

Tropomyosin and Troponin Are Required for Ovarian Contraction in the *Caenorhabditis elegans* Reproductive System^V

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Ovulation in the nematode *Caenorhabditis elegans* is coordinated by interactions between the somatic gonad and germ cells. Myoepithelial sheath cells of the proximal ovary are smooth muscle-like cells, but the regulatory mechanism of their contraction is unknown. We show that contraction of the ovarian muscle requires tropomyosin and troponin, which are generally major actin-linked regulators of contraction of striated muscle. RNA interference of tropomyosin or troponin C caused sterility by inhibiting ovarian contraction that is required for expelling mature oocytes into the spermatheca where fertilization takes place, thus causing accumulation of endomitotic oocytes in the ovary. Tropomyosin and troponin C were associated with actin filaments in the myoepithelial sheath, and the association of troponin C with actin was dependent on tropomyosin. A mutation in the actin depolymerizing factor/cofilin gene suppressed the ovulation defects by RNA interference of tropomyosin or troponin C. These results strongly suggest that tropomyosin and troponin are the actin-linked regulators for contraction of ovarian muscle in the *C. elegans* reproductive system.

INTRODUCTION

In reproductive systems of multicellular organisms, somatic gonads provide the proper environment for germ cells for their development, transport, and fertilization. Ovulation in the nematode *Caenorhabditis elegans* requires a signal from sperm that induces ovary contraction and oocyte maturation, which are followed by spermathecal dilation and fertilization (Ward and Carrel, 1979; McCarter *et al.*, 1997, 1999; Hubbard and Greenstein, 2000). Myoepithelial sheath cells of the proximal ovary are morphologically smooth muscle-like cells with distinct thick and thin filaments that are organized into a nonstriated manner (Strome, 1986; Ardizzi and Epstein, 1987; Hall *et al.*, 1999) and are required for ovulation of mature oocytes (McCarter *et al.*, 1997). However, the same myosin heavy chain isoforms are expressed in the myoepithelial sheath and the striated body wall muscle (Ardizzi and Epstein, 1987), suggesting that the sheath cells are physiologically similar to striated muscle. Intense contraction of the myoepithelial sheath is induced by sperm in the absence of oocytes (McCarter *et al.*, 1997). Purified major sperm protein induces contraction through an Eph receptor (Miller *et al.*, 2001; Miller *et al.*, 2003), but its downstream regulation of cytoskeletal activity is not understood.

To date, only several cytoskeletal proteins are implicated in sheath contraction. The UNC-54 myosin heavy chain is a component of the thick filaments in the myoepithelial sheath (Ardizzi and Epstein, 1987), but its mutation causes only weak ovulation defects (McCarter *et al.*, 1997). The MYO-3 myosin heavy chain is also a component of the thick fila-

ments in the myoepithelial sheath (Ardizzi and Epstein, 1987) and may have a partially redundant function with UNC-54. However, mutations in the *myo-3* gene are homozygous lethal (Waterston, 1989), and its function is not investigated in the sheath cells. Disturbance of the functions of MUP-2 troponin T (Myers *et al.*, 1996), PAT-3 β -integrin (Lee *et al.*, 2001), or talin (Cram *et al.*, 2003) also causes ovulation defects, but their subcellular localization in the sheath cells is not examined in detail. In addition, perturbation of β -integrin or talin causes defects in the gonadal morphogenesis (Lee *et al.*, 2001; Cram *et al.*, 2003), suggesting that the ovulation defects might be partly due to structural defects rather than defects in the regulation of contraction.

Tropomyosin (TM) is a major actin-associated protein among eukaryotes. In striated muscle, TM is coupled with troponin (TN) and transmits the calcium signal to activate actin-myosin interaction (Gordon *et al.*, 2000). Mutations in the human TM gene are associated with nemaline myopathy and familial hypertrophic cardiomyopathy (Michele and Metzger, 2000; Tubridy *et al.*, 2001). In both muscle and nonmuscle cells, TM stabilizes actin filaments by protecting them from disassembly (Cooper, 2002). Null or severe loss-of-function mutations of a TM gene are lethal in yeast (Balasubramanian *et al.*, 1992; Drees *et al.*, 1995), mice (Blanchard *et al.*, 1997; Rethinasamy *et al.*, 1998), and *C. elegans* (Williams and Waterston, 1994; Anyanful *et al.*, 2001). Thus, TM is an important regulator of actin-dependent processes in a variety of cells. In contrast, TN is expressed only in striated muscle in vertebrates and functions as an actin-linked calcium switch to activate actomyosin interaction (Gordon *et al.*, 2000). However, there are two instances for the presence of TN in smooth muscle in other animals: adult body wall muscle of sea squirt (Endo and Obinata, 1981) and adductor muscle of scallop (Ojima and Nishita, 1986; Nishita *et al.*, 1997). Although these smooth muscle TNs have activity to regulate actin-myosin interaction in a calcium-dependent

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manner in vitro, their physiological roles in muscle contraction are yet to be established.

In this study, we demonstrate that TM and TN are the components of thin filaments in the sheath cells of the *C. elegans* ovary and are required for ovarian contraction during ovulation. We also find that the ovulation defects by RNA interference (RNAi) of TM or TN are suppressed by a mutation of actin depolymerizing factor (ADF)/cofilin, which enhances actin filament turnover (reviewed in Bamberg, 1999; Bamberg *et al.*, 1999; Ono, 2003). Thus, our results indicate that these cytoskeletal regulators play crucial roles in the *C. elegans* reproduction.

MATERIALS AND METHODS

Nematode Strains

Wild-type *C. elegans* strain N2 was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). *unc-60 (r398)* (McKim *et al.*, 1988) was obtained from D. Baillie (Simon Fraser University, Burnaby, BC, Canada). The strain DH1033 expressing YP170::GFP (Grant and Hirsh, 1999) was obtained from B. Grant (Rutgers University, Piscataway, NJ). Nematodes were grown at 20°C as described previously (Brenner, 1974).

RNA Interference Experiments

Nematodes were treated with RNAi for CeTM or *pat-10* by feeding as described previously (Ono and Ono, 2002). Phenotypes were analyzed in their F1 generation. Worm motility was quantified as described previously (Epstein and Thomson, 1974; Ono *et al.*, 1999). Two different vectors for RNAi of CeTM were used: TM1 was used to suppress the CeTMI and CeTMII isoforms and TM2 to suppress all four CeTM isoforms (Ono and Ono, 2002). To construct a vector for *pat-10* (RNAi), a 1087-base pair genomic DNA fragment of the *pat-10* gene (F54C1.7) was amplified from genomic DNA of wild-type *C. elegans* by polymerase chain reaction by using a forward primer 5'-GATCAGATCTGGCTGAGGATATCGAAGAGATTC and a reverse primer 5'-GATCGCTAGCTTGAAGATTGTAGATCAGCGCTG. The amplified fragments were digested by *Bgl*III and *Nhe*I at the sites introduced in the polymerase chain reaction primers and cloned into L4440 (provided by A. Fire, Stanford University, Stanford, CA) at the cloning site between two oppositely oriented T7 promoters (Timmons and Fire, 1998).

Time-Lapse Nomarski Microscopy

Control worms were anesthetized in 0.1% tricaine, 0.01% tetramisole in M9 for 30 min and mounted on 2% agarose pads (McCarter *et al.*, 1997). Tricaine/tetramisole paralyzes body wall movement but does not block several rounds of oocyte maturation and ovulation. They were set on a Nikon Eclipse TE2000 inverted microscope and observed with a 40× CFI Plan Fluor objective (numerical aperture 1.4). Images were captured at room temperature by a SPOT RT Monochrome charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI) and recorded every 15 s for 60–100 min by the IPLab imaging software (Scanalytics, Fairfax, VA).

