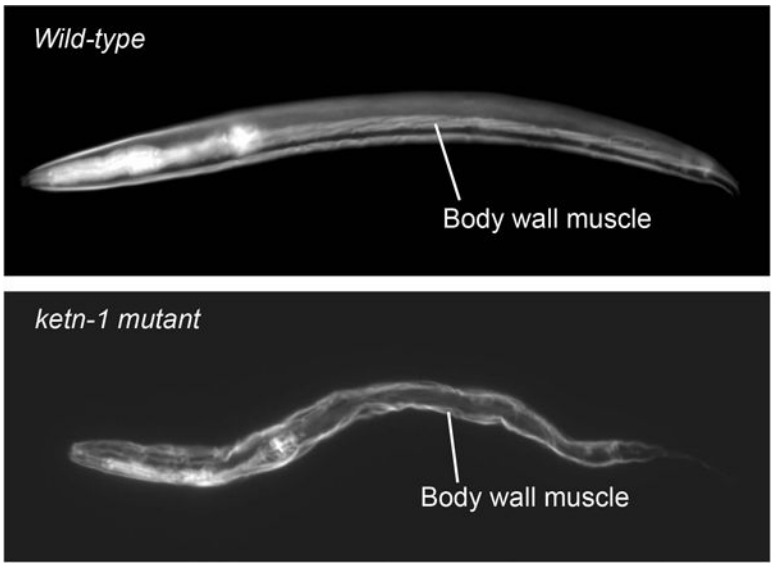
Author contributions

KO, ZQ, and RCJ performed experiments and analyzed data. DLB and SO conceived and designed research and analyzed data. KO and SO prepared figures. RCJ, DLB, and SO drafted and edited manuscript. All authors approved final version of manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.



Keywords

Actin; *Caenorhabditis elegans* (*C. elegans*); genetics; immunoglobulin domain; sarcomere; striated muscle

Introduction

Sarcomeric assembly of actin and myosin in myofibrils is essential for producing contractile forces in muscle cells. Although a number of sarcomere components and regulatory factors have been identified, the mechanism of myofibril assembly remains largely unclear [1–3]. In particular, precise regulation of actin in polymerization, depolymerization, bundling, and localization, is required for proper sarcomere assembly, but extensive investigation is still needed to understand how actin-binding proteins control these processes in a spatially and temporally specific manner in muscle cells [2].

At the physiological level, some of the muscle tissues are critical for morphogenesis, body movement, and function of the cardiovascular system. In humans, severe defects in sarcomeric proteins cause death due to respiratory failure [4] or heart failure [5]. Thus, a subset of muscle sarcomere genes are also essential for viability in animals. In the nematode *Caenorhabditis elegans*, body wall muscle is the major striated muscle tissues, which are responsible for body movement [6–8]. During late embryonic development, muscle contraction is required for body elongation [9], and defects in embryonic muscle contraction result in paralysis and developmental arrest before the body is completely elongated [10, 11]. Based on this phenotype, some of the muscle sarcomere genes, including integrin (*pat2/pat-3*) [12], integrin-linked kinase (*pat-4*) [13], troponin C (*pat-10*) [14] and tropomyosin (*lev-11*) [10, 15], have been identified as essential genes. On the other hand, unbiased screens for lethal phenotypes have identified hundreds of essential genes in *C. elegans* [16], and recent whole genome sequencing revealed molecular identities of many of these essential genes [17–19]. These analyses demonstrated previously unappreciated essential

functions of some of the genes, such as identification of *let-144* and *let-548* as *tln-1* talin, which was previously reported only as a component of the muscle adhesion complexes [20] and a regulator of muscle contractility [21]. Therefore, molecular characterization of additional essential genes in *C. elegans* may reveal novel genes that are involved in muscle sarcomere assembly.

In this study, we used whole genome sequencing for *C. elegans* mutants and determined that *let-330* is allelic to *ketn-1*, a gene encoding kettin that is a large actin-binding protein of 472 kDa [22, 23]. *let-330* was identified as the largest target of ethyl methanesulfonate mutagenesis on the left arm of Chromosome V [24]. *let-330* is homozygous lethal at early to mid larval stages [24], but its molecular identity and cellular phenotypes had not been characterized. Kettin was originally identified in insects as a large muscle protein of 500–700 kDa [25], which contains >30 immunoglubulin (Ig) domains with similarity to the connectin/titin family of giant elastic proteins [26, 27]. *C. elegans* kettin (Ce-kettin) has 31 Ig domains, binds to actin filaments *in vitro*, and localizes to a portion of muscle thin filaments [22]. Knockdown of Ce-kettin by RNA interference causes only mild disorganization of sarcomeric thin filaments probably due to insufficient depletion of the Ce-kettin protein, which is enhanced by tetramisole-induced muscle hypercontraction [22, 28]. Thus, the function of kettin in *C. elegans* was unclear. Although one study concluded that kettin is required for viability in *Drosophila* [27], other studies reported that kettin in arthropods is encoded by the connectin/titin gene (*sallimus* in *Drosophila*) as one of many splice variants including connectin/titin and zormin [29–32], suggesting that the reported lethal phenotype may be caused by a combination of deficiencies of kettin and other splice variants of the connectin/titin-related proteins. However, in *C. elegans*, the kettin gene (*ketn-1*) is distinct from other connectin/titin-related genes, including *unc-22* [33], *unc-89* [34], and *ttn-1* [35]. Therefore, *C. elegans* is a suitable model organism to analyze kettin-specific functions. Our phenotypic analyses indicate that *let-330/ketn-1* mutants are lethal at the first larval stage and exhibit severe disorganization of sarcomeric actin filaments in the body wall muscle.

