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## **C. ELEGANS MODEL FOR STUDYING TROPOMYOSIN AND TROPONIN REGULATIONS OF MUSCLE CONTRACTION AND ANIMAL BEHAVIOR**

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### **1. BACKGROUND**

There are two muscle tissues in the nematode *Caenorhabditis elegans*: the pharynx for feeding and the body wall for locomotion. These correspond to cardiac and skeletal muscles in vertebrates, respectively. Study of the muscle genes of *C. elegans* can be classified into three stages; first, mutant isolation and gene mapping, second, cloning and sequencing of the gene, and third, complete sequences of all genes. Many uncoordinated mutant animals have been isolated (Brenner 1974, Waterston, 1988, Moerman and Fire 1997) and the complete amino acid sequence of myosin heavy chain, twitchin, and paramyosin, (invertebrate specific core protein of thick filament), and were the first determined in any animals by analyzing the *unc-54*, *unc-22*, and *unc-15* mutants, respectively (Kam *et al.*, 1983, Benian *et al.*, 1989, Kagawa *et al.*, 1989). Tropomyosin and troponin components are also present but as with actin and myosin heavy chain in the worm, there are some differences in gene structure and sequence compared to those in other animals (Kagawa *et al.*, 1995, Myers *et al.*, 1996, Moerman and Fire 1997). Deficiencies of body wall troponin C or tropomyosin in *C. elegans* cause the Pat (paralyzed arrest at embryonic two-fold stage) phenotype (Williams and Waterston 1994, Terami *et al.*, 1999) and those of troponin T cause Mup (muscle position abnormal) phenotype (Myers *et al.*, 1996). After determining the complete genome sequences of the nematode (The *C. elegans* Sequence Consortium, 1998), we can find out how isoforms are related to each other. Only one troponin C gene, *pat-10/tnc-1*, is expressed in the body wall muscles and the gene defect causes a developmental arrest of the animals (Terami *et al.*, 1999). This is the first report analyzing a troponin C mutant. Recently we analyzed the tissue expression patterns of the four troponin I genes and their interaction with two troponin C isoforms (Ruksana *et al.*, 2005). It now possible to compare how the troponin complex works in muscle contraction in invertebrates since Ebashi, Ohtsuki and their school had described these systems in vertebrates (Ohtsuki *et al.*, 1986). In this paper we review how the mutant genes for tropomyosin and the troponin components of *C. elegans* affect animal behavior. A summary of expression, mutation and RNA interference results on these isoforms are provided in Table 1.

#### **2.1. Tropomyosin mutant shows lethal or Ca<sup>2+</sup> signaling defect**

In contrast to one gene encoding one protein in most other muscle genes in *C. elegans* the tropomyosin gene, *tmx-1/lev-11*, encodes four isoforms, which are two each for the pharynx and

body wall muscles produced under the control of distal and internal promoters and splicing choice, respectively (Kagawa *et al.*, 1995, Anyanful *et al.*, 2001). The *lev-11(st557)* mutant animal, which was isolated as a Pat animal (Williams and Waterston 1994) produces no tropomyosin due to a mutation at the splice donor site of exon 1 (Kagawa *et al.*, 1997). Another mutant *lev-11(x12)* animal, which was isolated as a levamisole-resistant animal (Lewis *et al.*, 1980) has an amino acid substitution of glutamic acid to lysine at position 234 of tropomyosin (Kagawa *et al.*, 1997).

