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Molecular evolution of troponin I and a role of its N-terminal extension in nematode locomotion

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Summary

The troponin complex, composed of troponin T (TnT), troponin I (TnI), and troponin C (TnC), is the major calcium-dependent regulator of muscle contraction, which is present widely in both vertebrates and invertebrates. Little is known about evolutionary aspects of troponin in the animal kingdom. Using a combination of data mining and functional analysis of TnI, we report evidence that an N-terminal extension of TnI is present in most of bilaterian animals as a functionally important domain. Troponin components have been reported in species in most of representative bilaterian phyla. Comparison of TnI sequences shows that the core domains are conserved in all examined TnIs, and that N- and C-terminal extensions are variable among isoforms and species. In particular, N-terminal extensions are present in all protostome TnIs and chordate cardiac TnIs but lost in a subset of chordate TnIs including vertebrate skeletal-muscle isoforms. Transgenic rescue experiments in *C. elegans* striated muscle show that the N-terminal extension of TnI (UNC-27) is required for coordinated worm locomotion but not in sarcomere assembly and single muscle-contraction kinetics. These results suggest that N-terminal extensions of TnIs are retained from a TnI ancestor as a functional domain.

Keywords

Actin; contraction; muscle; myosin; troponin

Introduction

Across various organisms and muscle types, contraction is regulated by several distinct mechanisms (Lehman and Szent-Gyorgyi 1975). In vertebrates, striated (skeletal and cardiac) muscle is regulated through a Ca^{2+} -sensitive troponin-tropomyosin system on thin

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filaments (Squire and Morris 1998). In contrast, the major regulatory system for vertebrate smooth (or non-striated) muscle is through the phosphorylation of myosin regulatory light chain (Somlyo and Somlyo 1994), with additional regulation through actin-binding proteins, such as calponin and caldesmon (Wang 2001; Winder et al. 1998). In comparison to vertebrates, invertebrate muscles are diverged in both morphology and physiology, and these simple correlations cannot be applied (Hooper et al. 2008). For example, molluscan striated and smooth muscles have both troponin-tropomyosin (Goldberg and Lehman 1978; Ojima and Nishita 1986; Tanaka et al. 2005) and myosin that is regulated by direct binding of Ca^{2+} to essential light chain (Szent-Gyorgyi et al. 1999). In the nematode *Caenorhabditis elegans*, the troponin-tropomyosin system controls contraction of both striated muscle (Burkeen et al. 2004; Ruksana et al. 2005; Terami et al. 1999) and non-striated muscle (Myers et al. 1996; Obinata et al. 2010; Ono and Ono 2004). Muscle regulation has been experimentally studied in limited species. Therefore, we currently have a partial view of divergence and conservation of the regulatory mechanisms of muscle contraction across the animal kingdom. Further information on the comparative aspects of the muscle regulatory mechanisms should be important to gain insight in the evolution of various muscle types that have adapted to different functions.

Troponin is a key regulatory component of muscle contraction. It acts as a rapid Ca^{2+} -dependent on-off switch for the actin-myosin interaction (Ebashi 1984). The functional unit of troponin is a complex of three components: troponin T (TnT), troponin I (TnI), and troponin C (TnC). In the vertebrate troponin complex, TnI inhibits actin-myosin interaction at low Ca^{2+} concentrations in cooperation with tropomyosin. At high Ca^{2+} concentrations, Ca^{2+} binds to TnC and relieves the inhibitory function of TnI. TnT tethers the troponin complex to tropomyosin and promotes the inhibition of actomyosin interaction at low Ca^{2+} (Galinska-Rakoczy et al. 2008). Atomic structures of the core domain of the troponin complex show extensive intra- and intermolecular interactions among the troponin components (Takeda et al. 2003; Vinogradova et al. 2005). Ca^{2+} -dependent dynamic alterations in the troponin conformations are required for its switch function (Kowlessur and Tobacman 2012; Lehman et al. 2009). Troponin is present broadly in the animal kingdom, suggesting that molecular evolution of the troponin complex might be a key to understanding the origin of muscle and evolution of its regulatory systems. A recent comparative genomic study has shown that troponin genes emerged in bilaterians (animals with bilateral body plans) but are absent in cnidarians, ctenophores, and porifera (Steinmetz et al. 2012). However, how broadly troponin is present in bilaterian species has not been extensively studied.

In this study, we examined databases and literature and report that troponin is present in all representative bilaterian phyla except for the Ambulacrarian clade (Echinodermata and Hemichordata). Comparison of the TnI sequences shows conservation of the core domains and variation in the N- and C-terminal extensions. Particularly, N-terminal extensions are present in all protostome TnIs but absent in a subset of deuterostome TnIs, suggesting an isoform-specific evolutionary loss of the N-terminal extension from TnI. Functional analysis in the nematode *C. elegans* indicates that the N-terminal extension of TnI is important for coordinated locomotive behavior of worms. Thus, the results suggest that the N-terminal extension is retained in most of TnIs as a functionally important domain.