Results

Identification of let-330 as ketn-1, a gene encoding kettin

To determine molecular nature of the *C. elegans let-330* gene, we first used genetic mapping data to narrow down the genomic region for analysis. Using deficiency mapping, two deficiencies, *sDf27* and *sDf34*, failed to complement *let-330*, indicating that *let-330* is located in the region of overlap between *sDf27* and *sDf34* [24] (Fig. 1A). The breakpoints of *sDf27* and *sDf34* have been precisely located [36, 37]: the left end of *sDf27* is at 2,741,462bp on *LGV* while the right end of *sDf34* is at 3,336,652bp, thus *let-330* must be located in that overlapped region (Fig. 1A). Total 17 *let-330* alleles have been isolated and their allelic relationships have been confirmed by complementation tests [24]. We then sequenced two *let-330* alleles, *let-330(s1425)* and *let-330(s1429)*. Both of these alleles are homozygous lethal at early larval stages [24] (Fig. 1B). We found that *s1425* and *s1429* had nonsense mutations in *ketn-1*, which encodes kettin, a 472-kDa actin-binding protein with 31 Ig domains (Table 1 and Fig. 1E). Within the region of overlap between *sDf27* and *sDf34*,

these were the only mutations that altered protein sequences. Furthermore, we found that a deletion allele *ketn-1(tm2554)* (Fig. 1E), which was isolated by the *C. elegans* Deletion Mutant Consortium [38], was homozygous lethal with nearly identical phenotypes (Fig. 1C) to other severe *let-330* alleles (Fig. 1B). *ketn-1(tm2554)* had a 232-bp deletion plus an 11-bp insertion causing a frame shift near the 3'end of the protein coding region (Fig. 1 and Table 1). The severe phenotype of *ketn-1(tm2554)* suggests that the mRNA with the mutation is subjected to nonsense-mediated decay and fails to produce functional kettin protein. Trans-heterozygotes between *ketn-1(tm2554)* and *let-330(s1425)* or *let-330(s1429)* were early-laval lethal similarly to *let-330(s1425)* or *let-330(s1429)* homozygotes (Fig. 1D). Therefore, *ketn-1(tm2554)* failed to complement either *let-330(s1425)* or *let-330(s1429)*, confirming that *let-330* is identical to *ketn-1*.

Previously reported *let-330/ketn-1* strains were maintained in the presence of the *eT1(III;IV)* balancer that contained only recessive markers [24]. Therefore, *let-330/ketn-1* homozygotes could be distinguished only after they are nearly dead. To precisely distinguish *let-330/ketn-1* homozygous animals from heterozygous animals at early stages, we utilized the *nT1[qIs51]* balancer which contains green fluorescent protein (GFP) as a dominant marker [39]. The *ketn-1(tm2554)* allele, which hereafter represents *let-330/ketn-1* in this paper, was maintained as *ketn-1(tm2554)/nT1[qIs51]* heterozygotes, and *ketn-1(tm2554)* homozygotes were identified as GFP-negative animals in the following analyses (Fig. 1F–H). *ketn-1(tm2554)* homozygotes showed no detectable reactivity with anti-kettin monoclonal antibody MH44 (Fig. 1J), confirming that the kettin protein was absent and that the antibody was specific to kettin. All observed *ketn-1(tm2554)* homozygotes were arrested at a larval stage and barely moved (Fig. 1H). The arrested worms were 202±29 μm (mean±SD, n=20) in length, which was comparable to but slightly shorter than wild-type L1 (the first larval stage) larvae [262±22 μm (mean±SD), n=38] (Fig. 1I). Thus, the data indicate that *ketn-1(tm2554)* homozygotes are arrested at the L1 larval stage with mild defects in body elongation.

let-330/ketn-1 mutants have severe defects in muscle sarcomere assembly

ketn-1(tm2554) homozygotes had severely disorganized actin filaments in the body wall muscle (Fig. 2A and B). In wild-type L1 worms, actin filaments were organized in myofibrils in a striated pattern (Fig. 2A). However, in the *ketn-1* mutant, actin filaments were assembled into thicker and fewer disorganized bundles than wild-type (Fig. 2B). Tropomyosin, one of major actin-associated proteins in sarcomeres [40, 41], showed similar patterns: it was organized in a striated pattern in wild-type (Fig. 2C) but disorganized in thick bundles in the *ketn-1* mutant (Fig. 2D). Similarly, sarcomeric organization of myosin was disturbed by the *ketn-1* mutation. At the L1 stage, two rows of body wall muscle cells are present in each muscle quadrant, and each muscle cell contains two bands of myosin [42], as detected by an anti-MYO-3 antibody. Therefore, in wild-type L1 worms, four distinct bands of myosin were clearly observed (Fig. 2F and K) with an alternate pattern of actin bands to form sarcomeric structures (Fig. 2E, G, and K). In *ketn-1* mutant, myosin and actin were often clumped together with significant overlaps (Fig. 2H–J and L). Sarcomeric organization of actin and myosin was further analyzed quantitatively by measuring Pearson's coefficient (PC), which is close to 1 when two images overlap perfectly and <0