Fluorescence Microscopy

Staining of whole animals with tetramethylrhodamine-phalloidin (Sigma-Aldrich, St. Louis, MO) was performed as described previously (Ono, 2001). 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (Sigma-Aldrich) was included at 0.1 µg/ml in the phalloidin solution to stain DNA. For immunofluorescent staining of the *C. elegans* gonads, gonads were dissected by cutting adult worms at the level of the pharynx as described previously (Rose *et al.*, 1997), attached on poly-lysine-coated slides by a freeze-crack method (Epstein *et al.*, 1993), and fixed with 4% formaldehyde in cytoskeleton buffer (10 mM MES-KOH, pH 6.1, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA) containing 0.32 M sucrose for 30 min at room temperature. They were permeabilized with methanol for 5 min at -20°C and stained with anti-actin antibody (C4; MP Biomedicals, Irvine, CA), DAPI, and anti-CeTM (Ono and Ono, 2002), or anti-PAT-10 antibody (Terami *et al.*, 1999) (provided by H. Kagawa, Okayama University, Okayama, Japan). Alexa 488-conjugated goat anti-guinea pig IgG or goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies. For staining of the gonads only by tetramethylrhodamine-phalloidin, the dissected gonads were fixed with 4% formaldehyde in cytoskeleton buffer containing 0.32 M sucrose for 30 min at room temperature and incubated with 0.2 µg/ml tetramethylrhodamine-phalloidin in phosphate-buffered saline containing 0.5% Triton X-100, 30 mM glycine, 1 mM EDTA, and 0.05% sodium azide for 1 h at room temperature. Samples were observed with a Nikon Eclipse TE2000 inverted microscope with a 40× CFI Plan Fluor objective (numerical aperture 1.4). Images were captured at room temperature by a SPOT RT Monochrome charge-coupled

Table 1. RNAi phenotypes for CeTM and *pat-10*

Strain	RNAi	Motility (beats/30 s, <i>n</i> = 10)	Brood size (<i>n</i> = 10)	Emo phenotype (%, <i>n</i> = 200)
Wild-type	Control	105 ± 5.5	314 ± 33	0
	<i>CeTMI,II</i>	46 ± 6.8	0 ± 0	100
	<i>CeTMI,II,III,IV</i>	13 ± 5.9	0 ± 0	100
	<i>pat-10</i>	0.6 ± 1.1	0 ± 0	100
<i>unc-60(r398)</i>	Control	48.9 ± 11	251 ± 46	0
	<i>CeTMI,II</i>	47 ± 12	271 ± 39	0
	<i>CeTMI,II,III,IV</i>	39 ± 5.2	72 ± 50	59
	<i>pat-10</i>	1.7 ± 1.3	8.3 ± 11	10

device camera (Diagnostic Instruments) and edited by the IPLab imaging software (Scanalytics) and Adobe Photoshop 6.0 with no gamma adjustment.

Western Blot

The worm lysates were prepared and Western blot performed as described previously (Ono and Ono, 2002). Primary antibodies used were anti-PAT-10 (Terami *et al.*, 1999), anti-CeTM (Ono and Ono, 2002), and anti-actin (C4; MP Biomedicals) antibodies.

RESULTS

RNA Interference of Tropomyosin Causes Sterility

We previously showed that suppression of TM by RNAi caused paralysis or reduced motility and disorganization of actin filaments in body wall muscle of *C. elegans* (Ono and Ono, 2002). In addition, we found that suppression of TM caused strong sterility (Table 1). The TM1 RNAi construct suppresses the CeTMI and CeTMII isoforms of the four TM isoforms present in worms [*CeTMI,II* (RNAi)] and reduced the total level of TM protein to 50% (see Figure 7 of Ono and Ono, 2002; Figure 3b). Decreased motility of the affected worms to approximately one-half the speed of control worms was also observed as reported previously (Ono and Ono, 2002) (Table 1). In addition, the *CeTMI,II* (RNAi) worms produced no progeny (Table 1), whereas control worms produced ~300 progeny per worm (Table 1). Suppression of all four TM isoforms by the TM2 RNAi construct [*CeTMI,II,III,IV* (RNAi)] reduced the TM protein level to ~10% (see Figure 7 of Ono and Ono, 2002; Figure 3b) and caused a more severe motility defect than that by TM1 as reported previously (Ono and Ono, 2002) (Table 1). Nonetheless, the sterile phenotypes induced by both *CeTMI,II* (RNAi) and *CeTMI,II,III,IV* (RNAi) were equally severe (Table 1). These results strongly suggest that TM is required for the reproductive system of *C. elegans* and that CeTMI and CeTMII are the TM isoforms that play major roles in this event or that the reproductive system is more sensitive to the level of TM than body wall muscle.

Tropomyosin Is Essential for Ovulation

Examination of the adult gonads in *CeTM* (RNAi) worms revealed that the oocytes at the very proximal ends of the ovary became endomitotic and no embryos resided in the uterus (compare Figure 1, a and b). In control worms, the oocytes were located in the proximal ovary (Figure 1a) and were arrested at meiosis with condensed chromosomes (Figure 1c, arrows). However, in both *CeTMI,II* (RNAi) and *CeTMI,II,III,IV* (RNAi) worms, oocytes at the proximal gonads had a large accumulation of DNA (Figure 1d, arrowhead), indicating that there was endomitotic replication of

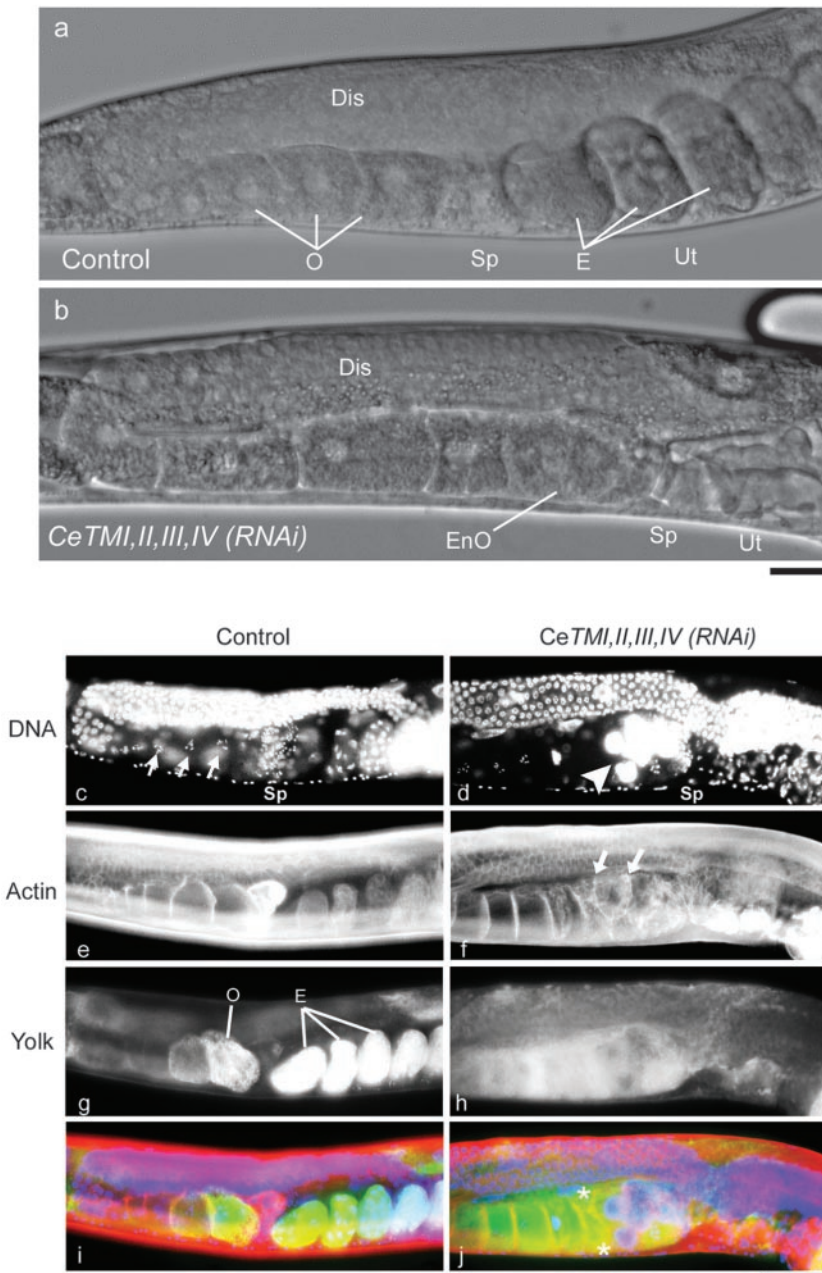


Figure 1. RNA interference of tropomyosin causes ovulation defects and endomitotic DNA replication in the oocytes. (a and b) Nomarski images of the adult hermaphroditic gonad of control (a) and *CeTMI,II,III,IV (RNAi)* (b) worms. Germ cells are proliferated in the distal gonad (Dis) and developed into oocytes (O) in the proximal gonad. A mature oocyte is ovulated and fertilized in the spermatheca (Sp) and initiates embryogenesis in the uterus (Ut). In the *CeTMI,II,III,IV (RNAi)* worm, the proximal oocytes (EnO) had ambiguous nuclear-cytoplasmic boundary, which is characteristic of endomitotic oocytes, and there was no embryos in the uterus. Bar, 20 μm. (c–j) Visualization of DNA by DAPI staining showed that control oocytes had condensed chromosomes (c, arrows), whereas the proximal oocytes in the *CeTMI,II,III,IV (RNAi)* worm had large accumulations of DNA (d, arrowhead). Staining of actin filaments with rhodamine-phalloidin demonstrated that oocytes were regularly compartmentalized in the control ovary (e) but that they had irregular cell compartments in the *CeTMI,II,III,IV (RNAi)* ovary (f, arrows). Transgenically expressed GFP-tagged yolk protein YP170 accumulated in control oocytes and embryos by endocytosis (g). The oocytes in the *CeTMI,II,III,IV (RNAi)* worm also had the yolk protein (h), suggesting that endocytosis was normal. Merged in i and j are DNA (blue), actin (red), and yolk (green). Asterisks in j indicate anuclear cells. Bar, 50 μm.