Results and Discussion

Troponin Evolved Early in the Bilateria

Steinmetz and colleagues analyzed sequences of various muscle proteins from broad species and found that the troponin complex is absent in cnidarians and ctenophores, despite the fact that they have striated muscle (Steinmetz et al. 2012), suggesting that troponin has evolved in bilaterians. We reexamined this view by analyzing existing literature and sequence data broadly in representative bilaterian species (Fig. 1). Troponin components have been characterized at genomic and/or mRNA levels in many species. However, in some cases, troponin or troponin-like proteins have been characterized only at protein levels based on their troponin-like biochemical properties with no known sequence information. Therefore, we assessed both molecular and biochemical data in databases and literature and examined whether a component(s) of the troponin complex is present or absent within bilaterian phyla (Fig. 1).

Troponin components have been identified widely in both protostomes and deuterostomes (Fig. 1, troponin is present in phyla indicated by red), suggesting that troponin emerged shortly after evolution of bilaterians. Within the Deuterostomia, multiple isoforms of the three troponin components have been extensively characterized in the Chordata including vertebrates (Jin et al. 2008), urochordates (sea squirts) (Obinata and Sato 2012), and cephalochordates (e.g. amphioxus) (Dennisson et al. 2010). Within the Protostomia, in the Arthropoda, all three troponin components have been characterized biochemically or genetically in several crustaceans and insects, including the fruit fly *Drosophila melanogaster* (Bullard and Pastore 2011). In the Nematoda, all three troponin components have been genetically characterized in *Caenorhabditis elegans* (Kagawa et al. 2007) and biochemically in *Ascaris suum* (Donahue et al. 1985; Kimura et al. 1987). In the Mollusca, the three troponin components from sea scallop have been characterized both molecularly and biochemically (Goldberg and Lehman 1978; Ojima and Nishita 1986; Tanaka et al. 2005). However, in other phyla, components of troponin have been reported only based on cDNA or genomic sequences and/or immunoreactivity with antibodies. In the Annelid, a TnT-like protein was detected in the earthworm *Eisenia fortida* by anti-rabbit TnT antibody (Royuela et al. 1996), and a TnI sequence was found in the genome of the polychete worm *Capitella teleta* (Table 1). In the Tardigrada, a TnI-like protein was detected by anti-nematode TnI antibody (Obinata et al. 2011), and a TnC cDNA has been cloned from the water bear *Hypsibius* (Prasath et al. 2012). In the acoel flatworm, which has been recently classified in the new phylum Xenacoelomorpha (Philippe et al. 2011), a TnI cDNA has been cloned from *Symsagittifera roscoffensis* (Chiodin et al. 2011). In addition, TnI-like sequences have been annotated in the genome of the parasitic flatworm *Schistosoma japonicum* (Platyhelminthes) and the bdelloid rotifer *Adineta vaga* (Rotifera) (Table 1).

By contrast, within the Deuterostomia, our homology search in the genome of the sea urchin *Strongylocentrotus purpuratus* (Sea Urchin Genome Sequencing Consortium et al. 2006) failed to detect a troponin component. This is consistent with earlier biochemical studies showing the absence of a troponin-like protein in muscles of sea urchin (Obinata et al. 1974), sea cucumber (Lehman and Szent-Gyorgyi 1975), and sea lily (Obinata et al. 2014),

suggesting strongly that the phylum Echinodermata lacks troponin (Fig. 1). Interestingly, a recent biochemical study has demonstrated that the acorn worm *Balanoglossus misakiensis* also lacks troponin in its muscle (Sonobe et al., personal communication), suggesting that the phylum Hemichordata also lacks troponin (Fig. 1). These observations are indeed consistent with the current views that the Echinodermata and Hemichordata are related phyla forming the clade Ambulacraria (Blair and Hedges 2005; Halanych 1995). Thus, the widespread presence of troponin in the Bilateria (Fig. 1) suggests that troponin has emerged in a common ancestor of the Deuterostomia and the Protostomia but was lost in the Ambulacrarian lineage (Fig. 1).

Phylogenetic Relationships of Troponin I Sequences Suggest That the N-terminal Extension Was Lost in a Subset of Isoforms in the Deuterostomia

Next, we compared molecular phylogenetic relationships among the TnI sequences (Fig. 2 and Table 1). We selected one species from each phylum in which troponin has been detected (Fig. 1), with the exception of the Chordata, in which we included two species: the vertebrate *Mus musculus* (mouse) and the urochordate *Halocynthia roretzi* (sea squirt). The Tardigrada was not included because a TnI has not been cloned. Alignment of 19 TnI sequences from 9 species using Clustal Omega (Sievers et al. 2011) agreed with the phylogenetic relationship of the organisms (Fig. 2). TnI sequences in the Protostomia Ecdysozoa, the Protostomia Lophotrochozoa, and the Deuterostomia were grouped (Fig. 2). The multiple TnI isoforms in the Nematoda, the Arthropoda, and the Chordata branched after each phylum diverged (Fig. 2), suggesting that the TnI isoforms derive from a single ancestor and evolved after each phylum was established. Likewise, within the Chordata, TnI isoforms in vertebrates and urochordates branched after these groups split, suggesting that these isoforms are derived from a single common ancestor rather than multiple isoform-specific ancestors. Although phylogenetic positioning of the acoel flatworm (Xenacoelomorpha) is still under debate (Egger et al. 2009; Lowe and Pani 2011), our unbiased analysis suggests that TnI from *Symsagittifera* (acoel flatworms) is an early branch within the Deuterostomia TnI group (Fig. 2), which agrees with the placement based on extensive molecular phylogenetic analysis (Philippe et al. 2011).

