Published in final edited form as:
Cell Motil Cytoskeleton. 2006 November ; 63(11): 659–672.

Dual Roles of Tropomyosin as an F-Actin Stabilizer and a Regulator of Muscle Contraction in *Caenorhabditis Elegans* Body Wall Muscle

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Abstract

Tropomyosin is a well-characterized regulator of muscle contraction. It also stabilizes actin filaments in a variety of muscle and non-muscle cells. Although these two functions of tropomyosin could have different impacts on actin cytoskeletal organization, their functional relationship has not been studied in the same experimental system. Here, we investigated how tropomyosin stabilizes actin filaments and how this function is influenced by muscle contraction in *Caenorhabditis elegans* body wall muscle. We confirmed the antagonistic role of tropomyosin against UNC-60B, a muscle-specific ADF/cofilin isoform, in actin filament organization using multiple UNC-60B mutant alleles. Tropomyosin was also antagonistic to UNC-78 (AIP1) in vivo and protected actin filaments from disassembly by UNC-60B and UNC-78 in vitro, suggesting that tropomyosin protects actin filaments from the ADF/cofilin-AIP1 actin disassembly system in muscle cells. A mutation in the myosin heavy chain caused greater reduction in contractility than tropomyosin depletion. However, the myosin mutation showed much weaker suppression of the phenotypes of ADF/cofilin or AIP1 mutants than tropomyosin depletion. These results suggest that muscle contraction has only minor influence on the tropomyosin's protective role against ADF/cofilin and AIP1, and that the two functions of tropomyosin in actin stability and muscle contraction are independent of each other.

Keywords

tropomyosin; ADF/cofilin; AIP1; myofibrils; actin dynamics

INTRODUCTION

Proper regulation of disassembly and stabilization of actin filaments are generally important for assembly, maintenance, and reorganization of actin cytoskeletal structures. Myofibrils, stable contractile apparatuses in muscle, are also subjected to constant exchange of actin subunits within the thin filaments [Imanaka-Yoshida et al., 1993; Komiyama et al., 1993; Littlefield et al., 2001], which is probably required for maintaining their organized structures. Actin depolymerizing factor (ADF)/cofilin enhances actin dynamics by severing actin filaments and accelerating monomer dissociation from the pointed ends [Bamburg, 1999; Bamburg et al., 1999], and its functional significance in muscle cells have been demonstrated in several organisms [Obinata et al., 1997; Ono 2003a]. On the other hand, tropomyosin is one of the major F-actin binding proteins in muscle and provides stability to actin filaments [Cooper, 2002; Gunning et al., 2005]. Tropomyosin slows down dissociation of actin

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Contract grant sponsor: National Science Foundation; Contract grant number: MCB-0110464; Contract grant sponsor: National Institute of Health; Contract grant number: R01 AR48615.

monomers from actin filaments [Lal and Korn, 1986; Hitchcock-DeGregori et al., 1988; Broschat et al., 1989; Broschat, 1990] and protects actin filaments from severing by gelsolin [Fattoum et al., 1983; Ishikawa et al., 1989a,b; Nyakern-Meazza et al., 2002] and ADF/cofilin (see below).

Several biochemical studies have demonstrated that ADF/cofilin and tropomyosin compete for binding to F-actin and antagonistically regulate actin filament dynamics *in vitro* [Bernstein and Bamburg, 1982; Mabuchi, 1982; Nishida et al., 1984, 1985]. A genetic study in the nematode *Caenorhabditis elegans* showed that tropomyosin has an inhibitory role for ADF/cofilin-mediated actin filament dynamics in the body wall muscle *in vivo* [Ono and Ono, 2002]. In *C. elegans*,the *unc-60* gene encodes two ADF/cofilin isoforms, UNC-60A and UNC-60B, which are generated by alternative pre-mRNA splicing [McKim et al., 1994; Anyanful et al., 2004]. UNC-60B is specifically expressed in body wall muscle, and mutations in *unc-60B* cause severe disorganization of actin filaments [Waterston et al., 1980; Zengel and Epstein, 1980; Ono et al., 1999, 2003]. *C. elegans* tropomyosin (CeTM) is encoded by the *lev-11/tmy-1* gene which produces multiple splice variants [Williams and Waterston, 1994; Kagawa et al., 1995]. RNA interference of CeTM induces disorganization of actin filaments in wild-type back-ground, but has minimal effects on an *unc-60B* mutant [Ono and Ono, 2002], suggesting that CeTM protects actin from disassembly by UNC-60B. Actin disassembly is also regulated by the *unc-78* gene encoding actin interacting protein 1 (AIP1) in body wall muscle [Ono, 2001; Mohri et al., 2006]. AIP1 is a conserved regulator of actin dynamics and enhances disassembly of actin filaments only in the presence of ADF/cofilin [Ono, 2003b]. The UNC-78 protein has very strong activity to enhance disassembly of UNC-60B-bound actin filaments *in vitro* [Mohri and Ono, 2003; Ono et al., 2004]. However, a regulatory mechanism of the activity of UNC-78 is unknown.

Tropomyosin is also well-characterized as a regulator of muscle contraction [Perry, 2003; Brown and Cohen, 2005]. In striated muscle, tropomyosin anchors troponin to the thin filaments [Ebashi and Kodama, 1966] and transmits a calcium signal to the thin filaments to activate actomyosin interaction [Ebashi, 1984; Squire and Morris, 1998; Gordon et al., 2000]. The actomyosin contractile activity is necessary for proper assembly of striated myofibrils [Soeno et al., 1999; De Deyne, 2000; Ramachandran et al., 2003; Kagawa et al., 2006]. However, excessive contraction can be a mechanical stress that disrupts the myofibril organization [Lieber et al., 1991; Friden and Lieber, 1992]. In *C. elegans*, disorganization of muscle actin filaments due to mutations in *unc-87*, a calponin-repeat protein [Goetinck and Waterston, 1994] or RNA interference of kettin, an immunoglobulin-like repeat protein [Ono et al., 2006] can be suppressed by a myosin mutation that reduces muscle contraction, suggesting that these F-actin-binding proteins also stabilize actin filaments. Thus, tropomyosin physically stabilizes actin filaments, but it also activates cycling of contraction and relaxation, which could lead to a mechanical stress to the myofibrils. However, these two functions of tropomyosin have not been examined in parallel in the same experimental system.

In this study, we investigated how tropomyosin functionally interacts with the ADF/cofilin-AIP1 actin disassembly system and how muscle contraction affects this interaction. We found that tropomyosin is antagonistic to ADF/cofilin and AIP1 *in vitro* and *in vivo*, and that muscle contraction has only a weak effect on this interaction *in vivo*. The results suggest that tropomyosin has two independent roles as a stabilizer of actin filaments and as a regulator of muscle contraction.