when two images are exclusive [43]. Therefore, if actin and MYO-3 are organized in a sarcomeric pattern, PC values will be low. However, the bands of actin and MYO-3 are projected towards the cytoplasm vertically from the plasma membrane [6], and they will appear overlapped if a cell is viewed from a tilted angle. Due to the round shape of the worm body, micrographs could not always be taken from a perfectly vertical angle. As a result, PC values for actin and MYO-3 were quite variable in wild-type (Fig. 2M). Nonetheless, in *ketn-1* mutant, PC values were consistently high (Fig. 2M), indicating a higher degree of overlaps between actin and MYO-3 in *ketn-1* than in wild-type. These observations demonstrate that kettin is essential for sarcomeric organization of actin and myosin in the body wall muscle cells.

Kettin is not required for assembly of dense bodies, but vinculin promotes coupling of actin with kettin

In *C. elegans* body wall muscle, dense bodies, which are one type of integrin-based adhesion structures similar to the Z-discs in cross-striated muscles, link the plasma membrane to thin filaments of myofibrils [7], and mutations in the dense-body proteins often cause severe sarcomere disorganization and embryonic lethality [10]. Since kettin localizes to a part of the thin filaments proximal to the dense bodies [22], we tested whether *let-330/ketn-1* mutation affects assembly of the dense bodies at the L1 stage. α-actinin (ATN-1) is concentrated in the cytoplasmic portion of the dense bodies in adult muscle [44] but its expression is undetectable in embryonic muscle by immunofluorescence microscopy using anti-ATN-1 antibody [42], which has been verified to be specific to ATN-1 [45]. Expression and subcellular localization of ATN-1 in larval stages had not been reported. We found that ATN-1 became detectable in body wall muscle at the L1 stage only in the most peripheral sarcomeres as linearly arranged dot-like structures, which are presumably dense bodies (Fig. 3A). This early localization pattern of ATN-1 was not significantly altered in *ketn-1(tm2554)* while actin was disorganized (Fig. 3B). Vinculin (DEB-1) is another dense-body component that localizes at the base near the plasma membrane [46]. DEB-1 is expressed from embryonic stages [46, 47] and localized in a punctate pattern at the L1 stage throughout all sarcomeres (Fig. 3C). Again, the overall DEB-1 localization pattern was not largely affected in *ketn-1(tm2554)*. These results suggest that kettin is not required for assembly of dense bodies.

Next, we tested whether localization of kettin is dependent on assembly of the dense bodies. Since insect kettin binds to α-actinin and actin filaments simultaneously and is implicated in reinforcement of the Z-discs [48], we examined whether kettin localization is dependent on α-actinin. The α-actinin gene *atn-1* is required for assembly of the cytoplasmic portion of the dense bodies, but *atn-1* null mutants are viable and exhibit only mild actin disorganization in adult muscle [45]. Kettin was expressed in embryonic body wall muscle [22] before α-actinin was expressed, and, at the L1 stage, kettin localized to a portion of sarcomeres in a punctate pattern (Fig. 4A). The striated organization of actin, as well as that of kettin, was indistinguishable between wild-type and *atn-1*-null mutant worms (Fig. 4A and B), indicating that α-actinin is not required for assembly of actin and kettin at the L1 stage. As kettin is also not required for α-actinin to localize to the dense bodies (Fig. 3B),

kettin and α-actinin should assemble independently to the myofibrils during larval development.

The vinculin gene *deb-1* is essential for dense-body assembly, and *deb-1* mutants are lethal at the L1 stage with defects in embryonic sarcomere assembly [46, 47]. We examined embryonic phenotypes after knockdown of DEB-1 (vinculin) by RNA interference. At the two-fold stage (~490-min old embryo), actin and kettin were assembled into continuously organized myofibrils in control embryos (Fig. 5A). In *deb-1(RNAi)* embryos, actin became aggregated with a discontinuous appearance, whereas kettin was not aggregated and was continuously organized in a very similar pattern to control embryos (Fig. 5B). The degree of colocalization between actin and kettin was decreased by *deb-1(RNAi)* (Fig. 5E). In more advanced three-fold embryos (~550-min old embryos), striation of actin and kettin became evident in control embryos (Fig. 5C). In *deb-1(RNAi)* embryos, actin became more clearly aggregated, whereas kettin remained continuously organized (Fig. 5D). The decreased colocalization between actin and kettin in *deb-1(RNAi)* was quantitatively consistent in three-fold embryos (Fig. 5E). These results indicate that kettin localization is independent of vinculin and suggest that vinculin facilitates coupling of actin with kettin within embryonic myofibrils.