The sequence alignment indicates that the central ~150-amino-acid region of TnI including four helices (H1 – H4) and the inhibitory region (IR) are highly conserved (Fig. 3A and Fig. S1). Ca^{2+} -independent interactions between H1 and the C-lobe of TnC, and H2 with TnT, are important for formation of a stable core (Takeda et al. 2003; Vinogradova et al. 2005). H3 functions as a switch region by binding to the N-lobe of TnC in a Ca^{2+} -dependent manner. The inhibitory region (IR) binds to actin and is sufficient to inhibit actomyosin interaction (Luo et al. 2000; Syska et al. 1976; Talbot and Hodges 1981). Therefore, the homology suggests that the functions of these regions in the troponin complex formation and Ca^{2+} -dependent regulation of actomyosin interaction are conserved.

In contrast, the N- and C-terminal sequences are variable (Fig. 3A and Fig. S1). The C-terminal tails outside of the H4 sequences are highly variable in length and sequence (Fig. S1). The most extreme TnI is the insect flight-muscle-specific TnI (also known as TnH, not included in the alignment) that has a ~150-residue long proline-alanine-rich extension

(Bullard and Pastore 2011). The C-terminal sequences among chordate TnIs are relatively conserved (*e. g.* three mouse isoforms and sea squirt cardiac and skeletal isoforms in Fig. S1). Among the four TnI isoforms in *C. elegans*, three isoforms (UNC-27, TNI-1, and TNI-3) have similar C-terminal tails, but TNI-4 does not have a tail (Fig. S1). Likewise, the C-terminal tails are present in *Halocynthia* adult isoforms but absent in all three larval isoforms (Fig. 3 and Fig. S1). Interestingly, TnIs from two distant species, *C. elegans* (Nematoda) and *Capitella* (Annelida) have similar C-terminal tails that are highly enriched in glutamic acids (Fig. S1), suggesting the presence of a common ancestor. These observations suggest that the C-terminal tail was present in the ancestral TnI and variably truncated or retained during evolution.

The N-terminal extensions (NTEs) of TnIs are also variable but exhibit a different type of evolution than the C-terminal tails. NTEs are present in all examined TnIs in the Protostomia Ecdysozoa and Lophotrochozoa and cardiac isoforms in the Deuterostomia Chordata (Fig. 2, indicated by red) but absent in non-cardiac TnIs in the Deuterostomia (Fig. 2, indicated by blue). The lack of an NTE in the acoel flatworm *Symsagittifera* TnI is particularly interesting because truncation of the NTE appears to be a deuterostome-specific event (Fig. 2). However, in the absence of the genomic sequence of acoel flatworms, we cannot rule out the possibility that another TnI isoform with an NTE exists. Although the length of NTEs range from 30 to 130 amino acids, NTEs commonly contain high percentages of charged amino acids (Fig. 3B, blue and red indicate acidic and basic residues, respectively). The NTE sequences of TnIs from *C. elegans* and *Drosophila* are strikingly conserved (Fig. 3B). *Schistosoma* TNI has a longer NTE than *C. elegans* and *Drosophila* TNIs, but it is highly enriched in charged residues, and a part of the *Schistosoma* sequence aligns well with those of *C. elegans* and *Drosophila* (Fig. 3B). The occurrence of NTEs with a similar feature in broad species strongly suggests that an NTE was present in an ancestral TnI and lost specifically in the Deuterostomia lineage with an exception of cardiac isoforms in the Chordata. In mammals, three TnI isoforms are encoded by three independent genes (Hastings 1997). However, in sea squirts (urochordates), adult cardiac and body-wall isoforms are produced by alternative splicing of pre-mRNA encoded by a single gene (MacLean et al. 1997; Yuasa et al. 2002). Thus, the presence or absence of NTE has been accomplished by different strategies in vertebrates and urochordates.

Troponin I N-terminal Extension Is Required for Regulation of Locomotive Behavior in the Nematode *Caenorhabditis elegans*

In addition to the core TnI domains, NTEs are present in all examined protostome TnIs and chordate cardiac TnIs, suggesting that NTEs are important for the function of troponin. The NTE of mammalian cardiac TnI isoform has a modulatory function for Ca^{2+} sensitivity of the thin filaments (Solaro 2003; Solaro et al. 2008). A biochemical study on molluscan TnI has shown that the NTE is not required for the basic function of TnI but may enhance Ca^{2+} -dependent actomyosin ATPase *in vitro* (Tanaka et al. 2005). However, functional significance of NTEs of invertebrate TnIs has not been studied *in vivo*. Therefore, we examined an *in vivo* role of the NTE in *C. elegans* body wall muscle, which is obliquely striated muscle with highly organized sarcomeres (Ono 2014), using a transgenic technique.