**Table 1** Summary of gene, tissue expression and phenotype of tropomyosin and troponin isoforms in *C. elegans*

Gene / isoform	Cosmid (LG)	Tissue or Organ	Mutation <sup>a</sup>	RNAi <sup>a</sup>
<u>Tropomyosin<sup>b</sup></u>				
<i>lev-11/tmy-1</i> / TM-I,II	Y105E8B.1 (I)	BW <sup>b</sup>	Lev <sup>c</sup>	Unc <sup>d</sup>
<i>lev-11/tmy-1</i> / TM-III,IV	Y105E8B.1 (I)	Pharynx <sup>b,f</sup>	Pat <sup>e</sup>	Emb <sup>f</sup> , Ste <sup>g</sup>
<u>Troponin C</u>				
<i>pat-10/tnc-1</i>	F54C1.7 (I)	BW <sup>h</sup>	Pat <sup>e</sup>	Ste <sup>g</sup>
<i>tnc-2</i>	ZK673.7 (II)	Pharynx <sup>h</sup>	ND	Lva <sup>g,i</sup>
<u>Troponin I</u>				
<i>ttn-1</i>	F42E11.4 (X)	BW <sup>h,k</sup>	ND	Unc <sup>d</sup>
<i>unc-27/ ttn-2</i>	ZK721.2 (X)	BW <sup>h,k</sup>	Unc <sup>i,k,l</sup>	Unc <sup>d</sup>
<i>ttn-3</i>	T20B3.2 (V)	BW <sup>h,k</sup> , Vulva <sup>i</sup>	ND	Con, Egl, Pvl <sup>j</sup>
<i>ttn-4</i>	WO3F8.1 (IV)	Pharynx <sup>d,k</sup>	ND	Gro <sup>j</sup> , Emb <sup>j</sup>
<u>Troponin T</u>				
<i>mup-2/tnt-1</i>	T22E5.5 (X)	BW <sup>m</sup>	Mup, Ste <sup>m</sup>	WT <sup>i</sup> , Mup <sup>n</sup>
<i>tnt-2</i>	F53A9.10 (X)	ND	ND	WT <sup>i</sup> , Lva <sup>n</sup>
<i>tnt-3</i>	C14F5.3 (X)	ND	ND	WT <sup>i</sup>
<i>tnt-4</i>	T08B1.2 (V)	Pharynx <sup>n</sup>	ND	WT <sup>i</sup> , Lva <sup>n</sup> ,

<sup>a</sup>Designations for phenotypes: Lev, levamisole-resistant; Unc, uncoordinated; Pat, paralyzed arrest at embryonic two-fold stage; Emb, embryonic lethal; Ste, sterile; Con, constipated; Egl, egg laying-defective; Pvl, protruding vulva; Mup, muscle positioning; WT, wild type; ND, not determined. <sup>b</sup>The tropomyosin gene, *lev-11/tmy-1* encodes four isoforms (Kagawa *et al.*, 1995).

<sup>b</sup>Kagawa *et al.*, 1995; <sup>c</sup>Lewis *et al.*, 1980; <sup>d</sup>Ono and Ono, 2002; <sup>e</sup>Williams and Waterston, 1994; <sup>f</sup>Anyanful *et al.*, 2001; <sup>g</sup>Ono and Ono, 2004; <sup>h</sup>Terami *et al.*, 1999; <sup>i</sup>Kamath *et al.*, 2003; <sup>j</sup>Ruksana *et al.*, 2005; <sup>k</sup>Burke *et al.*, 2004; <sup>l</sup>Brenner, 1974; <sup>m</sup>Myers *et al.*, 1996; <sup>n</sup>Wombase (on TnT by Allen *et al.*).

Exon 1 encodes the N-terminal part of CeTMI and CeTMII isoforms that are only expressed in body wall muscles. Mutant animals lacking CeTMI and CeTMII show a Pat phenotype suggesting that tropomyosin in body wall muscle is essential for the late development of *C. elegans*. As levamisole is a potent agonist of acetylcholine *lev-11(x12)* mutant animals cannot transmit the Ca<sup>2+</sup> signal from the post-synaptic membrane to cause actin-myosin sliding step due to the position of the mutant tropomyosin. The mutation site of *lev-11(x12)* is in exon 7 a constitutive exon for all isoforms and causes the Glu234Lys substitution in tropomyosin (Kagawa *et al.*, 1997). How this mutation affects interactions between troponin and tropomyosin or tropomyosin and actin is not

currently known. This mutant will be useful in solving how tropomyosin functions in  $\text{Ca}^{2+}$  signal transduction.