DNA in the oocytes, which became highly polyploid. This phenotype resembles the previously described *endomitotic oocytes* in gonadal arms phenotype (*Emo*) that is observed when ovulation is defective (Iwasaki *et al.*, 1996). The *Emo* phenotype of *CeTM (RNAi)* worms was very severe, such that oocytes or embryos were not observed in the spermatheca or uterus (Figure 1b and Table 1), suggesting that there was no ovulation. In addition, visualization of the actin filaments revealed that the endomitotic oocytes had irregular cell compartments (Figure 1f, arrows) and that some cells were anuclear (Figure 1j, asterisks), suggesting that these oocytes underwent aberrant cell division in the ovary. The two RNAi treatments, *CeTMI,II (RNAi)* and *CeTMI,II,III,IV (RNAi)*, caused nearly identical *Emo* phenotype (Table 1). Therefore, the phenotypes shown in the Figures 1 and 2 are after treatment with only *CeTMI,II,III,IV (RNAi)*.

Before the oocytes became endomitotic, there were no morphological abnormalities in the germ cells in *CeTM (RNAi)* worms. Sperm was properly stored in the spermatheca (Figure 1d) and sperm that was supplied by mating with control males did not rescue the RNAi phenotype (our unpublished data). Developing oocytes were morphologically normal before they became endomitotic (our unpublished data). In addition, the endocytic process of the yolk proteins in oocytes seemed to be normal (Figure 2, g and h), although the actin cytoskeleton plays important roles in endocytosis (Schafer, 2002; Engqvist-Goldstein and Drubin, 2003). We followed the dynamics of a green fluorescent protein (GFP)-tagged yolk protein (YP170) in control, *CeTMI,II (RNAi)*, or *CeTMI,II,III,IV (RNAi)* worms. In control worms, the yolk protein accumulated in maturing oocytes and early embryos (Figure 1g). *CeTM (RNAi)* worms also

Control

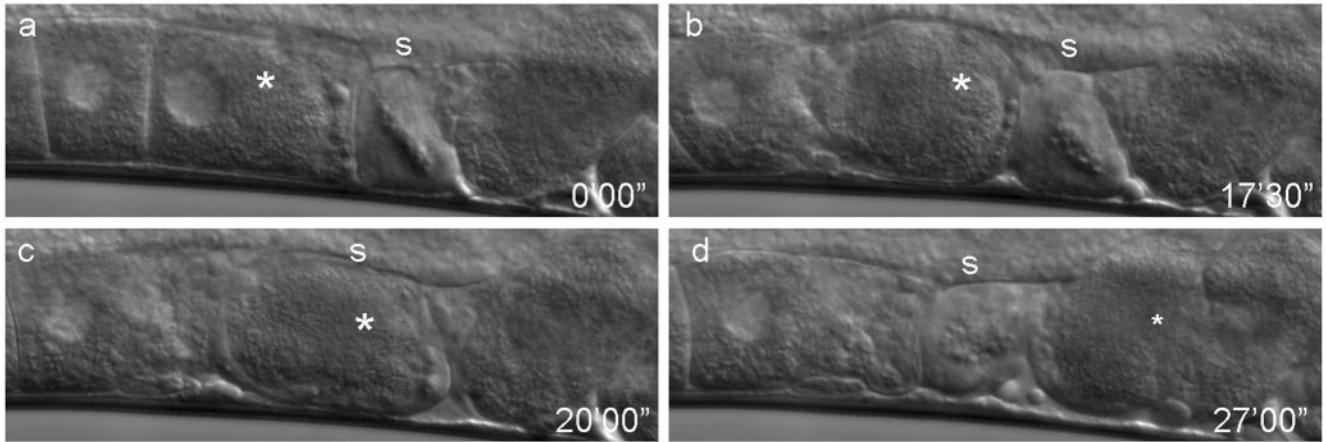
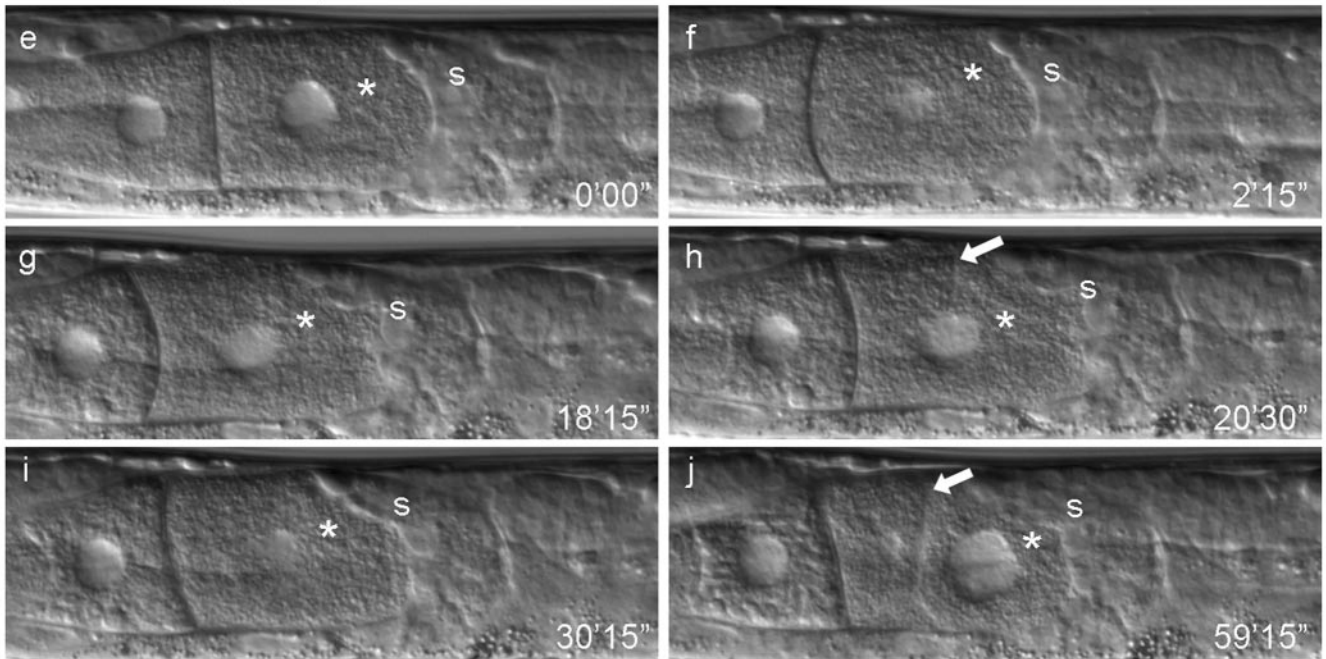
*CeTMI,II,III,IV (RNAi)*

Figure 2. Ovulation, but not oocyte maturation, is defective in the tropomyosin-RNAi worms. Ovulation processes of control (a–d) and *CeTMI,II,III,IV (RNAi)* (e–j) worms were recorded by time-lapse Nomarski microscopy (also see Videos 1 and 2). In a control worm, the most proximally located oocyte (a, asterisk) became mature and showed morphological change into a round shape and nuclear envelope breakdown (b). It was pushed by intense contraction of the ovary and fertilized in the spermatheca (c). Then, embryogenesis was initiated in the uterus after ovulation (d). In the *CeTMI,II,III,IV (RNAi)* worm, the most proximal oocyte (e, asterisk) became mature (f), but it was not ovulated due to weak contraction of the ovary (g). In the absence of ovulation, the nuclear envelope reappeared (g, asterisk) and a cleavage furrow was formed (h, arrow). The furrow was dynamic and sometimes regressed (i). However, when the cleavage is complete, the daughter cell on the left became anuclear and the large nucleus is segregated into the other cell (j). Positions of the spermatheca are indicated by “s”. Numbers indicate time (minute seconds) from the first frame. Bar, 20 μ m.

had the yolk protein in the cytoplasm of the oocytes (Figure 1h) and did not show a typical endocytosis-defective phenotype (Grant and Hirsh, 1999).