Among the four *C. elegans* TnI genes, UNC-27 is the major TnI isoform expressed in the body wall muscle (Burkeen et al. 2004; Ruksana et al. 2005). In wild-type, actin filaments are regularly organized in a striated pattern in the body wall muscle (Fig. 4A). In *unc-27*-null mutant [*unc-27(e155)*], sarcomeric actin filaments were disorganized, which is likely due to unregulated actomyosin contraction (Fig. 4D). When green fluorescent protein (GFP)-tagged UNC-27 [wild-type (WT)] was expressed in the body wall muscle of the *unc-27* mutant using the muscle-specific *myo-3* promoter, striated actin organization was restored nearly to the appearance of wild-type (Fig. 4G). GFP-UNC-27(WT) co-localized with actin filaments in a striated pattern (Fig. 4H and I). These data indicate that GFP-UNC-27(WT) incorporated into the thin filaments and was functional to maintain sarcomeric actin organization. Expression of GFP-UNC-27(N), which lacks the NTE (residues 2–29), also restored striated actin organization (Fig. 4J), and GFP-UNC-27(N) co-localized with sarcomeric actin filaments (Fig. 4K and L). We isolated three independent transgenic strains for GFP-UNC-27(WT) and GFP-UNC-27(N) and found no significant differences in GFP localization and worm motility among the isolates (our unpublished observations). Expression of GFP-UNC-27(WT) and GFP-UNC-27(N) were confirmed by Western blot with anti-TnI antibody (Fig. 4M) and anti-GFP antibody (Fig. 4O). Quantitative analysis of the band intensity of the GFP-fusion proteins (normalized to the relative levels of actin) indicates that the protein levels of GFP-UNC-27(WT) and GFP-UNC-27(N) were not significantly different ($P = 0.66$) (Fig. 4P). Therefore, we concluded that the NTE of *C. elegans* UNC-27 is not required for incorporation of UNC-27 into sarcomeres and maintenance of sarcomeric actin organization.

Next, we examined roles of UNC-27 in locomotive behavior of worms. *C. elegans* crawl on a flat surface in a sinusoidal motion and swim in liquid in a swinging motion. These locomotive behaviors require coordinated contraction and relaxation of muscle cells in different parts of the body (Cohen and Sanders 2014). Worm motility, as determined by beat frequency in liquid, was significantly impaired in the *unc-27* mutant (Fig. 5). Motility of the *unc-27* mutant worms was considerably restored by expression of GFP-UNC-27(WT) but not by expression of GFP-UNC-27(N) (Fig. 5). Motility of *unc-27* expressing GFP-UNC-27(WT) was not as fast as wild-type (Fig. 5). These transgenes were maintained as extrachromosomal arrays, which are sometimes lost in a subset of cells (Mello et al. 1991). We manually selected worms that express GFP in most muscle cells (roughly >80 %) for analysis, but such mosaicism might have caused the incomplete rescue of the motility. The protein levels of GFP-UNC-27(WT) and GFP-UNC-27(N) were comparable (Fig. 4P), suggesting that the presence or absence of the NTE in UNC-27 confers the difference in worm motility.

We further quantified amplitude of body bending during touch-induced backward locomotion. This parameter has previously been used to detect locomotive phenotypes in several muscle-affecting mutants (Moulder et al. 2010; Nahabedian et al. 2012). Backward locomotion was induced by gently touching the head of a worm (Fig. 6A), and body bending was observed within two sinusoidal waves with quantification of amplitude (A) normalized by the body length (L) (Fig. 6B). By comparing wild-type and *unc-27* mutant worms, the bodies of wild-type worms were more curved than those of *unc-27* worms (Compare Fig. 6B and D). Quantitative analysis shows that bending amplitude of wild-type was nearly twice as

much as that of *unc-27* (Fig. 6I). Expression of GFP-UNC-27(WT) in the *unc-27* mutant restored bending amplitude to the wild-type level (Fig. 6F and I), whereas expression of GFP-UNC-27(N) did not affect the phenotype (Fig. 6H and I). These observations strongly suggest that the NTE of UNC-27 is required for efficient sinusoidal locomotive behavior of worms, which involves coordinated regulation of contraction and relaxation of body wall muscle.