MATERIALS AND METHODS

C. elegans Strains and Culture

N2 (wild-type) and *unc-54(s95)* [Moerman et al., 1982] were obtained from *Caenorhabditis* Genetics Center (Minneapolis, MN). *unc-60B(r398)* and *unc-60B(s1309)* [McKim et al., 1988] were provided by Dr. David L. Baillie (Simon Fraser University, Burnaby, Canada). The original *unc-60B(r398)* strain was outcrossed three times with N2 by Dr. Myeongwoo Lee (Baylor University, Waco, TX) and provided to us. *unc-78(gk27)* [Ono, 2001] was provided by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia (Vancouver, Canada). Double mutant strains, *unc-54(s95)*;*unc-60B(r398)*, *unc-54 (s95)*;*unc-60B(s1309)*, and *unc-54(s95)*;*unc-78(gk27)* were generated by standard crosses, and homozygosity of the mutant alleles was confirmed by complementation tests. All mutants were examined as homozygotes. Nematodes were grown under standard conditions at 20°C as described previously [Brenner, 1974].

RNA Interference Experiments

Nematodes were treated with RNA interference (RNAi) for CeTM by feeding *Escherichia coli* expressing double-stranded RNA under conditions described previously [Ono and Ono, 2002]. Control experiments were performed with the *E. coli* strain HT115 (DE3) that was transformed with an empty RNAi vector L4440 (provided by Dr. Andrew Fire, Stanford University, Stanford, CA) [Timmons and Fire, 1998; Timmons et al., 2001]. Two vectors for RNAi of CeTM, TM1 for *CeTMI, II(RNAi)* (targeting two CeTM isoforms) and TM2 for *CeT-MI,II,III,IV(RNAi)* (targeting four CeTM isoforms), were described previously [Ono and Ono, 2002, 2004]. Phenotypes were analyzed in their F1 generation.

Worm Motility Assay

Worm motility was quantified as described [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.

Phalloidin Staining

Staining of worms with tetramethylrhodamine-phalloidin was performed as described previously [Ono, 2001]. Samples were viewed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope with a CFI Plan Fluor ELWD 40× objective (dry, N.A.= 0.60). Images were captured by a SPOT RT Monochrome CCD camera (Diagnostic Instruments, Sterling Heights, MI) and processed by the IPLab imaging software (Scanalytics, Rockville, MD) and Adobe Photoshop 6.0.

Actin Pelleting Assay

Rabbit muscle actin [Pardee and Spudich, 1982], CeTM [Ono and Ono, 2002], recombinant UNC-60B [Ono and Benian, 1998], and recombinant glutathione S-transferase (GST)-tagged UNC-78 [Mohri et al., 2004] were purified as described previously. Actin pelleting assays were performed as described previously [Mohri et al., 2004] with slight modifications. F-actin (10 μM) was pre-incubated with or without 5 μM CeTM for 30 min in a buffer containing 0.1 M KCl, 2 mM $MgCl_2$, 20 mM HEPES-NaOH, 1 mM dithiothreitol, pH 7.5. Then, final 10 μM UNC-60B and 0-2 μM GST-UNC-78 were added to the reactions, and they were incubated for 15 min. They were ultracentrifuged at 80,000 rpm (285,000× g) for 20 min in a Beckman TLA-100 rotor. The supernatants and pellets were adjusted to the same volumes and analyzed by SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250 (National Diagnostics, Atlanta, GA) and scanned by a UMAX PowerLook III scanner at 300 dpi, and the band intensity was quantified by Scion Image Beta 4.02 (Scion, Frederick, MD).

RESULTS AND DISCUSSION

Re-Evaluation of the Effect of CeTM-RNA Interference on *unc-60B* ADF/Cofilin Mutants

We previously demonstrated that RNA interference (RNAi) of *C. elegans* tropomyosin (CeTM) caused disorganization of actin filaments in body wall muscle and sterility due to ovulation defects in wild-type back-ground. This treatment did not worsen the actin organization in body wall muscle [Ono and Ono, 2002] or alter the ovulation process [Ono and Ono, 2004] in *unc-60B(r398)*, a weak loss-of-function mutant of *unc-60B* (a muscle-specific ADF/cofilin isoform). However, preliminary experiments by Dr. M. Lee's group (Baylor University, Waco, TX, personal communication) suggested that the *unc-60B(r398)* strain might have an RNAi-defective phenotype. Therefore, we compared the original *unc-60B (r398)* strain and the *unc-60B(r398)* strain that had been outcrossed three times with wild-type for their phenotypes after the CeTM-RNAi treatment.

Worm motility was measured to quantify the contractile activity of body wall muscle [Epstein and Thomson, 1974]. Motility of the original *unc-60B(r398)* strain was not greatly affected by the CeTM-RNAi treatments [Ono and Ono, 2002], while that of the outcrossed *unc-60B (r398)* strain was reduced 25 and 50% by *CeTMI,II (RNAi)* (targeting two CeTM isoforms) and *CeT-MI,II,III,IV (RNAi)* (targeting four CeTM isoforms), respectively (Fig. 1). The original *unc-60B(r398)* strain showed nearly normal ovulation and produced progeny even after the CeTM-RNAi treatments [Ono and Ono, 2004], whereas the outcrossed *unc-60B(r398)* strain became sterile by the CeTM-RNAi treatments as shown by the absence of embryos on the culture plates (Figs. 2E and 2F). Organization of actin filaments in the body wall muscle was not significantly modified by the CeTM-RNAi treatments in the original *unc-60B(r398)* strain [Ono and Ono, 2002]. However, in the outcrossed strain, the actin organization was improved by the CeTM-RNAi treatments with less actin aggregates and more striated myofibrillar actin filaments (Figs. 3D-3F). *CeTMI,II,III,IV (RNAi)* had stronger suppression effects on the actin organization than *CeTMI,II (RNAi)* (Fig. 3, compare E and F), which is in reverse correlation to their effects on worm motility (Fig. 1).

