Sarcomeric actin organization is synergistically promoted by tropomodulin, ADF/cofilin, AIP1 and profilin in *C. elegans*

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Summary

Sarcomeric organization of thin and thick filaments in striated muscle is important for the efficient generation of contractile forces. Sarcomeric actin filaments are uniform in their lengths and regularly arranged in a striated pattern. Tropomodulin caps the pointed end of actin filaments and is a crucial regulator of sarcomere assembly. Here, we report unexpected synergistic functions of tropomodulin with enhancers of actin filament dynamics in *Caenorhabditis elegans* striated muscle. Pointed-end capping by tropomodulin inhibited actin filament depolymerization by ADF/cofilin in vitro. However, in vivo, the depletion of tropomodulin strongly enhanced the disorganization of sarcomeric actin filaments in ADF/cofilin mutants, rather than antagonistically suppressing the phenotype. Similar phenotypic enhancements by tropomodulin depletion were also observed in mutant backgrounds for AIP1 and profilin. These in vivo effects cannot be simply explained by antagonistic effects of tropomodulin and ADF/cofilin in vitro. Thus, we propose a model in which tropomodulin and enhancers of actin dynamics synergistically regulate elongation and shortening of actin filaments at the pointed end.

Key words: Actin dynamics, Myofibrils, Striated muscle, Pointed end

Introduction

In striated muscle, contractile proteins are assembled into sarcomeres that contain highly ordered arrangements of actin thin filaments and myosin thick filaments. Sarcomeric actin filaments are straight and uniform in length. This is in contrast to in vitro polymerized actin filaments that can be much longer and more variable in length than muscle thin filaments. Therefore, polymerization and depolymerization of actin must be precisely regulated during assembly and maintenance of striated myofibrils. However, the regulatory mechanism of assembly and maintenance of sarcomeric actin filaments is not clearly understood.

Several actin-associated proteins have been implicated in organized assembly of actin filaments in striated muscle (Littlefield and Fowler, 1998; Obinata, 1993). Nebulin is important for sarcomere assembly in vertebrate muscle (Bang et al., 2006; McElhinny et al., 2005; Witt et al., 2006). These cell-biological and genetic studies revealed that nebulin deficiency alters thin filament length but not uniformity of the length, which is inconsistent with the proposed function of nebulin as a ruler for thin filaments (Labeit et al., 1991). Rather, more recent studies suggest that nebulin regulates actin filament dynamics by interacting with capping proteins (Fowler et al., 2006; McElhinny et al., 2001; Pappas et al., 2008). Tropomodulin (Tmod) and capping protein cap pointed and barbed ends of actin filaments, respectively, and limit polymerization and depolymerization from filament ends (Gregorio et al., 1995; Schafer et al., 1995). Actin depolymerizing factor (ADF)/cofilin and actin interacting protein 1 (AIP1, also known as WDR1) enhance disassembly of actin filaments and are required for organized assembly of actin filaments in *Caenorhabditis elegans* muscle (Ono, 2001; Ono et al., 1999). Tropomyosin protects actin filaments from severing by ADF/cofilin and AIP1, and stabilizes sarcomeric actin organization (Ono and Ono, 2002; Yu and Ono, 2006). Recently, *Drosophila melanogaster* SALS (Bai et al., 2007) and vertebrate leiomodin (Chereau et al., 2008) have been identified as new regulators of actin polymerization in muscle. A number of mutations in genes coding for these actin-regulatory proteins, including nebulin, tropomyosin and cofilin, cause human hereditary myopathies (Laing, 2007), indicating that correct regulation of sarcomeric actin organization is crucial for normal function of striated muscle. In vivo, these actin regulators might function together to promote sarcomeric actin organization, but the functional relationship among these proteins is not completely understood.

Although both ends of sarcomeric actin filaments are capped, actin is still dynamically polymerized and depolymerized at these ends (Littlefield et al., 2001). To maintain constant filament length, capping of pointed ends by Tmod is particularly important (Gregorio et al., 1995; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001). Tmod caps the pointed end of actin filaments and inhibits both polymerization and depolymerization. Tropomyosin binds to Tmod and enhances capping activity (Fischer and Fowler, 2003). Gene knockout of tropomodulin 1 (Tmod1) in mice results in impaired assembly of myofibrils in the embryonic heart (Chu et al., 2003; Fritz-Six et al., 2003). However, the phenotypic consequences of Tmod inhibition are somewhat complex. Inhibition of Tmod in cardiac myocytes by antibody injection causes either elongation of actin filaments from their pointed ends (Gregorio et al., 1995) or disassembly of thin filaments (Mudry et al., 2003).

Concentrations of free actin monomers and activity of actin filament severing proteins could influence the dynamics of actin filaments when Tmod is inhibited. Therefore, we were motivated to investigate functional relationship between Tmod and enhancers of actin filament dynamics.

The body-wall muscle of the nematode *C. elegans* is obliquely striated muscle and has organized sarcomeres (Waterston, 1988). ADF/cofilin (UNC-60B) (Ono et al., 2003; Ono et al., 1999) and AIP1 (Ono, 2001) enhance actin filament dynamics and are required for assembly of sarcomeric actin filaments. Tropomyosin (Ono and Ono, 2002), kettin (Ono et al., 2006) and UNC-87 (a calponin-like protein) (Yamashiro et al., 2007) bind to the side of actin filaments and stabilize them. PFN-3 is a muscle-specific profilin isoform, but its function is still unclear because a *pfn-3* null mutant shows only minor muscle defects (Polet et al., 2006). Recently, *unc-94/tmd-1* has been demonstrated to encode Tmod that is expressed in the body-wall muscle (Stevenson et al., 2007). Thus, *C. elegans* is an excellent model system to examine how Tmod might functionally interact with other regulators of actin filament dynamics. In addition, the *C. elegans* genome does not have genes encoding homologs of nebulin, SALS or leiomodin, suggesting that the *C. elegans* muscle uses a conserved core mechanism to regulate sarcomeric actin organization. In this study, we found that Tmod promotes sarcomeric actin organization synergistically with enhancers of actin dynamics including ADF/cofilin, AIP1 and profilin. This in vivo effect cannot be simply explained by the antagonistic activity of Tmod against ADF/cofilin-mediated actin depolymerization observed in vitro. We propose that Tmod synergistically functions with enhancers of actin dynamics for organized assembly of sarcomeric actin filaments in vivo.

Results

An *unc-94/tmd-1* mutation causes severe disorganization of actin filaments in the body-wall muscle

Previous studies have demonstrated that point mutations in the *unc-94/tmd-1* gene or RNAi experiments targeting *unc-94/tmd-1* result in disorganization of sarcomeric actin filaments in body-wall muscle (Stevenson et al., 2007; Zengel and Epstein, 1980). However, the reported phenotypes are relatively mild, and the mutant or double-stranded RNA (dsRNA)-treated worms retain substantially organized sarcomeric actin filaments. This is in contrast to very severe defects in the cardiac muscle of tropomodulin-knockout mice (Chu et al., 2003; Fritz-Six et al., 2003). We found that a new *unc-94/tmd-1* allele, *tm724*, causes very severe defects in the actin filament organization of the body-wall muscle (Fig. 1). *unc-94/tmd-1(tm724)* has a deletion of 685 bp that removes most of the fourth exon that is common to the two splice variants (Fig. 1A). Even if the third exon splices onto the fifth exon, it is predicted to cause a frame shift, such that the resultant mRNA encodes only the N-terminal 126 amino acids, lacking the leucine-rich repeats and the C-terminal actin-capping site. By western blotting, the TMD-1 protein was significantly reduced in *unc-94/tmd-1(tm724)* worms (Fig. 1B), although weak bands around 50-60 kDa were inconsistently detected (our unpublished data). *unc-94/tmd-1(tm724)* was recessive and *unc-94/tmd-1(tm724)/+* heterozygotes were indistinguishable from wild-type worms (data not shown). These results suggest that this is a null- or strong loss-of-function allele, although the possibility that this allele has a neomorphic effect cannot be excluded.

unc-94/tmd-1(tm724) homozygotes had severe defects in the actin filament organization of the body-wall muscle in embryonic to adult stages (Fig. 1C). In wild-type embryos, actin filaments are assembled into myofibrils, and continuous accumulations of actin filaments were clearly detected in the body-wall muscle (Fig. 1Ca). However, in *unc-94/tmd-1(tm724)*, the actin arrays were often discontinuous and a number of aggregates were detected (Fig. 1Cb). Adult *unc-94/tmd-1(tm724)* worms appeared flaccid and moved more slowly than wild-type animals (Table 1). In addition, *unc-94/tmd-1(tm724)* hermaphrodites produced a number of arrested embryos and early larva (38% lethal, n=407). Time-lapse Nomarski observation revealed that 45% of arrested embryos (n=22) were paralyzed at about the 3.5-fold stage (~500 minutes after the first cell division) and failed to hatch, which is consistent with severe defects in the assembly of body-wall muscle (Williams and Waterston, 1994). Actin filaments in the adult body-wall muscle of