Because worm locomotion involves asynchronous muscle contraction and relaxation, we examined synchronous contraction and relaxation events by adapting an optogenetic technique in combination with a microfluidic device and real-time imaging, which had been developed to characterize neuronal circuits and synaptic transmission in *C. elegans* (Stirman et al. 2012; Stirman et al. 2011). Channelrhodopsin-2, a light-activatable cation channel from *Chlamydomonas*, was expressed in cholinergic motoneurons, which allows induction of synchronous whole-body muscle contraction by blue-light illumination (Nagel et al. 2005). The worms were trapped in microfluidic channels, and temporal changes in their body size after turning the light on (contraction) and off (relaxation) were measured. Then, rates of contraction and relaxation were determined (Stirman et al. 2010). Expression of channelrhodopsin-2 did not alter sarcomere organization and worm motility (our unpublished data). Recently, we utilized this method to characterize 16 muscle-affecting mutants and verified that this is an excellent non-invasive method for quantitative analysis of muscle contractility (Hwang et al. 2016).

Using this approach, we found that the *unc-27* mutation did not affect the rate of contraction (Fig. 7A) but significantly increased the rate of relaxation (Fig. 7B). Enhanced relaxation rate in the *unc-27* mutant was somewhat unexpected. TnI normally inhibits actomyosin contractility at low Ca^{2+} ; we therefore expected its absence to decelerate dissociation of actomyosin. However, troponin complexes from ascidian (Endo and Obinata 1981; Ohshiro et al. 2010) and molluscan (Tanaka et al. 2005) accelerate actomyosin ATPase in a Ca^{2+} -dependent manner. If UNC-27 is a component of an accelerator-type troponin complex, disruption of such a system might enhance the rate of relaxation. Expression of either GFP-UNC-27(WT) or GFP-UNC-27(N) in the *unc-27* mutant did not affect the rate of contraction (Fig. 7A) and rescued the faster relaxation rate of the *unc-27* mutant to the wild-type rate (Fig. 7B). Thus, the results demonstrated that the NTE of UNC-27 is not required for proper control of synchronous muscle contraction or relaxation. Our observations that both GFP-UNC-27(WT) and GFP-UNC-27(N) could rescue sarcomeric disorganization in the *unc-27* mutant at a cellular level (Fig. 4), suggesting that each muscle cell has functional contractile apparatuses, which are capable of producing maximum outputs during synchronous muscle contraction.

Previous phenotypic analysis of *unc-27* mutants suggest that they are constitutively contracted under normal culture conditions (Burkeen et al. 2004). In the optogenetic experiments, reduction of the body area upon light stimulation was slightly less in *unc-27* than in wild-type, suggesting that the *unc-27* mutant had been partially pre-contracted before light stimulation (Hwang et al. 2016). Basal contraction levels can potentially influence the rates of light-induced contraction and relaxation. To minimize the effect of basal contraction, an archeal protein halorhodopsin from *Natronomonas pharaonis* (NpHR), which can be

activated by green light to hyperpolarize membranes and silence cells (Zhang et al. 2007), could be used to relax muscle cells before light activation of ChR2.

The discrepancy in the results of synchronous contractility and worm locomotion might indicate that the NTE of UNC-27 is required for fine spatiotemporal tuning of muscle contractility. In the optogenetic synchronous experiments, all cholinergic neurons are simultaneously activated to induce maximum muscle contractile outputs. In contrast, in worm locomotion, muscle cells are regulated by both excitatory and inhibitory neurons in a coordinated manner, such that muscle cells can produce various levels of contractility. In mammalian cardiac TnI, the NTE binds to the N-terminal lobe of TnC and modulates the Ca^{2+} sensitivity of thin filaments (Howarth et al. 2007; Ward et al. 2004; Ward et al. 2003; Warren et al. 2009). Phosphorylation of the NTE by protein kinase A weakens its binding to TnC and enhances dissociation of Ca^{2+} from troponin C (Gaponenko et al. 1999). Thus, β -adrenergic stimulation induces protein kinase A phosphorylation of cardiac TnI within the NTE and this modification enhances cardiac muscle relaxation (Kentish et al. 2001; Zhang et al. 1995a; Zhang et al. 1995b). Mutations in the NTE of human cardiac TnI (TNNI3) are found in patients with dilated cardiomyopathy (Murphy et al. 2004) and hypertrophic cardiomyopathy (Arad et al. 2005), indicating functional significance of the NTE in cardiac muscle. However, proteolytic removal of the NTE from cardiac TnI occurs naturally in tail-suspended rats (Yu et al. 2001), and transgenic expression of cardiac TnI lacking NTE enhances diastolic function of the mouse heart (Barbato et al. 2005; Biesiadecki et al. 2010; Feng et al. 2008), suggesting that removal of the NTE might be an adaptive mechanism in altered cardiac conditions. These effects of NTE-truncation from mammalian cardiac TnI are quite different from those in *C. elegans*, in which muscle relaxation is enhanced by a null mutation of UNC-27 (TnI) and suppressed by full-length or NTE-truncated UNC-27 (this study). Thus, *C. elegans* troponin may have different biochemical properties from vertebrate troponin. Unfortunately, we have not been able to produce recombinant *C. elegans* troponin due to toxicity of UNC-27 in *E. coli* (our unpublished results). Nonetheless, further structure-function analysis of *C. elegans* troponin using both biochemical and genetic approaches should help to understand fundamental mechanisms of the troponin regulation of actomyosin interaction and evolution of troponin in the animal kingdom.