To observe the live activities of the gonads of *CeTM (RNAi)* worms by time-lapse Nomarski microscopy (Figure 2; Videos 1 and 2). In control worms, only the most proximal oocyte became mature, which was characterized by nuclear envelope breakdown and rounding up of the oocyte (McCarter *et al.*, 1999) (Figure 2, a and b, asterisks; Video 1). This

was accompanied by frequent and intense myoepithelial sheath contraction and spermathecal dilation that allowed the oocyte to enter the spermatheca where it was fertilized (McCarter *et al.*, 1999) (Figure 2c; Video 1). The fertilized egg was subsequently expelled into the uterus and initiated embryogenesis (Figure 2d; Video 1). In the *CeTMI,II,III,IV (RNAi)* worms, the proximal oocyte became mature normally (Figure 2, e and f, asterisks; Video 2), but neither intense contraction of the myoepithelial sheath nor dilation of the spermatheca took place. As a result, the oocyte re-

Table 2. Tropomyosin and troponin in *C. elegans*

Protein	Gene	Length (aa)	Mutant ^a	RNAi ^a
Tropomyosin	<i>lev-11/tmy-1</i> (Y105E8B.1 I)	256–301	Lev ^b , Pat ^c	Emb ^d , Unc ^e , Ste ^f
Troponin C	<i>pat-10/tnc-1</i> (F54C1.7 I)	161	Pat ^g	Ste ^{f,h}
	<i>tnc-2</i> (ZK673.7 II)	160	ND	Lva ^h
Troponin I	<i>tmi-1</i> (F42E11.4 X)	250	ND	WT ^h
	<i>unc-27/tmi-2</i> (ZK721.2 X)	242	Unc ⁱ	WT ^h
	<i>tmi-3</i> (T20B3.2 V)	260	ND	WT ^h
	<i>tmi-4</i> (W03F8.1 IV)	197	ND	Gro ^h
Troponin T	<i>mup-2/tnt-1</i> (T22E5.5 X)	405	Mup ^j , Ste ^j	WT ^h
	<i>tnt-2</i> (F53A9.10 X)	428	ND	WT ^h
	<i>tnt-3</i> (C14F5.3 X)	328–1216	ND	WT ^h
	<i>tnt-4</i> (T08B1.2 V)	347–375	ND	WT ^h

^a Designations for the phenotypes: Lev, levamisole-resistant; Pat, paralysed arrest at twofold; Unc, uncoordinated; Ste, sterile; Lva, larval arrest; Gro, slow growth; Mup, muscle positioning; WT, wild type; ND, not determined.

^b Lewis *et al.*, 1980.

^c Williams and Waterston, 1994.

^d Anyanful *et al.*, 2001.

^e Ono and Ono, 2002.

^f This study.

^g Williams and Waterston, 1994; Terami *et al.*, 1999.

^h Kamath *et al.*, 2003.

ⁱ Burkeen *et al.*, 2004.

^j Myers *et al.*, 1996.

mained in the gonad, rearranged its cortex into a square shape, reformed the nuclear envelope (Figure 2g; Video 2), and, surprisingly, initiated cytokinesis (Figure 2h, arrow; Video 2). The cleavage furrow was often dynamic and sometimes regressed (Figure 2i; Video 2). However, the nucleus was not divided. Thus, when the cleavage was complete, the nucleus was restricted to only one of the two daughter cells, leaving the other cell anuclear (Figure 2j, asterisk; Video 2). In the absence of ovulation, maturation of the second or third proximal oocyte was often observed (our unpublished data), indicating that a signal to induce oocyte maturation can reach distally located oocytes as demonstrated previously (Iwasaki *et al.*, 1996). Therefore, suppression of TM specifically caused a defect in the contractile activity of the ovary, but not in the process of oocyte maturation. The *CeTMI,II* (RNAi) (n = 5) and *CeTMI,II,III,IV* (RNAi) (n = 10) worms showed a nearly identical ovulation defect by time-lapse microscopy (Table 3).

PAT-10 Troponin C Is Required for Ovulation

A previous report by Myers *et al.* (1996) demonstrated that a mutation in the *mup-2* troponin T gene impaired contraction of the myoepithelial sheath and caused a very similar sterile phenotype to *CeTM* (RNAi). This observation strongly suggests that troponin is also a regulator of sheath contraction. We extracted available functional data on all genes for the *C. elegans* troponin components (two troponin Cs [TNCs], four troponin Is, and four troponin Ts) (Table 2) and found that the *pat-10/tnc-1* TNC gene was the only troponin component that caused sterility by RNAi (Kamath *et al.*, 2003). These data led us to hypothesize that PAT-10 TNC might be a regulatory component of contraction of the myoepithelial sheath cells. PAT-10 TNC was previously reported as a component of thin filaments in body wall muscle (Terami *et al.*, 1999), but its expression and localization in the gonad are not known. Mutations in the *pat-10* gene cause embryonic

Table 3. Dissection of the ovulation process by time-lapse recording

Strain	RNAi	Sheath contraction	Spermathecal dilation	Oocyte maturation ^a	Aberrant cytokinesis	Ovulation	n
Wild-type	Control	100 ^b	89	100	0	78	9
	<i>CeTMI,II</i>	0	0	100	100	0	5
	<i>CeTMI,II,III,IV</i>	0	0	100	100	0	10
	<i>pat-10</i>	0	43	100	71	43 ^c	7
<i>unc-60(r398)</i>	Control	83	83	83	0	83	6
	<i>CeTMI,II</i>	60	100	100	0	80	5
	<i>CeTMI,II,III,IV</i>	0	80	100	20	60	5
	<i>pat-10</i>	0	50	83	17	50	6

^a Maturation was characterized by nuclear envelope breakdown.

^b All values except “n” indicate percentages.

^c This includes two cases (29%) of abnormal ovulation, which cleaves the oocyte before completion.