## 2.2. A Pat animal has missed $\text{Ca}^{2+}$ binding to site II and troponin I binding to the H-helix of the body wall troponin C

Two different genes; *pat-10/tnc-1* and *tnc-2*, encode the two troponin C isoforms that are expressed in body wall and pharyngeal muscles, respectively (Terami *et al.*, 1999). Comparisons to other troponin C sequences show that body wall troponin C, TnC-1, has the potential for  $\text{Ca}^{2+}$  binding at sites II and IV. Using TnC-1 produced in bacteria, the apparent  $\text{Ca}^{2+}$  binding constants,  $K_{app}$ , of both sites have been confirmed by fluorescence titration method to be  $7.9 \times 10^5 \text{M}^{-1}$  and  $1.2 \times 10^6 \text{M}^{-1}$ , respectively (Ueda *et al.*, 2001). Study of the  $\text{Mg}^{2+}$ -dependence of the  $K_{app}$  showed that both  $\text{Ca}^{2+}$ -binding sites II and IV do not bind  $\text{Mg}^{2+}$  competitively. This means that the  $\text{Ca}^{2+}$  binding of nematode TnC-1 is low affinity, fast dissociation and  $\text{Ca}^{2+}$ -specific.

A troponin C mutant animal was isolated as one of the Pat mutants. These show paralyzed arrest phenotype at embryonic two-fold stage (Williams and Waterston 1994). We report that the *pat-10(st575)* animal has two mutations in the body wall troponin C; an Asp64Asn (D64N) substitution at site II and a deletion of the H-helix of the C-terminus (Terami *et al.*, 1999). Recombinant mutant troponin C proteins produced in bacteria were assayed by SDS-Page to determine the molecular size and by Western blot analysis to assay their interactions with troponin I.

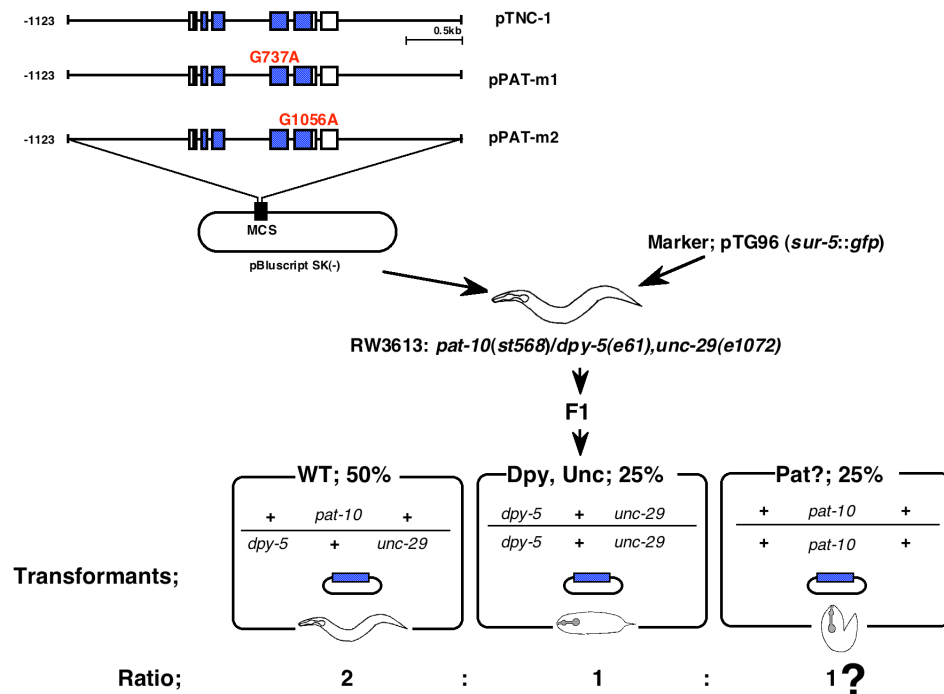


Figure 1 Experimental strategy of transgenic worms by injecting mutant gene of the troponin C. How designed mutant gene (top) affects on the animal is analyzed the phenotypic ratio by counting F2 animals (bottom). (Terami, PhD thesis, 2002)

TnC-1m1: D64N showed no change in mobility with or without  $\text{Ca}^{2+}$  suggesting that site II has lost  $\text{Ca}^{2+}$  binding. TnC-1m2: W153stop exhibited a mobility shift dependent on the presence or absence of  $\text{Ca}^{2+}$ , but did not bind troponin I due to missing H-helix.