Conclusion

Troponin proteins first appear in the evolution of the Bilateria. Our comparison of TnI sequences from representative animals demonstrated conservation of a core region and variations in N- and C-terminal extensions. In particular, the NTE remains in all examined protostome TnIs; however, truncation of NTE appears in a subset of deuterostome TnI isoforms. Functional analysis in the nematodes revealed an important role of the NTE of TnI in coordinated body locomotion but not in single-muscle contractility. This is in contrast to vertebrate TnIs that have diverged to skeletal muscle isoforms with no NTE and a cardiac isoform with an NTE with regulatory properties. From these observations, we hypothesize that an ancestral TnI had an NTE essential for coordination of body movement, and that functions of NTEs have evolved or been eliminated in deuterostomes as their body plans have diverged and increased in complexity. *C. elegans* could be a useful system to test functional conservation of NTE and other domains of TnI from other species by expressing

chimeric TnI proteins. Further functional studies on troponin and muscle-regulatory systems in diverse organisms may provide insight in the origin of troponin and fundamental mechanisms of actomyosin regulation.

Materials and Methods

Phylogenetic Analysis

Phylogenetic relationships of representative metazoan phyla (Fig. 1) were obtained from the National Center for Biotechnology Information (NCBI) Taxonomy Browser (U. S. National Library of Medicine), and a tree was drawn by TreeVector (Pethica et al. 2010). The tree was modified by moving the Platyhelminthes to the Lophotrochozoa according to the recent analysis (Riutort et al. 2012). Sequences for all TnIs except for *Adineta* TnI were obtained from the NCBI server with accession numbers indicated in Table 1. *Adineta* TnI was searched first by NCBI Basic Local Alignment Search Tool (BLAST) against the *Adineta* genomic sequence (Flot et al. 2013) using mouse cardiac TnI sequence, and an annotated protein sequence was obtained from *Adineta vaga* Genome Browser at Genoscope, Centre National de Séquençage (Evry, France). Sequence alignment was performed with Clustal Omega (Sievers et al. 2011) using MegAlign Pro (DNASTAR Inc., Madison, WI), and a tree was drawn by TreeVector.

Nematode Strains

Wild-type strain N2 and CB155 *unc-27(e155)* (Brenner 1974) were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). ZX460 *zxIs6[Punc-17::Chr2(H134R)::YFP + lin-15(+)]* (Liewald et al. 2008) was provided by Alexander Gottschalk (Goethe University, Frankfurt, Germany). Transgenic strains ON239 *unc-27(e155); ktEx170[Pmyo-3::GFP::UNC-27(WT)]* and ON242 *unc-27(e155); ktEx173[Pmyo-3::GFP::UNC-27(2-29)]* were constructed as described below. Following strains were generated by crossing the strains described above: ON295 *unc-27(e155); zxIs6*, ON313 *unc-27(e155); zxIs6; ktEx170*, and ON311 *unc-27(e155); zxIs6; ktEx173*. Nematodes were generally grown under standard conditions at 20 °C on Nematode Growth Medium agar plates with *Escherichia coli* OP50 as a food source (Stiernagle 2006). For optogenetic experiments, 100 μM all *trans*-retinal was added to the *E. coli* culture as a co-factor for channelrhodopsin-2 activation as described previously (Liewald et al. 2008).

Generation of Transgenic Nematode Strains

A full-length *unc-27* genomic fragment containing all protein coding regions and an N-terminally truncated *unc-27* genomic fragment lacking residues 2 – 29 were PCR-amplified from total genomic DNA from N2 and cloned at the *EcoRI-NheI* sites of pPD118.20, an expression vector with the *myo-3* promoter (Okkema et al. 1993) and the GFP coding sequence (provided by Andrew Fire, Stanford University, Stanford, CA). The entire coding regions were sequenced to confirm the presence of the inserts and the absence of PCR-induced errors. Transgenic nematodes were generated essentially as described previously (Mohri et al. 2006) except that *unc-27(e155)* was used as the parental strain. Transgenic worms were selected by expression of GFP as observed by fluorescence microscopy, and the transgenes were maintained as extrachromosomal arrays.

Fluorescence Microscopy

Staining of whole worms with tetramethylrhodamine-phalloidin to visualize actin filaments was performed as described (Ono 2001). GFP was observed by its own fluorescence. Samples were mounted with ProLong Gold (Life Technologies, Carlsbad, CA) and observed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope with a CFI Plan Fluor ELWD 40x (Dry; NA 0.60) or Plan Apo 60x (oil; NA 1.40) objective (Nikon Instruments, Tokyo, Japan). Images were captured by a SPOT RT monochrome CCD camera (Diagnostic Instruments, Sterling Heights, MI) and processed by IPLab imaging software (BD Biosciences, San Jose, CA) and Adobe Photoshop CS3 (Adobe, San Jose, CA).