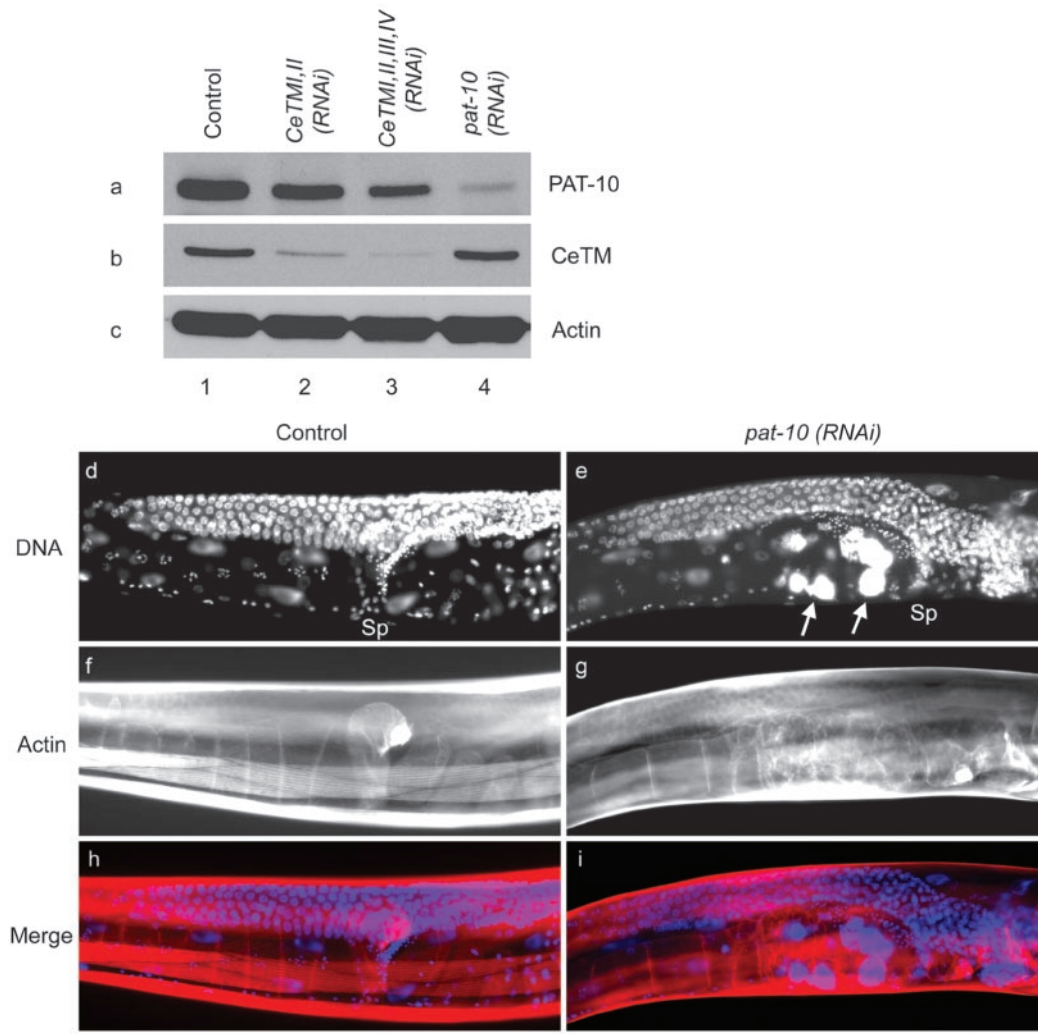


Figure 3. RNAi of *pat-10* troponin C results in ovulation defects. (a–c) Western blot analysis of PAT-10 (a), CeTM (b), and actin (c) after control RNAi with a blank vector (lane 1), *CeTMI,II (RNAi)* (lane 2), *CeTMI,II,III,IV (RNAi)* (lane 3), or *pat-10 (RNAi)* (lane 4). PAT-10 was significantly reduced by *pat-10 (RNAi)* (a, compare lanes 1 and 4) and slightly reduced by *CeTMI,II (RNAi)* (a, lane 2), *CeTMI,II,III,IV (RNAi)* (a, lane 3). In contrast, the level of CeTM was not altered by *pat-10 (RNAi)* (b, compare lanes 1 and 4). (c–i) Control (d, f, and h) or *pat-10 (RNAi)* (e, g, and i) worms were stained by DAPI (d and e) and rhodamine-phalloidin (f and g). Merged images of DNA (blue) and F-actin (red) are shown in h and i. Positions of the spermatheca (Sp) are indicated in d and e. Large accumulations of DNA in endomitotic oocytes are indicated by arrows in e. Bar, 50 μm .

arrest (Williams and Waterston, 1994) and do not allow us to investigate its role in the gonad that develops during larval stages (Hubbard and Greenstein, 2000).

We performed RNAi of *pat-10* by feeding and found that it allowed the treated worms to grow into adults and caused defective worm motility and sterility, which were very similar to the *CeTM (RNAi)* phenotypes (Figure 3). RNAi of *pat-10* significantly reduced the PAT-10 protein (Figure 3a, compare lanes 1 and 4) without affecting the level of CeTM (Figure 3b, compare lanes 1 and 4). The level of PAT-10 was slightly reduced by RNAi of CeTM (Figure 3a, lanes 2 and 3). The *pat-10 (RNAi)* worms were nearly paralyzed and produced no progeny (Table 1). Examination of the *pat-10 (RNAi)* gonads showed that endomitotic oocytes with large accumulations of DNA were present in the proximal gonad (100%, $n = 200$) (Figure 3e and Table 1). Time-lapse recording of the live *pat-10 (RNAi)* gonads showed that contraction of the proximal gonad was absent or very weak even after

the proximal oocyte became mature and the oocyte remained in the gonad (57%, $n = 7$) (Figure 4, a, c, e, and g; Video 3). Such oocytes underwent multiple rounds of nuclear envelope breakdown and reappearance (Figure 4, c and e, asterisks) and aberrant cytokinesis (71%, $n = 7$) (Figure 4, e and g, arrowheads). In some *pat-10 (RNAi)* worms (43%, $n = 7$), the spermatheca was able to dilate and allowed ovulation of the proximal oocyte in the absence of intense sheath contraction (Figure 4, b, d, f, and h; Video 4). However, in two of three such cases, the spermatheca contracted before ovulation was completed, which resulted in cleavage of the oocyte and ovulation of only a portion of the oocyte (Figure 4, f and h, arrows; Video 4), indicating that the spermatheca can dilate but its dilation and contraction are uncoordinated during ovulation. These results indicate that sterility by *pat-10 (RNAi)* is due to a strong defect in contraction of the myoepithelial sheath as observed for RNAi of CeTM.

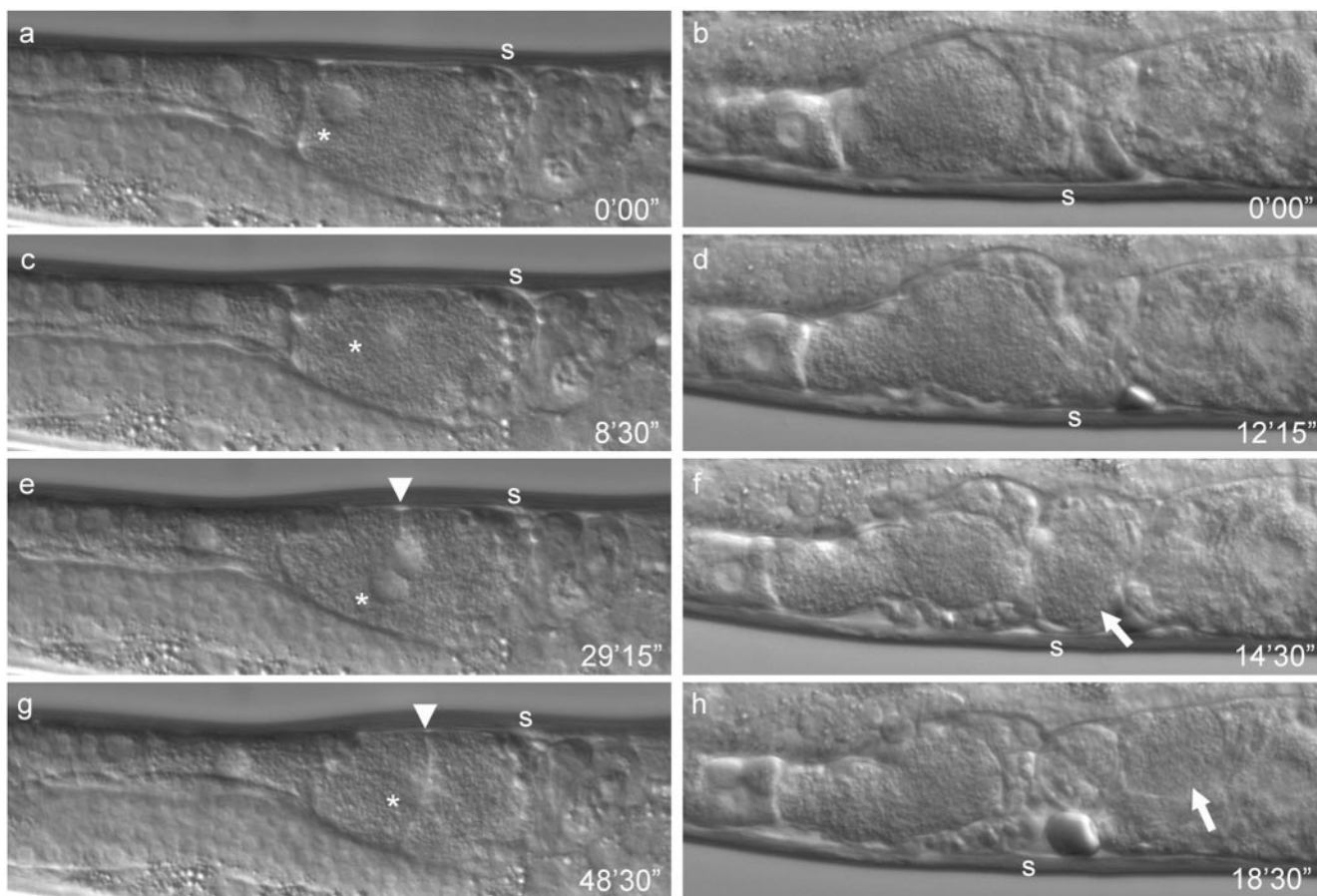


Figure 4. Time-lapse observation of ovulation in the *pat-10* (*RNAi*) worm. Ovulation process of two representative *pat-10* (*RNAi*) worms. In a, c, e, and g, ovulation was completely unsuccessful (also see Video 3). Nuclear envelope breakdown of the most proximal oocyte (c, asterisk) indicated oocyte maturation. However, due to lack of sheath contraction, the oocyte was not transported and nuclear envelope reappeared (e, asterisk). Aberrant cytokinesis of the oocyte was also observed (e and g, arrowheads). In b, d, f, and h, ovulation was partially successful (also see Video 4). After oocyte maturation (b), the spermatheca dilated (d), and the oocyte entered the spermatheca (f, arrow). However, only a portion of the oocyte was transported into the uterus (h, arrow). Numbers indicate time (minutes seconds) from the first frame. Bar, 20 μ m.