Discussion

This study demonstrated that *let-330/ketn-1* encoding kettin is an essential gene in the nematode *C. elegans*. No lethal mutation has been reported in other connectin/titin-related genes in *C. elegans*: *unc-22* (twitchin) [33], *unc-89* (obscurin) [34, 49], and *ttn-1* (a twitchin/titin hybrid protein) [35]. Thus, kettin has a specific function to support viability of the nematodes. A subset of body wall muscle-specific genes are essential and their mutant phenotypes are classified as Pat (Paralysed and Arrested at Two-fold) phenotypes [10]. In these mutants, body wall muscles fail to contract due to severe sarcomere disorganization, which also results in arrest of body elongation at the two-fold embryonic stage. *let-330/ketn-1* mutants exhibit milder phenotypes than Pat mutants since *let-330/ketn-1* homozygotes are arrested at the L1 larval stage with mild elongation defects. Many of the Pat genes encode components of the dense bodies, which are adhesion structures linking the plasma membrane with contractile apparatuses [50]. However, assembly of dense bodies appears relatively normal in *let-330/ketn-1* mutants, suggesting that some contractile apparatuses may assemble and function to suppress the severity of the phenotypes. Actin-myosin interactions without kettin might be sufficient to assemble functional myofibrils in early phases. Therefore, the *let-330/ketn-1* mutant phenotype is a novel class of muscle defects.

Previous genetic studies have shown that dense bodies are required for sarcomeric organization of the thin filaments [7]. The *let-330/ketn-1* mutations minimally affect dense bodies but cause severe disorganization of actin filaments. This phenotype suggests that kettin is required for stabilizing actin filaments to the dense bodies to maintain sarcomeres. This function is supported by the localization of kettin to a part of thin filaments near the dense bodies [22]. *Lethocerus* kettin binds directly to α-actinin and actin [25, 48] and may reinforce the α-actinin-actin bundles. However, in *C. elegans*, kettin assembles at the

myofibril regions before α-actinin is expressed (Fig. 3E) or when vinculin is depleted (Fig. 3F, H). Vinculin depletion decreases colocalization of kettin with actin filaments (Fig. 3I), suggesting that kettin binds to an unknown dense-body component(s) and anchor actin filaments to the dense bodies in embryonic and larval muscle.

Kettin is found only in invertebrates, but other actin-binding proteins in vertebrates may have similar functions to kettin in muscle cells. Kettin is composed nearly entirely of Ig domains, and some of them directly bind to actin filaments [22]. Insect kettin has been demonstrated to bundle actin filaments *in vitro* [48]. Therefore, kettin might be important to crosslink neighboring actin filaments to reinforce sarcomeric arrangements of thin filaments. Similarly, Ig domains act as actin-binding motifs in many vertebrate actin-binding proteins [51], including connectin/titin [52, 53], palladin [54], myotilin [55], and filamin [56, 57]. In particular, filamin shares similarities to kettin: human filamin is a large elongated homodimeric protein containing two N-terminal calponin-homology domains, 24 Ig domains, and a C-terminal dimerization domain [58]. Although the N-terminal calponin-homology domains are widely recognized as actin binding sites, Ig domains in the central region also bind to actin filaments [56, 57]. In mammalian muscle, filamin localizes to the Z-discs [59], and mutations in the human filamin gene *FLNC* cause several types of myopathies in skeletal muscle and cardiomyopathies [60–63]. Interestingly, *C. elegans* has two filamin genes [64] but their functions in muscle have not been characterized. Thus, further functional studies on kettin and filamin in *C. elegans* should provide insight into the fundamental roles of actin-binding proteins with Ig domains during assembly and maintenance of myofibrils.

Materials and methods

Worm strains

All *C. elegans* strains were cultured under standard conditions as described [65]. Strains used are: BC2226 *dpy-18(e364)/eT1(III); let-330(s1425) unc-46(e177)/eT1(V)* [24]; BC2230 *dpy-18(e364)/eT1(III); let-330(s1429) unc-46(e177)/eT1(V)* [24]; BC3952 *dpy-18(e364)/eT1(III); unc-46(e177)/let-500(s2165)eT1(V)* [17]; BC9223 *dpy-18(e364)/eT1(III); let-330(s1425) unc-46(e177)/let-500(s2165)eT1(V)* (generated in this study by crossing BC2226 and BC3952); BC9237 *dpy-18(e364)/eT1(III); let-330(s1429) unc-46(e177)/let-500(s2165)eT1(V)* (generated in this study by crossing BC2230 and BC3952); ON349 *ketn-1(tm2554)/nT1[qIs51]* (this study); RB1812 *atn-1(ok84)* [45]. ON349 *ketn-1(tm2554)/nT1[qIs51]* was generated by outcrossing the original *ketn-1(tm2554)/nT1[qIs51]* strain (FX18311, provided by Dr. Shohei Mitani, Tokyo Women's Medical University School of Medicine, Tokyo, Japan) four times. Complementation tests were performed by crossing BC9223 [*let-330(s1425)*] or BC9237 [*let-330(s1429)*] hermaphrodites and ON349 [*ketn-1(tm2554)*] males.