We conclude that the original *unc-60B(r398)* strain had a background mutation that weakens the effects of RNAi treatments, and that outcrosses successfully removed this mutation. Although the nature of the background mutation is unknown, the effect appears to be tissue- or gene-specific, because the protein levels of CeTM in total worm extracts were reduced in the original *unc-60B(r398)* strain by the CeTM-RNAi treatments [Ono and Ono, 2002], and RNAi of *pat-10* troponin C caused paralysis in the original *unc-60B(r398)* strain in a similar manner to wild-type [Ono and Ono, 2004]. Therefore, we need to re-evaluate our two previous conclusions based on the results obtained with the outcrossed *unc-60B(r398)* strain. First, we previously concluded that CeTM and UNC-60B antagonistically regulate the actin organization in the body wall muscle [Ono and Ono, 2002]. Our new results actually confirmed this conclusion with much more marked *in vivo* effects. *in vitro*, UNC-60B enhances actin filament dynamics [Ono and Benian, 1998; Ono et al., 1999; Yamashiro et al., 2005], while CeTM stabilizes actin filaments [Ono and Ono, 2002]. Thus, the result that RNAi of CeTM suppressed the *unc-60B* loss-of-function phenotype is consistent with their antagonistic roles on actin filament dynamics. Second, we previously concluded that CeTM and UNC-60B also antagonistically function during ovulation [Ono and Ono, 2004]. However, the new result that CeTM-RNAi caused sterility in the outcrossed *unc-60B(r398)* strain strongly suggests that the absence of ovulation defects in the original *unc-60B(r398)* strain was likely due to the background mutation but not the *unc-60B* mutation.

To determine if the suppression of the Unc-60B phenotype by the CeTM-RNAi treatments is an allele- or strain-specific event, we examined the effect of CeTM-RNAi on a different *unc-60B* allele. *unc-60B(s1309)* is a stronger loss-of-function allele than *unc-60B(r398)*

[McKim et al., 1988; Ono et al., 1999]. *unc-60B(r398)* produces a mutant UNC-60B protein with normal G-actin-binding but defective F-actin-binding and severing activities *in vitro* [Ono et al., 1999, 2001], while the mutant UNC-60B protein in *unc-60B(s1309)* shows severe defects in both G- and F-actin-binding and severing activities *in vitro* [Ono et al., 1999]. The *unc-60B (s1309)* homozygotes moved slower than *unc-60B(r398)* (Fig. 1, compare controls for *unc-60B (r398)* and *unc-60B (s1309)*). *CeTMI,II (RNAi)* significantly reduced motility of *unc-60B (s1309)* (Fig. 1 and Fig. 2H), and *CeT-MI,II,III,IV (RNAi)* caused nearly complete paralysis (Fig. 1, Fig. 2I). In contrast, actin organization in the body wall muscle of *unc-60B(s1309)* was improved by the CeTM-RNAi treatments (Figs. 3G-3I). Although the myofibrils are still disorganized, both *CeTMI,II (RNAi)* and *CeTMI,II,III,IV (RNAi)* similarly reduced formation of actin aggregates and enhanced organization of actin into a striated pattern (Figs. 3H and 3I). Thus, the antagonistic effects of CeTM and UNC-60B were also confirmed by a strong loss-of-function *unc-60B* allele, suggesting that their functional interaction is not allele- or strain-specific.

Interestingly, RNAi of CeTM and *unc-60B* mutations, in particular with a strong loss-of-function allele *unc-60B(s1309)*, synergistically reduced worm motility, (Fig. 1 and Figs. 2G-2I), while actin organization was improved by a combination of CeTM-RNAi and an *unc-60B* mutation (Figs. 3D-3I). These apparently paradoxical phenotypes suggest uncoupling of the regulation of actin organization from the regulation of muscle contraction. Although the combination of CeTM-RNAi and *unc-60B* mutations improved actin organization in the myofibrils (Figs. 3D-3I), other myofibrillar components that are required for contraction, such as troponin, might not be properly recruited to the thin filaments when CeTM is knocked down. Also, even if the actin filaments are apparently organized, dynamics of the myofibrillar proteins may not be properly regulated when CeTM and UNC-60B are impaired. In addition to dynamic exchange of actin subunits within myofibrils [Imanaka-Yoshida et al., 1993;Komiyama et al., 1993;Littlefield et al., 2001], even tropomyosin and troponin undergo constant turnover within mature myofibrils [Michele et al., 1999]. Such protein dynamics might be dependent on the activities of CeTM and UNC-60B to maintain normal contractile activity.

Suppression of the *unc-78* AIP1 Mutant Phenotype by CeTM-RNAi

UNC-78/AIP1 enhances disassembly of UNC-60B-bound actin filaments and is required for organized assembly of actin filaments in body wall muscle [Ono, 2001; Mohri and Ono, 2003; Mohri et al., 2006]. Therefore, we reasoned that UNC-78 might also be antagonistic to CeTM for actin organization in the body wall muscle. We tested functional interaction between UNC-78 and CeTM by examining CeTM-RNAi phenotypes in an *unc-78* mutant background. *unc-78(gk27)* is a null allele, and homozygous animals do not express the UNC-78 protein [Ono, 2001; Mohri and Ono, 2003]. *CeTMI,II (RNAi)* and *CeTMI,II,III,IV (RNAi)* reduced worm motility of *unc-78(gk27)* (Fig. 1), and the treated worms were nearly paralyzed on the culture plates (Figs. 2K and 2L). On the other hand, actin aggregates in the body wall muscle were diminished and striated organization was improved in the CeTM-RNAi-treated *unc-78 (gk27)* worms (Figs. 3J-3L). *CeTMI,II,III,IV (RNAi)* caused a stronger suppression effect than *CeTMI,II (RNAi)* (Fig. 3, compare K and L). These results are similar to the antagonism between CeTM and UNC-60B and strongly suggest that CeTM is also antagonistic to UNC-78 for organized assembly of actin filaments in the body wall muscle. Our results are consistent with the recently reported antagonism between AIP1 and tropomyosin for stability of actin cables in budding yeast [Okada et al., 2006]. Therefore, the antagonistic roles of tropomyosin and the ADF/cofilin-AIP1 actin disassembly system might be a conserved mechanism to regulate assembly and maintenance of actin cytoskeletal structures.

The antagonistic role of CeTM against the UNC-60B-UNC-78 system was further characterized *in vitro* by an actin pelleting assay (Fig. 4). Native CeTM was purified from a

crude thin filament fraction [Ono and Ono, 2002]. Therefore, the CeTM preparation is likely to be a mixture of multiple CeTM isoforms. However, the purified CeTM was resolved as a single band on SDS-PAGE, which corresponded to the size of high-molecular-weight isoforms, *CeTMI* and/or CeTMII [Kagawa et al., 1995]. F-actin (10 μM) was pre-incubated for 30 min with buffer alone or with 5 μM CeTM that should saturate F-actin binding [Ono and Ono, 2002], and then, UNC-60B and UNC-78 were added to the mixture. After incubating 15 min, extent of actin disassembly was determined by a pelleting assay as described in Materials and Methods. In the absence of CeTM in the pre-incubation, increasing concentrations of GST-UNC-78 (0-2 μM) enhanced disassembly of actin filaments and decreased the amounts of actin in the pellets as shown by SDS-PAGE (Fig. 4A, -CeTM; compare intensity of the actin bands in p). Densitometric quantification of actin in the pellets showed that less than 50% of actin sedimented in the presence of 2 μM GST-UNC-78, while more than 80% of actin was present in the pellet in the absence of GST-UNC-78 (Fig. 4B, black circles). However, when actin was pre-incubated with CeTM, actin disassembly was significantly inhibited (Fig. 4A, +CeTM; see that majority of actin was fractionated in p in the presence of 1.0 or 2.0 μM GST-UNC-78). Densitometric quantification indicated that more than 70% of actin sedimented in the presence of 2 μM GST-UNC-78 (Fig. 4B, white circles). The differences in the amounts of pelletable actin with and without CeTM were statistically significant ($P < 0.01$). Thus, these biochemical results show that CeTM binds to F-actin and protects it from actin disassembly by UNC-60B and UNC-78.