Tropomyosin and Troponin C Are the Components of the Nonstriated Thin Filaments in the Myoepithelial Sheath

By immunofluorescence microscopy, we found that CeTM and PAT-10 TNC colocalized with actin filaments in the myoepithelial sheath of the ovary (Figure 5). PAT-10 was expressed only in the myoepithelial sheath cells but not in the spermatheca and colocalized with the actin filaments in a nonstriated manner (Figure 5, a–c). In contrast, CeTM was expressed in both myoepithelial sheath and spermatheca and localized to the actin filaments in a nonstriated pattern (Figure 5, j–l). The *CeTMI,II,III,IV* (*RNAi*) treatment eliminated filamentous staining by anti-CeTM antibody but not diffuse staining of the gonad (Figure 5, m–o), suggesting that residual CeTM protein or nonspecifically recognized proteins are visualized. In addition, RNAi of CeTM reduced filamentous staining of PAT-10 (Figure 5, d–f), indicating that association of PAT-10 with actin is dependent on CeTM. In contrast, *pat-10* (*RNAi*) nearly completely eliminated PAT-10 in the gonad (Figure 5g) but did not affect the filamentous pattern of CeTM (Figure 5p). Thus, CeTM localizes to actin filaments independently of PAT-10. The organization of actin filaments in the myoepithelial sheath and spermatheca was not significantly altered by RNAi of CeTM (Figure 5, e and n) or *pat-10* (Figure 5, h and q) except for

minor disarrays of the filaments (also see Figure 6B), which might be due to excessive accumulation of endomitotic oocytes. Together, these observations strongly suggest that both CeTM and PAT-10 TNC are the components of nonstriated thin filaments in the myoepithelial sheath cells and regulate contraction.

ADF/Cofilin Is Antagonistic to Tropomyosin and Troponin in Ovulation

We previously reported that a mutation in *unc-60B*, encoding a muscle-specific ADF/cofilin isoform, suppresses the *CeTM* (*RNAi*) phenotype in the body wall muscle (Ono and Ono, 2002). We found that the *unc-60* (*r398*) mutation, which inhibits the filament severing activity of UNC-60B (Ono *et al.*, 1999, 2001), also suppressed the sterile phenotype by *CeTM* (*RNAi*) (Figure 6A and Table 1). The *unc-60* (*r398*) homozygotes with control RNAi treatment showed reduced motility and brood size compared with wild-type, but no ovulation defects (*Emo* phenotype) were detected (Figure 7, a–c, and Table 1; Video 5). The *CeTMI,II* (*RNAi*) treatment of *unc-60* (*r398*) had no effects on motility, brood size, and ovulation (compare Figure 6A, b and f, and Table 1), and *CeTMI,II,III,IV* (*RNAi*) only weakly affected motility and impaired brood size and ovulation to much lesser extents

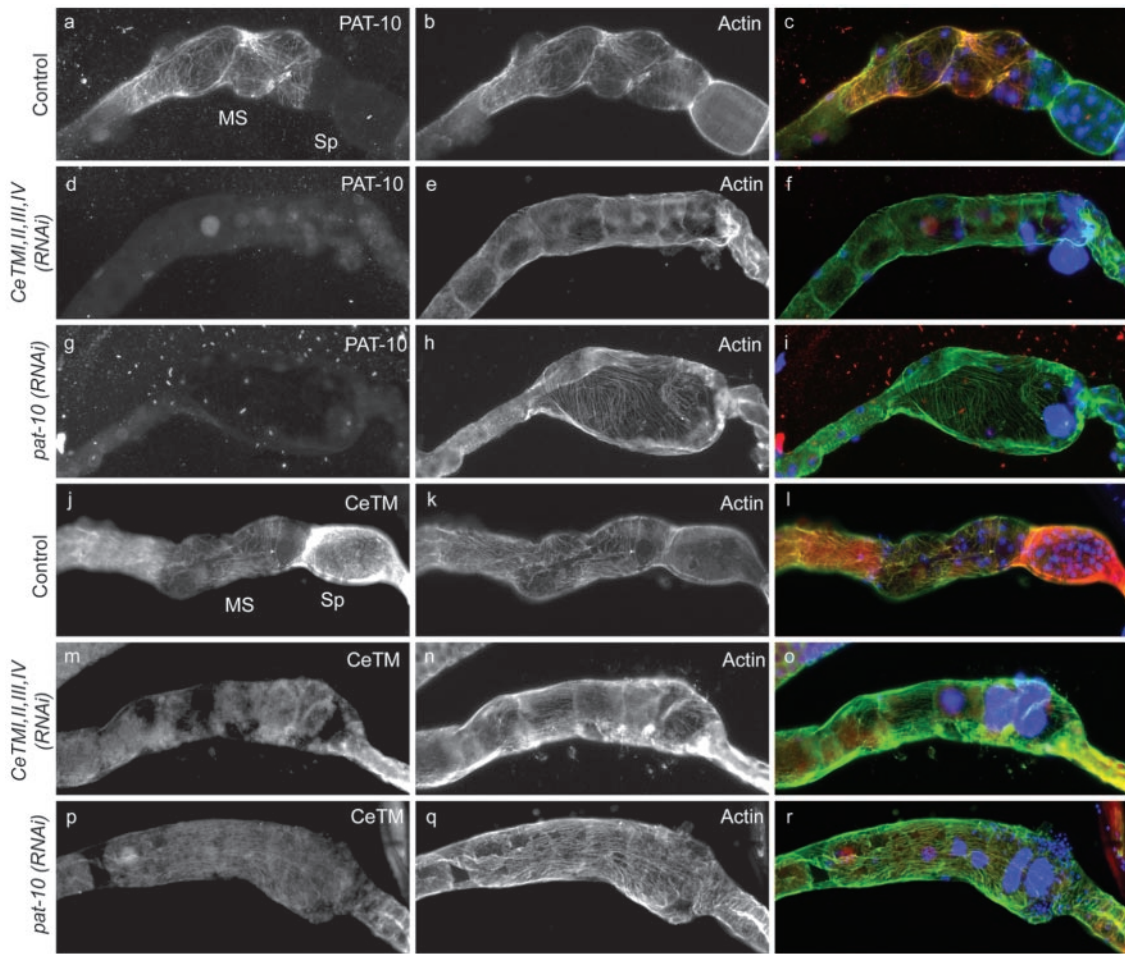


Figure 5. Localization of tropomyosin and PAT-10 troponin C to the thin filament network in the myoepithelial sheath. Hermaphroditic gonads were dissected from control (a–c and j–l), *CeTMI,II,III,IV (RNAi)* (d–f and m–o), or *pat-10 (RNAi)* (g–i and p–r) worms and stained with anti-PAT-10 (a, d, and g), or anti-CeTM (j, m, and p), and anti-actin antibody (b, e, h, k, n, and q). Merged images of PAT-10 or CeTM (green), actin (red), and DNA (blue) are shown in c, f, i, l, o, and r. PAT-10 was expressed in the myoepithelial sheath (MS) but not in the spermatheca (Sp) and colocalized with F-actin (a). CeTM was expressed in both myoepithelial sheath and spermatheca and colocalized with F-actin (j). Bar, 50 μ m.

than wild-type (compare Figure 6A, c and g, and Table 1). Nonetheless, the actin filament network in the myoepithelial sheath (Figure 6B) or the spermatheca (Figure 6C) was not significantly different between wild-type (Figure 6, Ba and Ca) and *unc-60 (r398)* (Figure 6Bb and Cb) and alteration of the actin filaments after the *CeTM (RNAi)* treatments were relatively minor (Figure 6B, c–f, for the myoepithelial sheath and C, c–f, for the spermatheca). Filamentous localizations of CeTM and PAT-10 TNC in the myoepithelial sheath were significantly reduced by *CeTM (RNAi)* in *unc-60 (r398)* as observed in wild-type (our unpublished data). Time-lapse observation of ovulation showed that the defect in contraction of the proximal gonads was partially suppressed in the RNAi-treated *unc-60 (r398)* worms (Figure 7, d–I, and Table 3; Videos 6 and 7). In addition, spermathecal dilation was not inhibited in the *unc-60 (r398)* worms (Figure 7, d–I, and Table 3; Videos 6 and 7), whereas it was strongly inhibited in wild-type (Figure 2 and Table 3). Thus, successful ovulation was observed at higher rates in the RNAi-treated *unc-60* mutant than wild type (Table 3). These results suggest that contractile activity of the gonad rather than assembly of the

contractile structure was altered by the *unc-60* mutation and *CeTM (RNAi)*.