Genomic DNA isolation and whole genome sequencing

Qin et al. [17] reported that identifying genomic variations in the *eT1* balanced mutations was simplified by replacing an *eT1* balancer with an *eT1* with a lethal mutation. Homozygous *eT1* hermaphrodites are fertile and contribute to the total sequenced genomic

DNA which makes the allelic ratios of lethal mutations difficult to predict and analyze. We replaced *dpy-18(e364)/eT1(III);let-330 unc-46(e177)/eT1(V)* with *dpy-18(e364)/eT1(III);let-330 unc-46(e177)/let-500(s2165)eT1(V)* to make BC9223 for *let-330(s1425)* and BC9237 for *let-330(s1429)*. *let-500(s2165)* blocks development early and drastically reduces the contribution of homozygous *eT1* DNA to sequence data [17]. We sequenced two *let-330* alleles using BC9223 and BC9237. Each of BC9223 and BC9237 was grown on two 10-cm petri dishes. Gravid adult worms were collected with M9 buffer (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.2 g $MgSO_4$ in 1 L of H_2O) followed by centrifugation at 1500 rpm for 2 mins at 4 °C. The worm pellets were washed three times with M9 buffer. The collected worms were placed on a rocker at room temperature for 2.5 hours. After centrifugation at 1500 rpm for 2 mins, the worm pellets were resuspended in 4 ml of M9 buffer and kept at −80 °C. Genomic DNA was purified by using QIAGEN DNeasy Blood & Tissue Kit (Catalog # 69504) and submitted to the British Columbia Cancer Agency Canada's Michael Smith Genome Sciences Centre for whole genome sequencing using the Illumina PET HiSeq technology to produce 150 bp long paired end reads. DNA amounts submitted for sequencing were 1.45 μg for BC9223 (*s1425*) and 1.22 μg for BC9237 (*s1429*).

Bioinformatics analysis of sequencing data

BWA [66] was used to align the sequencing reads against the *C. elegans* reference genome (version WS249). SAMtools [67] was applied to remove duplicate reads. Integrative Genomics Viewer (IGV) [68, 69] was employed to identify the breakpoints of large deletions, medium sized insertions, and translocations. Finally, CooVar [70] was applied to examine the effect of the variations on the coding sequences. The sequencing depths of BC9223 (*s1425*) and BC9237 (*s1429*) were 33 and 31, respectively.

Fluorescence microscopy

Staining of actin filaments in whole animals with tetramethylrhodamine-phalloidin was performed as described [71]. For immunofluorescent staining, worm embryos and L1 larvae were permeabilized by a freeze-crack method on poly-lysine-coated slides [72]. Staining of tropomyosin was performed by fixation with 4% paraformaldehyde for 10 min and permeabilization with 0.5% Triton X-100. All other samples were fixed with methanol at −20 °C for 5 min. Fixed samples were treated with primary antibodies in PBS with 1 % bovine serum albumin for 1 hr, washed with PBS, treated with secondary antibodies in PBS with 1 % bovine serum albumin for 1 hr, washed with PBS, and mounted with ProLong Gold Antifade Mountant (Thermo Fisher).

Rabbit anti-actin polyclonal antibody was purchased from Cytoskeleton Inc. (Catalog # AAN01; RRID:AB_10708070). Guinea pig anti-tropomyosin (LEV-11) polyclonal antibody was described previously [40]. Mouse anti-kettin monoclonal (MH44), mouse anti-α-actinin monoclonal (MH40), and mouse anti-vinculin monoclonal (MH24) antibodies [44] were provided by Dr. Pamela Hoppe (Western Michigan University, Kalamazoo, MI). Mouse anti-MYO-3 monoclonal antibody (Clone 5–6; RRID:AB_2147425) [73] was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, (Iowa City, IA). Secondary antibodies used were Alexa 488–conjugated goat anti-guinea pig immunoglobulin G (IgG) (Thermo

Fisher), Alexa 488-conjugated goat anti-mouse IgG (Thermo Fisher), and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Samples were observed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope (Nikon Instruments, Tokyo, Japan) with a CFI Plan Fluor ELWD 40× (dry; numerical aperture [NA] 0.60) or Plan Apo 60× (oil; NA 1.40) objective. Images were captured by a Hamamatsu ORCA Flash 4.0 LT sCMOS camera (Hamamatsu Photonics) and processed by NIS-Elements (Nikon Instruments) and Photoshop CS3 (Adobe).

Colocalization analysis

Quantitative image analysis was performed by ImageJ (National Institutes of Health, Bethesda, MD) using the JACoP plug-in [43]. Pearson's coefficients were analyzed in randomly selected sarcomere regions of 30 × 30 pixels. Student's t-test was performed using SigmaPlot 14.0 (Systat Software).

RNA interference experiments

RNA interference was performed by feeding *Escherichia coli* HT115(DE3) expressing double-stranded RNA under the conditions as described previously [40]. The RNAi clone for *deb-1* was described previously [74]. Control experiments were performed with the *E. coli* HT115(DE3) that was transformed with an empty RNAi vector L4440 (kindly provided by Dr. Andrew Fire, Stanford University, Stanford, CA) [75]. Phenotypes were characterized in their F1 generation at embryonic stages.