A series of amino acid substitution experiments destroying and shortening the H-helix in the C-terminal region of TnC-1 suggest that the length and direction of the H-helix are important for troponin I binding. Especially two residues F152 and W153 were found to be essential for  $\text{Ca}^{2+}$  binding and troponin I binding (Takaya and Kagawa, unpublished). These results confirm that the H-helix of TnC-1 is essential for  $\text{Ca}^{2+}$  binding at site IV and troponin I binding. The topographical position of the C-terminal of troponin C close to troponin I is consistent with the crystal structure of the troponin complex (Vassilyev *et al.*, 1998).

We have also analyzed transgenic animals to study how troponin C mutants affect the animal phenotype by injecting recombinant mutant genes with the marker plasmid, pTG96 (*sur-5::gfp*) (Fig. 1) and found that development of mutant animals of m1: D64N ( $\text{Ca}^{2+}$  binding site II of TnC-1) was almost to the same as wild type, but that adult transgenic animals dramatically lose their motility and show an egg-laying defect phenotype (Takaya, Terami and Kagawa, unpublished). This mutation is the first example of the miss-sense mutant worm that is homozygous for a mutant troponin C gene; the *pat-10(st575)* animal has two mutations in TnC-1 and is only kept in the heterozygous state (Williams and Waterston 1994, Terami *et al.*, 1999). On the other hand the mutation of m2: W153stop in TnC-1 caused the Pat phenotype in transgenic animals. Both *in vitro* and *in vivo* experiments indicate that the Pat phenotype of the worm comes from a loss of  $\text{Ca}^{2+}$  binding to site IV or of troponin I binding to the H-helix.

### 2.3. Pharynx and body wall muscles express one- and three-troponin I isoforms, respectively

Four troponin I isoforms of the worm were characterized and compared to those of other animals (Fig. 2). It is interesting that the three body wall troponin I isoforms have unique glutamate-rich C-terminal extensions. The C-termini of troponin T in *Drosophila* and crayfish *Astacus* also show conserved glutamate-rich extensions (Fyrberg *et al.*, 1990; Benoist *et al.*, 1998; Domingo *et al.*, 1998). It has been proposed that the C-terminal extension might enhance cooperation of troponin-tropomyosin complexes within thin filaments (White *et al.*, 1987). The *C. elegans* troponin I extensions may have a homologous function. Glutamate has a negative charge, which may contribute to protein-protein interactions under different  $\text{Ca}^{2+}$  concentrations. The C-terminal hydrophilic regions of the three body wall type troponin I isoforms could be important for interactions with TnC-1 or TnT in the worm body wall muscles.

Tissue expression patterns were determined using *lacZ/gfp/hfp* reporter gene assays. The *tmi-1*, *tmi-2/unc-27* and *tmi-3* genes, each encoding one troponin I isoform, are expressed in body wall, vulval and anal muscles at different intensities, but *tmi-4* was expressed solely in the pharynx. Knock down of *tmi-1* or *tmi-2* gene by RNA interference caused motility defects similar to the *unc-27(e155)* mutant, which is a *tmi-2/unc-27* null allele. An RNAi experiment for *tmi-3* produced egg-laying defects, while similar experiment with *tmi-4* RNAi caused arrest at gastrulation (Table 1). These show that body wall troponin I isoforms are important for animal motility and egg-laying and that the pharynx troponin I is essential for animal development (Ruksana *et al.*, 2005).