In contrast, the *unc-60 (r398)* mutation partially suppressed the effects of *pat-10 (RNAi)*. The paralyzed phenotype by *pat-10 (RNAi)* was not suppressed by the *unc-60 (r398)* mutation (Figure 6A, h and Table 1), suggesting that the RNAi effects on body wall muscle was not affected by the *unc-60* mutation. Brood size was greatly reduced (8.3 ± 11) but significantly more than wild type (0 ± 0). However, the ovulation defect was greatly suppressed in the *unc-60* mutants (Table 1). There were only minor disarrays of actin filaments in the myoepithelial sheath by *pat-10 (RNAi)* in both wild-type (Figure 6Bg) and *unc-60 (r398)* (Figure 6Bh). The actin filaments in the spermatheca were not significantly altered by *pat-10 (RNAi)* in wild-type (Figure 6Cg) and *unc-60 (r398)* (Figure 6Ch). Immunostaining of PAT-10 TNC in the gonads showed that the *pat-10 (RNAi)* treatment reduced the PAT-10 protein in *unc-60 (r398)* as well as in wild-type (our unpublished data). Time-lapse recording of the ovulation process in the *pat-10 (RNAi)*-treated *unc-60 (r398)* worms demonstrated that contraction of the proximal

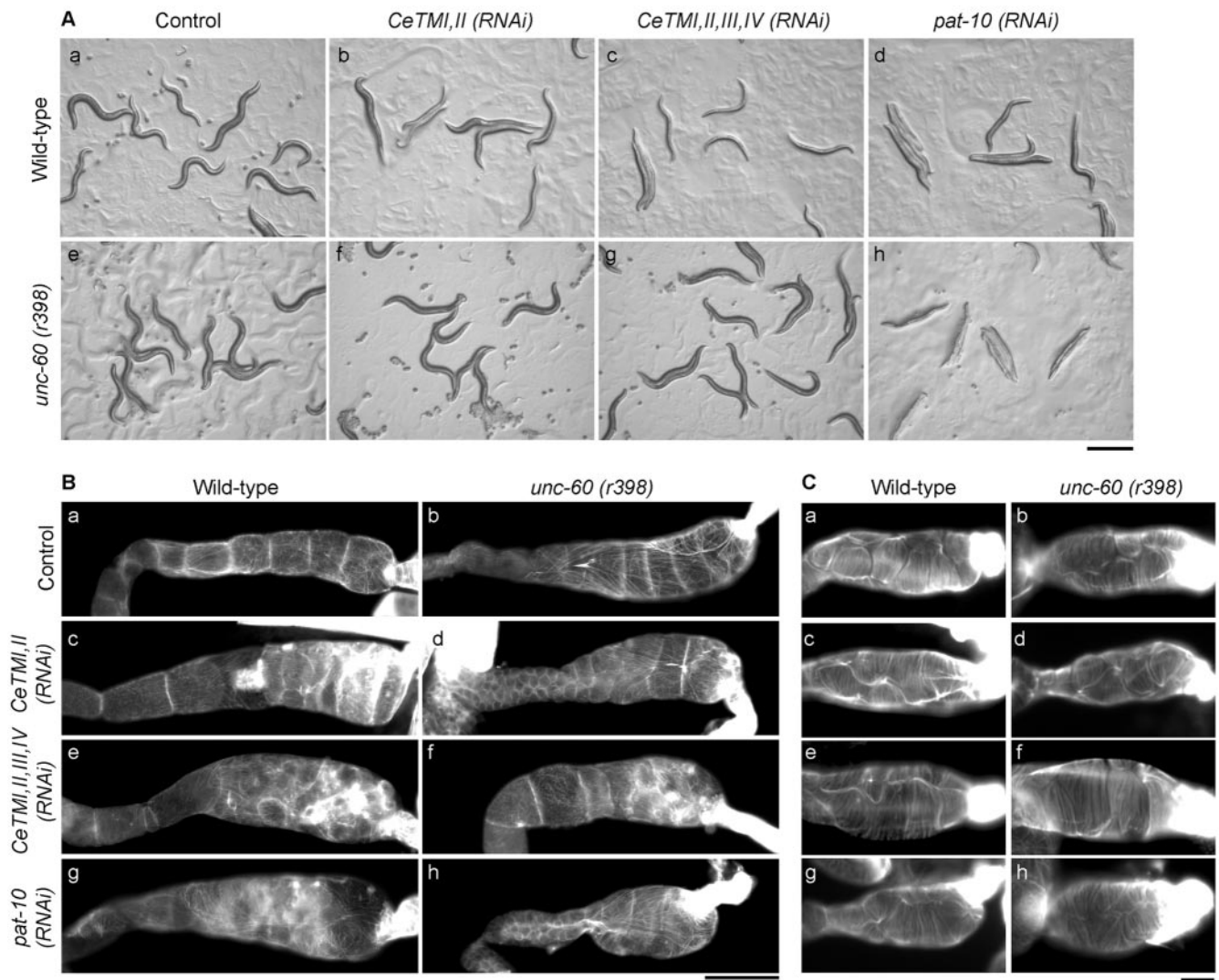


Figure 6. Suppression of ovulation defects by a mutation of *unc-60B* ADF/cofilin. (A) Micrographs of wild-type (a–d) or *unc-60 (r398)* (e–h) on agar plates after control (a and e), *CeTMI,II (RNAi)* (b and f) *CeTMI,II,III,IV (RNAi)* (c and g), or *pat-10 (RNAi)* (d and h) treatment. Bar, 0.5 mm. (B and C) The myoepithelial sheath (B) or the spermatheca (C) of dissected gonads from wild-type (a, c, e, and g) or *unc-60 (r398)* (b, d, f, and h) worms after control treatment (a and b), *CeTMI,II (RNAi)* (c and d), *CeTMI,II,III,IV (RNAi)* (e and f), or *pat-10 (RNAi)* (g and h) were stained by tetramethylrhodamine-phalloidin to visualize F-actin in the myoepithelial sheath. Note that intensely stained aggregates in c, e, f, and g were present in the oocytes not in the sheath cells due to aberrant cell division. Bars, 50 μ m (B) or 10 μ m (C).

gonad was still weak, but the spermatheca was able to dilate and allowed the oocyte to be expelled into the uterus (Figure 7, j–l; Video 8). In the *unc-60 (r398)* worms, early spermathecal contraction and cleavage of an ovulating oocyte were not observed. Thus, ovulation was successful in 50% of the observed worms (n = 6) (Table 3). Nonetheless, the low brood size of the *pat-10 (RNAi) unc-60 (r398)* worms suggests that fertilization was often unsuccessful or embryonic lethality occurred.

DISCUSSION

In this study, we identified TM and TNC as essential regulators of contraction of the myoepithelial sheath during ovulation in the *C. elegans* reproductive system. RNAi of *CeTM* or *pat-10* TNC strongly inhibited sheath contraction, which is essential for expelling a mature oocyte into the sper-

matheca for fertilization. Spermathecal dilation was strongly inhibited by *CeTM (RNAi)* but only weakly by *pat-10 (RNAi)*. This observation was supported by immunolocalization, demonstrating that *CeTM* localized to the actin filament network in the myoepithelial sheath and spermatheca, but *PAT-10* TNC was expressed only in the myoepithelial sheath. A mutation in the *unc-60B* ADF/cofilin gene suppressed ovulation defects by RNAi of *CeTM* or *pat-10*, suggesting that ADF/cofilin is antagonistic to TM and TN in ovulation.

Our results show that the nematode myoepithelial sheath is physiologically similar to striated muscle whose contraction is regulated by a TM–TN complex. Smooth muscles of vertebrates and most invertebrates lack troponin, and their contraction is generally regulated by phosphorylation of myosin light chain and/or Ca^{2+} -calmodulin regulation of caldesmon, an actin-associated protein (Marston, 1995;