Table 1. Motility of adult worms in various mutant backgrounds after RNAi treatment

Genotype	dsRNA	Worm motility (beating/30 second ± s.d.; n=10)
Wild-type	control	118±3.55
Wild-type	*unc-94/tmd-1*	103±3.08
unc-94/tmd-1(tm724)		73.3±9.98
unc-60B(r398)	control	72.0±10.1
unc-60B(r398)	*unc-94/tmd-1*	16.4±7.06
unc-60B(s1307)	control	66.3±15.4
unc-60B(s1307)	*unc-94/tmd-1*	18.5±12.7
unc-60B(s1309)	control	19.3±10.5
unc-60B(s1309)	*unc-94/tmd-1*	12.4±8.42
unc-60B(m35)	control	17.4±7.15
unc-60B(m35)	*unc-94/tmd-1*	12.0±7.83
unc-78(gk27)	control	89.2±4.49
unc-78(gk27)	*unc-94/tmd-1*	24.2±10.3
pfn-2(ok458)	control	114±6.12
pfn-2(ok458)	*unc-94/tmd-1*	105±3.22
pfn-3(tm1362)	control	114±4.54
pfn-3(tm1362)	*unc-94/tmd-1*	102±3.34
pfn-3(tm1362) pfn-2(ok458)	control	111±2.60
pfn-3(tm1362) pfn-2(ok458)	*unc-94/tmd-1*	98.7±3.95
unc-60B(r398); pfn-3(tm1362) pfn-2(ok458)	control	72.5±13.3
unc-60B(r398); pfn-3(tm1362) pfn-2(ok458)	*unc-94/tmd-1*	15.7±8.29
unc-60B(s1309); pfn-3(tm1362) pfn-2(ok458)	control	19.0±10.5
unc-60B(s1309); pfn-3(tm1362) pfn-2(ok458)	*unc-94/tmd-1*	12.2±4.26

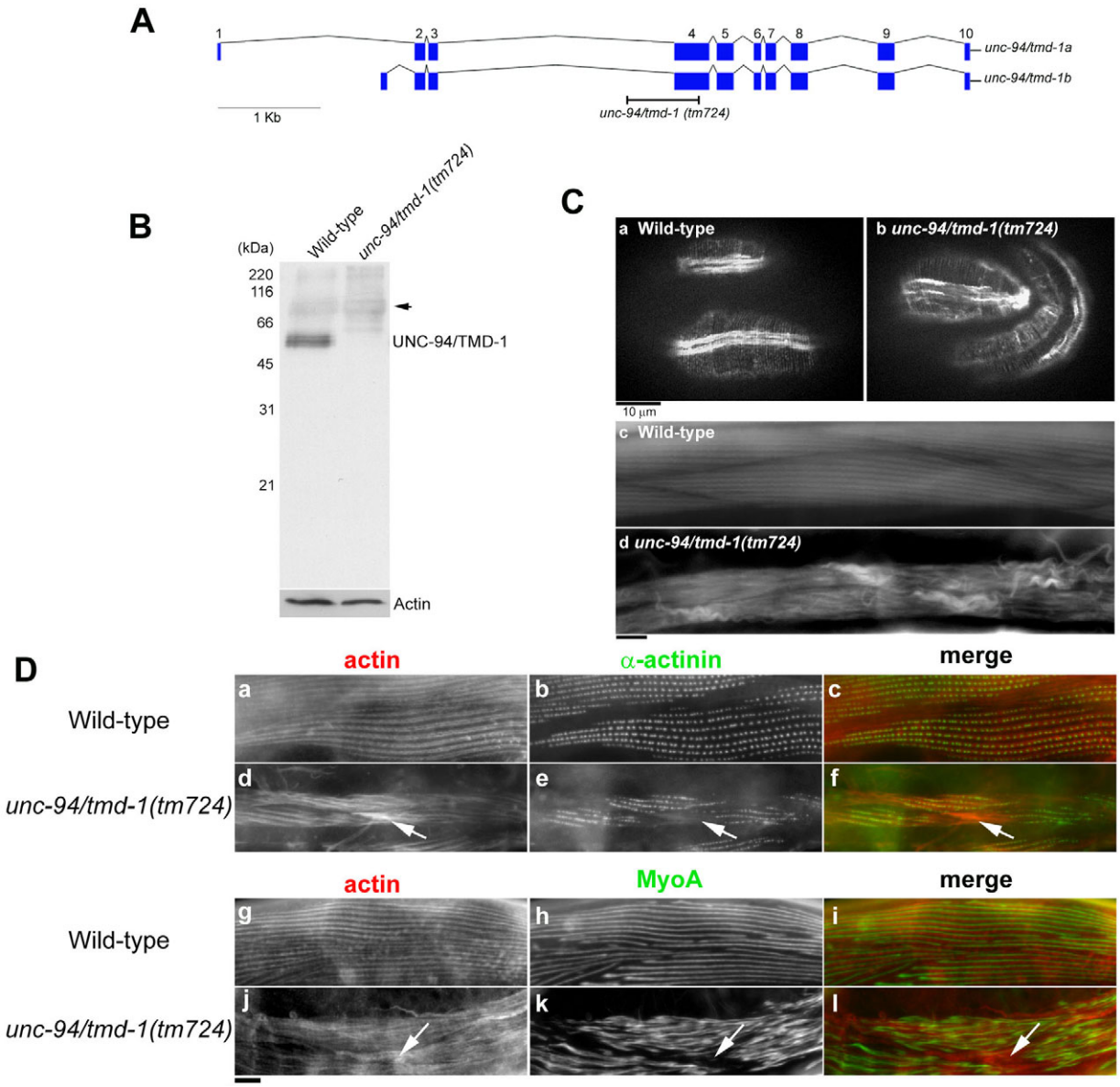

Fig. 1. Mutation of *unc-94/tmd-1* causes severe disorganization of actin filaments in body-wall muscle. (A) Genomic organization of the *unc-94/tmd-1* gene. Exons are indicated by boxes. Two alternatively spliced isoforms are generated as described (Stevenson et al., 2007). The position of the *unc-94/tmd-1(tm724)* deletion is indicated. (B) Western blot analysis of the TMD-1 protein in wild-type and *unc-94/tmd-1(tm724)* adult worms. The bands at ~68 kDa (arrow) are not related to the TMD-1 protein (Stevenson et al., 2007). Anti-actin antibody was used to monitor equal loading of the protein samples. (C) Actin filaments in embryos (a,b) and adults (c,d) were visualized by staining with tetramethylrhodamine-phalloidin in wild-type (a,c) and *unc-94/tmd-1(tm724)* (b,d). Bars, 10 µm. (D) Organization of α-actinin (a-f) and myosin (g-l) in body-wall muscle. Wild-type (a-c and g-i) or *unc-94/tmd-1(tm724)* (d-f and j-l) were immunostained for actin (a,d,g,j) and α-actinin (b,e) or MyoA myosin heavy chain (h,k). Merged images are shown in c, f, i and l (actin in red and α-actinin or MyoA in green). Arrows indicate positions of actin aggregates where α-actinin or myosin did not localize. Bar, 10 µm.

unc-94/tmd-1(tm724) were severely disorganized (Fig. 1Cd) as compared with striated actin organization in wild-type muscle (Fig. 1Cc). In *unc-94/tmd-1(tm724)*, the striated pattern of actin filaments was difficult to discern, and abnormal accumulations of actin filaments often formed near both ends of spindle-shaped muscle cells (Fig. 1Cd). Disorganized filament organization in *unc-94/tmd-1(tm724)* was also observed by electron microscopy (our unpublished data).

Other components of myofibrils were only mildly disorganized in *unc-94/tmd-1(tm724)* worms. Localization of α-actinin, a component of dense bodies, was only slightly affected in *unc-94/tmd-1(tm724)* animals (Fig. 1Dd-f). In wild-type worms, α-actinin localizes to dense bodies in a punctate pattern (Fig. 1Da-c). In *unc-94/tmd-1(tm724)* worms, a similar pattern of α-actinin was detected, although some neighboring dots often appeared continuous (Fig. 1De). α-actinin did not localize to the actin aggregates (Fig. 1Dd-f, arrows), which is similar to the observation in Tmod1-knockout mice (Fritz-Six et al., 2003). Striation of myosin thick filaments was less organized in *unc-94/tmd-1(tm724)* than wild-type animals (Fig. 1Dg-l). In *unc-94/tmd-1(tm724)* worms, bands of myosin heavy chain were often wider than those in wild-type, and spacing between myosin bands was irregular (Fig. 1Dk).