The biochemical results on the protective role of CeTM against actin disassembly by UNC-60B (ADF/cofilin) and UNC-78 (AIP1) are consistent with the *in vivo* data on the antagonism between CeTM and UNC-60B or UNC-78. AIP1 enhances disassembly of actin filaments in the presence of ADF/cofilin, while it has negligible effects on actin in the absence of ADF/cofilin [Aizawa et al., 1999; Okada et al., 1999; Rodal et al., 1999; Balcer et al., 2003; Mohri and Ono, 2003; Ono et al., 2004]. AIP1 has both actin- and ADF/cofilin-binding sites that are predicted to be necessary for its specific interaction with ADF/cofilin-bound actin filaments [Rodal et al., 1999; Mohri et al., 2004; Clark et al., 2006; Mohri et al., 2006; Okada et al., 2006]. Therefore, CeTM (tropomyosin) prevented decoration of actin filaments with UNC-60B (ADF/cofilin) as described previously [Ono and Ono, 2002], and, as a result, UNC-78 (AIP1) was unable to bind and disassemble CeTM-decorated actin filaments. Thus, one of the physiological functions of tropomyosin is to stabilize actin filaments by preventing them from disassembly by ADF/cofilin and AIP1.

Weak Suppression of the *unc-60B* and *unc-78* Phenotypes by a Myosin Mutation

Tropomyosin not only stabilizes actin filaments but also regulates muscle contraction. Reduction of motility in the CeTM-RNAi-treated worms is likely due to defective regulation of cycling of muscle contraction and relaxation. However, muscle contraction can cause a mechanical stress and destabilize the contractile apparatuses. Therefore, the suppression of the Unc-60B and Unc-78 phenotypes by CeTM-RNAi could also be due to reduced muscle contraction in addition to alteration in the actin dynamics. To examine the effect of muscle contraction on actin stability, muscle contraction was reduced by a mutation of the UNC-54 myosin heavy chain independently of CeTM, and its effect on the *unc-60B* or Unc-78 phenotypes was examined.

UNC-54 is the major myosin heavy chain in the body wall muscle [MacLeod et al., 1977; Miller et al., 1983]. The *unc-54(s95)* mutation impairs muscle contraction without affecting the structure of myofibrils [Moerman et al., 1982]. A missense mutation in *unc-54(s95)* converts Gly-118 to Arg that is located near the ATP-binding site of the myosin head [Dibb et al., 1985], suggesting that this mutation alters myosin ATPase activity but not myosin's ability to bind to actin and to assemble into thick filaments. This myosin mutation has been shown to

suppress disorganization of myofibrils due to loss of thin filament proteins, UNC-87 [Goetinck and Waterston, 1994] and kettin [Ono et al., 2006]. *CeT-MI,II (RNAi)* and *CeTMI,II,III,IV (RNAi)* reduced worm motility of *unc-54(s95)* (Fig. 1) with a stronger effect by *CeTMI,II,III,IV (RNAi)* than *CeTMI,II (RNAi)* (Fig. 1 and compare Figs. 2N and 2O). *CeTMI,II (RNAi)* and *CeTMI,II,III,IV (RNAi)* induced only minor disorganization of actin filaments in *unc-54(s95)* (Figs. 3N and 3O) as compared to wild-type in which *CeTMI,II (RNAi)* and *CeTMI,II,III,IV (RNAi)* caused formation of wavy actin bundles (Figs. 3B and 3C). These results suggest that disorganization of actin filaments by the CeTM-RNAi treatments is partly due to actomyosin contractility, and that this phenotype is suppressed by reduced myosin activity. This is also supported by the observation that *CeTMI,II (RNAi)* caused stronger actin disorganization in *unc-54(s95)* than *CeTMI,II,III,IV (RNAi)* (Fig. 3, compare N and O), which is in reverse correlation to their effects on worm motility (Fig. 1).

The *unc-54(s95)* mutation reduced worm motility of *unc-60B* or *unc-78* mutants (Fig. 1) and weakly suppressed disorganization of the actin filaments (Figs. 3M, 3P, 3S, and 3V). The actin organization was improved with diminished actin aggregates in *unc-54(s95);unc-60B(r398)* (Fig. 3, compare D and P), while it was only moderately improved and smaller actin bundles were still formed in *unc-54(s95);unc-60B(s1309)* (Fig. 3, compare G and S) or *unc-54 (s95);unc-78(gk27)* (Fig. 3, compare J and V). The phenotype of *unc-60B(r398)* was weaker than that of *unc-60B(s1309)*, which is probably why the extent of suppression by the myosin mutation was more noticeable in *unc-60B(r398)* than in *unc-60B(s1309)*. Importantly, worm motility was reduced by the myosin mutation to greater extents than by the CeTM-RNAi treatments (Fig. 1), yet the myosin mutation had much weaker suppression effects on actin organization than the CeTM-RNAi treatments (Figs. 3E, 3F, 3H, 3I, 3K, and 3L). This strongly suggests that reduced muscle contraction can only weakly suppress disorganized myofibrils when the actin disassembly system is defective, and that RNAi of CeTM suppresses this phenotype by altering the stability of actin filaments rather than by reducing contractile activity.