### 2.4. Body wall troponin I isoforms interact with only that of troponin C

*In vitro* overlay assays have been used to analyse interactions between the four troponin I isoforms to the two troponin C isoforms. The three body wall troponin I isoforms interacts with the body wall and pharyngeal troponin C isoforms, reciprocally, but the pharyngeal TnI-4 interacts only with the pharyngeal TnC-2. Our results suggest that body wall TnI genes have evolved following duplication of the pharynx gene and provide important data about gene duplication and functional differentiation of nematode troponin I isoforms (Ruksana *et al.*, 2005). Recently we confirmed that the N-terminal part of troponin I interacts with troponin C of the worm (Amin and Kagawa, unpublished). This molecular nature is similar to the molluscan troponin I that interacts with the

troponin C of the Akazara scallop (Tanaka *et al.*, 2005). The importance of the N-terminal function of troponin I could be common throughout the invertebrates.

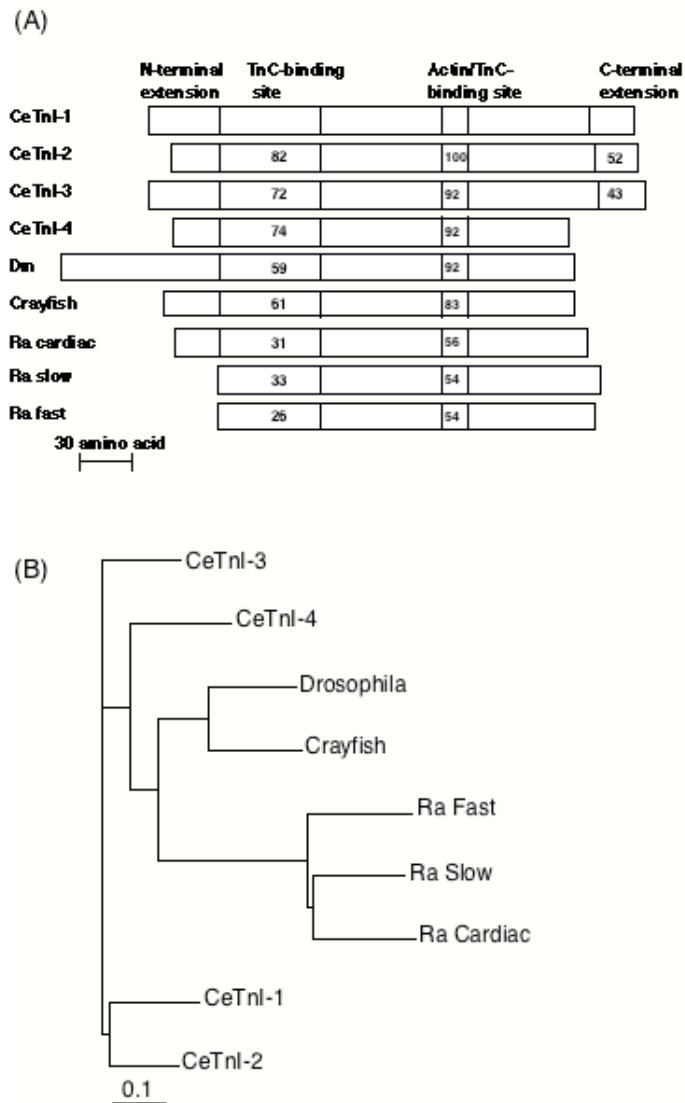


Figure 2. Structural homology of troponin I isoforms of invertebrates and vertebrates  
 (A) The two regulatory regions, TnC-binding and actin/TnC-binding sites showed conserved homology among invertebrates. Numbers represent percentage homology. Tnl-1, Tnl-2 and Tnl-3 of *C. elegans* had unique C-terminal extensions. (B) Phylogenetic alignment of four TNI isoforms of *C. elegans*; CeTNI-1, CeTNI-2, CeTNI-3 and CeTNI-4 and TNIs of *Drosophila*, crayfish, *Ciona* body wall, *Ciona* heart, rabbit cardiac (Ra cardiac), rabbit slow (Ra slow) and rabbit fast (Ra fast). The tree was derived using CLUSTALW and Tree View software. (from Ruksana *et al.*, 2005)