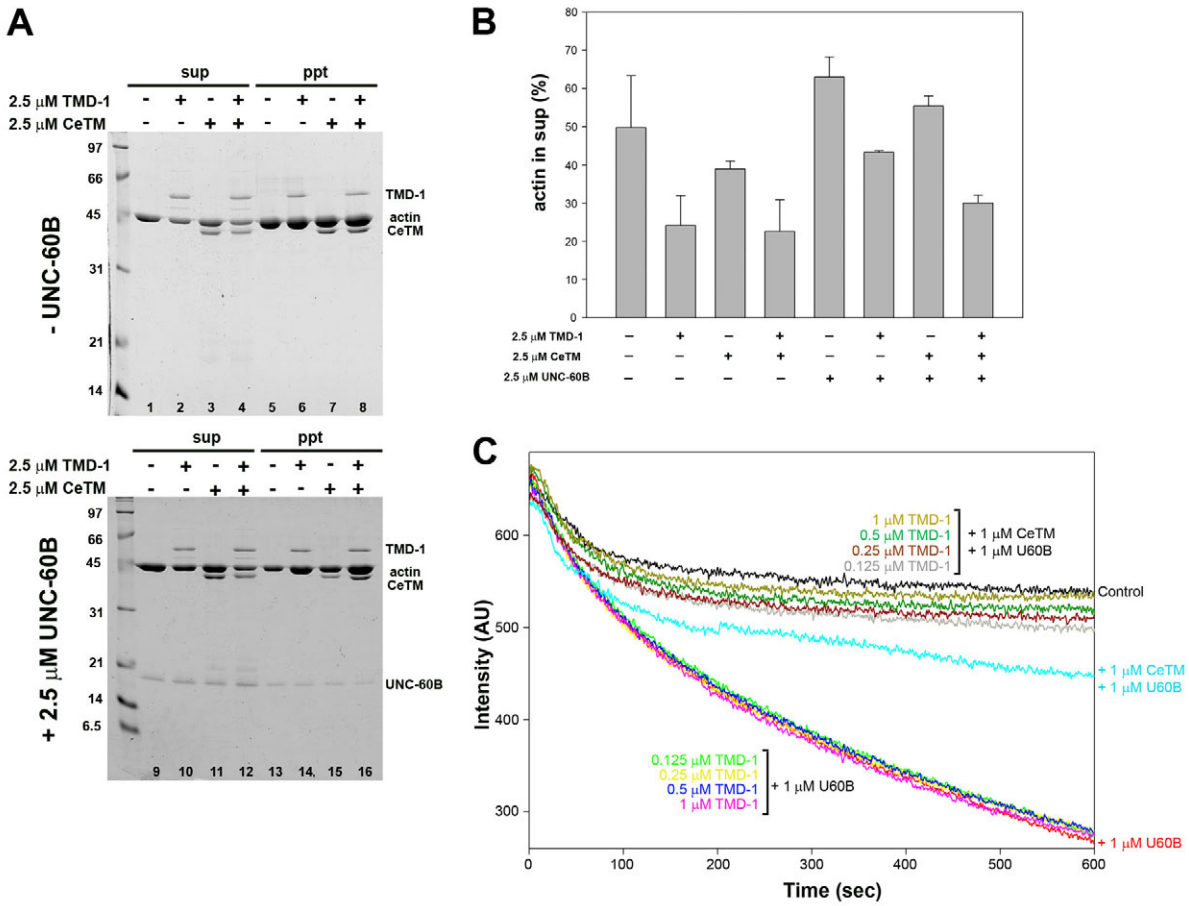

Fig. 2. TMD-1 inhibits UNC-60B (ADF/cofilin)-dependent depolymerization of actin filaments in vitro. (A,B) Depolymerization of CapZ-capped actin filaments in the presence of Lat-A was examined by a pelleting assay. CapZ-capped F-Ce-actin (5 μM actin, CapZ:actin ratio of 1:100) was incubated with various combinations of TMD-1, CeTM, and UNC-60B in the presence of 10 μM Lat-A for 15 minutes. The mixtures were ultracentrifuged and fractionated into supernatants (sup) and pellets (ppt) and analyzed by SDS-PAGE (A) and densitometric quantification of depolymerized actin (%) in the supernatants (B). Data are the mean ± s.d. of three experiments. (C) Kinetic measurements of depolymerization of CapZ-capped actin filaments. CapZ-capped F-Ce-actin (CapZ: actin ratio of 1:100; 10% labeled with pyrene) was diluted to 0.5 μM actin with 1 μM Lat-A in the presence of indicated combinations of UNC-60B, CeTM, and TMD-1. Depolymerization of actin was monitored by decrease in pyrene fluorescence.

Again, myosin was not found in the actin aggregates (Fig. 1Dj-l, arrows). These results suggest that mild disorganization of dense bodies and thick filaments is a secondary effect of severely disorganized actin filaments.

C. elegans tropomodulin protects against actin depolymerization from the pointed end mediated by ADF/cofilin in vitro

Tmod caps the pointed end of actin filaments and regulates actin dynamics at the pointed ends in muscle cells (Fischer and Fowler, 2003). However, ADF/cofilin enhances actin depolymerization at the pointed ends (Carlier et al., 1997; Maciver et al., 1998; Yamashiro et al., 2005) and is a crucial regulator of actin dynamics in muscle cells (Ono, 2003a; Ono, 2007). Therefore, we hypothesized that Tmod and ADF/cofilin have opposite roles in regulating actin dynamics at the pointed end.

We tested whether Tmod has a protective role against pointed-end depolymerization induced by ADF/cofilin in vitro. Bacterially expressed UNC-94/TMD-1 protein has typical Tmod-like pointed-end capping activity (E.A.C., S.Y., R. Zaidel-Bar, S.O. and J.H., unpublished). CapZ-capped *C. elegans* F-actin (hereafter referred to as F-Ce-actin) (CapZ:actin ratio of 1:100) was incubated for 15 minutes with latrunculin A (Lat-A) in the presence of TMD-1, *C. elegans* tropomyosin (hereafter referred to as CeTM), UNC-60B or combinations of these proteins, and ultracentrifuged to separate monomers and short oligomers from long filaments (Fig. 2A). In the presence of Lat-A, which sequesters G-actin, ~50% of actin spontaneously depolymerzed (Fig. 2A, lanes 1 and 5; Fig. 2B). TMD-1 strongly inhibited depolymerization to ~20% (Fig. 2A, lanes 2 and 6; Fig. 2B), while CeTM had only a weak inhibitory effect (Fig. 2A, lanes 3 and 7; Fig. 2B). The combination of TMD-1 and CeTM had a similar effect to TMD-1 alone (Fig. 2A, lanes 4 and 8; Fig. 2B). By contrast, UNC-60B enhanced depolymerization to ~70% (Fig. 2A, lanes 9 and 13; Fig. 2B). This effect was moderately inhibited by either TMD-1 alone (Fig. 2A, lanes 10 and 14; Fig. 2B) or CeTM alone (Fig. 2A, lanes 11 and 15; Fig. 2B) and strongly inhibited in the presence of both TMD-1 and CeTM (Fig. 2A, lanes 12 and 16; Fig. 2B). Thus, the combination of TMD-1 and CeTM strongly antagonizes UNC-60B-induced actin depolymerization in vitro.

Effects of UNC-60B, TMD-1 and CeTM on actin depolymerization were further examined by a kinetic assay. CapZ-capped F-Ce-actin

(CapZ: actin ratio of 1:100) was diluted to 0.5 μM actin in the presence of Lat-A and these proteins, and depolymerization was monitored by measurement of pyrene fluorescence. UNC-60B enhanced depolymerization (Fig. 2C, red curve) as previously reported (Yamashiro et al., 2005). TMD-1 alone (0.125–1 μM) did not inhibit UNC-60B-induced depolymerization (Fig. 2C), whereas CeTM alone showed a relatively strong inhibitory effect on UNC-60B-induced depolymerization (Fig. 2C, light blue curve). The combination of TMD-1 and CeTM inhibited depolymerization more strongly than either TMD-1 or CeTM alone (Fig. 2C). 0.125 μM TMD-1 plus 1 μM CeTM (Fig. 2C, gray curve) had a stronger effect than 1 μM CeTM alone (Fig. 2C, light blue curve), and 1 μM TMD-1 plus 1 μM CeTM (Fig. 2C, dark yellow curve) inhibited depolymerization to a rate similar to controls (capped F-actin alone and Lat-A). These results indicate that TMD-1 and CeTM synergistically inhibit actin depolymerization induced by UNC-60B in vitro.