Western Blot

Fifteen L4 or young adult worms per sample were picked and lysed in 15 μ l of SDS-lysis buffer (2 % SDS, 80 mM Tris-HCl, 5 % β -mercaptoethanol, 15 % glycerol, and 0.05 % bromophenol blue, pH 6.8), heated at 97 °C for 3 min and homogenized by brief sonication. The samples were resolved by SDS-PAGE by using a 12 % acrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; EMD Millipore, Billerica, MA). The membrane was blocked in 5 % nonfat milk in phosphate-buffered saline containing 0.1 % Tween 20 (PBS-T) overnight at 4 °C and incubated for 1 hr with rabbit anti-*Ascaris* TnI antibody (Nakae and Obinata 1993), which was diluted at 1:2000 in Signal Enhancer HIKARI (Nacalai USA, San Diego, CA). After washing with PBS-T, the membrane was reacted with horse radish peroxidase-labeled goat anti-rabbit IgG (1:2000-diluted) (Thermo Fisher Scientific Pierce Protein Biology, Rockford, IL). The reactivity was detected with a SuperSignal West Pico chemiluminescence reagent (Thermo Fisher Scientific Pierce Protein Biology). The membrane was treated with a buffer containing 2 % SDS, 100 mM β -mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8, at 50 °C for 30 min to remove bound probes and reprobed with mouse monoclonal anti-actin antibody (C4; MP Biomedicals, Irvine, CA) as a loading control.

To quantify protein levels of GFP-UNC-27(WT) and GFP-UNC-27(N), worm lysates (10 worms per sample) were subjected to Western blot essentially as described above using rabbit anti-GFP antibody (1:2000 dilution) (#600-401-215, Rockland Immunochemicals, Limerick, PA) and mouse monoclonal anti-actin antibody (1:3000 dilution). The reactivity was detected by SuperSignal West Femto (Thermo Fisher Scientific Pierce Protein Biology) for GFP and by SuperSignal West Pico for actin. Band intensity for the GFP-fusion proteins and actin was quantified using ImageJ. Data for the GFP-fusion proteins were normalized to relative band intensity of actin (actin in wild-type was set to 1.0). Statistical analysis (unpaired t-test) was performed using SigmaPlot 13 (Systat Software, Inc., San Jose, CA).

Locomotion Assays

Worm motility (beat frequency) in liquid was quantified as described previously (Epstein and Thomson 1974). Briefly, adult worms were placed in M9 buffer. Then, one beat was counted when a worm swung its head to either right or left. The total number of beats in 30 s was recorded. To measure bending amplitude, young animals were transferred to fresh NGM agar plates with a 2-min acclimation period and prodded at their heads with a platinum wire to induce backward locomotion. Movement of the worms were recorded as movies on a

standard stereo microscope with transmitted light. The movies were post-processed to extract the worm skeleton for quantitative measurements using custom software written in Matlab (the Mathworks, Inc., Natick, MA). The maximum bending amplitude was determined and normalized by the length of each worm. Statistical analysis (one-way ANOVA) was performed using SigmaPlot 13 (Systat Software, Inc., San Jose, CA).

Optogenetic Assays

Kinetic analysis of muscle contraction and relaxation was performed using a microfluidic device to trap worms and an optical system for light illumination and imaging as described previously (Stirman et al. 2010; Stirman et al. 2011) with an optimization for analysis of muscle contractility phenotypes as described (Hwang et al. 2016). Briefly, two-layer microfluidic devices made of polydimethylsiloxane (PDMS; Sylgard 184, Dow-Corning Corp., Midland, MI, USA) with 16 straight microchannels with membrane valves at the entrance and the exit were used to trap worms. After young adult animals were trapped in the channels, they were illuminated with blue light ($\lambda = 450\text{--}490\text{ nm}$; 0.3 mW/mm^2) for 15 s to induce channelrhodopsin-2 photoactivation. Movies were recorded using a CCD camera (Infinity 3-1, Luminera Corp., Canada) and post-processed using custom software written in Matlab. The projected body area of the worms in the segmented images was used as a read-out for the light-stimulated muscle contraction and relaxation. The body area measured from the images captured during 5 s before the blue light illumination was used as a baseline, and its change was plotted. The curves were fitted with the plateau followed by one phase decay or association equations for the kinetic analyses of the contraction or the relaxation processes, respectively. Curve fitting and statistical analysis were performed using Prism 5 (GraphPad Software, San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Evolution of troponin

Phylogenetic relationships of 14 representative phyla in the animal kingdom are shown. Troponin-positive and -negative phyla are indicated by red and blue, respectively. Major taxonomic groups are indicated at or near branch points.

Fig. 2. Molecular phylogenetic relationships of troponin I

Phylogenetic relationships of 19 TnI sequences from 9 species (8 phyla shown on the right) are shown. Three groups that match with major taxonomic groups are indicated by different colors: yellow (Protostomia, Ecdysozoa), pink (Protostomia, Lophotrochozoa), and green (Deuterostomia). N-terminal extensions are present in TnIs shown in red but absent in TnIs shown in blue. Entire sequence alignment is shown in Supplemental Fig. S1.