To further test the role of CeTM as a stabilizer of actin filaments, the *unc-54;unc-60B* or *unc-54;unc-78* double mutants were treated with *CeTMI,II (RNAi)* or *CeTMI,II, III,IV (RNAi)*. The double mutants were already paralyzed under the control conditions (Fig. 1 and Figs. 2P, 2S, and 2V), and either of the CeTM-RNAi treatments further reduced motility of *unc-54(s95);unc-60B(r398)* (Fig. 1 and Figs. 2P-2R) but did not have additional effects on motility of *unc-54(s95);unc-60B(s1309)* and *unc-54(s95);unc-78(gk27)* (Fig. 1 and Figs. 2S-2X). In all three double mutant strains, CeTM-RNAi reduced formation of actin aggregates and enhanced striated organization of actin filaments (Figs. 3P-3X). In the *unc-54 (s95);unc-60B(r398)* mutant, wavy actin bundles in control worms (Fig. 3P) were diminished and actin filaments were more clearly assembled into a striated pattern by *CeTMI,II (RNAi)* (Fig. 3Q) or *CeTMI,II,III,IV (RNAi)* (Fig. 3R). In the *unc-54(s95); unc-60B(s1309)* double mutant, *CeT-MI,II,III,IV (RNAi)* (Fig. 3U) showed a slightly stronger effect than *CeTMI,II (RNAi)* (Fig. 3T) in reducing long wavy actin aggregates that are found in the control worms (Fig. 3S). In the *unc-54(s95); unc-78(gk27)* double mutant, the *CeTMI,II (RNAi)* worms had thinner wavy actin bundles than the control worms (Fig. 3, compare W and V), while *CeTMI,II (RNAi)* apparently induced more extensive waviness to the actin bundles (Fig. 3W). However, *CeTMI,II,III,IV (RNAi)* significantly improved the actin organization with fewer abnormal actin bundles (Fig. 3, compare X and V).

These results demonstrate that RNAi of CeTM in already paralyzed double mutant worms can still cause significant suppression of the phenotypes in actin organization in the *unc-60B* and *unc-78* mutants. We observed several different patterns of actin disorganization. Typically, abnormal actin filaments became large aggregates (Figs. 3D, 3G, and 3J) or thin and wavy actin bundles (Figs. 3B, 3P, 3S, and 3W). Although we do not know how these abnormal structures are formed, there is tendency that severe defects in actin organization result in large

actin aggregates, while modest defects cause thin and wavy bundles. For example, formation of large actin aggregates in *unc-60B(r398)* (Fig. 3D) could be transformed into wavy actin bundles by *CeTMI,II (RNAi)* (Fig. 3E) or the *unc-54(s95)* mutation (Fig. 3P), suggesting that these phenotypes indicate severity in the defects of the actin-regulatory system. However, we cannot rule out the possibility that these phenotypes are caused by different mechanisms. Taken together, these observations support the function of CeTM as a stabilizer of actin filaments that is antagonistic to the ADF/cofilin-AIP1 actin disassembly system independently of its function as a regulator of muscle contraction.

CONCLUSION

Based on the current data and previous observations, we present a model of the relationship between actin dynamics and muscle contraction in organization of myofibrils (Fig. 5). Previous biochemical studies have shown that UNC-60B (ADF/cofilin) and UNC-78 (AIP1) cooperatively enhance actin dynamics by disassembling actin filaments [Ono et al., 1999;Mohri et al., 2006], while CeTM (tropomyosin) antagonistically stabilizes actin filaments [Ono and Ono, 2002]. These factors are expected to maintain the balance between dynamic and stable actin filaments (Fig. 5A), and stable actin filaments might be assembled into organized myofibrils by unknown mechanisms (Fig. 5A). As a separate function, CeTM activates actomyosin interaction and enhances muscle contraction (Fig. 5A). Moderate actomyosin contractility facilitates assembly of actin into myofibrils as shown in other experimental systems [Soeno et al., 1999;De Deyne, 2000;Ramachandran et al., 2003; Kagawa et al., 2006], although it has not been demonstrated in *C. elegans*. However, excessive contraction could disrupt the myofibril structure [Lieber et al., 1991;Friden and Lieber, 1992], and the actin filaments might be subjected to disassembly by the ADF/cofilin-AIP1 system (Fig. 5A).

When UNC-60B (ADF/cofilin) or UNC-78 (AIP1) is defective (Fig. 5B), the activity to disassemble actin is decreased, and stable actin filaments might be increased by CeTM. Although actin could still be assembled into myofibrils, excessive actin filaments may not be depolymerized and may form actin aggregates (Fig. 5B). Actomyosin contractility could partially disrupt myofibrils, and dissociated actin filaments may be incorporated into the actin aggregates. This hypothesis is supported by our results that the *unc-54(s95)* myosin mutation partially suppresses formation of actin aggregates in the *unc-60B* and *unc-78* mutants (Figs. 3P, 3S, and 3V). The mechanism of formation of actin aggregates is not known. However, actin aggregates in *unc-60B* mutants contain CeTM [Ono and Ono, 2002], but not α-actinin or vinculin [Ono et al., 2003], suggesting that CeTM might stabilize actin filaments in the aggregates. When CeTM is depleted in *unc-60B* or *unc-78* mutants (Fig. 5C), stable *vs*. dynamic actin filaments are better balanced, and proper amounts of actin might be integrated into myofibrils. Depletion of CeTM also reduces muscle contraction, which could reduce mechanical disruption of myofibrils. These models still need to be tested rigorously in *C. elegans* and other organisms. In particular, dynamics of actin filaments and other myofibrillar proteins are measurable in live cells using greenfluorescent protein-tagged proteins [Dabiri et al., 1999] and advanced microscopy techniques, such as fluorescence recovery after photobleaching [Lippincott-Schwartz et al., 2003]. These live cell studies should be very useful for testing these models.

Tropomyosin is associated with thin filaments in all muscle types and modulates contraction [Perry, 2003; Brown and Cohen, 2005]. In particular, in striated muscle, tropomyosin, together with troponin, is a core component of the actin-linked regulatory system for contraction [Ebashi, 1984; Squire and Morris, 1998; Gordon et al., 2000]. ADF/cofilin is also commonly expressed in muscle cells and believed to enhance reorganization of the actin filaments [Ono, 2003a]. Importantly, musclespecific ADF/cofilin isoforms are adapted for functions in muscle

cells in mammals [Ono et al., 1994; Mohri et al., 2000; Thirion et al., 2001; Vartiainen et al., 2002; Nakashima et al., 2005] and *C. elegans* [Ono and Benian, 1998; Ono et al., 1999; Yamashiro et al., 2005]. Therefore, tropomyosin is expected to be a common inhibitor of the ADF/cofilin-dependent actin dynamics in other muscle systems. In chick cardiac myocytes, tropomyosin and tropomodulin protect thin filaments from depolymerization [Mudry et al., 2003], most likely by inhibiting the action of ADF/cofilin. Mutations in tropomyosin are associated with several muscle diseases, including nemaline myopathy, hypertrophic cardiomyopathy, and dilated cardiomyopathy [Michele and Metzger, 2000; Tubridy et al., 2001; Clarkson et al., 2004; Chang and Potter, 2005]. Biochemical studies on some of the pathogenic mutations in tropomyosin showed that its activity to control the actomyosin interaction is impaired by the mutations [Golitsina et al., 1997; Moraczewska et al., 2000]. However, some of the pathogenic mutations may alter tropomyosin's activity to stabilize actin filaments. As a result, actin filaments might be exposed to the ADF/cofilin-AIP1 actin disassembly system, and the myofibrils might be disorganized. Thus, the dual functions of tropomyosin in contraction and actin stability could be crucial for maintaining normal muscle activity.