Tropomodulin synergistically functions with ADF/cofilin and AIP1 in actin filament organization in the *C. elegans* body-wall muscle

Our in vitro data shown in Fig. 2 indicate that Tmod and ADF/cofilin antagonistically regulate actin dynamics at the pointed end. However, Tmod and ADF/cofilin can cause similar effects in limiting the length of actin filaments: Tmod inhibits elongation at the pointed end to keep filaments short (Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001), and ADF/cofilin severs and depolymerizes actin filaments to generate short filaments (Ono, 2007). To understand the in vivo functional relationship between Tmod and ADF/cofilin in regulation of the actin cytoskeleton and also whether they are antagonistic or cooperative, we examined phenotypic consequences of single and double mutant or knockdown of TMD-1 and UNC-60B in *C. elegans* body-wall muscle. If TMD-1 and UNC-60B are antagonistic, double knockdown of these proteins is expected to suppress the phenotype. However, if they are cooperative, double knockdown is expected to enhance the phenotype.

We used RNA interference (RNAi) to knock down TMD-1 in *C. elegans* because *unc-94/tmd-1(RNAi)* causes only a mild phenotype compared with the *unc-94/tmd-1(tm724)* mutant and thus is suitable for detecting phenotypic enhancement or suppression when combined with other mutations. Western blot analysis indicated that *unc-94/tmd-1(RNAi)* caused 70–80% reduction of the TMD-1 protein (Fig. 3A). Therefore, the RNAi treatment did not entirely deplete TMD-1, which explains why the RNAi phenotype was much weaker than the *unc-94/tmd-1(tm724)* mutant phenotype. First, we found that mutation or knockdown of TMD-1 or UNC-60B drastically alters the localization of UNC-60B or TMD-1, respectively, in muscle cells. In wild-type worms, TMD-1 localizes to edges of the actin bands where the pointed ends are concentrated (Fig. 3Ba-c). However, in *unc-60B(r398)* animals (a weak loss-of-function mutant) TMD-1 was enriched in the actin aggregates, whereas localization of TMD-1 to the striated myofibrils was greatly diminished (Fig. 3Bd-f). However, UNC-60B colocalized with actin to myofibrils (Fig. 3Bg-i) as has been reported previously (Ono et al., 1999). *unc-94/tmd-1(RNAi)* caused only moderate disorganization of actin filaments with formation of actin aggregates (Fig. 3Bk). Nonetheless, UNC-60B was highly concentrated at the core of the actin aggregates and greatly reduced from the myofibrils (Fig. 3Bj-l). The strong influence of TMD-1 and UNC-60B on the localization of each other might be a secondary effect of actin filament disorganization. However, α-actinin and myosin did not accumulate in the actin aggregates of an *unc-94/tmd-1* mutant (Fig. 1D), suggesting that not all actin-binding proteins are enriched in the actin aggregates and that TMD-1 and UNC-60B are involved in the same actin-regulatory process.

To better understand how TMD-1 and UNC-60B function, we characterized phenotypes when both TMD-1 and UNC-60B were impaired. We found that a combination of the *unc-60B* mutation and *unc-94/tmd-1(RNAi)* synergistically enhanced the phenotype. *unc-60B* mutants moved much slower than wild-type worms (Fig. 3C). Strong loss-of-function alleles *m35* and *s1309* yielded much more severe motility defects than the weak loss-of-function alleles, *r398* and *s1307* (Fig. 3C) as has been reported previously (Ono et al., 1999). *unc-94/tmd-1(RNAi)* slightly affected motility in the wild-type background (Fig. 3C). However, in *unc-60B* mutant backgrounds, *unc-94/tmd-1(RNAi)* greatly enhanced the motility defects (Fig. 3C). Strong loss-of-function alleles, *unc-60B(m35)* and *unc-60B(s1309)*, seemed only slightly affected by *unc-94/tmd-1(RNAi)* in the motility assay (Fig. 3C), but the *unc-94/tmd-1(RNAi)*-treated *unc-60B* mutants were nearly immobile on agar plates (Fig. 3Dg,h). Because the worm-motility assay was performed in solution, worms may be less mobile on the surface of an agar plate than in fluid. Weak loss-of-function alleles, *unc-60B(r398)* and *unc-60B(s1307)*, were more profoundly affected by *unc-94/tmd-1(RNAi)* (Fig. 3C). Worm motility was remarkably reduced by *unc-94/tmd-1(RNAi)* in these mutant backgrounds (Fig. 3C), and the worms were nearly paralyzed on agar plates (Fig. 3Di,j). Examination of actin filament organization in the body-wall muscle revealed that *unc-94/tmd-1(RNAi)* enhanced disorganization of actin filaments in *unc-60B* mutants (Fig. 3E). In the wild-type background, *unc-94/tmd-1(RNAi)* induced only moderate disorganization of actin filaments with formation of actin aggregates (Fig. 3E, compare a and b, arrowheads). In *unc-60B* mutants, although actin filaments were severely disorganized in control dsRNA-treated worms (Fig. 3E, panels c,e,g,i), some actin filaments were still organized in a striated manner (Fig. 3E, panels c,e,g, and Fig. 3I, arrows). *unc-94/tmd-1(RNAi)* decreased striated organization of actin filaments and enhanced the formation of large wavy actin aggregates (Fig. 3E, panels d,f,h,j). Thus, mutations of UNC-60B and knockdown of TMD-1 synergistically enhanced defects in muscle contractility by increasing disorganization of actin filaments. These results suggest that TMD-1 and UNC-60B are synergistic rather than antagonistic in regulation of actin organization in vivo.

AIP1 cooperates with ADF/cofilin to enhance severing of actin filaments (Ono, 2003b). In *C. elegans*, *unc-78* encodes AIP1 and cooperates with *unc-60B* to promote organized assembly of actin filaments in body-wall muscle (Mohri et al., 2006; Mohri and Ono, 2003; Ono, 2001). Therefore, we also tested whether TMD-1 and AIP1 collaborate in sarcomeric actin organization. Similarly to *unc-60B* mutants, TMD-1 mislocalized to actin aggregates in an *unc-78*-null mutant [*unc-78(gk27)*] (Fig. 4Ad-f). By contrast, AIP1 localizes to the diffuse cytoplasm and the myofibrils in wild type (Fig. 4Ag-i), and this pattern was not significantly affected by *unc-94/tmd-1(RNAi)* (Fig. 4Aj-l). Worm motility was only slightly affected by *unc-94/tmd-1(RNAi)* in wild-type worms but remarkably reduced by *unc-94/tmd-1(RNAi)* in the *unc-78*-null background (Fig. 4B). As a result, *unc-94/tmd-1(RNAi); unc-78* worms appeared nearly paralyzed on an agar plate (Fig. 4Cd, compare with Fig. 4Ca-c). Disorganization of actin filaments was also enhanced by *unc-94/tmd-1(RNAi)*: aggregates of actin were larger and more extensive in *unc-94/tmd-1(RNAi); unc-78(gk27)* animals (Fig. 4Dd) than in *unc-78(gk27)* treated with control dsRNA (Fig. 4Dc). These results strongly suggest that TMD-1 and AIP1 act also synergistically in regulating actin organization in vivo.