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Abbreviations:

GFP	green fluorescent protein
Ig	immunoglobulin
PC	Pearson's coefficient
RNAi	RNA interference

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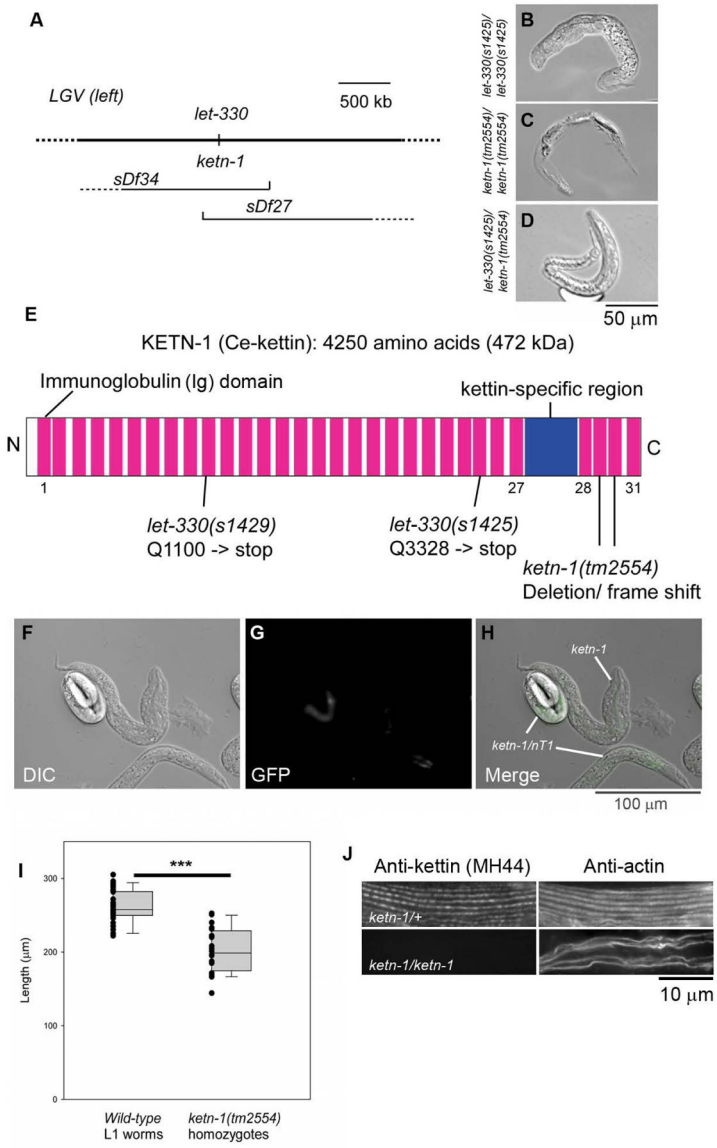

Figure 1.

Mutations in *let-330/ketn-1* encoding kettin cause lethality at the L1 larval stage. **(**A) Genetic mapping of *let-330*. *let-330* was mapped in the overlapping region between two deficiencies, *sDf27* and *sDf34* [24]. (B–D) *ketn-(2554)* failed to complement *let-330(s1425)*. *let-330(s1425)* homozygotes (B), *ketn-1(tm2554)* homozygotes (C), and *let-330(s1425)/ ketn-1(tm2554)* trans-heterozygotes (D) were arrested at the L1 larval stage. *ketn-1(tm2554)* also failed to complement *let-330(s1429)*. Bar, 50 μm. (E) Domain structure of Ce-kettin and mutation sites in *let-330/ketn-1*. Ig domains are shown in magenta, and a kettin-specific region in blue. (F–H) Characterization of *ketn-1(tm2554)* phenotypes. *ketn-1(tm2554)* was homozygous lethal and maintained as heterozygotes over the *nT1[qIs51]* GFP-marked balancer. Progeny from *ketn-1(tm2554)/nT1[qIs51]* were identified as *ketn-1(tm2554)* homozygotes (GFP-negative) and *ketn-1(tm2554)/nT1[qIs51]* (GFP-positive). *nT1[qIs51]* homozygotes did not develop into embryos. All observed *ketn-1(tm2554)* homozygotes were

arrested at a larval stage. Bar, 100 μm. (I) Length measurement of wild-type L1 larvae (n=38) and arrested *ketn-1(tm2554)* homozygous animals (n=20). Boxes represent a range of the 25th and 75th percentiles, with the medians marked by solid horizontal lines, and whiskers indicate the 10th and 90th percentiles. ***, $p < 0.0001$ by Student's t-test. (J) The kettin protein is absent in *ketn-1(tm2554)* homozygotes. *ketn-1(tm2554)/+* heterozygotes (top) or *ketn-1(tm2554)* homozygotes (bottom) were immunostained with anti-Ce-kettin monoclonal antibody (MH44) and anti-actin polyclonal antibody. The results also indicate that the anti-Ce-kettin antibody specifically recognized Ce-kettin.