Tropomyosin is also associated with contractile actomyosin structures in non-muscle cells, such as stress fibers [Pittenger et al., 1994; Lin et al., 1997; Gunning et al., 2005]. In motile cells, tropomyosin is generally absent from the leading edge where ADF/cofilin is concentrated [DesMarais et al., 2002], with exception of several tropomyosin isoforms [Bryce et al., 2003; Hillberg et al., 2006]. However, tropomyosin localizes to lamella, which is the proximal region of membrane protrusions, and is proposed to recruit myosin and promote cell migration [Gupton et al., 2005]. Thus, during cell migration, the dual functions of tropomyosin might be important to convert dynamic actin filaments into a contractile structure and support actomyosin contraction. Further studies in other experimental systems might reveal general significance of the functions of tropomyosin in actin stability and actomyosin contractility.

ACKNOWLEDGMENTS

The authors thank Kanako Ono and Sawako Yamashiro for technical assistance. Some *C. elegans* strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institute of Health National Center for Research Resources.

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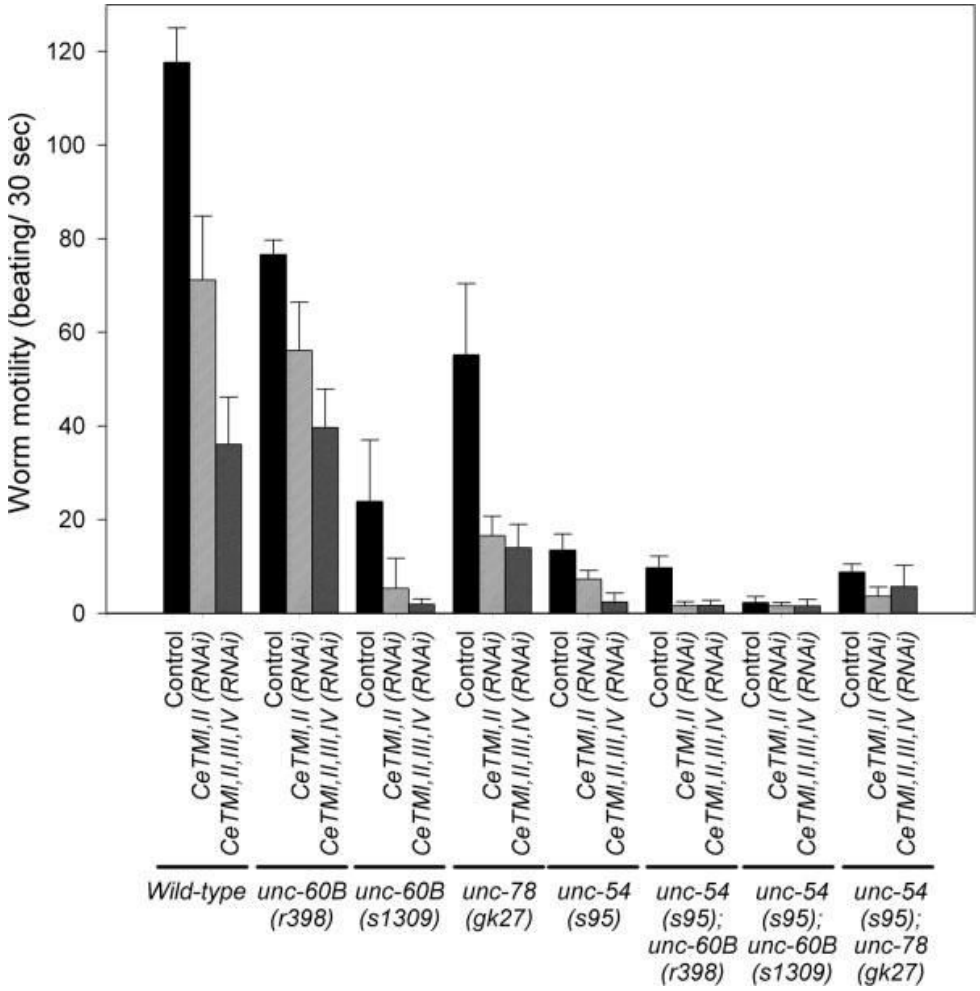

Fig. 1.

Effects of CeTM-RNAi and mutations in *unc-60B*, *unc-78*,and *unc-54* on worm motility. Motility of wild-type and other mutant worms with treatments with control RNAi, *CeTMI,II (RNAi)*,or *CeTMI,II,III,IV (RNAi)* was quantified as beating frequency. Data are means ± standard deviations (n = 10).