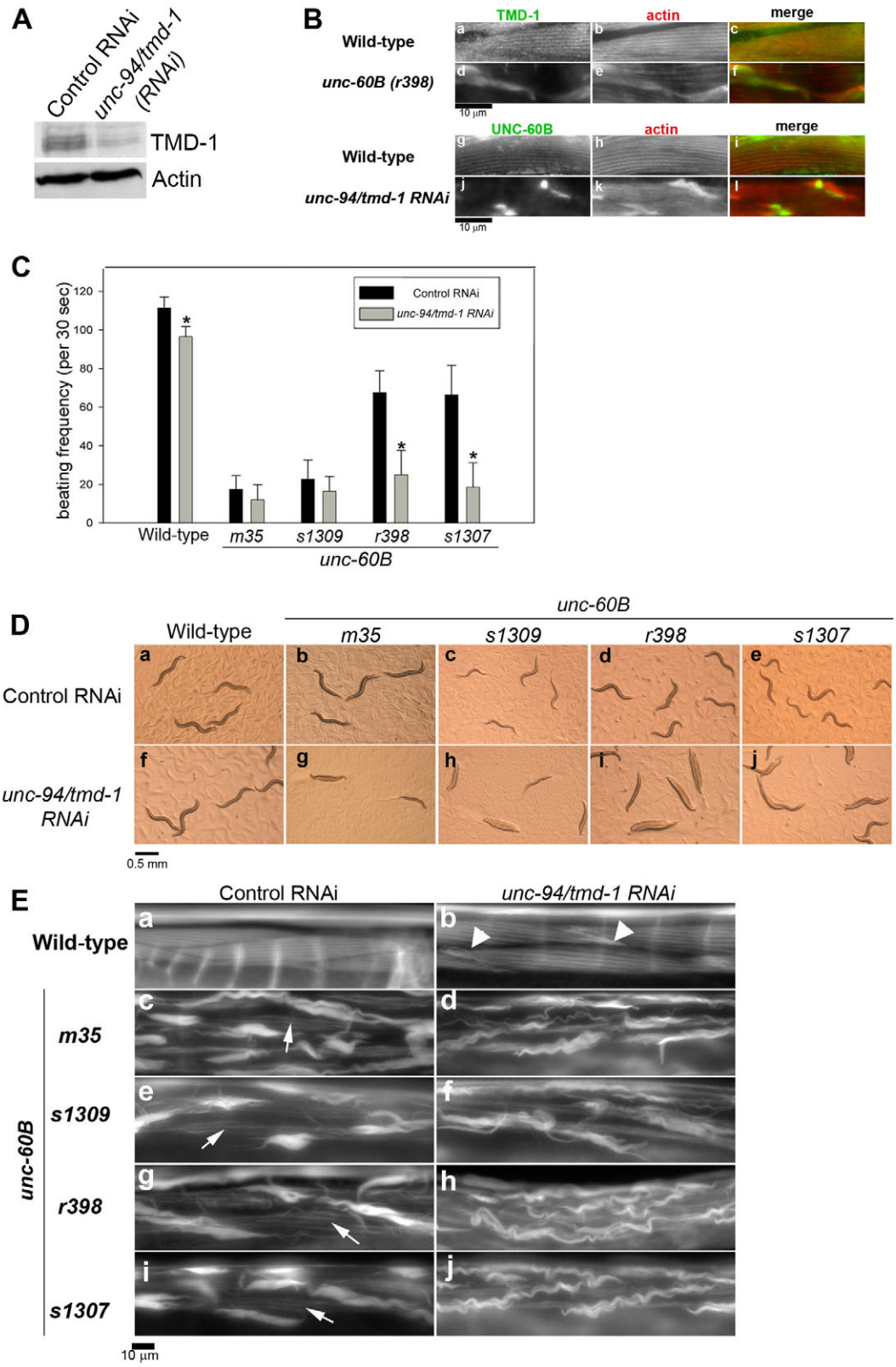

Fig. 3. *unc-94/tmd-1(RNAi)* strongly enhances actin disorganization in *unc-60B* mutant backgrounds. (A) Effect of *unc-94/tmd-1(RNAi)* on the TMD-1 protein level. Wild-type worms were treated with control dsRNA or *unc-94/tmd-1(RNAi)* and the TMD-1 protein level was determined by western blotting using anti-TMD-1 antibody. Anti-actin antibody was used to monitor equal loading of the protein samples. (B) Localization of TMD-1 and UNC-60B in *unc-60B* mutant and *unc-94/tmd-1(RNAi)* muscles, respectively. TMD-1 (a,d) and actin (b,e) were immunolocalized in the body-wall muscle of wild-type (a-c) and *unc-60B(r398)* (d-f). Merged images are shown in c and f (TMD-1, green; actin, red). UNC-60B (g,j) and actin (h,k) were immunolocalized in the body-wall muscle of wild-type (g-i) and *unc-94/tmd-1(RNAi)* (j-l). Merged images are shown in i and l (UNC-60B in green and actin in red). (C) Effects of *unc-94/tmd-1(RNAi)* on worm motility in *unc-60B* backgrounds. Wild-type and four *unc-60B* alleles were examined. *m35* and *s1309* are strong loss-of-function, whereas *r398* and *s1307* are weak loss-of-functinon. Data are the mean ± s.d., n=10. Asterisks indicate $P<0.005$ by t-test comparing control RNAi experiments and *unc-94/tmd-1(RNAi)* for each strain. (D) Appearance of wild-type (a, f) and *unc-60B* (b-e, g-j) worms treated with control dsRNA (a-e) or *unc-94/tmd-1(RNAi)* (f-j) on agar plates. (E) Actin filament organization in the body-wall muscle of wild-type (a and b) and *unc-60B* (c-j) treated with control dsRNA (a,c,e,g and i) or *unc-94/tmd-1(RNAi)* (b,d,f,h and j). Arrowheads in b indicate actin aggregates. Arrows in c, e, g and i indicate striated organization of actin filaments.

Tropomodulin synergistically functions with profilin in the organization of actin filaments the in *C. elegans* body-wall muscle

Profilin is another regulator of actin dynamics that can functionally interact with tropomodulin at the pointed end. Profilin prevents the association of actin monomers with the pointed end and inhibits their elongation when the barbed end is capped (Pantaloni and Carlier, 1993). Therefore, profilin is expected to have an effect similar to that of tropomodulin on actin elongation at the pointed end. We tested the functional relationship of profilin and

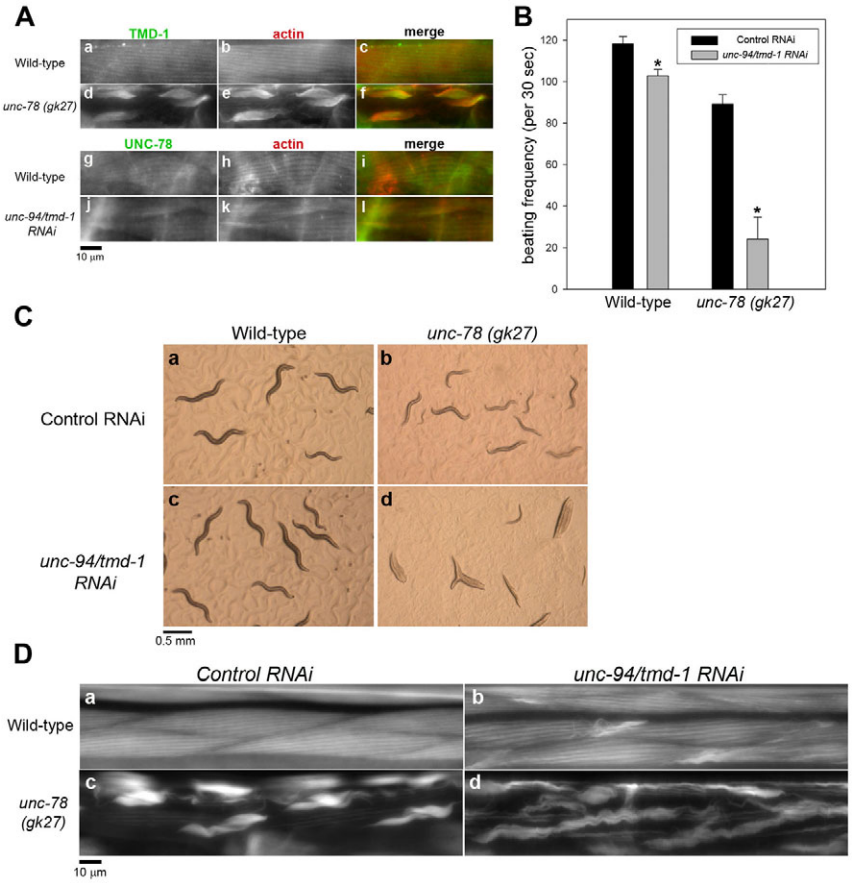

Fig. 4. *unc-94/tmd-1(RNAi)* strongly enhances actin disorganization in an *unc-78*-null background. (A) Localization of TMD-1 and UNC-78 in *unc-78* mutant and *unc-94/tmd-1(RNAi)* muscles, respectively. TMD-1 (a,d) and actin (b,e) were immunolocalized in the body-wall muscle of wild-type (a-c) and *unc-78(gk27)* (d-f). Merged images are shown in c and f (TMD-1, green; actin, red). UNC-78 (g,j) and actin (h,k) were immunolocalized in the body-wall muscle of wild-type (g-i) and *unc-94/tmd-1(RNAi)* (j-l). Merged images are shown in i and l (UNC-78 in green and actin in red). (B) Effects of *unc-94/tmd-1(RNAi)* on worm motility in an *unc-78* null background. Wild-type and *unc-78(gk27)* were examined. Data are the mean ± s.d., n=10. Asterisks indicate $P<0.005$ by t-test comparing control RNAi experiments and *unc-94/tmd-1(RNAi)* for each strain. (C) Appearance of wild-type (a,c) and *unc-78(gk27)* (b,d) worms treated with control dsRNA (a,b) or *unc-94/tmd-1(RNAi)* (c,d) on agar plates. (D) Actin filament organization in the body-wall muscle of wild-type (a,b) and *unc-78* (c,d) treated with control dsRNA (a,c) or *unc-94/tmd-1(RNAi)* (b,d).

tropomodulin by examining phenotypic consequences on actin organization when both profilin and tropomodulin were depleted.