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Fig. 4. Effect of truncation of the N-terminal extension of troponin I on sarcomeric actin organization

(A–L), Micrographs of adult body wall muscle from wild-type (A–C), *unc-27* (D–F), *unc-27; GFP-UNC-27(WT)* (G–I), or *unc-27; GFP-UNC-27(ΔN)* (J–L) are shown for filamentous (F-) actin stained with tetramethylrhodamine-phalloidin (left) and GFP (middle). Merged images are shown on the right column (F-actin in red and GFP in green). Bar, 50 μm. (M, N) Protein levels of TnI (M) and actin (N) were examined by Western blot. Lysates from 15 worms with indicated genotypes were loaded per sample and reacted with anti-*Ascaris* TnI antibody (M). Note that this antibody reacts with all four TnI isoforms, and showed significant reactivity with endogenous TnI with apparently higher molecular weight in the *unc-27* mutant background as described previously (Obinata et al. 2010). (O, P) Quantitative analysis of the protein levels of GFP-UNC-27 (WT) and GFP-UNC-27(ΔN). Worm lysates (10 worms per sample) from *unc-27; GFP-UNC-27(WT)* and *unc-27; GFP-UNC-27(ΔN)* were subjected to Western blot with anti-GFP and anti-actin antibodies (O). Five samples for each strain and one sample for wild-type were examined. Band intensity of the GFP-fusion proteins was normalized by the actin levels and shown in the graph (P). Data are means ± standard errors, n = 5. Transgenically expressed GFP-UNC-27 (WT) and GFP-UNC-27(ΔN) were not at significantly different levels: n. s., not significant ($P > 0.05$).

Fig. 5. Effect of truncation of the N-terminal extension of troponin I on worm motility in liquid
 Worm motility of indicated strains was examined in liquid as beat frequency (beating per 30 sec). Data are means $\pm$ standard errors, $n = 10$. Results of one-way ANOVA are shown: n. s., not significant ($P > 0.05$); ***, $P < 0.001$.

Fig. 6. Effect of truncation of the N-terminal extension of troponin I on bending amplitude during backward locomotion

Backward locomotion was induced by touching the head (A, C, E, and G). Then, amplitude of body bending (indicated as “A” in B) was measured and normalized by body length (indicated as “L” in B) (B, D, F, and H). Bar, 100 μm. Normalized bending amplitude (A/L) is shown in a box plot (I). Each box represents the 25th and 75th percentiles with a line at the median, and error bars indicate the 10th and 90th percentiles. n = 10. **, $P < 0.001$; ***, $P < 0.0001$.

Fig. 7. Effect of truncation of the N-terminal extension of troponin I on rates of contraction and relaxation examined by optogenetics

(A) Representative images of an optogenetic experiment show that contraction of a worm expressing channelrhodopsin-2 in a microfluidic channel (60 μm wide) is induced by turning blue light on, and relaxation induced by turning blue light off. (B, C) Rate constants (s^{-1}) for contraction (B) and relaxation (C) were measured from changes in the body area and expressed in box plots. $n = 30$. ***, $P < 0.0001$.

Table 1

Troponin I sequences examined in the phylogenetic analysis

Phylum	Species	Common name	Protein	Accession number ^a	Amino acids
Chordata	<i>Mus musculus</i>	Mouse	TNNI1 (slow skeletal)	NP_001106173	187
			TNNI2 (fast skeletal)	NP_033431	182
			TNNI3 (cardiac)	NP_033432	211
	<i>Halocynthia roretzi</i>	Sea squirt	Adult body wall muscle TNI	BAB83810	182
			Adult cardiac TNI	BAB83811	260
			Larval alpha TNI	BAB83807	149
			Larval beta TNI	BAA19427	149
Xenacoelomorpha	<i>Symsagittifera roscoffensis</i>	Acoel flatworm	Larval gamma TNI	BAB83808	150
			TNI	AEG66943	175
			TNI	BAE43657	293
Mollusca	<i>Chlamys nipponensis</i>	Scallop (clam)	TNI	ELU06642	346
Annelida	<i>Capitella teleta</i>	Polychete worm	TNI	CAX73588	218
Platyhelminthes	<i>Schistosoma japonicum</i>	Flatworm	TNI	GSADV00022792001 ^b	209
Rotifera	<i>Adineta vaga</i>	Bdelloid rotifer	TNI	AAF48803	208
Arthropoda	<i>Drosophila melanogaster</i>	Insect (fruit fly)	wupAa	AFH07445	260
Nematoda	<i>Caenorhabditis elegans</i>	Roundworm	wupAh	CAA91466	250
			TNI-1	CCD73101	242
			UNC-27	CAB04737	260
			TNI-3	CCD65507	194
			TNI-4		

^a All accession numbers except for *Adineta* TNI are valid in the National Center for Biotechnology Information Browser (<http://www.ncbi.nlm.nih.gov/>).^b The number is valid in the *Adineta vaga* Genome Browser (<http://www.genoscope.cns.fr/adineta/cgi-bin/gbrowse/adineta/>).