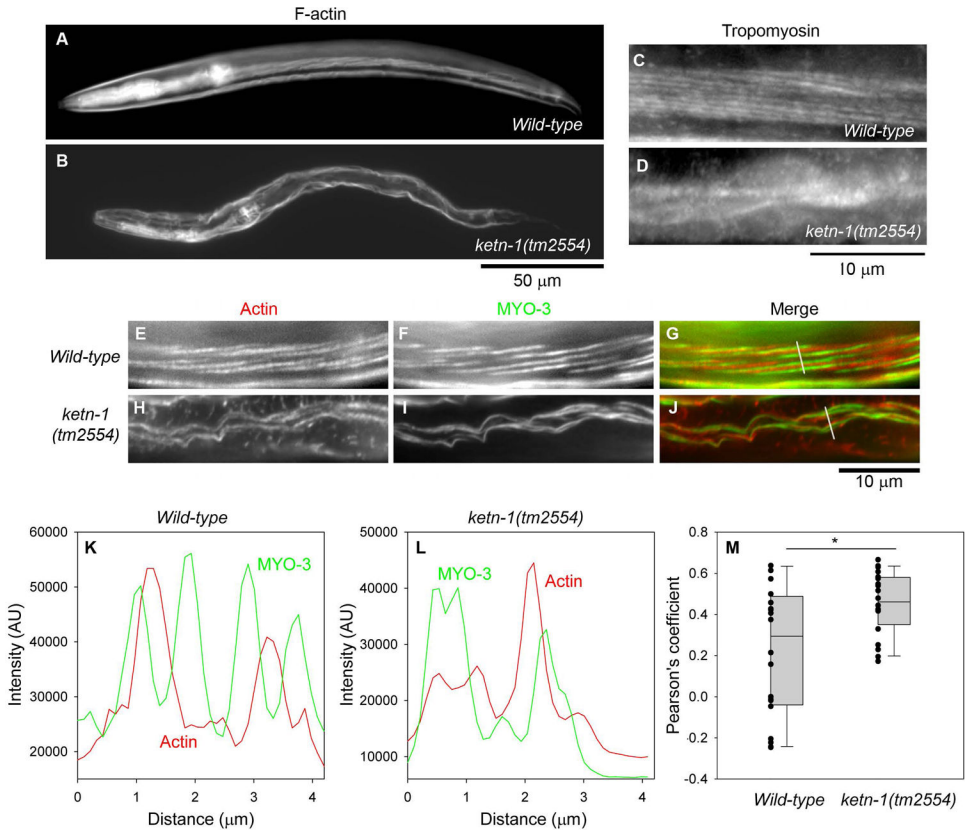

Figure 2.

Mutations in *let-330/ketn-1* cause disorganization of actin and myosin in the body wall muscle. **(**A, B) Wild-type (A) or *ketn-1(tm2554)* homozygous (B) L1 larvae were stained with tetramethylrhodamine-phalloidin to visualize actin filaments. Bar, 50 μm. (C, D) The body wall muscle regions of wild-type (C) or *ketn-1(tm2554)* homozygous (D) L1 larvae stained for tropomyosin. Bar, 10 μm. (E-M) The body wall muscle region of wild-type (E-G) or *ketn-1(tm2554)* homozygous (H-J) L1 larvae immunostained for actin (E, H) and MYO-3 myosin heavy chain (F, I). Merged images are shown in G and J (actin in red and MYO-3 in green). Bar, 10 μm. Line scans of fluorescence intensity (arbitrary unit) at the white lines in G and J (from top to bottom) are shown in K and L, respectively. (M) Colocalization of actin and MYO-3 was quantitatively analyzed by measuring Pearson's coefficient (n = 20). Boxes represent a range of the 25th and 75th percentiles, with the medians marked by solid horizontal lines, and whiskers indicate the 10th and 90th percentiles. *, $p < 0.05$ by Student's t-test.

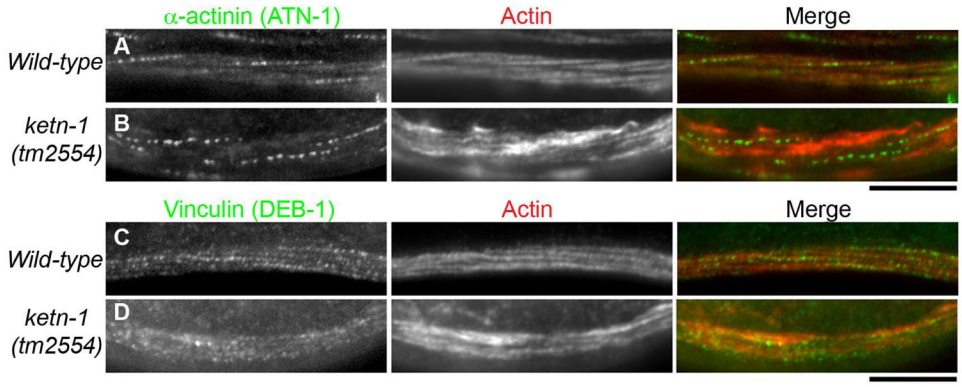

Figure 3.

α-actinin and vinculin assemble in dense bodies independently of kettin in larval muscle. Immunofluorescent localization of ATN-1 (α-actinin) and actin (A, B) or DEB-1 (vinculin) and actin (C, D) in wild-type (A, C) or *ketn-1(tm2554)* homozygous (B, D) L1 larvae. The body wall muscle regions are shown. Merged images are shown with actin in red and α-actinin or vinculin in green. Bars, 10 μm.