C. elegans has three profilin genes, *pfn-1*, *pfn-2* and *pfn-3* (Polet et al., 2006). Although PFN-3 is the only profilin isoform that was detected in the body-wall muscle, PFN-2 might also have a function in muscle because depletion of both PFN-2 and PFN-3 causes mild disorganization of sarcomeric actin. The latter is more severe than the phenotypes that result from the depletion of a single profilin isoform (Polet et al., 2006). In control RNAi experiments, no actin aggregates were detected in *pfn-2* or *pfn-3* single-mutant or *pfn-3 pfn-2* double mutant animals (Fig. 5A,Ba,c,e,g). (Note that both *pfn-3* and *pfn-2* are on the X chromosome, and that *pfn-3* is located 5′ to *pfn-2*. In such a case, a double mutant is described as *pfn-3 pfn-2* in a standard practice in *C. elegans* genetics.) When TMD-1 was depleted by dsRNA, actin aggregates were found in ~50% of treated wild-type worms (Fig. 5A,Bb), the *pfn-2* mutation did not enhance this phenotype (Fig. 5A,Bd). However, in the *pfn-3*-null background, actin aggregates were found in 100% of *unc-94/tmd-1 (RNAi)*-treated worms (Fig. 5A), although the appearance of actin aggregates was similar to that in wild-type or *pfn-2* animals (Fig. 5Bf). Thus, the *pfn-3* mutation enhances the *unc-94/tmd-1 (RNAi)* phenotype. In the *pfn-3 pfn-2* double mutant, more intense and larger actin aggregates were formed in 100% of *unc-94/tmd-1 (RNAi)* worms (Fig. 5Bh) as compared with *unc-94/tmd-1 (RNAi)*-treated *pfn-3* single-mutant worms (Fig. 5Bf). Therefore, the *pfn-2* mutation enhances the *unc-94/tmd-1 (RNAi)* phenotype in *pfn-3* mutants but not in wild-type worms, suggesting that *pfn-2* has a partially redundant function together with *pfn-3*, whereas *pfn-3* appears to have a major role. Despite the fact that actin disorganization was enhanced in the profilin mutants, we did not detect significant differences in motility of *unc-94/tmd-1 (RNAi)*-treated worms compared with wild-type and profilin mutants (Table 1). This suggests that the functional interaction between Tmod and profilin is only moderately important in muscle. Taken together, these results suggest that tropomodulin and profilin collaborate in the regulation of actin filament organization in *C. elegans* body-wall muscle, which is consistent with a model in which they have similar effects on actin elongation at the pointed end (Littlefield and Fowler, 1998).

Mutation of profilin does not enhance the ADF/cofilin mutant phenotype

Profilin and ADF/cofilin synergistically enhance actin filament turnover in vitro (Didry et al., 1998), and their cooperative function in vivo has been demonstrated in yeast (Nakano and Mabuchi, 2006; Wolven et al., 2000). Under these biochemical and cellular conditions, free actin barbed ends are constantly generated, so that treadmilling of actin filaments is efficiently enhanced by profilin and ADF/cofilin. However, in striated muscle, sarcomeric actin filaments are straight – without any branching – and their barbed ends are tightly capped by capping protein. A functional relationship between profilin and ADF/cofilin under such conditions where free barbed ends are limited has not been investigated.

We examined the phenotype of the *unc-60B; pfn-3 pfn-2* triple mutant and found that the profilin mutation does not enhance the *unc-60B*-mutant phenotype (Fig. 6). When *unc-60B(r398)* (weak loss-of-function) or *unc-60B(s1309)* (strong loss-of-function) were combined with *pfn-3(null) pfn-2(null)*, the extent of actin

A

Strain	Percentages of adult worms with actin aggregates (%) Control RNAi	*unc-94/tmd-1 RNAi*
Wild-type	0 (0/92)	44.0 (44/100)
pfn-2 (null)	0 (0/128)	50.4 (60/119)
pfn-3 (null)	0 (0/107)	100 (110/110)
pfn-3 (null) pfn-2 (null)	0 (0/101)	100 (97/97)

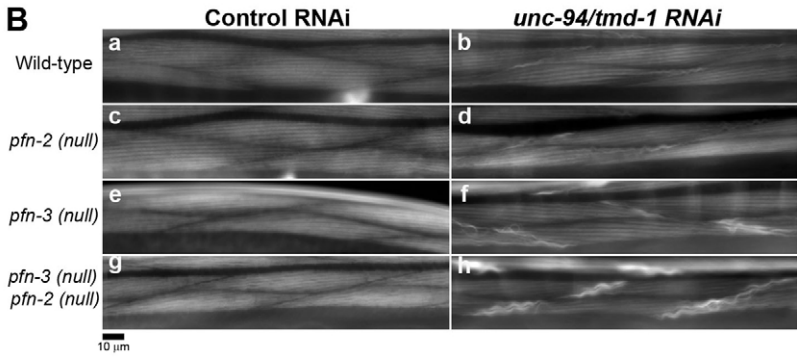

Fig. 5. *unc-94/tmd-1(RNAi)* enhances the formation of actin aggregates in profilin-mutant backgrounds. (A) Frequency of adult worms that have actin aggregates in the body-wall muscle. Adult worms were stained with tetramethyl-Rhodamine–phalloidin, and worms with actin aggregates in their muscle were scored. Wild-type, *pfn-2(null)* and *pfn-3(null)* single mutants, and a *pfn-3 pfn-2* double mutant were treated with control dsRNA or *unc-94/tmd-1(RNAi)*. (B) Actin filament organization in the body-wall muscle of wild-type (a,b), *pfn-2(null)* (c,d), *pfn-3(null)* (e,f) and *pfn-3(null) pfn-2(null)* (g,h) treated with control dsRNA (a,c,e,g) or *unc-94/tmd-1(RNAi)* (b,d,f,h).

disorganization was almost identical to each of the *unc-60B* single mutants (Fig. 6, compare E with G, and I with K). The presence of the *pfn-3 pfn-2* mutation did not alter worm motility of *unc-60B* mutants (Table 1). In addition, the *pfn-3 pfn-2* mutation neither enhanced nor suppressed the effects of *unc-94/tmd-1(RNAi)* in the *unc-60B*-mutant background (Fig. 6F,H,J,L, and Table 1). These results suggest that a synergistic function between profilin and ADF/cofilin is not detectable in *C. elegans* striated muscle and does not have a significant impact on the organization of sarcomeric actin.

Discussion

In this study, we have characterized phenotypes of an *unc-94/tmd-1*-mutant phenotype and the functional relationships of tropomodulin with enhancers of actin-filament dynamics in *C. elegans* striated muscle. The *unc-94/tmd-1(tm724)*-mutant phenotype was more severe than the previously reported *unc-94/tmd-1* loss-of-function or RNAi phenotypes (Stevenson et al., 2007). Actin filaments in the *unc-94/tmd-1(tm724)* worms were severely disorganized in the body-wall muscle, indicating that Tmod is a crucial regulator of actin filament organization in *C. elegans* striated muscle. Our genetic analysis suggests that Tmod is cooperative, rather than antagonistic, with ADF/cofilin, AIP1 and profilin in regulating actin filament organization. These in vivo functional relationships cannot be explained simply by their antagonistic effects on the rate of actin turnover. Rather, Tmod, ADF/cofilin, AIP1 and profilin might collaborate to regulate lengths of actin filaments and might promote the organized assembly of sarcomeric thin filaments.