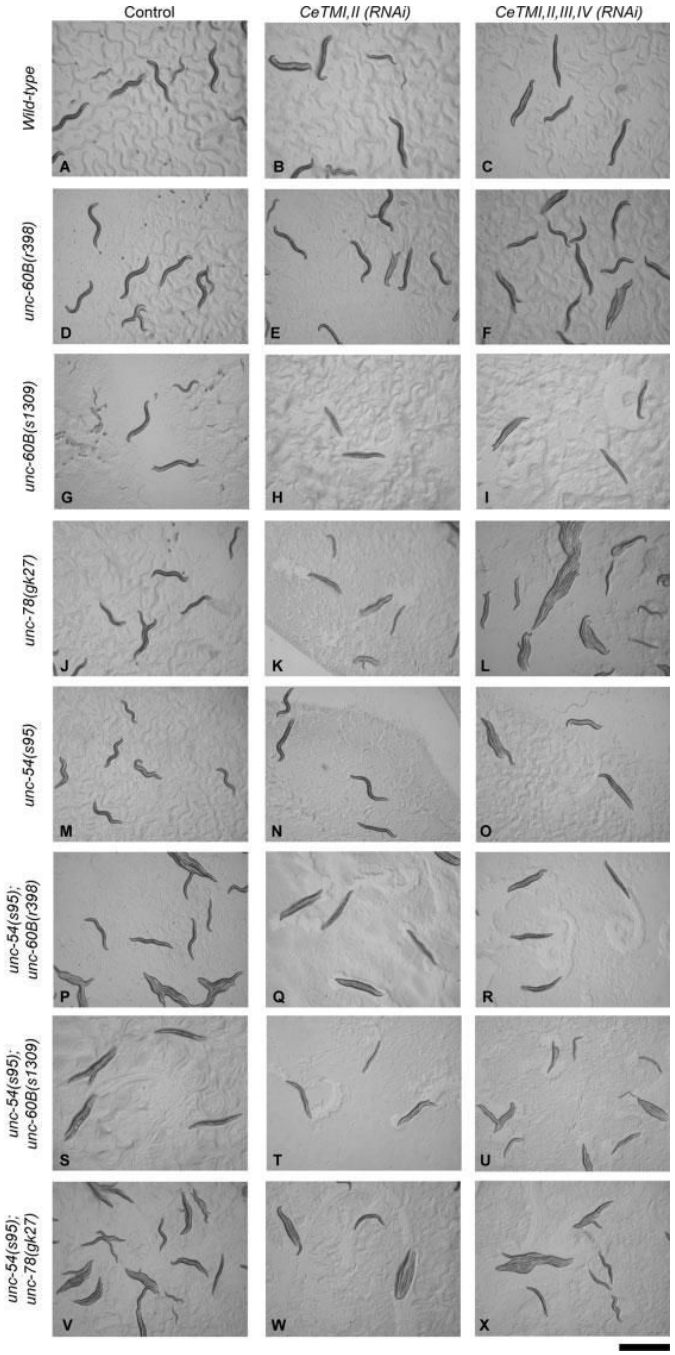

Fig. 2.

Effects of CeTM-RNAi and mutations in *unc-60B*, *unc-78*, and *unc-54* on worm movement. Micrographs of live adult worms on culture plates are shown. Wild-type worms showed typical sinusoidal movement (A). However, reduction in muscle contractility and paralysis were represented by increase in the appearance of more straight worms. Bar, 1.0 mm.

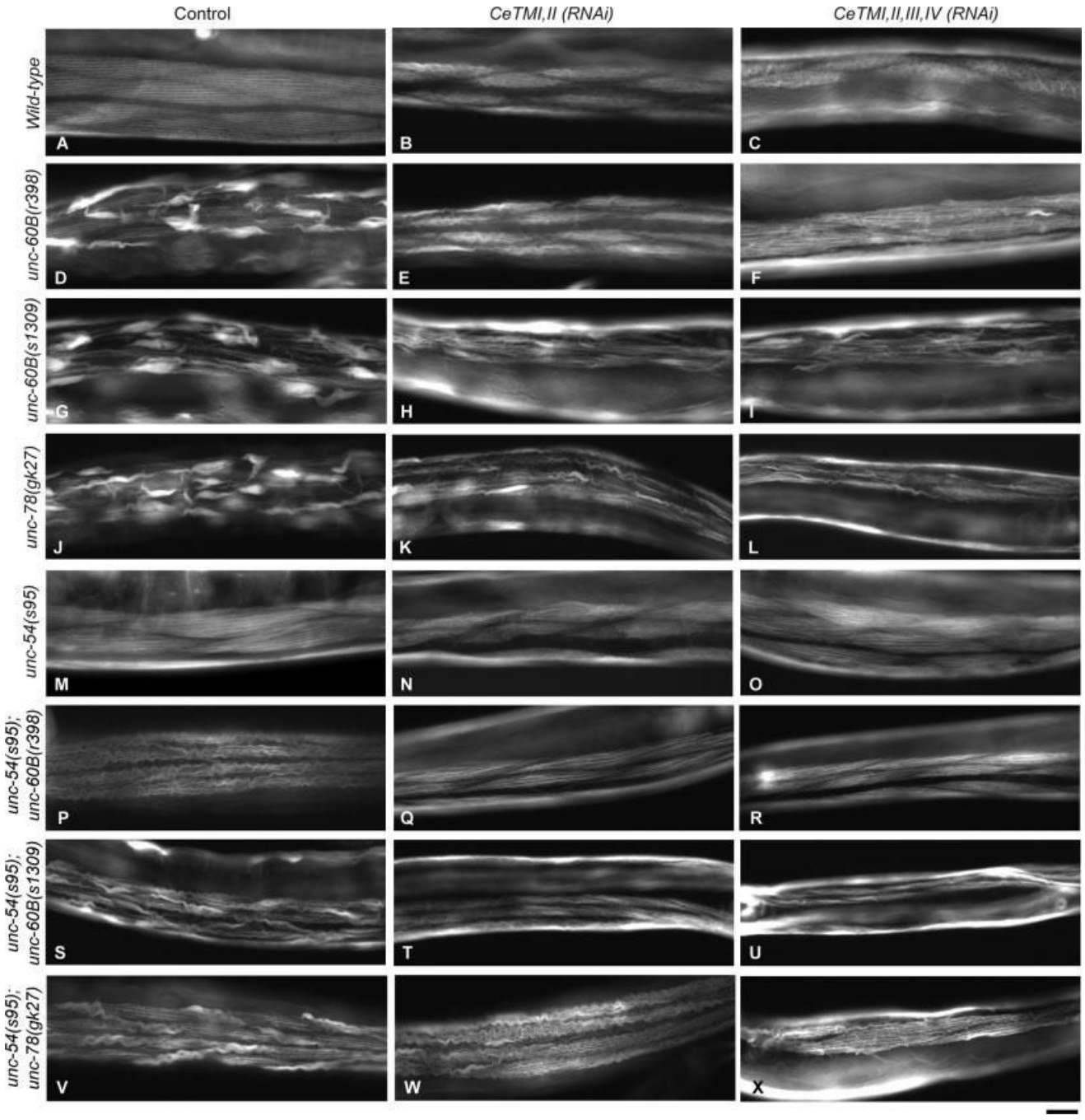

Fig. 3.

Effects of CeTM-RNAi and mutations in *unc-60B*, *unc-78*, and *unc-54* on actin organization in the body wall muscle. Adult worms were stained with tetramethylrhodamine-phalloidin to visualize F-actin. Wild-type muscle showed organized striated pattern of actin filaments (A). *CeTMI,II (RNAi)* or *CeTMI,II,III,IV (RNAi)* increased formation of wavy actin bundles in wild-type (B and C), while mutations in *unc-60B* (D and G) or *unc-78* (J) caused aggregation of actin filaments. The actin aggregates in *unc-60B* or *unc-78* mutants were suppressed by *CeTMI,II (RNAi)* (E, H, and K) or *CeT-MI,II,III,IV (RNAi)* (F, I, and L). The *unc-54* mutation partially suppressed the phenotype in the *unc-60B* (P and S) or *unc-78* (V) mutants. *CeTMI,II*

(RNAi) or CeTMI,II,III,IV suppressed formation of actin aggregates in the *unc-54*;*unc-60B* (Q, R, T, and U) or *unc-54*;*unc-78* (W and X) double mutants. Bar, 20 μm.