## 2.5. Troponin T mutant shows muscle position abnormal

Mutants for a troponin T heat-sensitive allele, *mup-2(e2346ts)* and for a putative null, *mup-2(up1)* are defective in embryonic body wall muscle cell contraction and sarcomere organization (Myers *et al.*, 1996). The troponin T gene abundantly expresses in body-wall muscles. The *mup-2(up1)* mutation causes a termination codon near the NH<sub>2</sub> terminus (Glu94) and *mup-2(e2346ts)* is a termination codon in the C-terminal invertebrate specific tail (Trp342) (Myers *et al.*, 1996). These results indicate that the body-wall troponin T is also essential for muscle formation. At least three troponin T genes are expressed in the body wall and one is in the pharynx of the worm (WormBase, from a report of Allen *et al.*). This expression profile is similar to that of the troponin I isoforms. How isoforms function in muscle cells and interact with other troponin components will be known in future.

### 3. DISCUSSION

Mutants of tropomyosin and three troponin components of *C. elegans* have been isolated and characterized (Table 1). The isolated mutants can be divided into two groups; one group are missense alleles having single amino acid substitutions in the protein, the other groups are nulls producing no product. The null mutant phenotypes are consistent with the results obtained in gene knock down experiments using RNAi. Recently we have been able to use the experimental data of the total genome sequence together with the mutant genes, and phenotypes of RNAi animals (WormBase). In combination with biochemical and biophysical approaches on tropomyosin and troponin mutants, we have been able to determine the relationships between the molecular nature of the proteins and the behavior of the animals. Interestingly, the mutants isolated map to one of the genes expressed in body wall muscles. Body wall troponin I and troponin T of the worm are encoded by three different genes, but by only one troponin C gene. This is the reason that troponin C mutant animal is isolated under control of genetic skills, analyzing the F2 ratio of the mutant animals that are kept in the heterozygous state (Williams and Waterston, 1994, Terami *et al.*, 1999). This is also applicable to isolating null mutants of the pharynx type isoform because only one gene encodes one isoform. There are three muscle tissues of the body wall type; body wall for locomotion, vulva for egg-laying and anus for defecation. Isolated mutants of troponin I and troponin T are found only in the abundant body wall isoform genes of *tmi-2/unc-27* and *mup-2/tnt-1*, which are expressed in body wall muscles, respectively (Table 1). Some differences of expression control and tissue localization of the three troponin I genes are detected between these three muscle groups, but functional differences have not been found in comparisons of the amino acid sequences (Ruksana *et al.*, 2005). It is of interest to know how three isoforms of troponin I and troponin T interact each other and are utilized in different tissues. How minor isoforms can function and interact to other components in different tissues is also of interest in understanding the molecular interactions and evolutionary relationships.

Sequence comparisons between the different isoforms and with other animals indicate that tropomyosin and troponin components have common interaction sites to other proteins and additional unique sequences in their molecules. The binding sites for troponin C and actin/troponin C are common for all isoforms of troponin I (Figure 2). The N-terminal extension is found in vertebrate cardiac and all invertebrate troponin I isoforms. There is a C-terminal hydrophilic regions in the three troponin I isoforms of *C. elegans* (Ruksana *et al.*, 2005). This may be important for interactions with other proteins. Recently the crystal structure of the core troponin complex has been determined. The authors stated that the interaction sites between troponin and tropomyosin/actin might be unique to each molecule of the complex in different tissues and animals (Takeda *et al.*, 2003). Ohtsuki and Morimoto and their colleagues have established an *in vitro* physiological testing system for studying interactions between exchanged troponin components in myofibrils (Shiraishi *et al.*, 1992). Using our system, a reverse genetic approach in which a designed gene can be introduced into the animal is possible (Figure 2). With this we can determine which part of the molecule functions in muscle and subsequently contributes to animal behavior. These experiments offer two approaches to troponin function; one is to compare proteins from different species and another is related to mutant proteins. The former is useful in studying how molecules are changed during evolution. The later is applicable to understanding how mutations disturb tissue functions of animals

or even humans. As we have mentioned, troponin mutants that cause functional defects of these proteins in the nematode will only be apparent in animals homozygous for the mutants. Even in some human disorders in human symptoms will be detected by a combination of genome or gene sequencing approaches of the gene and phenotypic analysis of the transgenic model animal.

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