Tmod is a conserved regulator of sarcomeric actin organization

The severe defects in striated myofibril organization in the *unc-94/tmd-1(tm724)* mutant are consistent with myofibril defects in the heart of *Tmod1*-null mice (Chu et al., 2003; Fritz-Six et al., 2003). *Tmod1* is predominantly expressed in the mouse heart during embryogenesis, and knockout of *Tmod1* results in embryonic lethality due to a severe defect in heart development (Chu et al., 2003; Fritz-Six et al., 2003). Aggregates of F-actin are formed in *Tmod1*-null muscle, and they do not contain α-actinin, a component of the Z-line (where actin filaments are anchored) (Fritz-Six et al., 2003). Similarly, aggregates of F-actin are formed in *unc-94/tmd-1*-mutant *C. elegans* muscle, which does not contain α-actinin,

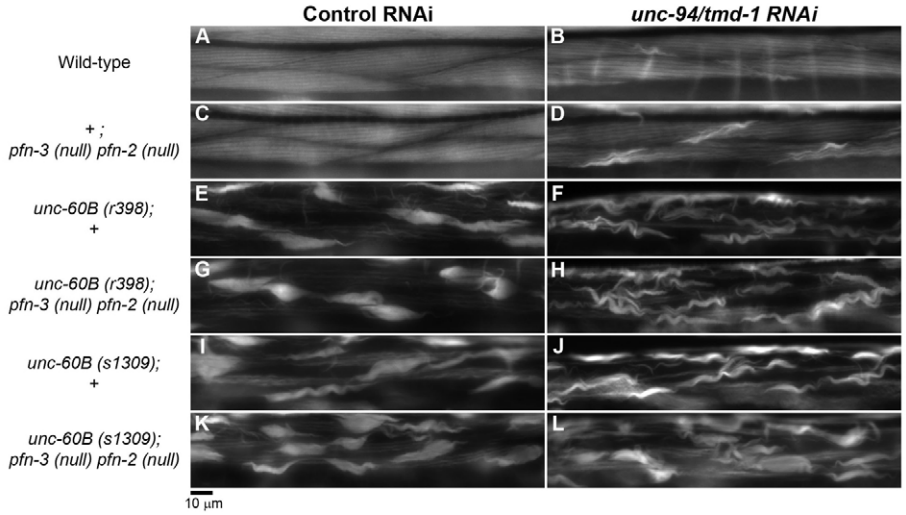

Fig. 6. Mutation of profilin does not enhance the *unc-60B* phenotype. Actin filament organization in the body-wall muscle of wild-type (+;+) (A,B), *+; pfn-3(null) pfn-2(null)* (C,D), *unc-60B(r398); +*(E,F), *unc-60B(r398); pfn-3(null) pfn-2(null)* (G,H), *unc-60B(s1309); +* (I,J) and *unc-60B(s1309); pfn-3(null) pfn-2(null)* (K,L) treated with control dsRNA (A,C,E,G,I,K) or *unc-94/tmd-1(RNAi)* (B,D,F,H,J,L).

suggesting that disorganization of actin is caused by similar mechanisms in *Tmod1*-null muscle of mice and *unc-94/tmd-1* worm mutants. One possible cause for the formation of actin aggregates is the excessive elongation of actin filaments from the pointed end. In striated muscle, actin dynamics at the pointed end is important to regulate the lengths of thin filaments (Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001). Inhibition of Tmod allows actin elongation from the pointed end (Gregorio et al., 1995); excessively long thin filaments may become unstable, dissociate from myofibrils and form aggregates. Another possibility is the detachment of actin filaments following the depletion of TMD-1. TMD-1 localizes to cell-cell junctions in the body-wall muscle (Stevenson et al., 2007) and has a role in formation of adherens junction in epidermal cells (our unpublished data). Although the precise mechanism of actin-aggregate formation is not clear, the *C. elegans* phenotype strongly suggests that TMD-1 is crucial for the assembly and maintenance of sarcomeric actin organization.

Tmod synergistically functions with ADF/cofilin and AIP1

The synergistic effects of TMD-1 and UNC-60B (ADF/cofilin) on sarcomeric actin organization are paradoxical, given their antagonistic effects on actin depolymerization at the pointed end in vitro. However, if one assumes that the major functions of TMD-1 and UNC-60B are to regulate the length of thin filaments, they could have similar effects. Tmod caps the pointed end and limits elongation, thereby keeping the filament short. ADF/cofilin severs and depolymerizes actin filaments, and shortens the filaments. Therefore, TMD-1 and UNC-60B could cooperate to prevent the formation of excessively long thin filaments – a model that would be consistent with our prediction that the actin aggregates are formed from excessively long thin filaments as mentioned above. However, because the actin filaments are severely disorganized in *unc-94/tmd-1* and *unc-60B* mutants, investigation of length distribution of thin filaments is technically difficult. Perhaps, temperature-sensitive mutants or the conditional activation or inactivation of TMD-1 and UNC-60B will be useful in the future to determine their roles in the regulation of the length of thin filaments.

The synergistic effects of TMD-1 and AIP1 also support our model that Tmod and ADF/cofilin-AIP1 do not simply compete for actin pointed end dynamics in vivo. AIP1 is a conserved WD-repeat protein that promotes actin filament disassembly in the presence of ADF/cofilin (Ono, 2003b; Ono, 2007). AIP1 enhances fragmentation rather than pointed-end depolymerization by ADF/cofilin (Ono et al., 2004). A severing-defective mutant of UNC-60B (ADF/cofilin) can still induce actin depolymerization from filament ends, but this activity is not enhanced by AIP1 (Mohri and Ono, 2003), indicating that AIP1 does not affect actin depolymerization from filament ends. Therefore, TMD-1 and AIP1 are not likely to interact at the pointed end. Thus, our results support a model in which ADF/cofilin and AIP1 promote the severing of actin filaments to maintain the length of thin filaments in cooperation with the inhibitory effect of Tmod on actin elongation.

Tmod synergistically functions with profilin

The synergy between Tmod and profilin revealed a new function of profilin in muscle and suggests that profilin acts as an inhibitor of pointed-end elongation in vivo – a fact that had been previously only demonstrated in vitro. Profilin binds to G-actin and inhibits its association with the actin pointed end, whereas it promotes barbed-end elongation especially in the presence of formin (Kovar et al., 2003; Pantaloni and Carlier, 1993; Romero et al., 2004). When

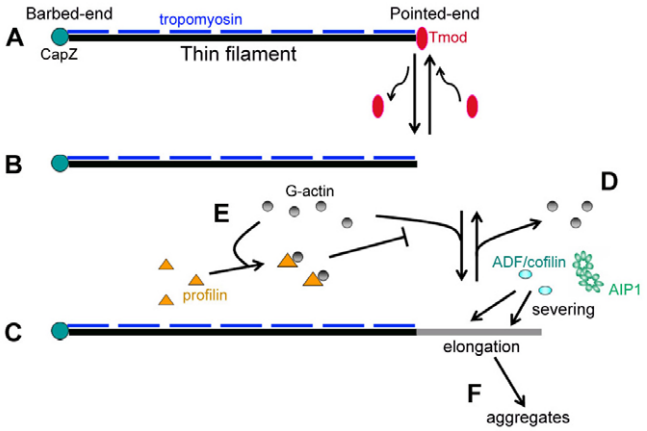

Fig. 7. Model of synergistic regulation of sarcomeric actin organization by Tmod, ADF/cofilin, AIP1 and profilin. A stable thin filament has CapZ at the barbed end, Tmod at the pointed end, and tropomyosin on the side (A). Dynamic behavior of Tmod at the pointed end transiently generates a free pointed end (B) and allows elongation (C). This tropomyosin-free region of the filament is subjected to severing by ADF/cofilin and AIP1 (D), and depolymerized actin is captured by profilin, which prevents elongation from the pointed end (E). When this machinery is impaired, excessively elongated filaments may become unstable and form aggregates (F).

the barbed end is capped, profilin simply inhibits actin polymerization. In striated muscle cells, the actin barbed end is tightly capped by capping protein, and a constant generation of free barbed ends by Arp2/3-mediated branching or severing is expected to be minor. Therefore, profilin is likely to sequester G-actin in muscle cells and inhibit actin elongation from the pointed end. When Tmod is depleted, actin can elongate from the pointed end, but profilin can inhibit monomer association with the pointed end. However, if both Tmod and profilin are depleted, actin can elongate excessively from the pointed end, which might cause disorganization of the sarcomeric structure. Nonetheless, the synergy between Tmod and profilin is weaker than that between Tmod and ADF/cofilin. In striated muscle, the cellular concentration of G-actin is low, near the critical concentration level (Shimizu and Obinata, 1986). Therefore, the rate of actin polymerization should be slow, and the inhibitory effect of profilin on actin polymerization might be only a supportive mechanism to limit the length of actin filaments.