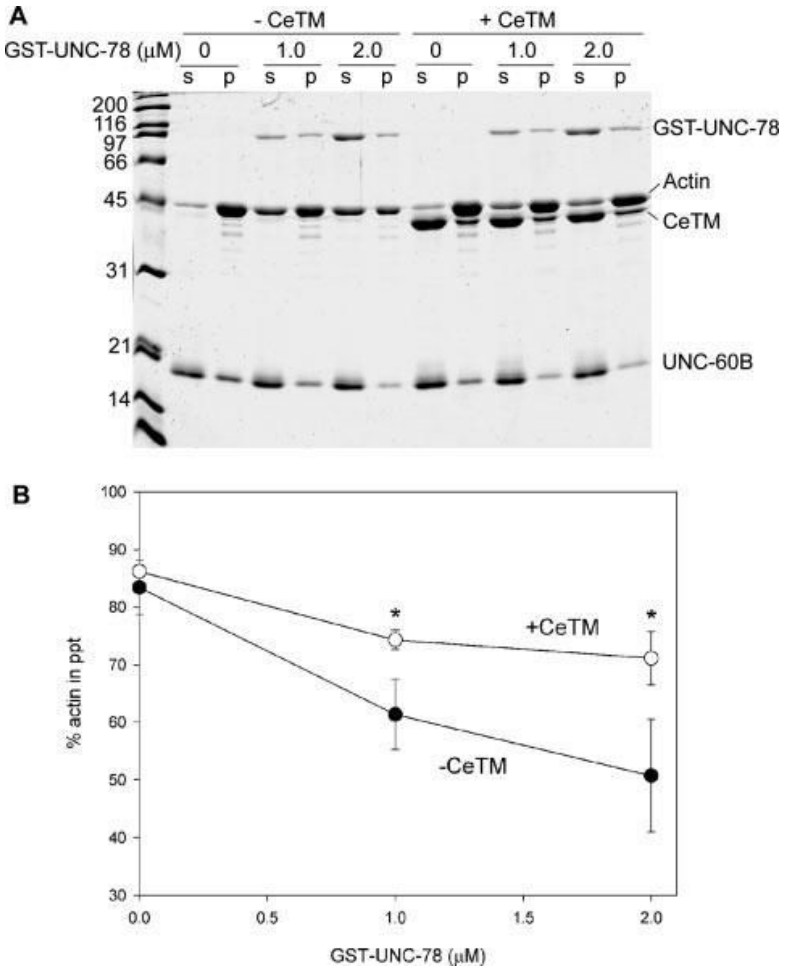

Fig. 4.

An inhibitory effect of CeTM on actin disassembly by UNC-60B and UNC-78. (A) 10 μM F-actin was pre-incubated with or without 5 μM CeTM for 30 min, and then, final 10 μM UNC-60B and 0-2 μM GST-UNC-78 were added. After incubating 15 min, the reactions were ultracentrifuged and fractionated into supernatants (s) and pellets (p). (B) Quantitative data of the actin pelleting assays. Percentages of actin in the pellets are plotted as a function of the concentrations of GST-UNC-78. Black circles are data without CeTM, and white circles are data with 5 μM CeTM. Data are means ± standard deviations of three experiments. Asterisks indicate $P < 0.01$ by the t-test.

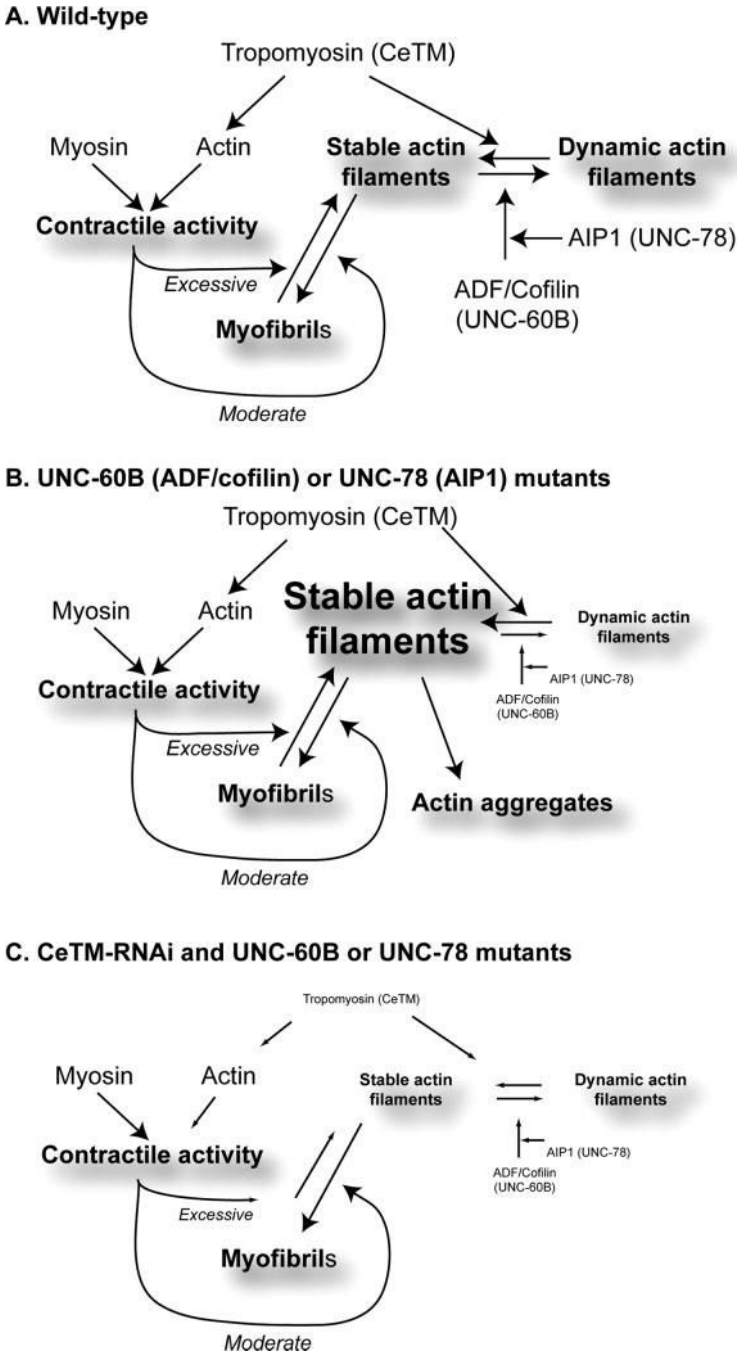

Fig. 5.

Models of the dual functions of tropomyosin in actin stability and actomyosin contractility in assembly and maintenance of myofibrils. Models are proposed for wild-type (A), UNC-60B (ADF/cofilin) or UNC-78 (AIP1) mutants, or CeTM-RNAi and UNC-60B or UNC-78 mutants (C).