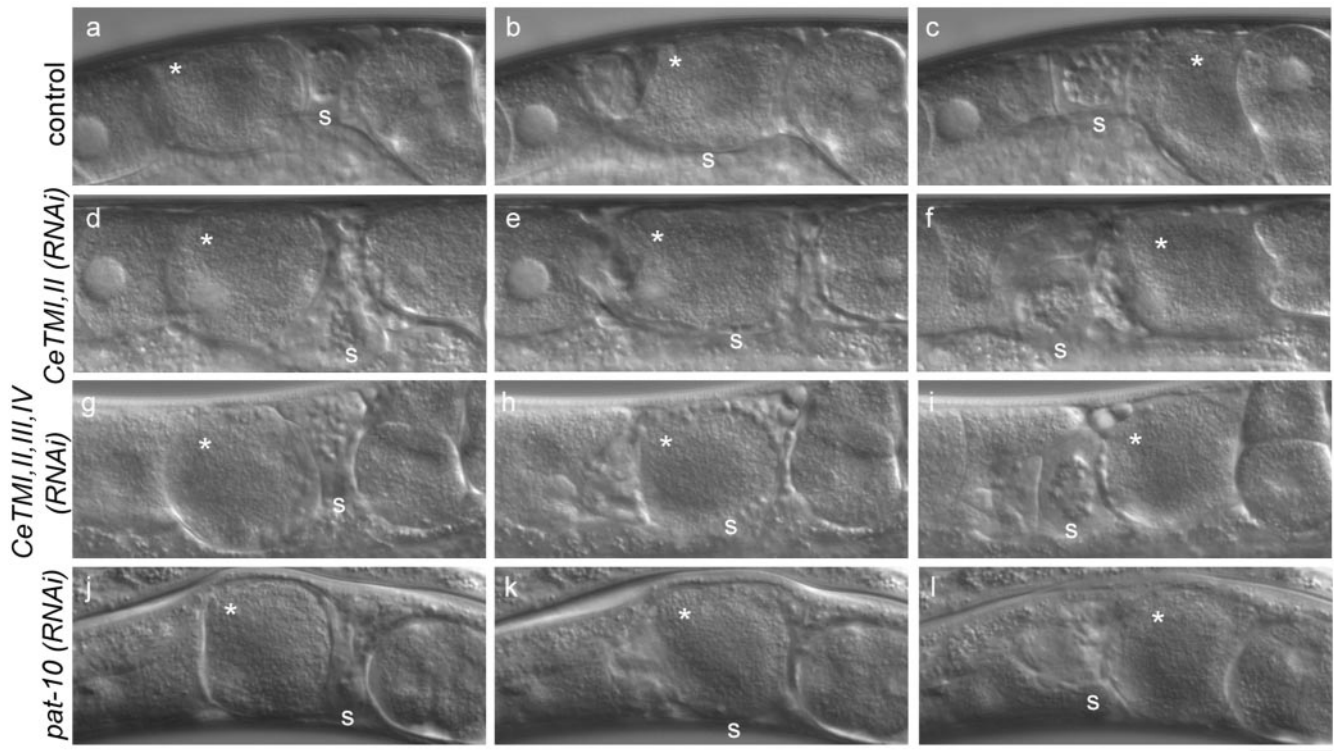


Figure 7. Ovulation processes in the *unc-60B* mutant after RNAi treatment. Time-lapse observation of ovulation processes in the *unc-60* (*r398*) worms after control treatment (a–c; also see Video 5), *CeTMI,II* (RNAi) (d–f; also see Video 6), *CeTMI,II,III,IV* (RNAi) (g–i; also see Video 7), or *pat-10* (RNAi) (j–l; also see Video 8). Oocytes (asterisks) are shortly after maturation (a, d, g, and j), transported in the spermatheca (b, e, h, and k), and expelled into the uterus (c, f, i, and l). Positions of the spermatheca are indicated by s. Bar, 20 μm .

Wang, 2001). However, no caldesmon homolog is found in the *C. elegans* genome sequence, and, currently, no candidates for myosin-linked regulators of sheath contraction, such as myosin light chain, calmodulin, and myosin light chain kinase, have been characterized. Rho-kinase and myosin phosphatase also regulate smooth muscle contraction in vertebrates by modulating myosin activity (Kureishi *et al.*, 1997; Uehata *et al.*, 1997; Hartshorne *et al.*, 1998) and their *C. elegans* orthologues LET-502 and MEL-11 are involved in ovulation (Wissmann *et al.*, 1999). However, they are expressed in the spermatheca but not in the myoepithelial sheath and implicated in spermathecal contraction and dilation (Wissmann *et al.*, 1999). Major sperm protein (Miller *et al.*, 2001) and an Eph receptor (Miller *et al.*, 2003) are identified as the upstream signals for sheath contraction. Therefore, based on the established function of troponin as a thin filament-linked calcium-switch for muscle contraction (Gordon *et al.*, 2000), it is likely that intracellular calcium is elevated by major sperm protein and Eph, which is then detected by troponin to activate actomyosin interaction.

The primary effect of RNAi of CeTM or *pat-10* was on contraction of the myoepithelial sheath. In addition, spermathecal dilation was severely impaired by *CeTM* (RNAi) and partially by *pat-10* (RNAi). However, CeTM was expressed in the spermatheca, but PAT-10 was not. Therefore, the spermathecal defect in *pat-10* (RNAi) worms might be a secondary effect of defective sheath contraction. It is proposed that the myoepithelial sheath pulls dilating spermatheca and facilitates dilation (McCarter *et al.*, 1997). Genetic studies show that spermathecal dilation is mediated by an inositol triphosphate pathway (Clandinin *et al.*, 1998; Bui and Sternberg, 2002), suggesting strongly that calcium is the

second messenger. Nonetheless, absence of troponin in the spermatheca suggests that spermathecal dilation is regulated by a different mechanism from sheath contraction. As briefly mentioned above, Rho-kinase and myosin phosphatase regulate spermathecal activity (Wissmann *et al.*, 1999), but the functional relationship between calcium and the Rho-kinase-myosin phosphatase pathway is not understood.

The myoepithelial sheath after RNAi of CeTM or *pat-10* did not seem to constrict the enclosed endomitotic oocytes, suggesting that the sheath cells are relaxed. However, if the function of the TM–TN complex is to inhibit the actomyosin interaction when the Ca^{2+} concentrations are low, perturbation of TM or TN is expected to cause hypercontraction. Therefore, it is possible that the activity of myosin is regulated by a separate mechanism, which may prevent hypercontraction when the TM–TN system is inhibited. Indeed, biochemical studies on isolated *C. elegans* myosin and actin demonstrated that *C. elegans* has both actin- and myosin-linked regulatory systems for the actomyosin activity (Harris *et al.*, 1977). Regulatory myosin light chains often play critical roles in the regulation of myosin activity, although the regulatory mechanisms are various. In addition to the phosphoregulation of myosin light chain in vertebrate smooth muscle, molluscan regulatory myosin light chains directly binds to Ca^{2+} and activate the myosin motor activity (reviewed in Szent-Györgyi, 1996). In *C. elegans*, two regulatory light chain genes, *mlc-1* and *mlc-2*, are expressed in body wall muscle (Rushforth *et al.*, 1998), but their biochemical properties and expression in the myoepithelial sheath are not understood. Alternatively, the possibility that TNC directly regulates myosin activity is not excluded be-

cause TNC is homologous to myosin light chains and calmodulin that are known to regulate myosin activity.

A mutation in the *unc-60B* ADF/cofilin gene suppressed the ovulation defects by RNAi of *CeTM* or *pat-10*. Our phenotypic analysis suggests that this suppression is due to alteration in the spermathecal activity in the *unc-60B* mutant. This is consistent with our previous immunolocalization of the UNC-60B protein in the spermatheca but not in the myoepithelial sheath (Ono *et al.*, 2003). We previously demonstrated that tropomyosin is antagonistic to UNC-60B-dependent actin filament dynamics in body wall muscle (Ono and Ono, 2002). Similar antagonistic mechanism in the spermatheca may explain why the ovulation defects of *CeTM* (RNAi) worms are suppressed by the *unc-60B* mutation. However, our observation that sheath contraction of *CeTM* (RNAi) worms was partially restored in the *unc-60B* mutant suggests that UNC-60B is expressed in the myoepithelial sheath at low levels and functions as a regulator of actin organization. In contrast, partial suppression of the ovulation defect of *pat-10* (RNAi) worms by the *unc-60B* mutation is probably due to enhanced spermathecal dilation in the *unc-60B* mutants. PAT-10 and UNC-60B are not enriched in the same cells in the gonad, and contraction of the myoepithelial sheath was not restored in the *unc-60B* mutants. Also, the paralyzed phenotype of *pat-10* (RNAi) worms was not suppressed by the *unc-60B* mutation (Figure 6h), suggesting distinct roles of PAT-10 and UNC-60B in muscle contraction and actin filament dynamics, respectively.

It was somewhat surprising that RNAi of *CeTM* caused aberrant cytokinesis in the endomitotic oocytes, because TM is essential for cytokinesis in fission yeast (Balasubramanian *et al.*, 1992). Our RNAi treatment did not completely eliminate the *CeTM* protein. Therefore, residual *CeTM* might be sufficient to support cytokinesis. Alternatively, an uncharacterized *CeTM* isoform(s) might be expressed in the oocytes. The *CeTM* gene, *tmy-1/lev-11*, undergoes alternative splicing to produce multiple isoforms (Kagawa *et al.*, 1995; Anyanful *et al.*, 2001). However, a nonmuscle isoform of *CeTM* has not been identified. Our anti-*CeTM* antibody weakly stains early embryos, but *CeTM* (RNAi) did not affect embryonic cytokinesis (our unpublished data). In vertebrates, >40 TM isoforms are produced and play distinct roles in different cellular events (Gunning *et al.*, 1998). Therefore, an uncharacterized *CeTM* isoform(s) might not be suppressed by our RNAi constructs and support cytokinesis in the endomitotic oocytes.

Although the mechanisms of ovulation-fertilization are various among different metazoan species, *C. elegans* is an attractive model to study communication between somatic gonad and germ cells. In this study, we identified TM and TN as essential regulators of cytoskeletal activity in the somatic gonad. Further genetic and cell biological studies on this system should reveal how sperm and oocytes influence activity of the TM-TN-actin system of the somatic gonad and how the gonadal activity affects oocyte maturation and fertilization.

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