Functional relationship between ADF/cofilin and profilin in striated muscle

We observed that profilin null mutations did not enhance the ADF/cofilin mutant phenotype. However, in vitro, profilin and ADF/cofilin synergistically enhance actin turnover (Blanchoin and Pollard, 1998; Didry et al., 1998), and their synergistic roles in vivo have been demonstrated in yeast (Nakano and Mabuchi, 2006; Wolven et al., 2000). ADF/cofilin preferentially disassembles ADP-actin from or near the pointed end, and profilin catalytically enhances exchange of ADP with ATP and promotes assembly at the barbed end. Therefore, profilin and ADF/cofilin can synergistically enhance actin treadmilling when the barbed end is free or when new barbed ends are constantly generated. However, as mentioned above, most of the actin barbed ends are expected to be capped in striated muscle, and profilin and ADF/cofilin may not function synergistically under these conditions. When the activity of ADF/cofilin is weakened by mutation, actin monomers will not

be supplied by depolymerization, so that the presence or absence of profilin may not have a major impact on pointed-end elongation and actin-filament organization.

A model of synergistic functions of Tmod and enhancers of actin dynamics in sarcomeric actin organization

Taken together, we propose a model in which Tmod collaborates with ADF/cofilin, AIP1 and profilin to promote organized assembly of sarcomeric actin filaments (Fig. 7). When a thin filament is decorated by tropomyosin and capped by Tmod, the filament is protected from severing and depolymerization by ADF/cofilin and AIP1 (Fig. 7A). However, Tmod in myofibrils is dynamic and allows elongation from the pointed end (Littlefield et al., 2001) (Fig. 7A,B). This elongation generates a tropomyosin-free part of the filament (Fig. 7C), where ADF/cofilin and AIP1 can sever and inhibit excessive growth of the filament (Fig. 7D). Profilin supports this action by sequestering monomeric actin from the pointed end (Fig. 7E). A defect in this machinery can cause excessive elongation of the filament leading to loss of integrity and stability of the thin filament. Such an unstable filament might be released from myofibrils and contribute to formation of actin aggregates (Fig. 7F). Although this model still needs to be tested rigorously, Tmod, tropomyosin, ADF/cofilin, AIP1 and profilin are strong candidates for regulators of a conserved core mechanism of sarcomeric actin organization in striated muscle.

Materials and Methods

Nematode strains

Nematodes were grown at 20°C as described previously (Brenner, 1974). Wild-type strain N2 was obtained from the *Caenorhabditis* Genetic Center (Minneapolis, Minnesota, USA). *unc-94/tmd-1(tm724)I* was obtained from Shohei Mitani (National BioResource Project, Tokyo Women's Medical University School of Medicine, Tokyo, Japan) and outcrossed six times. *unc-60B(r398)V, unc-60B(s1309)V*, *unc-60B(m35)V* and *unc-60B(s1307)V* have been reported previously (McKim et al., 1988). *unc-78(gk27)X* has been reported previously (Ono, 2001). *pfn-2(ok458)X* and *pfn-3(tm1362)X* have been reported previously (Polet et al., 2006). The *pfn-3(tm1362) pfn-2(ok458)* double mutant was generated by crossing *pfn-3(tm1362)* and *pfn-2(ok458)* single mutants, and isolating a recombinant that carry both mutations on the same chromosome. All mutants were used as homozygotes in this study.

Western blotting

Western blot analysis of TMD-1 was performed essentially as described (Ono and Ono, 2002). Briefly, 20 adult worms were lysed in SDS-lysis buffer, and subjected to SDS-PAGE and blotting. Rabbit anti-TMD-1 antibody (Stevenson et al., 2007) was diluted at 1:2000 in Signal Enhancer HIKARI (Nacalai USA, Inc.) and used to detect TMD-1 protein. Mouse anti-actin monoclonal antibody (C4, MP Biomedicals) was used to demonstrate equal loading of the samples.

Proteins

C. elegans actin (Ce-actin) (Ono, 1999) and *C. elegans* tropomyosin (CeTM) (Ono and Ono, 2002) were purified from wild-type N2 strain as described previously. Bacterially expressed recombinant UNC-60B was prepared as described (Ono and Benian, 1998). Bacterially expressed recombinant chicken CapZ (a gift from Takashi Obinata, Chiba University, Japan) was prepared as described (Soeno et al., 1998). Pyrene-labeled rabbit muscle G-actin was prepared as described previously (Kouyama and Mihashi, 1981). Recombinant TMD-1 was expressed in *E. coli* with an N-terminal 6×His-tag and purified by metal-affinity chromatography and ion-exchange chromatography (E.A.C., S.Y., R. Zaidel-Bar, S.O. and J.D.H., unpublished).

Assays for F-actin depolymerization from pointed ends by pelleting

G-Ce-actin (10 μM) in G-buffer (2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT) was mixed with 100 nM CapZ, and then polymerization was initiated by adding salts and buffers to final concentrations of 2 mM $MgCl_2$, 0.1 M KCl and 20 mM Hepes-NaOH, pH 7.5. After polymerization for overnight, the CapZ-capped actin filaments (5 μM actin) was incubated with combinations of 2.5 μM each of TMD-1, CeTM, and UNC-60B in the presence of 10 μM latrunculin A (Lat-A; Biomol) in F-buffer (0.1 M KCl, 2 mM $MgCl_2$, 1 mM DTT and 20 mM Hepes-NaOH, pH 7.5) for 15 minutes. The mixtures were ultracentrifuged in a Beckman TLA100 at 446,000 ***g*** for 15 minutes. The supernatants and pellets were adjusted to the same volumes and analyzed by SDS-PAGE. Protein bands were stained with Coomassie Brilliant Blue R-250 (National Diagnostics), band intensity was quantified using ImageJ.

Monitoring kinetics of actin depolymerization from pointed ends

Unlabeled G-Ce-actin (4.5 μM) was mixed with pyrene-labeled rabbit muscle G-actin (0.5 μM) in G-buffer with 50 nM CapZ, and then polymerization was initiated by adding salts and buffers to final concentrations of 2 mM $MgCl_2$, 0.1 M KCl, and 20 mM HEPES-NaOH, pH 7.5. After incubation overnight, the CapZ-capped actin filaments were diluted to 0.5 μM actin with F-buffer with 1 μM UNC-60B, 1 μM CeTM and various concentrations of TMD-1 in the presence of 1 μM Lat-A. Changes in the pyrene fluorescence (excitation at 366 nm and emission at 384 nm) were monitored with a Perkin-Elmer LS50B fluorescence spectrophotometer.

RNA interference experiments

RNAi against *unc-94/tmd-1* was performed by feeding worms with *E. coli* that expressed double-stranded RNA (MRC GeneService clone I-2F12) as described previously (Ono and Ono, 2002). L4 larvae were treated with dsRNA, and phenotypes were characterized in the F1 generation.

Fluorescence microscopy

Immunofluorescent staining of adult nematodes was performed as described previously (Finney and Ruvkun, 1990). Primary antibodies used were rabbit anti-TMD-1 (Stevenson et al., 2007), mouse monoclonal anti-actin (C4, MP Biomedicals), rabbit anti-UNC-60B (Ono et al., 1999), rabbit anti-UNC-78 (Mohri and Ono, 2003), mouse monoclonal anti-α-actinin (MH40) (Francis and Waterston, 1985) and mouse monoclonal anti-MyoA (5-6) (Miller et al., 1983). Secondary antibodies used were Alexa-Fluor-488-conjugated goat anti-mouse IgG (Invitrogen) and Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Staining of adult worms using tetramethyl-Rhodamine–phalloidin was performed as described previously (Ono, 2001).

Samples were observed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope with CFI Plan Fluor ELWD 40× (dry, NA 0.60) objective. Images were captured by a SPOT RT monochrome CCD camera (Diagnostic Instruments) and processed by IPLab imaging software (BD Biosciences) and Adobe Photoshop CS3.

Motility assay

Worm motility was quantified as described previously (Epstein and Thomson, 1974). Briefly, adult worms were placed in M9 buffer. Then, one beat was counted when a worm swung its head to either the right or left. The total number of beats in 30 seconds was recorded.

We thank Paul Sims for performing ultrastructural observation of the *unc-94/tmd-1* mutant phenotype. Monoclonal antibody 5-6 was developed by Henry Epstein (University of Texas Medical Branch, Galveston, TX) and was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Some *C. elegans* strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources. D.L.B. holds a Canadian Research Chair in Genomics. This work was supported by grants from NSERC (Canada) and CIHR (Canada) to D.L.B., NIH grant GM058038 and a grant from Muscular Dystrophy Association to J.D.H., and NIH grant AR48615 to S.O.

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