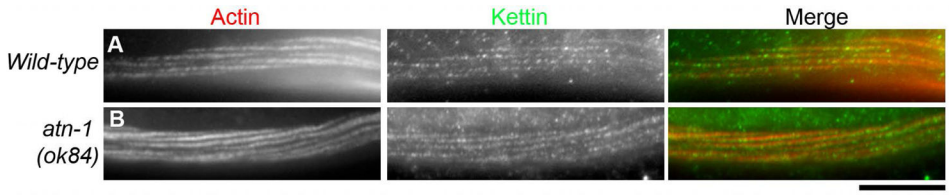

Figure 4.

α-actinin is not required for sarcomeric assembly of actin and kettin in larval muscle. Immunofluorescent localization of actin and kettin in wild-type (A) or *atn-1-null* [*atn-1(ok84)*] (B) at the L1 stage. The body wall muscle regions are shown. Merged images are shown with actin in red and kettin in green. Bars, 10 μm.

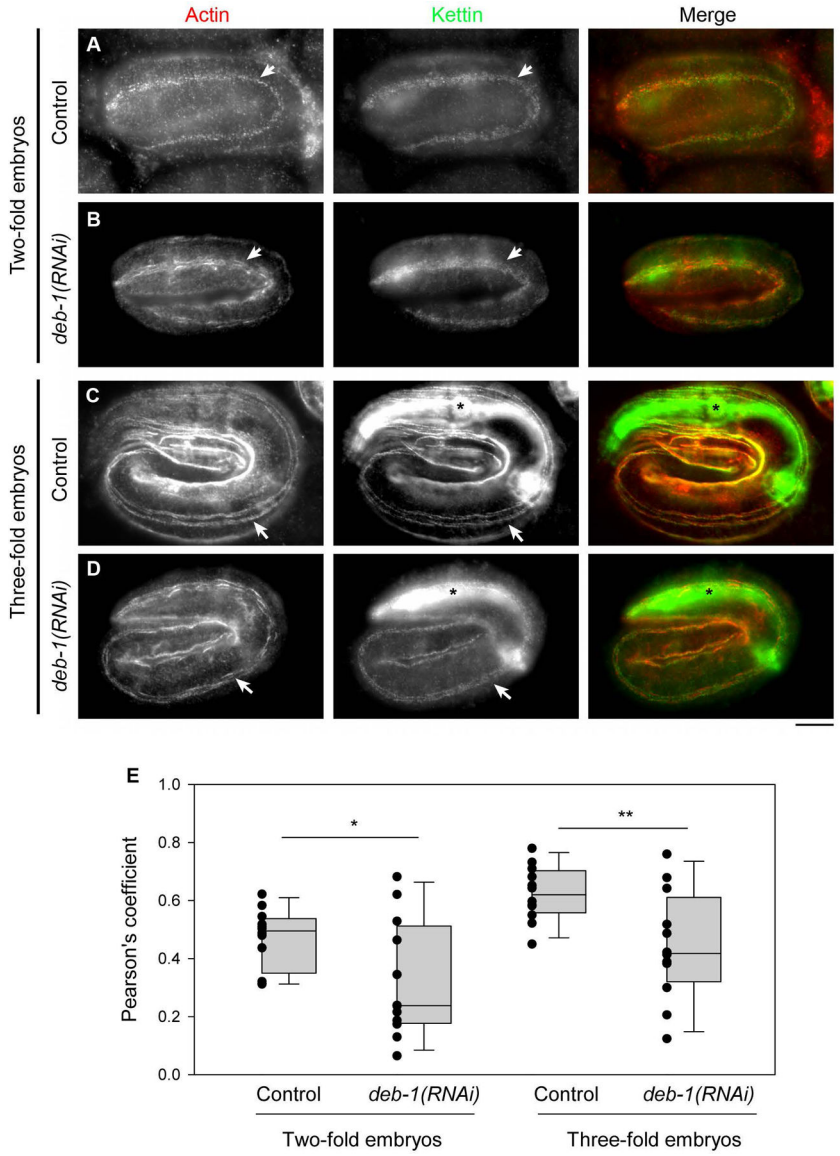

Figure 5.

Vinculin depletion reduces co-localization of kettin and actin. (A–D) Immunofluorescent localization of actin and kettin in control RNAi (A, C) or *deb-1(RNAi)* (B, D) embryos at the two-fold (A, B) and three-fold (C, D) stages. Arrows indicate representative rows of body wall muscle. Merged images are shown with actin in red and kettin in green. Bars, 10 μm. Note that strong staining of kettin was observed in the pharynx at the three-fold stage (asterisks in C and D). (E) Colocalization of actin and kettin was quantitatively analyzed by measuring Pearson's coefficient (n = 12). Regions where pharyngeal staining of kettin was present were omitted from the analysis. Boxes represent a range of the 25th and 75th percentiles, with the medians marked by solid horizontal lines, and whiskers indicate the 10th and 90th percentiles. *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$ by Student's t-test.

Table 1.

Summary of *let-330/ketn-1* mutations.

Allele	Position	Mutation	Amino acid change
s1425	V:2786406	A to G	Q3328 to stop
s1429	V:2793975	A to G	Q1100 to stop
tm2554	V:2783427–2783658	Deletion plus 11 bp insertion	Frame shift after G4034